Redirected Perforin-Dependent Lysis of Colon Carcinoma by Ex Vivo Genetically Engineered CTL

Phillip K. Darcy, Nicole M. Haynes, Marie B. Snook, Joseph A. Trapani, Loretta Cerruti, Stephen M. Jane and Mark J. Smyth

*J Immunol* 2000; 164:3705-3712; doi: 10.4049/jimmunol.164.7.3705

http://www.jimmunol.org/content/164/7/3705

**References**

This article cites 41 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/164/7/3705.full#ref-list-1

**Subscription**

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

**Permissions**

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

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Redirected Perforin-Dependent Lysis of Colon Carcinoma by Ex Vivo Genetically Engineered CTL

Phillip K. Darcy,2* Nicole M. Haynes,* Marie B. Snook,† Joseph A. Trapani,* Loretta Cerruti,† Stephen M. Jane,† and Mark J. Smyth*

The redirection of autologous lymphocytes to predefined tumor target Ags has considerable potential for the immunotherapeutic treatment of cancer; however, robust experimental systems for comparing various approaches have not been developed. Herein, we have generated a single chain variable domain anti-carcinoembryonic Ag (CEA) Fcε receptor I γ-chain fusion (scFv anti-CEA) receptor and demonstrated high-level expression of this chimeric receptor in naïve mouse T lymphocytes by retroviral gene transduction. These gene-modified CTL were able to lyse CEA+ targets and secrete high levels of IFN-γ following Ag stimulation. Depletion studies demonstrated that specific tumor cell cytotoxicity was mediated by gene-modified CD8+ T cells. Importantly, in increasingly stringent tests of efficacy in vivo, transduced CTL were sequentially shown to reject CEA+ colon carcinoma cells in a Winn assay and then reject established s.c. colon carcinoma in scid or syngeneic mice. Furthermore, using gene-targeted and scFv anti-CEA receptor-transduced donor CTL, perforin and IFN-γ were demonstrated to be absolutely critical for the eradication of colon carcinoma in mice. In summary, we have developed a highly efficient gene transfer system for evaluating chimeric receptor expression in cytotoxic lymphocytes. This series of experiments has revealed the utility of scFv anti-CEA chimeras in providing mouse T cells the capacity to reject colon carcinoma in an Ag- and perforin-specific manner. The Journal of Immunology, 2000, 164: 3705–3712.

Adoptive transfer of activated lymphocytes can be highly effective in the destruction of tumor burdens in some patients with melanoma and renal carcinoma (1, 2); however, in general, adoptive immunotherapy has been unsuccessful in the treatment of more prevalent cancers such as ovarian, breast, and colon carcinoma. Three problems have commonly precluded more universally effective adoptive immunotherapy including 1) difficulty in isolating and culturing cytotoxic and tumor-specific T cells from all tumor types; 2) poor tumor localization of ex vivo-modified lymphocytes upon adoptive transfer; and 3) IL-2 toxicity.

To address these problems, one alternative approach explored in recent years has been to genetically manipulate CTL ex vivo to express receptors for tumor-associated Ags (TAA) that can activate CTL effector function upon Ag ligation (3–7). This alternative approach has several advantages over strategies relying strictly upon cellular or humoral immunity. First, CTL and other effector lymphocytes can be redirected to a variety of tumor types, particularly those for which very few specific CTL clones have ever been isolated. Second, gene-engineered T cells kill tumor cells in a non-MHC-restricted and tumor Ag-specific manner, and thus this approach is not compromised, like others, by tumor loss of MHC alleles. Third, CTL grafted with chimeric receptors can be targeted not only to protein TAA, but also to tumor-associated gangliosides and carbohydrates (8).

Several single chain variable domain (scFv) receptors reactive to TAA on ovarian, breast, and colon carcinoma have been functionally expressed in mouse T cell lines such as MD45 and in both mouse and human lymphocytes (4–10). Ag-specific activation via these chimeric scFv receptors resulted in cytokine release and specific lysis of tumor cells in mice (4, 6, 11). Despite these advances, progress toward understanding the important features and limitations of this approach have been constrained by the lack of a robust system for efficiently expressing chimeric genes in primary lymphocytes. In this study, we have developed such a robust system and used it to demonstrate the efficacy of a scFv-γ chimeric receptor recognizing carcinoembryonic Ag (CEA) to redirect T cell-mediated rejection of colon carcinoma. The general utility and effectiveness of this system of gene transduction was highlighted by the efficacy of redirected T cells to reject tumor in a variety models requiring stringent CTL/tumor localisation, specificity, and potency. Furthermore, transduction of T lymphocytes from various gene-targeted mice enabled us to begin to dissect which effector and inflammatory cytokine molecules are critical, such as perforin and IFN-γ, respectively, in achieving tumor eradication. This protocol and these experiments provide the basis on which to rationally test and improve gene-modified lymphocyte therapies of cancer.

Materials and Methods

Cell culture

The human colorectal carcinoma cell lines COLO 205 and Lovo, mouse (C57BL/6) colon adenocarcinoma MC-38 and its CEA+ transfectant MC-38-CEA2 (12) (kindly provided by Dr. Jeff Schlom, National Institutes of Health, Bethesda, MD), and the C57BL/6 sarcoma cell line 24JK (kindly

*Cellular Cytotoxicity Laboratory, The Austin Research Institute, Heidelberg, Victoria, Australia; and †Rotary Bone Marrow Research Laboratory, Royal Melbourne Hospital, Parkville, Victoria, Australia

Received for publication October 6, 1999. Accepted for publication January 21, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

M.J.S. and J.A.T. are currently supported by National Health and Medical Research Council of Australia Principal Research Fellowships. S.M.J was supported by a Wellcome Trust Senior Research Fellowship. P.K.D., M.B.S., and this work were supported by project grants from the National Health and Medical Research Council of Australia and Anti-Cancer Council of Victoria.

2 Address correspondence and reprint requests to Dr. Phillip Darcy, Cellular Cytotoxicity Laboratory, The Austin Research Institute, Studley Road, Heidelberg, 3084, Victoria, Australia. E-mail address: p.darcy@ari.unimelb.edu.au

3 Abbreviations used in this paper: TAA, tumor-associated Ag; CEA, carcinoembryonic Ag; FasL, Fas ligand; IFN-γ, IFN-γ-deficient; Pε, perforin-deficient; scFv, single chain variable domain; TNFα, TNF-deficient.
provided by Dr. Patrick Hwu, National Institutes of Health) were maintained in RPMI 1640 or DMEM at 37°C and 5% CO2 supplemented with the following additives: 10% (v/v) FCS, 2 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). The retroviral packaging cell lines GP+E86 and PA317 and the fibroblast cell line NIH3T3 were cultured in DMEM with additives. PA317 cells transfected with recombinant retroviral DNA were maintained in DMEM supplemented with 0.5 mg/ml G418 (Life Technologies). Transduced T cells were cultured in DMEM containing 100 U/ml of human recombinant IL-2 (kindly provided by Chiron, Emeryville, CA).

Mice
Inbred C57BL/6 (B6), BALB/c, and BALB/c scid/scid (scid) mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. C57BL/6 gld (Fax ligand (Fasl) mutant) and C57BL/6 Tnf-/-deficient (B6.TNF-/-) mice (13) were obtained from the Cen- tenary Institute of Cancer Medicine (Life Technologies, Sydney, Australia). C57BL/6 IFN-γ-deficient (B6.INF-γ-/-) (14) mice were obtained from Ge- nentech (South San Francisco, CA). These gene targeted mice and C57BL/6 perforin-deficient (B6.Po) mice (15) were bred at the Austin Research Institute Biological Research Laboratories. Mice of 4–8 wk of age were used in all experiments that were performed according to animal experimental ethics committee guidelines.

Chimeric receptor gene construction
A 767-bp fragment of DNA encoding for scFv of anti-CEA and a marker epitope from c-myc was amplified by PCR from the vector MFE-2 (16) and subcloned into XhoI/BstZII-digested pRSVscFv (a kind gift from Zelig Eshhar, Weizmann Institute, Rehovot, Israel). The scFv anti-CEA construct was composed of the VH and VL domains of the anti-CEA mAb joined via a flexible linker; a membrane proximal hinge region of human CD8 and the transmembrane and cytoplasmic regions of the human FcyR chain. The scFv anti-CEA chimeric receptor was then digested with SnaBI/ XhoI and subcloned into the Hpal/Xhol site of the retroviral vector pLXSN (a kind gift from Dusty Miller, Seattle, WA) containing the murine Moloney leukemia virus long repeat and a neomycin resistance gene under the control of an SV40 promoter.

Retroviral gene transfer of primary mouse T lymphocytes
The LXSN retroviral vectors (plus the anti-CEA scFv receptor) were transferred into the amphotropic PA317 packaging cell line by CaPO4 precipitation. The supernatants were then collected and used to infect the ecotropic packaging cell line GP+E86 for 24 h in the presence of 4 μg/ml polybrene (Sigma, St. Louis, MO). Stable ecotropic packaging cell lines were obtained after G418 selection. High receptor-expressing clones were isolated by RNA Slot-blot analysis as described previously (17), and viral titer was determined on the basis of neomycin resistance of infected isolated by RNA Slot-blot analysis as described previously (17), and viral titer was determined on the basis of neomycin resistance of infected NIH3T3 cells. GP+E86 clones producing 10^6 viral-producing packaging cells in DMEM supplemented with 0.5 mg/ml G418 (Life Technologies). Transduced T cells were cultured in DMEM containing 100 U/ml of human recombinant IL-2 (kindly provided by Chiron, Emeryville, CA).

Expression of chimeric scFv anti-CEA receptor in mouse T cells
The scFv anti-CEA chimeric receptor gene was composed of the VH and VL regions of the anti-CEA mAb joined via a flexible linker; a membrane proximal hinge region of human CD8 and the transmembrane and cytoplasmic regions of the human FcyR chain. It was subcloned into the retroviral vector pLXSN and high titer virus-producing GP+E86 clones were used to transduce enriched naive mouse T cells from BALB/c mice as described in Materials and Methods. Viral titers of GP+E86 clones ranged from 5 x 10^6 to 2 x 10^7 cfu/ml determined after infection of NIH3T3 fibroblast cells, and thus subsequent T cell infections were performed using the highest virus-producing clone. Naive mouse T cells were transduced by coculturing with virus in the presence of activating agents PHA and IL-2 and polybrene for 72 h. Following cocultivation, expression of the scFv receptor on the surface of the T cells was determined by flow cytometry using an anti-c-myc tag mAb. High-level expression of the scFv receptor (between 40 and 70% for many infections) was detected on T cells (59 ± 10%, n = 10) (Fig. 1A). Expression of the scFv receptor could not be detected on T cells transduced with the LXSN retroviral vector alone (Fig. 1B). Consistent with preferential proliferation of CD8+ spleen T cells in response to PHA/IL-2, the transduced T cell population consisted of predominantly TCRβ+CD8^high and TCRβ+CD4^CD8^low/- T cells (mean CD8^high T cells 86 ± 11%, n = 10).

Ag-specific binding, cytokine release, and cytotoxicity by redirected T cells
In all in vitro and in vivo assays where the specificity of mouse T cells expressing scFv anti-CEA receptor against human CEA-expressing tumor cells was examined, a suitable control human CEA- tumor (i.e., that was nonadherent and grew well in scid mice) was not available. Consequently, we used CEA- mouse 24JK sarcoma cells with the rationale that, if anything, they would be more likely to interact non-specifically with mouse effector CTL than a human tumor target. The ability of mouse T cells expressing scFv anti-CEA to specifically bind to CEA-expressing

Ag-specific binding, cytokotoxicity, and IFN-γ secretion
The binding capacity of gene-modified mouse T lymphocytes was deter- mined in a rosetting assay as described (10). The cytotoxicity capacity of transduced T cells was determined in a 6-h3^1 Cr release assay as described (9). Mouse IFN-γ secretion by scFv-modified mouse T lymphocytes upon CEA ligation was detected by ELISA. Mouse T cells (transduced with LXSN alone or LXSN plus anti-CEA scFv receptor) were cultured with either 5 x 10^5 Lovo (CEA+ ) or 24JK (CEA-) sarcoma cells for 24 h in 24-well plates at a 1:1 E:T ratio. Following incubation, supernatants were harvested and spun to remove cell debris, and levels of IFN-γ were measured by ELISA (PharMingen) according to the suppliers specifications.

Adoptive transfer
Two different adoptive transfer models were employed. First, mouse 24JK sarcoma cells (5 x 10^5) and/or human COLO 205 colon carcinoma cells (5 x 10^5) were injected s.c. into opposite flanks of groups of five scid mice. Spleen T lymphocytes (5 x 10^7) from B6, B6.Po, B6.gld, B6.INF-γ-/-, or B6.TNF-/- mice (transduced with LXSN vector alone or LXSN plus scFv anti-CEA) were injected i.v. into groups of 5 scid mice at 6 h (day 0), days 0 and 1, or day 3 after tumor inoculation. Second, mouse MC-38 colon adenocarcinoma cells (5 x 10^5) and/or their CEA- transfectants, MC-38-CEA2 (5 x 10^5) were injected s.c. into opposite flanks of groups of five B6 mice. Spleen T lymphocytes (5 x 10^7) from B6 mice (transduced with LXSN vector alone or LXSN plus scFv anti-CEA) were injected i.v. into groups of 5 B6 mice at 6 h (day 0), days 0 and 1, or day 3 after tumor inoculation. In both models above, subsequent tumor growth was moni- tored daily and measured by a caliper square along the perpendicular axes of the tumors. The data were recorded as the mean tumor size (product of the two perpendicular diameters) ± SEM.

Results
Expression of chimeric scFv anti-CEA receptor in mouse T cells
The scFv anti-CEA chimeric receptor gene was composed of the VH and VL regions of the anti-CEA mAb joined via a flexible linker; a membrane proximal hinge region of human CD8 and the transmembrane and cytoplasmic regions of the human FcyR chain. The scFv anti-CEA chimeric receptor was then digested with SnaBI/ XhoI and subcloned into the Hpal/Xhol site of the retroviral vector pLXSN (a kind gift from Dusty Miller, Seattle, WA) containing the murine Moloney leukemia virus long repeat and a neomycin resistance gene under the control of an SV40 promoter.
target cells was first demonstrated in a rosetting assay. Transduced BALB/c T cells conjugated with the CEA+ colon carcinoma cell line Lovo (44 ± 2%, n = 3) (Fig. 1D), but not the CEA− sarcoma cell line 24JK (Fig. 1F). T cells transduced with the control LXSN vector were unable to conjugate to either cell line (Fig. 1, C and E). The ability of transduced mouse BALB/c T cells to secrete IFN-γ was demonstrated after specific interaction with CEA+ Lovo target cells (1062 ± 15 pg/ml, n = 3), but not CEA− 24JK sarcoma cells (<20 pg/ml). T lymphocytes transduced with LXSN vector alone secreted < 20 pg/ml IFN-γ after interaction with Lovo or 24JK cells.

We then evaluated the ability of transduced T cells to mediate specific target cell lysis in a 6-h 51Cr release assay. BALB/c T lymphocytes expressing the scFv anti-CEA receptor were able to lyse the CEA+ colon carcinoma cells Lovo and COLO 205, but not the CEA− 24JK sarcoma cell line (Fig. 2A). T cells transduced with the LXSN vector were unable to lyse Lovo, COLO 205, or 24JK cells (Fig. 2A). Lysis of both COLO 205 and Lovo (data not shown) was CEA specific, because intact anti-CEA mAb, but not isotype control mAb, inhibited lysis by gene-modified T cells (Fig. 3, A, C, E, and F). Transduced T cells expressing scFv anti-CEA receptor from all strains of mice consisted of similar ratios of CD8+ T cell population, because depletion of CD8+ cells with magnetic beads completely abrogated lysis of both COLO 205 cells (Fig. 2B) and Lovo cells (data not shown). B6 T lymphocytes that expressed the scFv anti-CEA receptor equivalently to BALB/c T cells (Fig. 3A) were next examined in a syngeneic setting. These effectors were able to lyse the CEA+ MC-38-CEA2 mouse colon adenocarcinoma cells, but not the CEA− parental MC-38 cells (Fig. 2C). T cells transduced with the LXSN vector were unable to lyse MC38-CEA2 or MC-38 cells (Fig. 2C).

Tumor cell lysis by transduced mouse T cells was perforin dependent

CTL-mediated lysis occurs via two major pathways: either involving granule exocytosis (perforin and granzymes) or a FasL/Fas interaction (20). We have previously demonstrated that CTL cell lines engineered with scFv can be activated to lyse colon carcinoma cells in a FasL-dependent manner (9). To determine what mechanism of lysis is used by spleen T cells engineered with a scFv chimera, enriched spleen T cells from B6, B6.Po, and B6.gld (FasL mutant) were transduced with scFv anti-CEA receptor. Flow cytometry determined a high level of receptor expression (40–68% between infections) in T lymphocytes from B6 (49 ± 4%, n = 5), B6.gld (45 ± 4%, n = 5), and B6.Po (40 ± 3%, n = 5) (Fig. 3, A, C, and E). Transduced T cells expressing scFv anti-CEA receptor from all strains of mice consisted of similar ratios of TCRb+ CD8hi and TCRb+ CD4+ CD8lo mice, were demonstrated to equivalently bind to CEA+ colon carcinoma cell lines, and comparably secreted IFN-γ after specific Ag stimulation (data not shown). Importantly, transduced T cells from both B6 and B6.gld, but not B6.Po, mice were able to specifically lyse both CEA+ targets, Lovo and COLO 205, in a 6-h 51Cr release assay (Fig. 3, B, D, and F). By contrast, transduced T cells were unable
release assays were performed at least twice using triplicate samples. Control mAb. Transduced T cells after depletion of the CD8 population (CD8<sup>-</sup> CD8<sup>+</sup>) were also examined (CD8<sup>+</sup> depl.). The experiment was performed twice and error bars represent the SE of triplicate samples. C. Enriched splenic T cells from B6 mice were transduced with the scFv anti-CEA-γ (filled) or pLXSN (open symbols) and tested for lytic activity against CEA<sup>-</sup> MC-38-CEA2 (squares) or CEA<sup>+</sup> MC-38 (triangles) cells. The spontaneous lysis was <10% in all assays, and all <sup>3</sup>Cr release assays were performed at least twice using triplicate samples.

FIGURE 2. Ag-specific lysis of colon carcinoma cells by transduced T cells. The ability of T cells expressing the scFv anti-CEA-γ receptor to mediate specific cytolysis of colon carcinoma cells was determined by a 6-h <sup>3</sup>Cr release assay (A). T cells cocultured with GP+E86 cells producing the pLXSN retrovirus alone were unable to lyse Lovo (B), COLO 205 (C), or 24JK (D). B. BALB/c T cells expressing scFv anti-CEA or pLXSN were tested for specific lysis of COLO 205; an E:T ratio of 20:1 is shown. In some wells, the assay was performed in the presence of 1 μg/ml anti-CEA mAb or an isotype control Ab (+control mAb). Transduced T cells after depletion of the CD8<sup>-</sup> T cell population (<2% CD8<sup>-</sup>) were also examined (CD8<sup>-</sup> depl.). The experiment was performed twice and error bars represent the SE of triplicate samples. C. Enriched splenic T cells from B6 mice were transduced with the scFv anti-CEA-γ (filled) or pLXSN (open symbols) and tested for lytic activity against CEA<sup>-</sup> MC-38-CEA2 (squares) or CEA<sup>+</sup> MC-38 (triangles) cells. The spontaneous lysis was <10% in all assays, and all <sup>3</sup>Cr release assays were performed at least twice using triplicate samples.

FIGURE 3. Colon carcinoma cell lysis by transduced mouse T cells is perforin dependent. Enriched splenic T cells from B6 (A and B), B6.gld (C and D), and B6.P<sup>o</sup> (E and F) mice were transduced by coculture with GP+E86 clones producing the retroviral vector pLXSN alone or recombinant retroviral vector harboring the scFv anti-CEA-γ fusion protein. pLXSN (single line)- or scFv anti-CEA (solid line)-transduced T cells were stained with the mouse anti-tag mAb and with PE-labeled sheep anti-mouse Ig or scFv-transduced T cells with the PE-labeled secondary Ab alone (dashed line) and analyzed by flow cytometry. The cytotoxicity of T cells expressing the scFv anti-CEA-γ receptor against CEA<sup>-</sup> colon carcinoma cell lines Lovo (B) and COLO 205 (C) and the CEA<sup>-</sup> 24JK sarcoma cell line (A) was measured. T cells cocultured with GP+E86 cells producing the pLXSN retrovirus alone were unable to lyse Lovo (B), COLO 205 (C), or 24JK (D). The spontaneous lysis was <10% in all assays, and all <sup>3</sup>Cr release assays were performed at least twice using triplicate samples.

Efficacy of redirected T cells after adoptive transfer into scid mice bearing tumors

The simplest in vivo assay to determine the ability of transduced mouse T cells to control tumor growth is a Winn assay in scid mice (21). B6 T cells transduced with scFv anti-CEA receptor rejected the growth of COLO 205 tumor cells, but not 24JK cells, at an E:T ratio of 1:1 in all mice (data not shown). Preincubation of COLO 205 cells with pLXSN-transduced T cells did not inhibit tumor cell growth. To further test the efficacy of redirected T cells in a more stringent model requiring T cell localization to the site of tumor challenge, transduced T cells were adoptively transferred i.v. into scid mice with established s.c. colon carcinoma. Transduced B6 T cells were injected at either 6 h (day 0), days 0 and 1, or on day 3 after COLO 205 tumor inoculation. COLO 205 tumors were measurable within 2 days of tumor inoculation. Adoptive transfer of T cells transduced with LXSN vector alone had no effect on COLO 205 tumor growth (Fig. 4). By contrast, mice treated on days 0 and 1 with T cells expressing scFv anti-CEA receptor completely rejected COLO 205 tumor growth in two of five mice and partially inhibited tumor growth in the remaining three mice (Fig. 4). In addition, COLO 205 tumor growth was temporarily delayed (for 3 days) in groups of mice receiving T cells expressing scFv anti-CEA receptor on day 0 or day 3 alone. Escaping tumors still retained significantly high levels of CEA expression (data not shown). These initial data suggested that multiple i.v. injections or larger numbers of gene-modified CTL would be required to completely regress established tumors.

Perforin-specific control of colon carcinoma in scid mice

To test whether the granule exocytosis or FasL pathway was critical for tumor rejection in vivo, transduced B6.P<sup>o</sup> T cells were transferred. Adoptively transferred B6.P<sup>o</sup> T cells expressing scFv anti-CEA receptor did not inhibit the growth of s.c. COLO 205 tumors, indicating that perforin was critical for effective tumor rejection (Fig. 5). T cells from B6.gld mice that were transduced with scFv anti-CEA receptor inhibited the growth of COLO 205 tumors to a similar degree to transduced B6 T cells. These data suggested that FasL was not an important CTL effector for CTL-mediated tumor rejection.
molecule in the control of this colon carcinoma (in support of the in vitro data), despite the fact that in vitro ligation of Fas can mediate the death of COLO 205 tumor cells (P. K. Darcy, unpublished data).

The role of IFN-\(\gamma\) in control of colon carcinoma in scid mice

To examine whether effector cell inflammatory mediators were also important in redirected T cell-mediated rejection of colon carcinoma, T cells from B6.IFN-\(\gamma\)-o and B6.TNF-o mice were transduced with scFv anti-CEA receptor. High-level expression of the scFv anti-CEA receptor was observed in B6. IFN-\(\gamma\)-o (42 ± 3%, \(n = 5\)) and B6.TNF-o (45 ± 2%, \(n = 5\)) as demonstrated in B6 T cells. Transduced B6.TNF-o T cells inhibited the growth of COLO 205 tumors to a similar degree to transduced B6 T cells. These data suggested that effector cell TNF was not important in the control of this colon carcinoma. By contrast, a notable observation supported by a repeat experiment (data not shown, also \(n = 5\)) was that despite early treatment and reduction in tumor growth, scFv-transduced T cells from B6.IFN-\(\gamma\)-o mice were unable to cause tumor eradication. These data suggested that effector cell IFN-\(\gamma\) may play a minor but significant role in the control of colon carcinoma (Fig. 5).

Ag-specific control of colon carcinoma in scid mice

To demonstrate Ag-specific tumor eradication, additional experiments were performed with mice inoculated with CEA\(^+\) COLO 205 tumor cells in the right flank and CEA\(^-\) 24JK tumor cells in the left flank. T cells expressing scFv anti-CEA receptor specifically reduced COLO 205 tumor growth (two of five eradicated) (Fig. 6A), while 24JK grew unaffected (Fig. 6B). Control nontransduced, but activated, T cells did not reduce COLO 205 or 24JK tumor growth. Nevertheless, perforin-dependent eradication of some CEA\(^+\) tumors in a s.c. site upon early treatment was impressive evidence that the transduced CTL could localize to the site of tumor inoculation.

Correlation between T cell transfer number and inhibition of tumor growth

Two additional experiments were performed to assess the possible limitations of efficacy of T cells expressing scFv anti-CEA receptor. In the first, multiple adoptive transfers were provided in an attempt to eradicate all COLO 205 tumors. Four sequential transfers of redirected T cells enabled four of five mice to survive tumor free; however, this was not a large improvement over two sequential transfers producing three tumor-free mice in a group of five (Fig. 7A). Due to the nature of i.v. injection, the maximum number of sequential T cell transfers practically achieved was four (from...
tumor growth (Fig. 8, B). MC-38 tumor grew unaffected (Fig. 8). Control LXSN vector, while the neighboring growth (two of five eradicated) (Fig. 8). or as late as day 2, specifically inhibited MC-38-CEA2 tumor cells expressing anti-CEA receptor administered on days 0 and 1, by inoculating mice with CEA mouse colon carcinoma. Ag-specific efficacy was again examined tively transferred i.v. into syngeneic B6 mice with established s.c. To further test the efficacy of redirected mouse T cells in a more Efficacy of redirected T cells in syngeneic mice bearing mouse of anti-tumor T cells given.

Correlation between timing and number of T cells trans-ferred and rejections achieved. The growth of human COLO 205 colon carcinoma cells (CEA−) in groups of five scid mice injected s.c in the right flank with 5 × 10^6 COLO 205 (■). Groups receiving T cells were as follows: (A) mice were injected i.v. with 5 × 10^6 B6 T cells (pLXSN) on day 0 and 1 (■), 5 × 10^6 B6 T cells (α-CEA) on day 0 and 1 (○, dashed line), or 5 × 10^6 B6 T cells (α-CEA) on day 0, 1, 2, and 3 (●, dashed line); (B) mice were injected i.v. with 5 × 10^6 B6 T cells (α-CEA) on day 0, 1, and 2 (○) or 1.5 × 10^7 B6 T cells (α-CEA) on day 0 (□). In a second experiment, three sequential injections of 5 × 10^6 CEA-specific T cells were compared with one single bolus dose of 1.5 × 10^7 T cells (Fig. 7B). Not surprisingly, a larger dose of cells earlier (6 h after tumor inoculation) was more effective than the same total dose delivered over three successive days (6 h, day 1, day 2). In all experiments to date, the indications have been that greater tumor inhibition can be achieved with earlier and larger T cell transfers. These data are consistent with a previous study using T cells transgenic for a scFv (22), where the prolonged survival of the experimental group was proportional to the number of anti-tumor T cells given.

Efficacy of redirected T cells in syngeneic mice bearing mouse colon adenocarcinoma

To further test the efficacy of redirected mouse T cells in a more immunologically stringent model, transduced T cells were adop-tively transferred i.v. into syngeneic B6 mice with established s.c. mouse colon carcinoma. Ag-specific efficacy was again examined by inoculating mice with CEA+ MC-38-CEA2 tumor cells in the right flank and CEA− MC-38 tumor cells in the left flank. B6 T cells expressing anti-CEA receptor administered on days 0 and 1, or as late as day 2, specifically inhibited MC-38-CEA2 tumor growth (two of five eradicated) (Fig. 8A), while the neighboring MC-38 tumor grew unaffected (Fig. 8B). Control LXSN vector alone transduced T cells did not reduce MC-38-CEA2 or MC-38 tumor growth (Fig. 8, A and B).

Discussion

The approach of merging T cell function with Ab specificity to create engineered receptors composed of scFv fused to TCR/CD3 signaling components is now close to a decade old (4–6, 23). Despite the promise of this approach and a period of great activity in engineering various scFv reactive with target Ags on tumors (24), only a few studies have evaluated the anti-tumor efficacy of redirected T cells in vivo (4, 25). Initial studies used electropora-tion and other standard transfection techniques to introduce chimeric genes into T cell lines and hybridomas (26–28). More recently, retrovirus infection has been successfully used to express scFv chimeras in human PBL and cell lines (7, 29, 30). Neverthe-less, the major limitation to testing the efficacy and mechanism of action of redirected T cells in vivo has been the difficulty in ex-pressing scFv receptors in primary mouse T lymphocytes. In this study, we have demonstrated a reproducible and high level expres-sion of a scFv chimeric receptor in mouse T lymphocytes by ret-roviral transduction. We have successfully expressed a number of chimeric scFv reactive with CEA or other TAA in T cells using this method (P. K. Darcy, unpublished data). The ability to effi-ciently and reproducibly express scFv receptor (and other) genes in primary CD8+ effector cells is a significant step forward to achiev-ing rigorous examination of various redirected T cell therapeutic strategies.

We have demonstrated the efficacy of the scFv anti-CEA-γ re-ceptor to redirect CTL to CEA-expressing colon carcinoma in three settings in vivo. From the Winn assay to targeting established s.c. syngeneic colon carcinoma, each model was increasingly string-ent and biologically relevant. Indeed, the efficacy of scFv-engi-neered and -transferred T cells in our study has demonstrated that these effector T cells can even eradicate colon carcinomas that grow quite rapidly in a syngeneic host. Furthermore, there was no requirement for the addition of exogenous IL-2. In the adoptive transfer models (in scid and syngeneic mice), the specificity of the redirected CTL was demonstrated by the rejection of CEA− colon carcinoma and the normal tumor growth of CEA− tumor inoculated on the opposite flank. This is the first study to demonstrate in this fashion the targeted specificity of CTL-expressing scFv. Further specificity controls included the inability of either T cells acti-vated with IL-2/PHA (as for infection) or T cells infected with the LXSN retroviral vector alone to affect tumor growth. In all models evaluated, CTL-expressing scFv anti-CEA rejected colon
cancer in a CEA-specific manner. Total tumor eradication in mice correlated with both the commencement of treatment and the number of transduced T cells administered.

Only one other study has shown that mouse T lymphocytes retrovirally transduced with a chimeric scFv can reject tumors in vivo (25). However, it is important to note two points when examining that study: 1) large numbers (up to $5 \times 10^7$ total) of transduced T cells were injected directly into the established tumor; and 2) the tumors expressing human erbB-2 receptor only grew in BALB/c mice that had been tolerized with Ag as newborns. In this sense, our study has more definitively demonstrated the ability of transferred T cells expressing scFv to specifically localize and prevent the growth of a s.c. syngeneic tumor that grows rapidly in the naive host. Moreover, we have demonstrated the robustness and utility of our system by determining critical molecules involved in the rejection process. In both xenogeneic and syngeneic settings, donor-transduced T cells have been adaptively transferred from gene-targeted mice. Consistent with other studies that suggested that CTL-mediated tumor regression or disease protection was perforin dependent (31–33), we have clearly shown that gene-modified T cells expressing scFv anti-CEA-mediated cytotoxicity and tumor rejection via a perforin-dependent pathway. By contrast, transduced T cells from FasL mutant or TNF-deficient mice were able to reject colon carcinomas cells following adoptive transfer. This is not to say that in some models FasL or TNF might be important effector molecules in the rejection process. Sherman et al. have demonstrated that a fusion protein consisting of a scFv Her-2/neu-specific mAb linked to IL-2 could mediate formation of stable conjugates between T cells expressing the IL-2 receptor and tumor cells expressing Her-2/neu, resulting in lysis through the FasL-Fas pathway (34). In addition, we have previously shown that the mouse hybridoma cell line MD45 expressing either the scFv anti-CEA-γ or anti-erbB2-γ could specifically lyse target cells in vitro by a FasL-dependent mechanism (9, 10). Yet clearly the most appropriate test of effector mechanism is in vivo using primary T cells and solid syngeneic tumors.

One interesting observation realized from the in vivo analysis of various gene-targeted T cells was the importance of IFN-γ in achieving effective tumor eradication. Clearly IFN-γ-deficient T cells could inhibit colon carcinoma growth, but complete tumor eradication was not achieved. There has been previous evidence to suggest that secretion of T cell cytokines such as IFN-γ may enhance the lytic capacity of effector cells through up-regulation of both Ag and Fas expression on colon carcinoma cells (35). Indeed, we have demonstrated that human IFN-γ treatment of COLO 205 increases CEA and Fas expression (9). The exact role of IFN-γ in tumor eradication remains unclear, but herein effector cell mouse IFN-γ exerted its action against human colon carcinomas growing in scid mice. In the known absence of murine IFN-γ activity on human IFN-γ receptors and a lack of mature T and B cells in the scid mouse, these experiments raise the possibility of an indirect mechanism involving either an innate arm of immunity or effects of IFN-γ on tumor angiogenesis. It remains to be determined whether the addition of exogenous mouse IFN-γ can enhance redirected T cell lysis in our models. Whatever the mechanism, it would appear Ag specific because coimmunized control CEA+ tumor cells grew unaffected. It had been postulated that grafted CTL might secrete important cytokines following Ag ligation (22) and clearly our data support the concept that such activity was important for tumor eradication.

It has also been noted that to be effective in adoptive immunotherapy, scFv expressing T cells would need to be preactivated (22). In our preparation, T cells have to be preactivated to get them cycling for retroviral transduction. Although the levels of scFv receptor expressed on T cells did not approximate endogenous TCR/CD3 complex, these cells appeared relatively able to mediate cytotoxicity and cytokine secretion after scFv receptor ligation. While adhesion and accessory molecules clearly play an important role in T cell activation and redirected T cell-mediated lysis (36), these molecules appeared to be adequately expressed in as much as scFv anti-CEA-expressing mouse CTL were effective against both CEA+ human colon carcinoma and mouse adenocarcinoma cell lines in vitro and in vivo. The importance of molecules such as T cell CD2, CD11a/CD18, and CD54 will now be evaluated in our models using gene-targeted mice as donors or blocking mAbs in vivo. It appeared, at least for this scFv receptor, that transduced effector cells must eventually die rather than differentiating into memory cells, because mice (scid or B6) surviving tumor in the first instance, subsequently succumbed to a secondary s.c. challenge (data not shown). Future studies using other scFv receptors better designed to improve the survival and differentiation of transduced T cells may be required to improve anti-tumor efficacy and resist tumor rechallenge.

The issue regarding the most optimal signaling domain for any given population of effector cells remains controversial. Many studies have only assessed the functional capacity of scFv receptors linked to either γ, ζ, or downstream intracellular protein tyrosine kinases in vitro (7, 10, 37–39). In this study, we have demonstrated that the FeCγ-γ signaling chain could induce in vivo effector function in purified primary mouse CTL. Tumor studies are currently in progress to compare the efficacy of FeCγ-γ and TCR/CD3-ζ signaling domains linked to the same scFv when expressed in mouse CTL.

Generally CEA serves as a good model target TAA for testing new immune therapies (40, 41). However, soluble monovalent CEA Ag may bind to scFv anti-CEA receptor-grafted CTL without cross-linking the receptor, thereby preventing tumor cell recognition and rendering the CTL refractory to Ag-driven activation. This limitation may restrict the scFv receptor approach to patients expressing low levels of soluble CEA, and indeed our previous studies in vitro have shown such a correlation between inhibition of lytic capacity and soluble CEA levels (9). Examining this problem in experimental settings in vivo is more problematic; however, the development of scFv that preferentially recognize the membrane-bound form of CEA over the soluble form (42) offers an alternative solution. Studies will now be conducted using these robust CEA+ xenogeneic and syngeneic tumor rejection models to further evaluate the mechanism of tumor rejection and to optimize the homing, proliferation, and persistence of retrovirally transduced mouse T cells.

Acknowledgments
We thank the staff of the Austin Research Institute Biological Research Laboratories for their maintenance and care of the mice in this project. We also acknowledge helpful discussions with Drs. Geoffrey Pietersz, Ricky Johnstone, and Michael Kershaw.

References