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Rejection of Syngeneic Colon Carcinoma by CTLs Expressing Single-Chain Antibody Receptors Codelivering CD28 Costimulation

Nicole M. Haynes,* Joseph A. Trapani,* Michele W. L. Teng,* Jacob T. Jackson,* Loretta Cerruti,† Stephen M. Jane,† Michael H. Kershaw,* Mark J. Smyth,2* and Phillip K. Darcy2,3*

A new strategy to improve the therapeutic utility of redirected T cells for cancer involves the development of novel Ag-specific chimeric receptors capable of stimulating optimal and sustained T cell antitumor activity in vivo. Given that T cells require both primary and costimulatory signals for optimal activation and that many tumors do not express critical costimulatory ligands, modified single-chain Ab receptors have been engineered to codeliver CD28 costimulation. In this study, we have compared the antitumor potency of primary T lymphocytes expressing carcinoembryonic Ag (CEA)-reactive chimeric receptors that incorporate either TCR-ζ or CD28/TCR-ζ signaling. Although both receptor-transduced T cell effector populations demonstrated cytolysis of CEA+ tumors in vitro, T cells expressing the single-chain variable fragment of Ig (scFv)-CD28-ζ chimera had a far greater capacity to control the growth of CEA+ xenogeneic and syngeneic colon carcinomas in vivo. The observed enhanced antitumor activity of T cells expressing the scFv-CD28-ζ receptor was critically dependent on perforin and the production of IFN-γ. Overall, this study has illustrated the ability of a chimeric scFv receptor capable of harnessing the signaling machinery of both TCR-ζ and CD28 to augment T cell immunity against tumors that have lost expression of both MHC/peptide and costimulatory ligands in vivo. The Journal of Immunology, 2002, 169: 5780–5786.

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Given the refractive nature of colon carcinoma to conventional T cell therapies (4, 27), we have investigated the utility of scFv receptors that incorporate CD28 costimulatory signals to treat carcinomaembryonic Ag (CEA)-overexpressing colon cancer. In this study, the therapeutic efficacy of primary mouse T lymphocytes expressing scFv-α-CEA receptors containing the -CD28-ζ, or -CD28 signaling domains was evaluated in a syngeneic mouse tumor model. We describe a significant advance to adoptive immunotherapy by demonstrating that T cells activated by a scFv-α-CEA-CD28-ζ chimera have an enhanced capacity to control the growth of established syngeneic tumors, through a mechanism that requires both perforin (pfp) and IFN-γ.

Materials and Methods

Cell culture

The human colorectal carcinoma cell lines COLO 205, mouse (C57BL/6 (B6)) colon adenocarcinoma MC-38 and its CEA transfectant MC-38-CEA2, and the B6 sarcoma cell line 24JK (kindly provided by Dr. P. Hwu, National Institutes of Health, Bethesda, MD) were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 5% CO2, 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Grand Island, NY). All tumor cell lines used in this study did not express the CD80 and CD86 costimulatory ligands as determined by flow cytometry. The retroviral packaging cell lines GP+E86 and PA317 and the fibroblast cell line NIH3T3 were cultured in DMEM with additives. GP+E86 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 human IL-2 (kindly provided by Chiron, Emeryville, CA).

Mice

Inbred B6, BALB/c, and BALB/c scid/scid (SCID) mice were purchased from The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) and housed in filter-bottom cages. Mice were maintained in a temperature- and humidity-controlled environment under specific pathogen-free conditions with a 12-h light/dark cycle. All mice used in this study were between 6–16 wk of age and 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 coding for scFv of anti-CEA and a marker epitope from c-myc were amplified by PCR from the MFE-23 vector (28) and subcloned into XhoI/BstEII-digested pRSVscFv-Y (a kind gift from A. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) containing the long terminal repeat and a neomycin resistance gene under the control of an SV40 promoter.

Retroviral gene transfer of mouse spleen T lymphocytes

Stable GP+E86 ecotropic packaging cell lines expressing the scFv-anti-CEA-ζ, -CD28, or -CD28-ζ receptors were isolated as previously described (13, 15). Stable GP+E86 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 human IL-2 (kindly provided by Chiron, Emeryville, CA).

Flow cytometry

Detection of cell surface chimeric receptor expression on mouse T lymphocytes was achieved by indirect immunofluorescence with c-myc tag Ab purified from supernatants of mouse 9E10 cells (29), followed by staining with a PE-labeled anti-mouse Ig mAb (BD Biosciences, San Jose, CA). Background fluorescence was assessed using a purified IgG isotype Ab (3S193, Ludwig Institute for Cancer Research, Melbourne, Australia). Cell surface phenotype of transduced cells was determined by direct staining with Quantum-R-Red-labeled anti-TCRβ (clone H57-597; BD Pharmingen, San Diego, CA), FITC anti-CD4 (H12919; Sigma-Aldrich), and Quantum-Red-labeled anti-CD8 (R-3762; Sigma-Aldrich) mAbs as previously described (13). Cell surface phenotype of tumor cell lines was determined by indirect immunofluorescence with anti-human CEA (11-1, Ref. 30), anti-mouse or -human CEA (mouse, 1G10; human, BB1; Sigma-Aldrich), anti-CD86 (mouse, GL1; human, 233G1; Sigma-Aldrich) mAb, followed by staining with a fluorescein-labeled anti-Ig mAb. As previously described, T cell populations were >85% CD8+ (13, 15).

Ag-specific binding and direct cytotoxicity

The binding capacity of gene-modified mouse T lymphocytes was determined in a rosetting assay as described (13). The cytolytic capacity of transduced T cells was determined in a 6-h 51Cr-release assay. Briefly, receptor-modified and mock-transduced T cells from BALB/c or B6 mice were cocultured in a flat-bottom 96-well microtiter plate with medium 15Cr-labeled target cells (either CEA-stable GP+E86 and PA317 and the fibroblast cell line NIH3T3 were cultured in DMEM with additives. GP+E86 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 human IL-2 (kindly provided by Chiron, Emeryville, CA).

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Chimeric receptor gene construction

A 767-bp fragment of DNA coding for scFv of anti-CEA and a marker epitope from c-myc were amplified by PCR from the MFE-23 vector (28) and subcloned into XhoI/BstEII-digested pRSVscFv-Y (a kind gift from Z. Ishhar, Weizmann Institute, Rehovot, Israel). The chimeric gene constructs were composed of the scFv-anti-CEA mAb, a membrane proximal hinge region of human CD8, and one of the transmembrane and cytoplasmic regions of the human TCR-ζ chain (scFv-anti-CEA-ζ), the transmembrane and cytoplasmic regions of the mouse CD28 signaling chain (scFv-anti-CEA-CD28), or the transmembrane and cytoplasmic regions of the mouse CD28 signaling chain fused to the cytoplasmic region of TCR-ζ (scFv-anti-CEA-CD28-ζ) (Fig. 1A). For detection purposes, each receptor contained a c-myc tag epitope at the C terminus of the Vζ region. The scFv anti-CEA chimeric receptors were digested with Smal/Bsu36I and subcloned into the Hpal/XhoI restriction sites of the retroviral vector pLXSN (a kind gift from D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) containing the long terminal repeat and a neomycin resistance gene under the control of an SV40 promoter.

Retroviral gene transfer of mouse spleen T lymphocytes

Stable GP+E86 ecotropic packaging cell lines expressing the scFv-anti-CEA-ζ, -CD28, or -CD28-ζ receptors were isolated as previously described (13, 15). GP+E86 clones producing ~107 CFU/ml were used for transduction of mouse spleen T lymphocytes. Spleen cells from mice were initially depleted of RBCs by hypotonic lysis with NH4Cl and enriched by passing through a nylon wool column as described previously (13, 15). Enriched T lymphocytes (105) were then cocultivated for 72 h with 5 x 106 virally-producing packaging cells in DMEM supplemented with 4 µg/ml polybrene, 5 µg/ml PHA (Sigma-Aldrich, St. Louis, MO), and 100 U/ml rIL-2. After cocultivation, T cells were separated from adherent packaging cells, washed with DMEM, and cultured in DMEM supplemented with 100 U/ml rIL-2. The cells were subsequently analyzed for transduction efficiency by flow cytometry and used for in vitro and in vivo experiments.
The incorporation of the ζ intracellular sequence distal to CD28 was required for effective functional expression of the scFv-CD28-ζ receptor, as previously demonstrated (23, 26). Surface expression of the scFv-α-CEA receptors in transduced CD8+ primary splenocytes was determined by flow cytometry using an anti-c-myc tag mAb directed at the tag epitope located within the extracellular domain of these receptors (Fig. 1, B–E). Consistent and equivalent levels of expression of the scFv-CD28-ζ, -CD28, and -ζ chimeric receptors were reproducibly detected on T cells (n = 5). The cytolytic capacities of T cells expressing the scFv-α-CEA-CD28-ζ (T-scFv-CD28-ζ), scFv-α-CEA-CD28 (T-scFv-CD28), or scFv-α-CEA-ζ (T-scFv-ζ) receptors were evaluated against CEA+CD80+CD86+ tumor cells in standard 6-h 51Cr release assays. T-scFv-CD28-ζ and T-scFv-ζ cells demonstrated an equivalent ability to specifically conjugate to (binding assays; data not shown) and lyse the CEA+ human COLO 205 colon carcinoma and mouse MC-38-CEA2 colon adenocarcinoma cell lines (Fig. 1, F and H). Equivalent levels of cytolysis were also mediated by both transduced T cell effector populations after 16 h (data not shown). Lysis of the CEA− mouse 24JK sarcoma and mouse MC-38 cell lines was not detected, demonstrating the Ag specificity of cytolysis (Fig. 1, G and I). T-scFv-CD28 cells were unable to lyse any of the tumor cell lines tested. These data indicated that the scFv-CD28-ζ chimera was functional and that fusion of the CD28 and ζ cytoplasmic domains neither enhanced nor diminished the cytolytic function. Previous studies have demonstrated greatly enhanced capacity of T-scFv-CD28-ζ cells to secrete Tc1 cytokines upon Ag ligation in vitro (23–26); however, our in vitro studies indicated only modest enhancement of proliferation and cytokine secretion by T cells engineered with the scFv-αCEA-CD28-ζ chimera in response to CEA-expressing tumor targets (data not shown).

**Superior efficacy of T-scFv-CD28-ζ cells in rejecting mouse and human colon carcinoma in SCID mice**

Previous studies have not tested the ability of T-scFv-CD28-ζ cells to reject tumor in vivo. Thus, the capacity of the scFv-CD28-ζ, -CD28, and -ζ chimeric receptors to stimulate T cell antitumor function against CEA+CD80+CD86+ tumor targets was evaluated in adoptive transfer assays using SCID mice. Transduced T-scFv-CD28-ζ, -CD28, and -ζ cells (5 x 10⁶) were injected i.v. into SCID mice 6 h (day 0) and 24 h (day 1) after s.c. inoculation of

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**FIGURE 1.** Expression of the chimeric scFv-anti-CEA-CD28-ζ, -CD28, and -ζ receptors in mouse T lymphocytes. A, Schematic representation of the scFv-anti-CEA-CD28-ζ, -CD28, and -ζ receptors. Each construct was composed of the VH and VL regions of the anti-CEA mAb joined by a flexible linker, a membrane-proximal hinge region of human CD8a and the transmembrane (TM) and cytoplasmic regions of the human TCR-ζ chain, mouse CD8 signaling chain, or CD28 fused to the intracellular domain of ζ. A c-myc tag epitope was incorporated into the C terminus of the VL region for expression analysis. Expression of the scFv-anti-CEA-CD28-ζ (B), -CD28 (C), and -ζ (D) chimeric receptors or pLXSN retrovirus (E) in primary mouse T lymphocytes was analyzed by flow cytometry. Cells were stained with an anti-tag mAb (solid line) or an IgG1 isotype control mAb (dashed line). Ag-specific cytotocicity of scFv-ζ-, -CD28-, and -CD28-ζ-transduced T cells was evaluated in 6-h ⁵¹Cr release assays. Enriched splenic B6 T cells transduced with the scFv-CD28-ζ (open circles) or scFv-ζ (filled circles) chimeras equivalently lysed the CEA+ tumors, COLO 205 (F) and MC-38-CEA2 (H), but not the CEA− tumors, 24JK (G) and MC-38 (I). T cells transduced with the CEA-CD28 chimeric receptor (open triangles) or pLXSN retrovirus alone (open squares) were unable to lyse the CEA− tumor cells. The spontaneous lysis was <10% in all assays. Results are expressed as percent specific ⁵¹Cr release ± SE of triplicate samples and are representative of at least two experiments.

**FIGURE 2.** Enhanced efficacy of scFv-anti-CEA-CD28-ζ-transduced T cells in rejecting colon carcinomas in SCID mice. A, Growth of the CEA+ human COLO 205 colon carcinoma cells (5 x 10⁶) (right flank) and CEA− 24JK mouse sarcoma cells (10⁶) (left flank) (B) or 3 x 10⁶ CEA+ mouse MC-38-CEA2 (right flank) (C) and CEA− MC-38 (left flank) (D) adenocarcinoma cells injected s.c. into groups of 10 SCID mice. Mice were injected i.v. with two doses of 5 x 10⁶ BALB/c T cells transduced with the pLXSN vector alone (open squares), scFv-CD28 chimera (open triangles), scFv-ζ chimera (filled circles), or scFv-CD28-ζ chimera (open circles) on days 0 and 1 after tumor inoculation. Growth of the CEA+ and CEA− tumors in mice receiving no T cell transfer was also monitored (filled squares). For all experiments, results are represented as the mean tumor size (mm²) ± SEM. Arrows depict the days of T cell transfer, and the number of tumor eradication is shown in parentheses. Tumor growth inhibition of T-scFv-CD28-ζ and T-scFv-ζ cells was statistically compared by Mann-Whitney U test; *, p ≤ 0.05.
CEA+ COLO 205 or MC-38-CEA2 tumor in the right flank and CEA- 24JK or MC-38 tumor in the left flank. As previously demonstrated (15), T cells expressing the scFv-ζ chimera were capable of mediating Ag-specific antitumor responses against the COLO 205 (5 of 10 eradications) and MC-38-CEA2 (3 of 10 eradications) tumor targets (Fig. 2, A and C). However, T-scFv-CD28-ζ cells were shown to mediate a more effective antitumor response, with the complete eradication of 7 of 10 COLO 205 and MC-38-CEA2 tumors (Fig. 2, A and C), and increased ability to control the growth of escaping tumors. However, more striking was the effect of T-scFv-CD28-ζ cells, which stimulated a more effective antitumor response with the complete eradication of 7 of 10 COLO 205 and MC-38-CEA2 tumors (Fig. 2, A and C). The antigenic specificity of both the scFv-ζ and CD28-ζ chimeras was demonstrated by the lack of antitumor activity against the CEA- tumors growing in the opposite flank of these mice (Fig. 2, B and D). Ligation of the scFv-CD28 chimera did not elicit a T cell antitumor response against either CEA-negative or -positive tumors (Fig. 2). Mice with tumors eradicated by T cell treatment were monitored for between 50 and 100 days after tumor inoculation, and all remained tumor free.

Enhanced efficacy of T-scFv-CD28-ζ cells in controlling syngeneic mouse adenocarcinoma

We next compared the specificity and antitumor potency of T-scFv-ζ and T-scFv-CD28-ζ cells against i.p. or s.c. MC-38-CEA2 tumors in syngeneic B6 mice. In the first model, the i.p. transfer of two doses of $5 \times 10^5$ (10^6 total) T-scFv-CD28-ζ cells, 6 h and 24 h after i.p. inoculation of MC-38-CEA2 tumor, resulted in 80% survival (8 of 10 mice; Fig. 3). In contrast, i.p. transfer of $10^5$ T-scFv-ζ cells ($5 \times 10^5$; days 0 and 1) resulted in 40% survival (4 of 10 mice; Fig. 3). Strikingly, T-scFv-CD28-ζ cells were significantly more potent than T-scFv-ζ cells in controlling the growth of MC-38-CEA2 tumors, in that the adoptive transfer of $10^5$ ($5 \times 10^5$; days 0 and 1) T-scFv-CD28-ζ cells resulted in 60% survival of mice (12 of 20 mice), compared with 25% survival for T-scFv-ζ cells (5 of 20; $p \leq 0.05$; Fig. 3). Both T-scFv-CD28-ζ and T-scFv-ζ cells had no effect on the i.p. growth of CEA+ MC-38 tumors (data not shown), and T-scFv-CD28 cells did not elicit an antitumor response against the i.p. growth of MC-38-CEA2 tumors (Fig. 3).

In the second and more stringent model, transduced T cells were adoptively transferred i.v. into B6 mice 6 h (day 0) and 24 h (day 1) after s.c. inoculation of mice with CEA+ MC-38-CEA2 tumor in the right flank and CEA- MC-38 tumor in the left flank. As was observed in the xenogeneic SCID mouse model, T-scFv-ζ cells were capable of significantly inhibiting the growth of CEA+ tumors (5 of 10 eradications; Fig. 4A). Although i.v. transfer of T-scFv-CD28-ζ cells eradicated only 6 of 10 MC-38-CEA2 tumors, these effector T cells were far more effective at controlling

![Figure 3](http://www.jimmunol.org/) Superior antitumor response by scFv-anti-CEA-CD28-ζ-transduced T cells against i.p. syngeneic colon adenocarcinoma. The survival of groups of 10–20 B6 mice inoculated i.p. with $3 \times 10^6$ MC-38-CEA2 tumor cells. Mice were injected i.p. with two doses of $5 \times 10^5$ B6 T cells transduced with the pLXSN vector alone (open squares), scFv-CD28 chimera (open diamonds), scFv-ζ chimera (filled circles), or scFv-CD28-ζ chimera (open circles) or with two doses of $5 \times 10^5$ B6 T cells transduced with the scFv-ζ chimera (filled triangles) or scFv-CD28-ζ chimera (open triangles) on days 0 and 1 after tumor inoculation. Survival of mice receiving no T cell treatment was also monitored (closed squares). Results are represented as the percentage of survival, and arrows depict the days of T cell transfer. Tumor-free mice treated with the same dose of T cells were compared and statistically evaluated by Fisher’s exact test; *, $p \leq 0.05$.

![Figure 4](http://www.jimmunol.org/) Optimal growth inhibition of s.c. syngeneic colon adenocarcinoma by scFv-anti-CEA-CD28-ζ-transduced T cells. Growth of CEA+ mouse colon adenocarcinoma cells, MC-38-CEA2 ($3 \times 10^6$) (right flank) (A) and CEA- MC-38 parental cells ($10^6$) (left flank) (B) injected s.c. in groups of 10 B6 mice. Mice were injected i.v. with two doses of $5 \times 10^5$ B6 T cells transduced with the pLXSN vector alone (open squares), scFv-CD28 chimera (open triangles), scFv-ζ chimera (filled circles), or scFv-CD28-ζ chimera (open circles) on days 0 and 1 after tumor inoculation. Growth of the CEA+ and CEA- tumors in mice receiving no T cell transfer were also monitored (filled squares). C, Growth inhibition of 3-day established MC-38-CEA2 tumor injected s.c. into groups of 10 B6 mice. Mice were injected i.v. with a single dose ($10^6$) of B6 T cells transduced with the pLXSN vector alone (open squares), scFv-CD28 chimera (open triangles), scFv-ζ chimera (open circles), or scFv-CD28-ζ chimera (filled circles) on day 3 after tumor inoculation. Growth of MC-38-CEA2 tumors in mice receiving no T cell transfer was also monitored (filled squares). For all experiments, results are represented as the mean tumor size (mm$^3$) ± SEM. Arrows depict the days of T cell transfer, and the number of tumors eradicated is shown in parentheses. Tumor growth inhibition of T-scFv-CD28-ζ and T-scFv-ζ cells was statistically compared by Mann-Whitney U test; *, $p \leq 0.01$. 
the growth of tumors that had escaped rejection, similar to what was observed in the xenogenic SCID model (Fig. 4A). The antigenic specificity of both the scFv-ζ and scFv-CD28-ζ chimeras was demonstrated by the lack of antitumor activity against CEA-tumors growing in the opposite flank of these mice (Fig. 4B). Given the effectiveness of early treatment of mouse colon adenocarcinoma with T-scFv-CD28-ζ cells, we next compared the antitumor efficacy of a single dose (10^5) of T cells against 3-day established CEA+ MC-38-CEA tumors (mean size, ~10 mm^3). Although no complete tumor eradications were observed, the T-scFvCD28-ζ cells mediated statistically greater growth inhibition of MC-38-CEA tumors compared with T-scFv-ζ cells, which had minimal antitumor effect on these rapidly growing tumors (p ≤ 0.01; Fig. 4C). Treatment of mouse colon adenocarcinoma with T-scFvCD28-ζ cells had no effect on the s.c. growth of CEA-negative and -positive MC-38 tumors.

Perforin and IFN-γ are critical for the antitumor activity of T-scFv-CD28-ζ cells

We next wanted to determine the underlying mechanism(s) influencing the potent antitumor activity of T-scFv-CD28-ζ cells in vivo. T cells from B6 wild-type (WT), IFN-γ−/−, pfp−/−, and pfp−/−IFN-γ−/− mice were transduced with the scFv-ζ or scFvCD28-ζ chimeric receptors and injected i.v. into B6 mice 6 h and 24 h after their s.c. inoculation with MC-38-CEA2 tumor. Individual MC-38-CEA2 tumors grew rapidly in all untreated mice (Fig. 5A). As demonstrated previously, WT T-scFv-ζ cells were somewhat effective and eradicated 2 of 10 tumors (Fig. 5B); however, strikingly WT T-scFvCD28-ζ cells were far more effective, eradicating 8 of 10 MC-38-CEA2 tumors. Perforin was particularly important for effective tumor rejection, because pfp−/− T-scFv-ζ and T-scFv-CD28-ζ cells did not eradicate any tumors (Fig. 5, C and G). Interestingly, IFN-γ−/− T-scFvCD28-ζ cells were significantly less effective (2 of 10 tumors eradicated) than WT T-scFvCD28-ζ cells (8 of 10 eradications) in inhibiting the growth of the CEA+ MC-38-CEA2 tumors (p ≤ 0.005), suggesting that enhanced IFN-γ production was critical for the superior antitumor response mediated by T-scFv-CD28-ζ cells in vivo (Fig. 5, D and H). Together, pfp and IFN-γ accounted for the antitumor activity of both transduced T cell effector populations, in that MC-38CEA2 tumor growth was unaffected in mice, which received gene-modified T cells from pfp−/−IFN-γ−/− mice (Fig. 5, E and J). Similar results were also obtained with the transfer of scFv receptor-modified BALB/c-WT, IFN-γ−/−, pfp−/−, and IFN-γ−/−pfp−/− T cells into SCID mice bearing human COLO 205 s.c. tumors (data not shown), indicating that both pfp and IFN-γ are important general antitumor effector pathways used by T-scFv-CD28-ζ cells.

Discussion

To address the problem of tumor escape, which includes down-regulation of MHC/peptide molecules and/or costimulatory ligands (1, 17), we have gene-engineered primary T cells with chimeric scFv receptors, which can stimulate both primary and costimulatory signals for optimal T cell activation. In this study, we have significantly advanced the examination of the scFv receptor approach by demonstrating enhanced in vivo function of primary mouse T lymphocytes expressing a scFv-CD28-ζ chimera reactive with the CEA. We have illustrated for the first time the ability of the scFv-CD28-ζ receptor to synergistically harness the costimulatory functions of CD28 and the cytolytic capacity of ζ to evoke a vigorous T cell-mediated antitumor response against both xenogeneic and syngeneic colon adenocarcinomas in vivo. This work has extended previous studies, which have only demonstrated superior function of the scFv-CD28-ζ receptor in vitro (23–26). Furthermore, we have also obtained similar data using T-scFvCD28-ζ cells recognizing the erbB2 tumor-associated Ag (data not shown), demonstrating the potentially broad therapeutic utility of this approach for the treatment of cancer.

An important observation from our studies using gene-targeted mice was that the enhanced antitumor efficacy of the scFv-CD28-ζ chimera in vivo was critically dependent on the Ag-specific, CD28-mediated secretion of IFN-γ by gene-engineered T cells.
Although a role for pfp in the direct lymphocyte-mediated cytology of tumors has been well documented (13, 31), as was the case for T cells expressing either chimeric receptor, the exact role of IFN-γ in T cell-mediated tumor immunity still remains unclear. There are a number of studies suggesting that IFN-γ can mediate its effect directly by enhancing Ag presentation on tumor cells through both MHC class I and II pathways (32, 33), increasing tumor cell susceptibility to apoptosis by up-regulation of Fas expression (33) and/or by inducing cell cycle arrest (34, 35). Interestingly, the comparable efficacy of T-scFv-CD28-ζ cells against both mouse adenocarcinoma, MC-38-CEA2, and human colon carcinoma, COLO 205, in SCID mice suggested that IFN-γ was not having a direct effect on these tumors. In addition to having direct effects, IFN-γ has been shown to have indirect mechanisms of action, including the recruitment and activation of endogenous immune effector cells (36), the regulation of leukocyte-endothelium interactions (36, 37), and/or induction of anti-angiogenesis (38–40). Although we have demonstrated the key need for T cell IFN-γ secretion, the production of other Tc1 cytokines may also contribute to the antitumor response mediated by the T-scFv-CD28-ζ cells. Additional adoptive transfer assays using WT cells and T cells from other gene-targeted mice will be directed at defining the effector molecules and host cells contributing to the improved efficacy of T-scFv-CD28-ζ cells. Furthermore, the ability of scFvCD28-ζ chimera to transduce costimulatory signals that can enhance T cell proliferation in vitro (5), even after sequential antigenic restimulation (26), may further account for the superior antitumor efficacy of T-scFv-CD28-ζ cells in vivo. We have not yet tested the proliferative and survival advantage of T cells expressing this scFv-CD28-ζ chimeric receptor in vivo; however, another study has indicated that mouse T cells retrovirally engineered with TCR genes could survive up to 80 days after transfer into SCID mice (41). Although encouraging, it will now be interesting to evaluate these functional parameters in immunocompetent tumor-bearing mice.

Similar to chimeric receptors containing the TCR-ζ and FcεRI-γ signaling chains, engagement of a chimera containing the intracellular domain of CD28 has also been shown to transduce costimulatory signals equivalent to those mediated upon ligation of endogenous CD28 receptors (42, 43). Although the ability of CD28 to transduce signals distinct from the TCR remains unresolved, CD28 may influence immediate and sustained TCR signaling by the recruitment of phosphatidylinositol-3-kinase and tyrosine kinase Itk and activation of Src family kinases such as Lck, mediated by different motifs within the cytoplasmic tail (44–47). Furthermore, recent studies have also identified specific adapter molecules such as VAV/SLP-76 within the CD28 signaling pathway that may play a role in regulating cytokine gene transcription (48). It will now be interesting to evaluate whether the fusion of the CD28 and ζ signaling molecules in the one chimera can mimic the synergistic signaling activities of endogenous TCR and CD28 or provide alternative signaling pathways. Given the independent potency of T cell signaling subunits and the modular design of chimeric receptors, there is further scope to engineer chimeras with varying signaling potency and function. The incorporation of the Src kinase Lck into chimeras containing ζ or CD28-ζ signaling domains was shown to stimulate enhanced T cell activity compared with a receptor containing only the ζ chain (23). Furthermore, a chimeric receptor containing the cytosolic protein tyrosine kinase Syk was also shown to be capable of stimulating T cells to produce cytokines and lyse target cells expressing an appropriate Ag in vitro (49). Incorporation of kinase activity may prove useful in redirecting the specificity of T cells with impaired downstream TCR signaling, such as those in cancer-bearing hosts (50).

Overall, this study has demonstrated the therapeutic value of providing T cells with primary and costimulatory signals using a single scFv chimeric receptor that recognizes a tumor-associated Ag in vivo. This approach to deliver costimulation to T cells avoids the problems associated with having to coexpress two chimeras in primary T lymphocytes. Unlike bispecific mAbs, which can rapidly dissociate from the T cell surface, the stable surface expression of scFv-CD28-ζ chimeric receptors can serve to increase the strength and time of interaction between T cells and tumor cells, thereby enhancing T cell responsiveness. Importantly, in terms of clinical application, T-scFv-CD28-ζ cells eliminated tumors in the absence of exogenous IL-2 administration and, given that similarly transduced human T cells produce IL-2 (24), this approach may eliminate IL-2 toxicity associated with other adoptive immunotherapies using lymphokine-activated killer cells and tumor-infiltrating lymphocytes (27). In addition, given the non-MHC-restricted reactivity of scFv receptors, this approach would not be affected by tumor loss of MHC/peptide molecules, which could severely compromise approaches redirecting T cell specificity using only TCR genes. Ultimately, these scFv-CD28-ζ chimeric receptors must now be humanized and expressed in human primary T lymphocytes, and their antitumor efficacy must be evaluated in tumor-bearing SCID mice. This optimized chimeric receptor design will have a significant impact on the overall utility of passive T cell-based immunotherapy for the treatment of cancers, which have proven resistant to common treatment regimes.

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References


CLARIFICATION


The authors of the above manuscript wish to acknowledge the similarity in experimental approach and therapeutic strategy underpinning the above paper and a separate study published by the same authors in *Blood* entitled “Single-chain recognition receptors that costimulate potent rejection of established experimental tumors.” *Blood* 2002;100:3155–3163. The authors regret, and wish to apologize to the readership that due to inadvertent error, the papers were not cross-referenced or sent to both sets of reviewers at the time of submission.