Cancer Gene Therapy (2003) 10, 779–790

www.nature.com/cgt

Stable expression of chimeric anti-CD3 receptors on mammalian cells for stimulation of antitumor immunity

Kuang-Wen Liao,[1,a] Bing-Mae Chen,[1,a] Tang-Bi Liu,[1] Shey-Cherng Tzou,[1] Ya-Min Lin,[2] Kai-Feng Lin,[1] Chien-I Su,[1,3] and Steve R Roffler[1]

[1]Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan, Republic of China; [2]Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan, Republic of China; and [3]Department of Life Sciences, National Tsing Hua University, Hsin-Chu 30043, Taiwan, Republic of China.

Expression of CD80 or CD86 costimulatory molecules on tumor cells can produce rejection of immunogenic but not poorly immunogenic tumors. We have previously shown that anti-CD3 single-chain antibodies expressed on the surface of cells can directly activate T cells. We therefore investigated whether anti-CD3 ''receptors'' could enhance CD86-mediated rejection of poorly immunogenic tumors. Expression of anti-CD3 receptors on cells was increased by introduction of membrane-proximal ''spacer'' domains containing glycosylation sites between the single-chain antibody and the transmembrane domain of the chimeric receptors. Removal of glycosylation sites in the spacer reduced surface expression due to increased shedding of chimeric receptors from the cell surface. Induction of T-cell proliferation by anti-CD3 receptors did not correlate with the expression level of chimeric protein, but rather depended on the physical properties of the spacer. Anti-CD3 receptors effectively induced T-cell cytotoxicity, whereas coexpression with CD80 or CD86 was required for generating T-cell proliferation and IL-2 secretion. Although expression of CD86 did not significantly delay the growth of poorly immunogenic B16-F1 tumors, expression of anti-CD3 receptors with CD86 produced complete tumor rejections in 50% of mice and induced significant protection against wild-type B16-F1 tumor cells. Our results show that spacer domains can dramatically influence the surface expression and the biological activity of chimeric antibody receptors. The strong antitumor activity produced by anti-CD3 receptors and CD86 on tumor cells indicates that this strategy may be beneficial for the gene-mediated therapy of poorly immunogenic tumors.
Cancer Gene Therapy (2003) **10,** 779–790. doi:10.1038/sj.cgt.7700637

Keywords: T lymphocytes; single-chain antibodies; CD80; CD86; CD3; immunotherapy

Augmentation of T-cell immunity to induce immunological rejection of tumors is a promising method of cancer treatment.[1] Activation of naive T cells requires both specific binding of T-cell receptors (TCRs) to peptides presented by MHC molecules as well as costimulation by CD80 or CD86.[2,3] These costimulatory molecules interact with CD28 on T cells to induce T-cell proliferation, stimulate IL-2 secretion and prevent T-cell anergy.[4] The importance of costimulatory molecules in antitumor immunity is highlighted by the observation that tumor cells engineered to express CD80 or CD86 are often rejected.[5–7] Many tumors, however, display defects in antigen processing and presentation including loss or downregulation of proteosome subunits, TAP-1, TAP-2, β2-microglobulin or MHC class I heavy chain.[8–12] Tumors that express low amounts of MHC class I molecules or process antigens poorly cannot effectively trigger T cells and transduction with CD80 or CD86 fails to induce tumor rejection.[13,14]

An alternative strategy to activate T cells that is less dependent on the expression of tumor-associated antigens or MHC molecules by tumor cells may extend the utility of immunotherapy to poorly immunogenic tumors. We and others have previously shown that cells modified to express chimeric anti-CD3 receptors can activate naive T cells.[15–17] Expression of anti-CD3 surface receptors should reduce the dependency of T-cell activation on MHC expression, allowing effective therapy by CD80 or CD86 in poorly immunogenic tumors that are normally refractive to modification with costimulatory molecules.

Translation of chimeric receptor therapy to the clinic requires efficient expression of biologically active receptors. We previously showed that the transmembrane (TM) domain employed to anchor chimeric receptors affected the rate of receptor transport to the cell surface[18] and greatly influenced surface expression.[19] These studies indicated that chimeric surface receptors were also shed from the cell surface. Receptor shedding reduces the level of active receptor on the cell surface and soluble receptors

Received March 20, 2003.

Address correspondence and reprint requests to: Dr Steve Roffler, Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan, Republic of China. E-mail: sroff@ibms.sinica.edu.tw

[a]These authors contributed equally to this work.

can act as receptor antagonists.[20] It is therefore important to reduce shedding of chimeric receptors from cells.

In the present study, we addressed four issues concerning the expression and function of single-chain antibody receptors. We first investigated whether antibody expression on cells could be increased by introduction of specific ''spacers'' between the antibody and TM domains of chimeric receptors. As glycosylation has been shown to be important for retention of endogenous receptors on the cells,[21,22] we next examined the hypothesis that spacer glycosylation could reduce shedding of chimeric receptors. Third, we investigated the functional effect of anti-CD3 receptor design on T-cell activation. Finally, we tested the hypothesis that expression of membrane-bound anti-CD3 receptors could extend the utility of CD86-mediated tumor rejection to poorly immunogenic tumors.

Materials and methods

Reagents

Rat anti-HA mAb 3F10 against an epitope of the hemagglutinin (HA) protein and mouse anti-c-myc mAb (9E10) were from Roche Biochemical Co. (Mannheim, Germany). Rat anti-mouse CD90.2-FITC, rat anti-mouse CD4-PE, rat anti-mouse CD8-PE, hamster anti-mouse CD80-biotin and rat anti-mouse CD86-biotin were purchased from PharMingen (San Diego, CA). Goat anti-human IgG Fc-FITC, goat anti-mouse $F(ab')_2$-FITC, goat ant-rat Ig-FITC, goat anti-rat IgG-horse radish peroxidase and streptavidin–FITC were from Organon Teknika (Turnhout, Belgium). Polyclonal rabbit serum against AFP and *E. coli* β-glucuronidase was produced as previously described.[19]

Cell lines and tissue culture

BALB/3T3 cells were obtained from the National Health Research Institutes (Hsin Chu, Taiwan). HT29 human colon carcinoma cells and B16-F1 melanoma cells were from the American Type Culture Collection (Manassas, VA). 145.2C11 hybridoma cells were generously provided by Dr Jeffrey A Bluestone, Ben May Institute for Cancer Research, Illinois. Cells were cultured in Dulbecco's minimal essential medium (Sigma, St Louis, MO) supplemented with 10% bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. All cells were free of mycoplasma as determined by a PCR-based mycoplasma detection kit (American Type Culture Collection).

Plasmid construction

The construction of pAFP-DAF, p2C11-B7 and p2C11-γ1-B7 has been described.[15,19] The *Xba*I DNA fragments of murine CD80 and CD86 (kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Harvard Medical School, Boston) were subcloned into the pcDNA3.1/zeo(+) vector (Invitrogen, Leek, The Netherlands) to obtain pCD80/zeo and pCD86/zeo. A cDNA fragment encompassing the IgG-like C type domain, TM domain and cytoplasmic tail of murine CD80 was PCR-amplified from pCD80/zeo with the primers 5′-aaagtcgacgctgacttctctacccccaacataact-3′ and 5′-aactcgagctaaaggaagacggtctgttcagc-3′. The PCR product was cut with *Sal*I and *Xho*I and inserted into p2C11-B7 in place of the B7 TM domain and cytoplasmic tail to obtain p2C11-e-B7 (Fig 1a). A DNA fragment corresponding to amino acids 35–142 of human biliary glycoprotein 1 (BGP) was PCR-amplified from IMAGE clone 2277741 with the primers 5′-ccaccgtcgaccagctcactactgaatccatg-3′ and 5′-ttggggtcgaccgggtatacatggaactgtcc-3′ and inserted into the unique *Sal*I site in p2C11-B7 to produce p2C11-BGP-B7. The three N-linked glycosylation sites present in the BGP spacer (N104, N111 and N115) were mutated to glutamine with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to produce transgenes lacking one (p2C11-BGP1-B7), two (p2C11-BGP2-B7) or all three (p2C11-BGP3-B7) glycosylation sites. A DNA fragment encompassing amino acids 21–400 of human CD44E was amplified with the primers 5′-tgagcgtcgaccagatcgatttgaatataacc-3′ and 5′-gatgatgtcgacttctggaatttggggtgtcc-3′ by RT-PCR from RNA isolated from HT29 cells. The PCR product was inserted into the unique *Sal*I site in p2C11-B7 to produce p2C11-CD44-B7. The *Sfi*I–*Sal*I DNA fragment encoding the phOx scFv and an myc epitope was cut from pHook-1 (Invitrogen) and inserted into p2C11-γ1-B7 in place of the 2C11 scFv to obtain phOx-γ1-B7. Likewise, the *Sfi*I–*Sal*I fragment encoding AFP was cut from pAFP-DAF and inserted into p2C11-BCP-B7 and p2C11-BGP3-B7 to generate pAFP-BGP-B7 and pAFP-BGP3-B7, respectively. All transgenes were completely sequenced.

Cell transfection

For transient transfection, 2.5×10^5 BALB/3T3 cells were cultured overnight in six-well plates before transfection with 3 μg plasmid and 10 μl lipofectamine according to the manufacturer's instructions (Gibco Laboratories, Grand Island, NY). BALB/3T3 cells transfected with p2C11-γ1-B7 or phOx-γ1-B7 were also selected in 0.5 mg/ml G418 (Calbiochem, San Diego, CA) and sorted for high expression to produce stable 2C11 and phOx cells. In addition, the scFv transgenes were cotransfected into BALB/3T3 cells with pB7-1/zeo or pB7-2/zeo. The transfected cells were selected in 0.5 mg/ml G418 and 0.5 mg/ml zeocin (Invitrogen) for 2 weeks before they were sorted for high expression to produce the stable transfectants 2C11/CD80, 2C11/CD86, phOx/CD80 and phOx/CD86. B16-F1 cells were transduced and selected (1.2 mg/ml G418, 0.4 mg/ml zeocin) in an analogous fashion.

Immunoblotting of chimeric proteins

BALB/3T3 fibroblasts were harvested 48 hours after transient transfection with anti-CD3 receptor transgenes. The cells were washed with PBS and 4×10^5 cells were boiled in nonreducing SDS-PAGE buffer. Proteins were electrophoresed on an 8% SDS-PAGE and transferred to a PVDF membrane. The blots were blocked with 5%

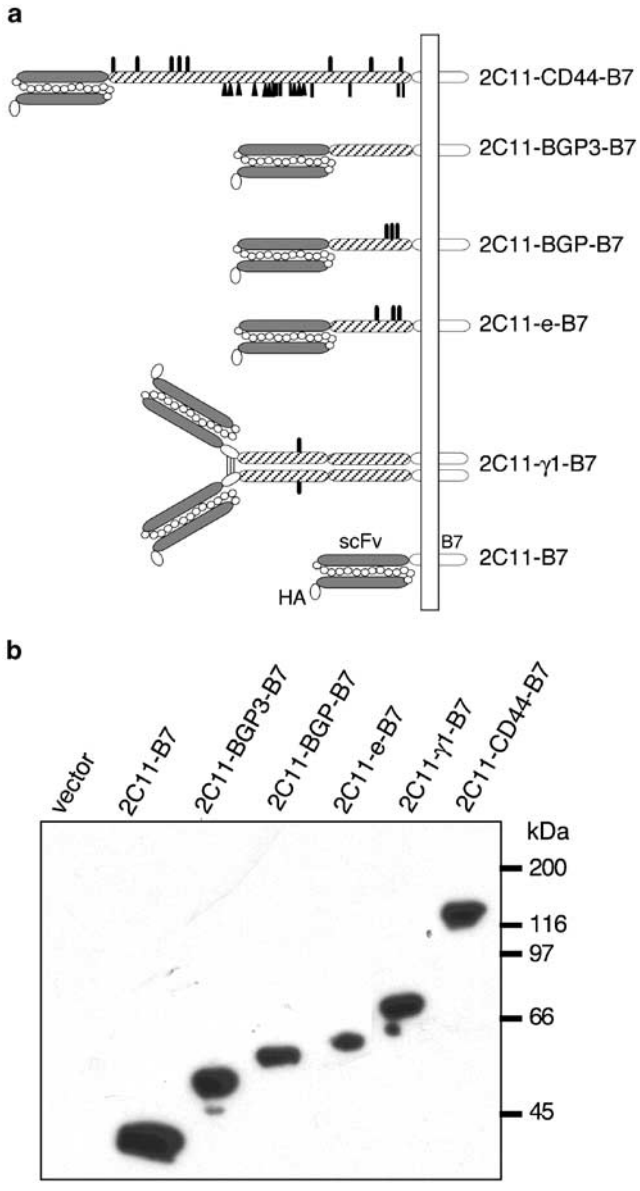

Figure 1 Structure and expression of anti-CD3 chimeric receptors. **(a)** The 2C11 scFv was fused to various extracellular "linker" domains followed by the TM and cytoplasmic domains of the murine B7-1 antigen. An HA epitope is present at the N-terminal of the 2C11 scFv in all chimeras. The chimeric protein 2C11-γ1-B7 can form disulfide-linked dimers, whereas all other proteins are monomers. Potential N-linked glycosylation sites are shown as black ovals and O-linked glycosylation sites are indicated as triangles and bars on 2C11-CD44-B7. The glycosylation sites were removed from 2C11-BGP3-B7. All proteins are drawn to scale and glycosylation sites are indicated in the proper positions. **(b)** Fibroblasts were transfected with empty vector or plasmids expressing anti-CD3 receptors. Transfectant lysates were separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-HA epitope antibody.

skim milk and probed with 0.25 μg/ml rat anti-HA antibody. The blots were extensively washed, incubated with horse radish peroxidase-conjugated goat anti-rat IgG (8 μg/ml), washed and visualized by ECL detection according to the manufacturer's instructions (Pierce, Rockford, IL).

Flow cytometer analysis

A total of 5×10^5 cells were washed and suspended in 50 μl DMEM containing 0.5% bovine serum. Expression of 2C11 and phOx scFv was measured by staining cells with rat anti-HA (5 μg/ml) followed by goat anti-rat IgG-FITC (4 μg/ml) at 4°C. CD80 and CD86 expression was detected with rat anti-mouse CD80-biotin (20 μg/ml) and rat anti-mouse CD86 biotin (20 μg/ml) followed by FITC-conjugated streptavidin (6.5 μg/ml). Chimeric proteins containing the γ1 domain were also stained with goat anti-human IgG Fc-FITC (2.5 μg/ml). The cells were washed and suspended in PBS containing 5 μg/ml propidium iodide before the surface immunofluorescence of 5000–10,000 viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA). Dead cells, identified by red propidium iodide fluorescence, were gated out. Fluorescence intensities were analyzed with Flowjo V3.2 (Tree Star, Inc., San Carlos, CA).

Immunoprecipitation of AFP from culture medium

BALB/3T3 fibroblasts were transiently transfected with pAFP-DAF, pAFP-BGP-B7 or pAFP-BGP3-B7 and the cells were reseeded in six-well plates 16 hours later. The cells were labeled for 11 hours with 300 μCi Pro-mix L-[^{35}S] (Amersham Pharmacia Biotech, Piscataway, NJ) in cysteine- and methionine-free DMEM containing 2% low IgG serum. The cells were then chased with 100 mM methionine for 1.5 hours. The culture medium was cleared twice with 2 μl normal rabbit serum and 30 μl Protein A Sepharose CL-4B gel (Pharmacia Biotech., Uppsala, Sweden) for 1 hour. AFP proteins were immunoprecipitated with 7.5 μg rabbit anti-AFP or control rabbit anti-β-glucuronidase antibody (IgG fractions) for 1 hour followed by 50 μl Protein A Sepharose CL-4B gel overnight. The protein A gel was washed five times with 1 ml wash buffer (20 mM sodium phosphate pH 7.4, 0.15 M NaCl, 0.68 M sucrose, 1% Triton X-100, 5 mM EDTA, 1 mg/ml BSA and protease inhibitor cocktail). The protein A gel was boiled in 30 μl reducing SDS-PAGE buffer before proteins were separated on a 10% SDS-PAGE. The polyacrylamide gel was treated with EN^{3}HANCE (NEN™ Life Science, Boston, MA) following the manufacturer's instructions before drying. Gels were exposed to Kodak X-ray film for 40 hours.

T-cell enrichment

Splenocytes isolated from BALB/c female mice were treated with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) to lyse RBCs and then allowed to attach to a culture plate for 2 hours. Nonadherent cells were transferred to a culture plate that was precoated with 100 μg of goat anti-mouse IgM, IgG, IgA antibody for 45 minutes at 37°C. Nonadherent cells

were loaded on a nylon wool column for 2 hours at 37°C. T cells were eluted from the column with DMEM.

T-cell adhesion assay

BALB/3T3 fibroblasts were transiently transfected with 2C11 transgenes. After 48 hours, 5×10^5 cells were allowed to attach in 24-well plates for 4 hours. Then, 5×10^6 nylon wool-enriched lymphocytes were added to each well with or without 50 μg/ml mAb 145.2C11. The cells were centrifuged for 5 minutes at 500 *g* and incubated for 1 hour at room temperature. Nonadherent cells were removed by gently washing the wells five times with DMEM/5% BCS. Adherent cells were fixed and stained with 0.5% methylene blue in 50% methanol and examined under a light microscope.

T-cell proliferation

Transiently or stably transfected BALB/3T3 fibroblasts were suspended in PBS and treated with 100 μg/ml mitomycin C for 2 hours at 37°C. The cells were then washed twice with PBS and once with medium. The transfectants and enriched T cells were incubated at a ratio of 1:10 (unless otherwise indicated) in quadruplicate in round-bottom 96-well plates for the specified times before 1 μCi ^{3}H-thymidine was added to the wells for 16 hours. The cells were harvested and the radioactivity was measured on a TopCount Microplate Scintillation Counter (Packard, Meriden, CT). Results are expressed as

$$\begin{aligned}&\text{thymidine incorporation (cpm)}\\&\quad = \text{cpmof test wells}\\&\qquad - \text{cpm of mitomycin treated transfectants}\end{aligned}$$

Purification of CD4$^+$ and CD8$^+$ T cells

A total of 3×10^7 T cells were washed with buffer (5 mM EDTA, 0.5% BSA in PBS) and suspended in 270 μl buffer. A volume of 30 μl of anti-CD4 beads or anti-CD8 beads (Miltenyi Biotec, Bergisch Gladbach, Germany) was added for 15 minutes at 10°C. The cells were washed and suspended in 0.5 ml of buffer. The cells were applied to a VS column (MACS system, Miltenyi Biotec) for positive selection or a BS column for negative selection. The cells were collected and a portion was double stained with rat anti-mouse CD90.2-FITC and rat anti-mouse CD4-PE or rat anti-mouse CD8-PE. After staining, the cells were analyzed to verify their purity by flow cytometer analysis.

IL-2 secretion

A total of 2.5×10^5 2C11, 2C11/CD80, 2C11/CD86 or phOx fibroblasts were cultured with 2.5×10^6 T cells in 2.5 ml of culture medium. A volume of 250 μl of the culture medium was harvested each day and the IL-2 concentration was measured by ELISA using the mouse IL-2 OptEIA kit according to the manufacturer's instructions (Pharmingen).

Induction of T-lymphocyte cytotoxicity

A total of 2.5×10^5 2C11, 2C11/CD80, 2C11/CD86 or phOx fibroblasts were incubated with 10^7 T cells in DMEM/5% serum for 3 days. In a similar fashion, 10^5 stably transfected B16-F1 cells were incubated with 2×10^6 purified T-lymphocytes in 12-well plates for 3 days. Nonadherent cells were removed and the wells were gently washed with medium. Adherent cells were harvested with trypsin, stained with Trypan Blue and counted under a light microscope. Cytotoxicity was calculated as

$$\begin{aligned}&\text{cytotoxicity}(\%) = 100 - 100\\&\times \frac{\text{number of viable transfectants incubated with lymphocytes}}{\text{number of viable transfectants incubated without lymphocytes}}\end{aligned}$$

In vivo *tumor growth*

C57BL/6 mice were obtained from the National Animal Center (Taipei, Taiwan) and maintained in the Institute of Biomedical Science, Academia Sinica. All animal experiments were carried out with ethical committee approval. The ethical guidelines that were followed met the standards required by the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia.[23] Groups of four to six C57BL/6 mice were s.c. injected with 2×10^6 B16-F1 cells or B16-F1 transfectant cells. Tumor dimensions were measured with calipers and tumor volumes were estimated as $0.5 \times \text{height} \times \text{width} \times \text{length}$. Mice were killed when tumor dimensions exceeded 2 cm^3. Naive mice as well as mice that survived the initial tumor challenge were rechallenged 50 days later with 5×10^4 parental B16-F1 cells. The survival time of tumor-free mice was taken as 50 days (last day of observation) for calculation of mean survival times.

Statistical analysis

Statistical significance of differences between mean values was estimated with Excel (Microsoft, Redmond, WA) using the independent *t*-test for unequal variances. Statistical analysis of animal survival was performed using SPSS for Windows software (version 10.0) (SPSS Inc., Chicago, IL). Survival curves were obtained by the Kaplan–Meier method, and the difference in survival times was analyzed with the log-rank test. *P*-values of less than .05 were considered to be statistically significant.

Results

Linker domains can enhance the expression of chimeric antibody receptors on cells

A panel of transgenes was constructed to examine the hypothesis that the presence of oligosaccharides in "linker" domains placed near the cell membrane could increase the expression of chimeric antibody receptors on cells. The prototypical transgene (p2C11-B7) encodes a chimeric receptor consisting of a murine immunoglobulin

kappa chain signal peptide, an HA epitope, an anti-CD3 single-chain antibody (2C11 scFv) and the TM and cytosolic tail of murine B7-1 (Fig 1a). p2C11-γ1-B7 contains the hinge-CH_2–CH_3 region of human IgG_1 (γ1 domain) between the scFv and the TM. We also inserted the sequence coding the first immunoglobulin-like V-type domain of biliary glycoprotein I (BGP-1) or the Ig-like C2-type and Ig-hinge-like domains of murine B7-1 between the scFv and TM cDNA as models of N-linked glycosylation domains. All three N-linked glycosylation sites were removed from 2C11-BGP3-B7 by site-directed mutagenesis. The effect of O-linked glycosylation was examined by fusing the cDNA of CD44E between the 2C11 scFv and TM. The expression of the anti-CD3 receptors was first examined by immunoblotting cell lysates prepared from transfected fibroblasts. Figure 1b shows that 2C11-B7 migrated with an apparent molecular mass of 36 kDa, in good agreement with the expected size of 37.5 kDa. 2C11-BGP3-B7 migrated at the predicted size of 48 kDa, whereas 2C11-BGP-B7 displayed a mass of 56 kDa due to the presence of oligosaccharide chains in the linker domain. Similarly, 2C11-e-B7 and 2C11-γ1-B7 displayed apparent molecular masses that were 11 and 7 kDa greater than predicted from the protein sequence due to N-linked glycosylation of their linker domains. 2C11-γ1-B7 migrated as a disulfide-linked dimer on a nonreduced gel (results not shown). 2C11-CD44-B7 migrated with an apparent molecular mass of 130 kDa as compared with the expected molecular weight of 83 kDa, confirming that the linker domain was extensively glycosylated.

The expression of anti-CD3 receptors on cells was increased by introduction of linker domains between the scFv and TM (Fig 2a). High surface expression of scFv was dependent on the presence of carbohydrate chains in the linker domain because 2C11-BGP3-B7 had low

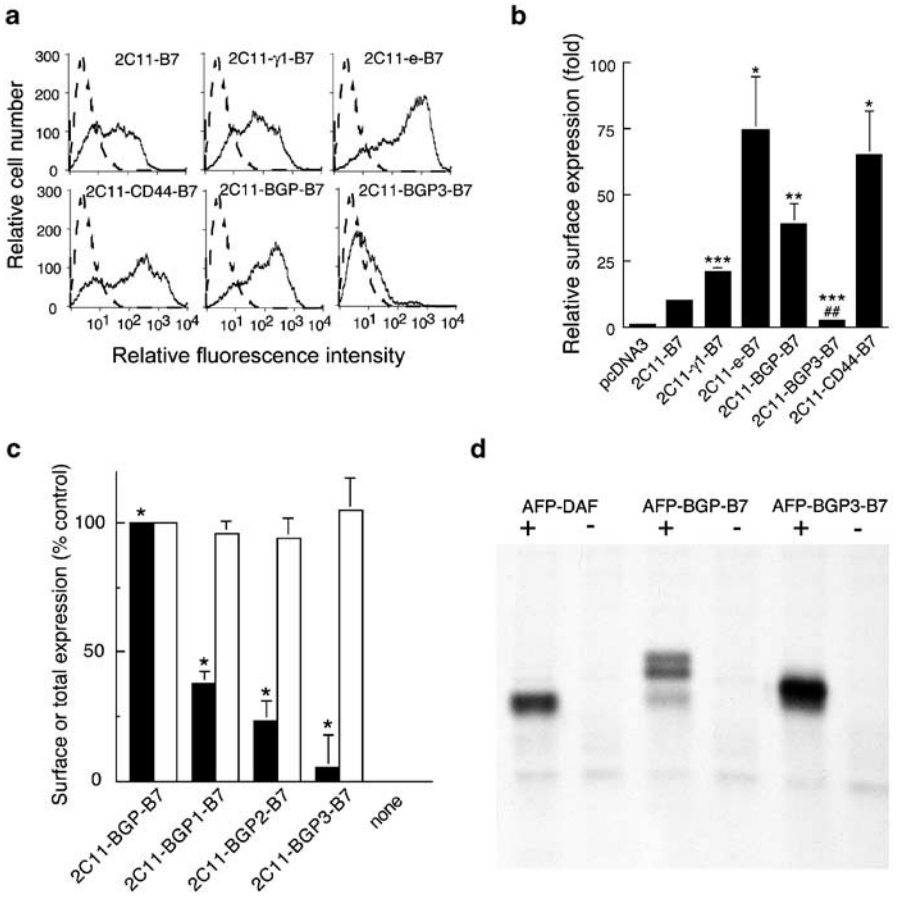

Figure 2 Linker domains increase the surface expression of chimeric receptors. **(a)** Immunofluorescence of transfected fibroblasts showing surface expression of anti-CD3 receptors (solid lines) relative to untransfected cells (dashed lines). **(b)** The mean fluorescent intensities ($n = 7$–8) of anti-CD3 receptor expression on cells are shown relative to cells transfected with vector alone (intensity = 1). Bars, SE. The expression of anti-CD3 receptors containing linker domains was greater or lower than 2C11-B7: $^{*}P \leq .05$; $^{**}P \leq .005$; $^{***}P \leq .0005$. The surface expression of 2C11-BGP3-B7 was lower than 2C11-BGP-B7: $^{\#\#}P \leq .005$. **(c)** The mean surface fluorescence intensities (solid bars) and total protein levels (open bars) of 2C11-BGP-B7 chimeras after removal of one, two or all three glycosylation sites are shown relative to wild-type 2C11-BGP-B7 ($n = 4$). Bars, SD. Significant differences between the surface expression of 2C11-BGP(n)-B7 *versus* 2C11-BGP(n-1)-B7 are shown: $^{*}P \leq .05$. **(d)** Transfected fibroblasts were labeled with ^{35}S-methionine and the culture supernatants were immunoprecipitated with anti-AFP antibody (+) or a negative control antibody (−), separated by SDS-PAGE and analyzed by autoradiography.

surface expression as compared with 2C11-BGP-B7. The mean results of between 3 and 11 such independent experiments are shown in Figure 2b. All the proteins were more highly expressed on cells as compared with 2C11-B7, except for 2C11-BGP3-B7, which displayed lower surface levels than 2C11-B7. The effect of N-linked glycosylation on surface expression was further examined by generating 2C11-BGP-B7 chimeras in which only one (2C11-BGP1-B7) or two (2C11-BGP2-B7) glycosylation sites were removed. Figure 2c shows that surface expression monotonically decreased as glycosylation sites were sequentially removed from the BGP linker. Reduced surface expression was not due to poor production of mutants because the overall levels of chimeric proteins in the cells were similar as determined by quantification of cell transfectant immunoblots (Fig 2c). We attempted to immunoprecipitate 2C11 scFv from the culture supernatant of transfected cells to examine whether glycosylation of the linker domain reduced shedding of scFv but failed, possibly due to rapid degradation of shed scFv. We therefore examined the effect of linker glycosylation on the shedding of a stable reporter protein. AFP could be specifically immunoprecipitated from the culture medium of cells transfected with plasmids directing the expression of AFP to the cell surface (Fig 2d). Expression of AFP as a GPI-anchored protein (AFP-DAF) resulted in detectable shedding of AFP as previously reported.[19] Low amounts of AFP were also shed into the culture medium when the BGP linker was employed (AFP-BGP-B7), but removal of the three N-linked glycosylation sites in the linker (AFP-BGP3-B7) resulted in greatly increased shedding of AFP, demonstrating that linker glycosylation reduced cleavage from the cell surface.

Linker domains influence T-cell activation by chimeric anti-CD3 receptors

The functional activity of the chimeric receptors was examined by determining whether anti-CD3 scFv could bind to CD3 on T cells. Figure 3a shows that T cells readily bound to 2C11-γ1-B7 and 2C11-CD44-B7 cells. Binding was specific because control scFv (phOx-γ1-B7) did not bind T cells. Addition of soluble 2C11 antibody blocked T-cell binding to the transfectants, demonstrating that the chimeric receptors bound CD3 on the T cells. Cells that displayed 2C11-e-B7 and 2C11-BGP-B7 bound T cells to a similar extent as 2C11-γ1-B7 whereas 2C11-B7 did not bind T cells, possibly due to steric hindrance of the receptor by surface proteins on the transfectant cells (results not shown).

The chimeric receptors were tested for their ability to induce T-cell proliferation. Figure 3b shows that 2C11-γ1-B7 induced the greatest proliferation of T cells. Even though 2C11-e-B7 and 2C11-BGP-B7 were expressed at higher levels on cells, they induced less splenocyte proliferation than did 2C11-γ1-B7. As expected from the T-cell binding results, 2C11-B7 did not induce significant proliferation of T cells. Surprisingly, 2C11-CD44-B7 did not induce T-cell proliferation even though it was highly expressed on cells and retained antigen-binding activity.

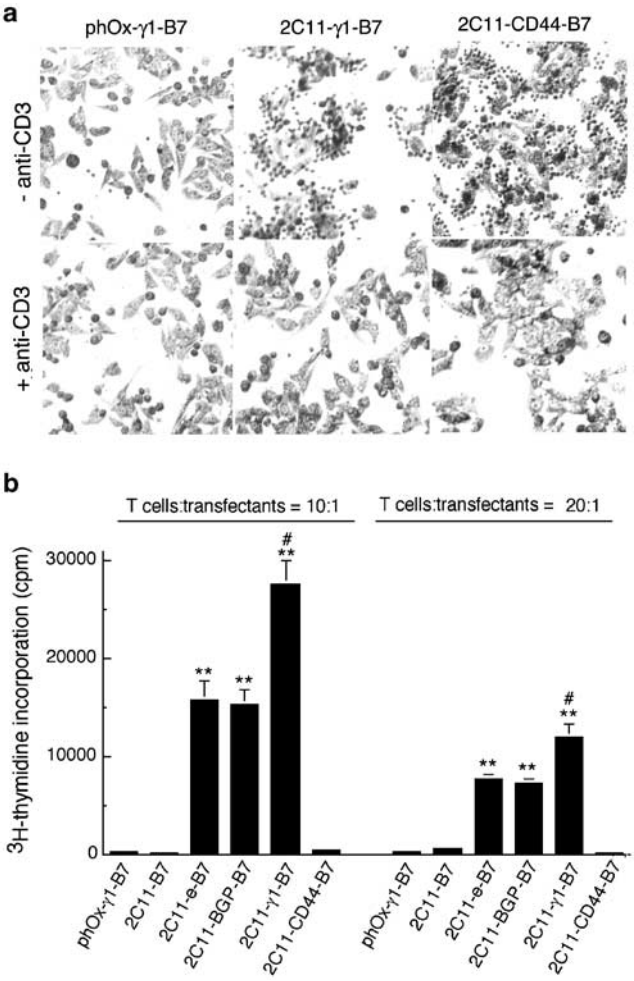

Figure 3 Functional activity of anti-CD3 receptors. **(a)** T cells were allowed to bind to transfected fibroblasts with (+ anti-CD3) or without (−anti-CD3) addition of soluble anti-CD3 antibody. **(b)** Transiently transfected fibroblasts (5×10^4) were incubated with naive T cells (T cell: transfectant ratio of 10:1 or 20:1) for 24 hours before T-cell proliferation was measured ($n=4$). Bars, SE. Several chimeric receptors produced greater proliferation than control phOx receptors: $^{**}P \leq .005$. 2C11-γ1-B7 produced greater T-cell proliferation than 2C11-e-B7 or 2C11-BGP-B7: $^{\#}P \leq .05$.

Providing CD80 costimulation in *trans* did not enhance 2C11-CD44-B7 stimulation of T cells above background levels even though CD80 enhanced T-cell proliferation induced by 2C11-γ1-B7, 2C11-e-B7 and 2C11-BGP-B7 by more than 10-fold (results not shown). Subsequent studies employed 2C11-γ1-B7 since it produced the greatest proliferation of T cells.

CD80 and CD86 enhance anti-CD3 receptor stimulation of lymphocyte proliferation

Stable BALB/3T3 transfectants were generated to investigate the influence of CD80 and CD86 on T-cell activation by anti-CD3 receptors. The cells were sorted to produce lines with similar levels of anti-CD3 (2C11) or control phOx receptors (results not shown). Cell lines

were also generated that coexpressed anti-CD3 or phOx receptors with CD80 or CD86. The ability of transfectants to stimulate the proliferation of purified T cells was examined by mixing naive T cells with mitomycin-killed transfectants. 2C11 cells induced significant ($P \leq .05$) but low levels of T-cell proliferation (Fig 4a). 2C11/CD80 and 2C11/CD86 cells, in contrast, induced robust proliferation of T cells that progressively increased for at least 4 days. As expected, phOx cells did not induce splenocyte proliferation regardless of whether CD80 or CD86 costimulation was provided. The density of BALB/3T3 transfectants required to induce T-cell proliferation was examined by adding nylon-wool-enriched T cells to decreasing numbers of transfectant cells. At least 16,000 2C11 cells were required to induce significant ($P \leq .05$) proliferation of T cells, whereas significant ($P \leq .005$) T-cell proliferation was induced by as few as 1000 2C11/CD80 or 2C11/CD86 cells (results not shown). In agreement with these results, the level of T-cell proliferation induced by 2C11-γ1-B7 in Figure 3b was greater than that observed in Figure 4a due to the lower cell density used in the latter experiment (8000 *versus* 30,000 transfectants).

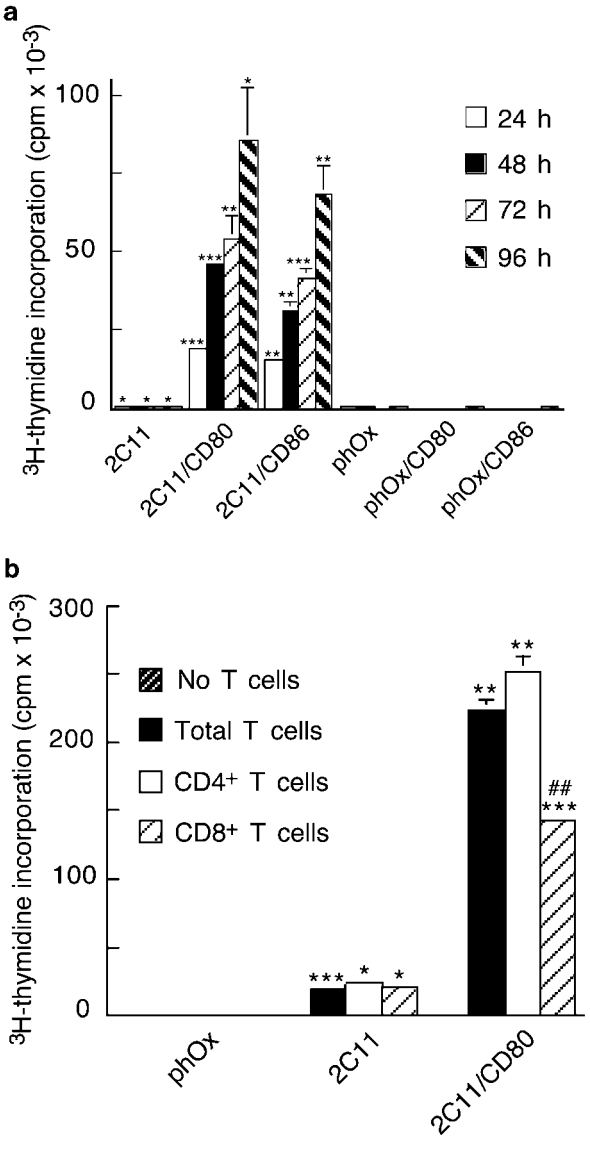

Figure 4 CD80 and CD86 promote anti-CD3 receptor stimulatory activity. **(a)** Transiently transfected fibroblasts (8×10^3) expressing anti-CD3 receptors produced greater proliferation of T cells (8×10^4) at each time point as compared to cell lines expressing the corresponding phOx receptors ($n = 4$): $^*P \leq .05$; $^{**}P \leq .005$; $^{***}P \leq .0005$. Bars, SE. **(b)** Transiently transfected fibroblasts (3×10^4) expressing anti-CD3 receptors with or without CD80 produced greater proliferation of total, $CD4^+$ or $CD8^+$ T cells (3×10^5) at 72 hours as compared to fibroblasts expressing phOx receptors ($n = 3$): $^*P \leq .05$; $^{**}P \leq .005$; $^{***}P \leq .0005$. $CD8^+$ T cells proliferated significantly less than $CD4^+$ T cells: $^{\#\#}P \leq .005$.

Stimulation of purified T-cell populations (purity > 98%) with cells expressing anti-CD3 receptors for 72 hours produced significantly greater proliferation of both $CD4^+$ and $CD8^+$ T cells as compared to stimulation with control phOx cells (Fig 4b). Stimulation with anti-CD3 and CD80 enhanced the proliferation of both $CD4^+$ and $CD8^+$ T cells, but the proliferation of $CD4^+$ T cells was enhanced to a greater degree ($P \leq .005$) than $CD8^+$ T cells. Similar results were found when specific T-cell populations were depleted. Stimulation by anti-CD3 receptors with CD80 or CD86 enhanced the proliferation of T cells depleted of either $CD4^+$ or $CD8^+$ T cells (results not shown). Depletion of $CD4^+$ T cells resulted in significantly reduced proliferation of the remaining T cells as compared to T cells depleted of $CD8^+$ T cells, indicating that $CD4^+$ T cells were more responsive to anti-CD3 receptors and CD80 or CD86 costimulation (results not shown).

Functional activation of T cells by anti-CD3 receptors and CD80 or CD86.

Culture of T cells with 2C11/CD80 or 2C11/CD86 cells induced the secretion of high levels of IL-2 (20–45 ng/ml) (Fig 5a). Stimulation of T cells by anti-CD3 receptors and CD86 produced significantly ($P \leq .05$) higher IL-2 concentrations than did anti-CD3 receptors and CD80. Anti-CD3 receptors alone produced about 1000 times less IL-2 than with costimulation. As expected, no IL-2 could be detected in the medium of lymphocytes cultured with phOx cells.

Figure 5b shows that over 95% of 2C11/CD80 and 2C11/CD86 cells and 58% of 2C11 cells were killed 72 hours after addition of naive T cells. Induction of lymphocyte cytotoxicity required expression of anti-CD3 receptors on cells because phOx cells were not killed. Expression of CD80 or CD86 on fibroblasts (phOx/CD80 and phOx/CD86) did not induce lymphocyte cytotoxicity.

In vivo *antitumor activity of anti-CD3 receptors and CD86*

The antitumor activity of anti-CD3 receptors was examined in a poorly immunogenic tumor model. B16-F1 melanoma cell lines were generated that stably

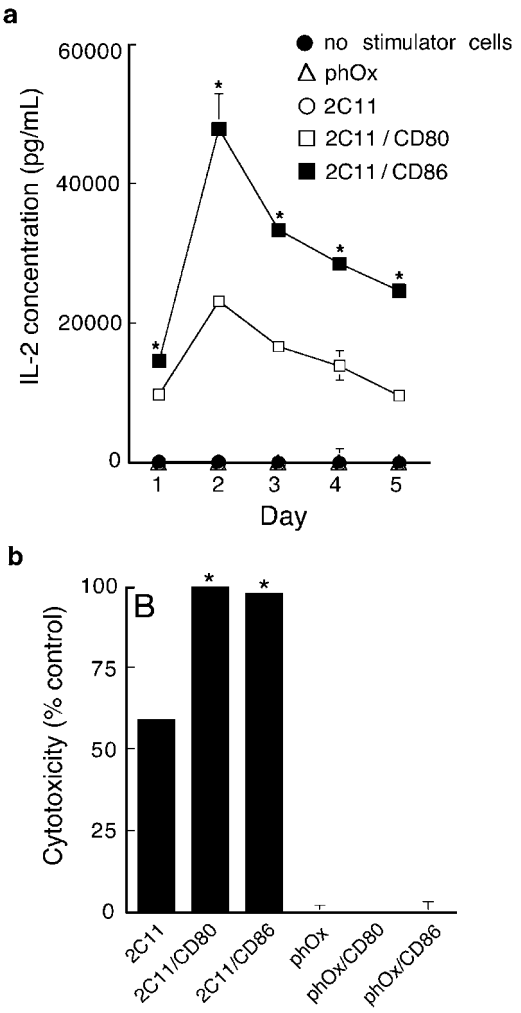

Figure 5 Anti-CD3 receptors require costimulation to induce IL-2 secretion but not cytotoxicity. **(a)** T cells (2.5×10^6) cultured with fibroblasts (2.5×10^5) expressing anti-CD3 receptors with CD80 or CD86 secreted high levels of IL-2 into the culture medium ($n=3$). 2C11/CD86 cells stimulated more IL-2 production than did 2C11/CD80 cells: $^*P \leq .05$. Bar, SE. **(b)** Naive T cells (10^7) killed cells (2.5×10^5) expressing anti-CD3 receptors but not phOx receptors ($n=3$). 2C11/CD86 and 2C11/CD80 cells were killed more completely than 2C11 cells: $^*P \leq .05$. Bars, SE.

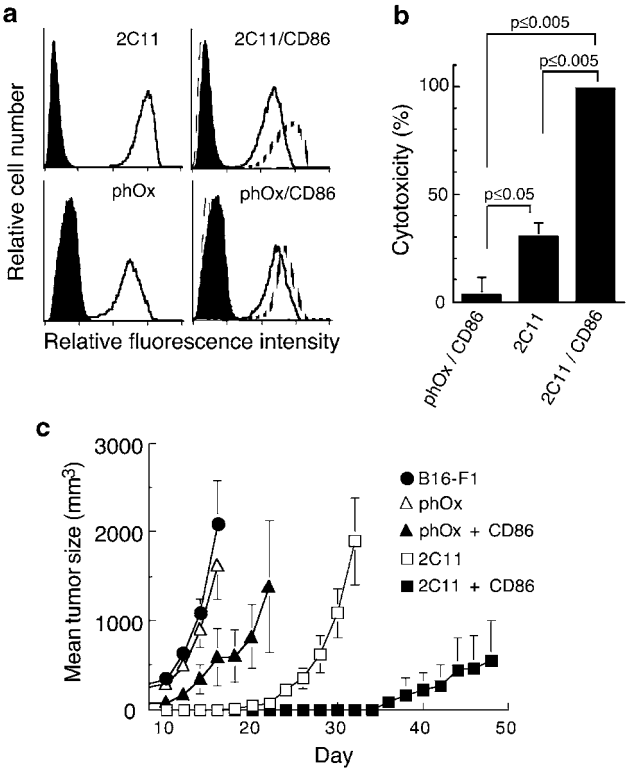

Figure 6 Anti-CD3 receptors suppress melanoma growth. **(a)** B16-F1 cells that expressed 2C11-γ1-B7 (2C11) or phOx-γ1-B7 (phOx) alone or with CD86 (2C11/CD86 and phOx/CD86) were stained for scFv (bold line), CD86 (striped lines) or negative control antibodies (filled curve for scFv and dashed line for CD86). **(b)** Naïve lymphocytes (2×10^6) and B16-F1 cells (10^5) were cultured together for 3 days ($n=3$). Significantly ($P \leq .005$) more B16-F1 cells expressing 2C11/CD86 were killed as compared to B16-F1 cells that expressed anti-CD3 receptors alone or phOx scFv and CD86. **(c)** Groups of four mice were s.c. injected with parental or transfectant B16-F1 cells. Mean tumor sizes are shown. Bars, SE.

expressed anti-CD3 or phOx receptors alone or with CD86 (Fig 6a). The ability of the tumor cell lines to induce CTL activity was examined by mixing naive T-lymphocytes and B16-F1 transfectants. Figure 6b shows that B16-F1 cells that expressed anti-CD3 receptors were significantly ($P \leq .05$) killed (30% killing) as compared to B16-F1 tumor cells that expressed phOx scFv and CD86 (3% killing). B16-F1 cells that expressed anti-CD3 and CD86 induced a significantly ($P \leq .005$) stronger CTL response (99% killing) as compared to anti-CD3 alone. Anti-CD3 receptors also induced tumor killing *in vivo*. Tumors that expressed phOx receptors grew as rapidly as parental B16-F1 cells in syngeneic mice (Fig 6c), demonstrating that the γ1 domain present in the chimeric scFv did not provide antitumor activity. Expression of CD86 with phOx receptors produced only modest delay of tumor growth, confirming that B16-F1 cells are poorly immunogenic. The formation of tumors was significantly suppressed ($P \leq .005$) by expression of anti-CD3 receptors as compared with parental B16/F1 tumors (Fig 6c). For example, the mean size of B16-F1 tumors was 2000 mm^3 on day 16 whereas 2C11 tumors were still unpalatable. Expression of both CD86 and anti-CD3 further suppressed tumor growth ($P \leq .005$) as compared to B16-F1 cells that expressed anti-CD3 alone with complete tumor rejections observed in two of four mice.

The survival of tumor-bearing mice was examined in a separate experiment. Mice injected s.c. with B16-F1 tumor cells had a mean survival time of 16.5 days (Fig 7a). Expression of control phOx receptors or phOx receptors with CD86 on B16-F1 cells modestly but significantly increased mean survival to 24 days. Expression of anti-CD3 receptors significantly ($P \leq .0005$ as

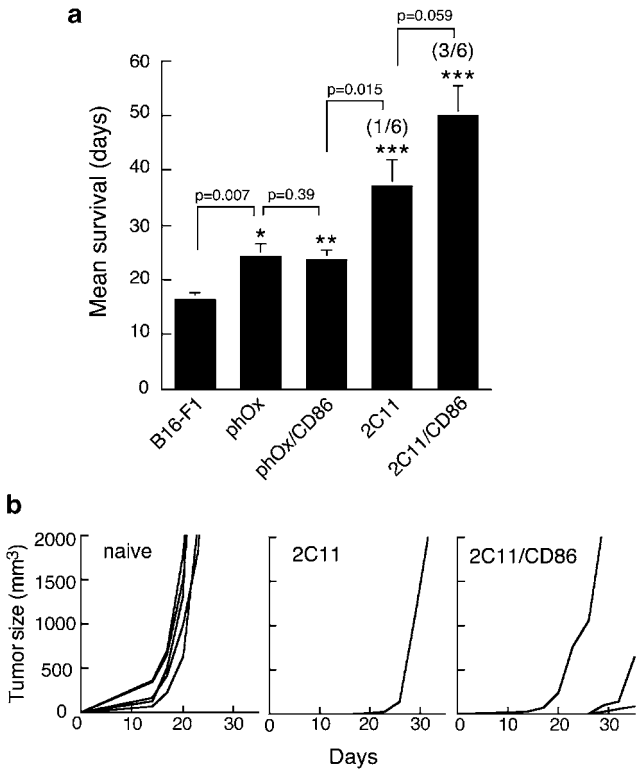

Figure 7 Anti-CD3 receptors expressed with CD86 increase animal survival. **(a)** Groups of six mice were s.c. injected with parental or transfectant B16-F1 cells. The mean survival and number of tumor-free mice (tumor-free/total) in each group are shown. Bars, SE. Significant increases in mean survival times as compared to B16-F1 mice are shown: $*P \leq .05$; $**P \leq .005$; $***P \leq .0005$. Significance (*P*-values) of the differences in the mean survival times between experimental groups is also indicated. **(b)** Naive mice and mice that rejected 2C11 or 2C11/CD86 transfectants were s.c. injected with parental B16-F1 cells. Results show the size of tumors in individual mice.

compared to B16/F1) increased survival time to 37 days with one tumor regression. Combination of anti-CD3 receptors and CD86 resulted in tumor rejection in three of six mice with a mean survival time of more than 50 days. 2C11/CD86 survival was significantly ($P \leq .0005$) longer than control B16-F1 mice but did not reach statistical significance ($P = .059$) as compared to the 2C11 group. The long-term survivors were challenged with parental B16-F1 tumor cells to determine if these mice possessed antitumor memory. The mouse that rejected 2C11 cells displayed delayed tumor formation, whereas the mice that rejected 2C11/CD86 cells exhibited markedly delayed growth of B16/F1 tumors as compared to naive mice, indicating that antitumor immunity was produced in these mice (Fig 7b).

Discussion

Expression of chimeric proteins on specific target cells may be useful for the therapy of diverse diseases. Surface expression may decrease systemic side effects and more efficiently elicit the biological activities of proteins such as enzymes, single-chain antibodies (scFv) and cytokines. Several chimeric surface proteins have recently been described including chimeric scFv receptors for T-cell activation[15,24–26] or deactivation,[27] membrane-bound cytokines,[28,29] prodrug-activating enzymes[30] and artificial Fc receptors.[31,32] Expression of engineered proteins on the surface of cells therefore represents a rich source for the development of novel therapeutics.

Realization of the full potential of chimeric surface receptors requires that high expression of biologically active proteins can be achieved. This is especially important for gene therapy since *in vivo* transduction efficiencies are typically low. We previously showed that the murine B7-1 TM and intact cytoplasmic tail allowed high expression of AFP[19] and scFv[15] due to rapid transport and stable retention of the proteins on the cell surface.[18] In the present investigation we tested the hypothesis that introduction of a spacer domain containing oligosaccharides at the juxtamembrane position of chimeric receptors could further increase surface expression by reducing shedding. The rationale for this hypothesis was based on the observation that many endogenous receptors contain serine- and threonine-rich domains near the cell membrane that are potential sites of O-linked glycosylation.[33] Furthermore, enzymatic deglycosylation of receptors[22] or pharmacological prevention of O-linked glycosylation[21] has been shown to result in rapid proteolysis and release of surface receptors. Our results clearly demonstrate that introduction of linker domains at the juxtamembrane position of chimeric receptors increased their accumulation on the cell membrane. In fact, the linker domains enhanced surface expression of scFv by four- to seven-fold as compared to 2C11-B7. It should be noted that 2C11-B7 already represented an improved surface receptor since it allowed about 5–10 times greater amounts of scFv to accumulate on cells as compared to the prototype scFv receptor (2C11-PDGFR).[15,18] The presence of oligosaccharide chains in the linker domain was mandatory for increased surface expression because removal of glycosylation acceptor sites (2C11-BGP3-B7) reduced surface expression to levels even below those observed without introduction of a linker (Fig 2b). Both O-linked (2C11-CD44-B7) and N-linked (2C11-BGP-B7, 2C11-γ1-B7 and 2C11-e-B7) glycosylation effectively reduced shedding. It seems likely that the introduction of oligosaccharide chains into the chimeric receptors hindered access to proteases since many integral membrane proteins are shed by proteolytic cleavage.[34–36]

Our results clearly demonstrate that the level of surface expression correlated with the degree of linker glycosylation (Fig 2c). Control of surface shedding by variation of glycosylation may be useful for tuning the retention of chimeric proteins on cells. Glycosylation sites can be added to chimeric receptors when it is beneficial to maximize surface expression and prevent release of soluble receptors. This could be especially important

when the cleaved receptor acts as an antagonist to the chimeric surface receptor. For example, expression of MIC on tumor cells can enhance tumor cell sensitivity to cytotoxic T-lymphocytes and NK cells, whereas soluble MIC prevents tumor cell recognition by effector cells.[20] Similarly, anti-CD3 antibodies linked to microbeads cause vigorous activation and expansion of T cells *in vivo*,[37] but soluble $F(ab')_2$ fragments of antibodies against both murine and human CD3 produce T-cell depletion[38] and immunosuppression.[39] In these cases, introduction of additional glycosylation sites to reduce shedding could minimize the antagonistic effects of the cleaved soluble receptors. On the other hand, glycosylation sites could be removed to generate a limited amount of soluble receptor, for example, to create a gradient of chemokine protein to promote chemotaxis of effector cells.

Although high expression of chimeric receptors is important for many applications, it is critical that the receptors retain biological activity. We found that the ability of anti-CD3 receptors to induce T-cell proliferation varied greatly. 2C11-γ1-B7 was about twice as potent as 2C11-BGP-B7 and 2C11-e-B7, whereas 2C11-CD44-B7 was unable to stimulate T cells even though all chimeric receptors were able to bind T cells. Receptor activity did not correlate with scFv expression levels on cells, showing that receptor design can dramatically influence its biological activity. The mechanism responsible for the lack of T-cell activation by 2C11-CD44-B7 is still unclear. Many models have been proposed for TCR activation.[40,41]Based on these models, several differences in the 2C11 scFv receptors could account for their divergent biological activities. First, formation of 2C11-γ1-B7 dimers may promote aggregation of TCRs. Second, the extensive glycosylation of the CD44E spacer in 2C11-CD44-B7 could hinder TCR clustering or prevent sufficient TCR density for efficient signaling. Third, the long CD44E spacer could prevent segregation of inhibitory receptors such as CD43 from the immune synapse based on the kinetic segregation model. We are currently investigating the mechanism responsible for the lack of T-cell activation by 2C11-CD44-B7.

Immunotherapy holds great promise for the prevention and treatment of cancer. Many tumors, however, display defects in antigen processing and presentation[42] and are poorly immunogenic even after transduction with cytokines or costimulatory molecules.[13,14] To overcome these limitations, we constructed chimeric antibody receptors to activate T cells at the local tumor environment directly. We found that expression of membrane-bound anti-CD3 receptors with CD80 or CD86 induced the proliferation of both $CD4^+$ and $CD8^+$ T cells, reduced the threshold for T-cell activation, stimulated prolific IL-2 production and generated T-cell cytotoxicity. More importantly, although expression of CD86 on poorly immunogenic tumor cells did not produce effective antitumor activity, expression of anti-CD3 antibody receptors with CD86 resulted in tumor rejections and produced a long-term protective antitumor memory response against parental tumors.

Signaling via the CD3 complex alone produced strong cytolytic activity *in vitro* (Figures 5b and 6b). Expression of both anti-CD3 receptors and CD86, however, was required for maximal cytolytic activity against B16-F1 tumor cells *in vitro* (Fig 6b) and for antitumor activity *in vivo* (Fig 6c). This result is consistent with recent studies showing that *in vivo* costimulation is required for effective tumor eradication by CTL.[43,44] Costimulation in our system may also be important for $CD4^+$ T-cell activation[45] and secretion of IL-2 (Fig 5a). IL-2 secreted by tumor-infiltrating $CD4^+$ lymphocytes can potentiate antitumor immunity[46] and generate inflammatory responses that increase tumor vascularization to enhance infiltration of effector cells into tumors.[47] Activated $CD4^+$ T cells can also prime dendritic cell to drive $CD8^+$ T-cell responses[48,49] as well as directly interact with $CD8^+$ T cells to provide help and promote memory cell differentiation.[50]

Development of long-term protective antitumor memory against parental tumor cells indicates that specific anti-B16 lymphocytes were activated in the tumors. Naive T cells are normally activated in lymphoid organs by dendritic cells that cross-present tumor antigens[51,52] and the clonally expanded tumor-specific lymphocytes then infiltrate tumors.[53,54] B16 melanoma cells express tumor-associated antigens such as tyrosinase-related protein 2 and gp-100 that can activate specific T cells in lymph nodes.[55,56] Infiltration of B16 tumors with antigen-specific T cells is likely ineffective, however, because B16 melanoma cells possess extensive defects in their antigen-processing machinery that results in profound downregulation of MHC class I surface expression.[57] Cells engineered to express anti-CD3 receptors and CD86, in contrast, can directly trigger tumor infiltrating lymphocytes to express their cytolytic activity and apparently promote their differentiation into memory T cells. Although the protective antitumor memory response against parental B16 tumors was incomplete, more potent antitumor memory may develop if anti-CD3 receptors and CD86 are employed in tumors without such profound defects in antigen processing.[57]

In summary, our results show that shedding and expression of chimeric receptors on cells can be controlled by introduction or removal of glycosylation sites. In addition, we found that receptor design profoundly impacted the functional activity of scFv receptors. Functional anti-CD3 receptors expressed on poorly immunogenic tumor cells enhanced the antitumor activity of CD86. These results suggest that coexpression of anti-CD3 receptors and CD86 molecules on tumor cells may be a useful therapeutic strategy especially suited to poorly immunogenic tumors that are normally refractive to immune stimulation. Further therapeutic efficacy may be possible by expressing anti-CD3 receptors with anti-CD28 scFv[25] to prevent downregulation of T-cell function via CTLA-4 ligation or anti-4-1BB scFv[26] to more completely activate T cells.

Abbreviations

2C11, anti-CD3 antibody; γ_1, H–CH_2–CH_3 region of human IgG_1; AFP, alpha fetoprotein; phOx, 4-ethoxy-methylene-2-phenyl-2-oxazolin-5-one; scFv, single-chain antibody; TM, transmembrane domain

Acknowledgments

We appreciate the technical assistance of Wesley Roy Balasubramanian. This study was supported by grants from the National Science Council, Taipei, Taiwan (NSC90-2318-B001-006-M51 and NSC91-3112-P001-026-Y).

References

1. Abken H, Hombach A, Heuser C, et al. Tuning tumor-specific T-cell activation: a matter of costimulation? *Trends Immunol*. 2002;23:240–245.
2. McHugh RS, Ahmed SN, Wang YC, et al. Construction, purification, and functional incorporation on tumor cells of glycolipid-anchored human B7-1 (CD80). *Proc Natl Acad Sci USA*. 1995;92:8059–8063.
3. Freeman GJ, Borriello F, Hodes RJ, et al. Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and interleukin 2 production. *J Exp Med*. 1993;178:2185–2192.
4. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Ann Rev Immunol*. 1996;14:233–258.
5. Hayakawa M, Kawaguchi S, Ishii S, et al. B7-1-transfected tumor vaccine counteracts chemotherapy-induced immunosuppression and prolongs the survival of rats bearing highly metastatic osteosarcoma cells. *Int J Cancer*. 1997;71:1091–1102.
6. Martin BK, Frelinger JG, Ting JP. Combination gene therapy with CD86 and the MHC class II transactivator in the control of lung tumor growth. *J Immunol*. 1999;162:6663–6670.
7. Fujii H, Inobe M, Kimura F, et al. Vaccination of tumor cells transfected with the B7-1 (CD80) gene induces the anti-metastatic effect and tumor immunity in mice. *Int J Cancer*. 1996;66:219–224.
8. Korkolopoulou P, Kaklamanis L, Pezzella F, et al. Loss of antigen-presenting molecules (MHC class I and TAP-1) in lung cancer. *Br J Cancer*. 1996;73:148–153.
9. Luboldt HJ, Kubens BS, Rubben H, et al. Selective loss of human leukocyte antigen class I allele expression in advanced renal cell carcinoma. *Cancer Res*. 1996;56:826–830.
10. Kaklamanis L, Townsend A, Doussis Anagnostopoulou IA, et al. Loss of major histocompatibility complex-encoded transporter associated with antigen presentation (TAP) in colorectal cancer. *Am J Pathol*. 1994;145:505–509.
11. Vitale M, Rezzani R, Rodella L, et al. HLA class I antigen and transporter associated with antigen processing (TAP1 and TAP2) down-regulation in high-grade primary breast carcinoma lesions. *Cancer Res*. 1998;58:737–742.
12. Restifo NP, Marincola FM, Kawakami Y, et al. Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J Natl Cancer Inst*. 1996;88:100–108.
13. Paul DB, Barth RF, Yang W, et al. B7.1 expression by the weakly immunogenic F98 rat glioma does not enhance immunogenicity. *Gene Therapy*. 2000;7:993–999.
14. Chen L, McGowan P, Ashe S, et al. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J Exp Med*. 1994;179:523–532.
15. Liao KW, Lo YC, Roffler SR. Activation of lymphocytes by anti-CD3 single-chain antibody dimers expressed on the plasma membrane of tumor cells. *Gene Therapy*. 2000;7:339–347.
16. Paul S, Regulier E, Poitevin Y, et al. The combination of a chemokine, cytokine and TCR-based T cell stimulus for effective gene therapy of cancer. *Cancer Immunol Immunother*. 2002;51:645–654.
17. Paul S, Regulier E, Rooke R, et al. Tumor gene therapy by MVA-mediated expression of T-cell-stimulating antibodies. *Cancer Gene Ther*. 2002;9:470–477.
18. Liao KW, Chou WC, Lo YC, et al. Design of transgenes for efficient expression of active chimeric proteins on mammalian cells. *Biotechnol Bioeng*. 2001;73:313–323.
19. Chou WC, Liao KW, Lo YC, et al. Expression of chimeric monomer and dimer proteins on the plasma membrane of mammalian cells. *Biotechnol Bioeng*. 1999;65:160–169.
20. Groh V, Wu J, Yee C, et al. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*. 2002;419:734–738.
21. Reddy P, Caras I, Krieger M. Effects of O-linked glycosylation on the cell surface expression and stability of decay-accelerating factor, a glycophospholipid-anchored membrane protein. *J Biol Chem*. 1989;264:17329–17336.
22. Ludwig A, Ehlert JE, Flad HD, et al. Identification of distinct surface-expressed and intracellular CXC-chemokine receptor 2 glycoforms in neutrophils: N-glycosylation is essential for maintenance of receptor surface expression. *J Immunol*. 2000;165:1044–1052.
23. Workman P, Twentyman P, Balkwill F, et al. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). *Br J Cancer*. 1998;77:1–10.
24. de Ines C, Cochlovius B, Schmidt S, et al. Apoptosis of a human melanoma cell line specifically induced by membrane-bound single-chain antibodies. *J Immunol*. 1999;163:3948–3956.
25. Winberg G, Grosmaire LS, Klussman K, et al. Surface expression of CD28 single chain Fv for costimulation by tumor cells. *Immunol Rev*. 1996;153:209–223.
26. Ye Z, Hellstrom I, Hayden-Ledbetter M, et al. Gene therapy for cancer using single-chain Fv fragments specific for 4-1BB. *Nat Med*. 2002;8:343–348.
27. Hwang KW, Sweatt WB, Brown IE, et al. Cutting edge: targeted ligation of CTLA-4 *in vivo* by membrane-bound anti-CTLA-4 antibody prevents rejection of allogeneic cells. *J Immunol*. 2002;169:633–637.
28. Graf MR, Jadus MR, Hiserodt JC, et al. Development of systemic immunity to glioblastoma multiforme using tumor cells genetically engineered to express the membrane-associated isoform of macrophage colony-stimulating factor. *J Immunol*. 1999;163:5544–5551.
29. Nagarajan S, Selvaraj P. Glycolipid-anchored IL-12 expressed on tumor cell surface induces antitumor immune response. *Cancer Res*. 2002;62:2869–2874.

30. Marais R, Spooner RA, Stribbling SM, et al. A cell surface tethered enzyme improves efficiency in gene-directed enzyme prodrug therapy. *Nat Biotechnol*. 1997;15: 1373–1377.
31. Stabila PF, Wong SC, Kaplan FA, et al. Cell surface expression of a human IgG Fc chimera activates macrophages through Fc receptors. *Nat Biotechnol*. 1998;16: 1357–1360.
32. Gruel N, Fridman WH, Teillaud JL. Bypassing tumor-specific and bispecific antibodies: triggering of antitumor immunity by expression of anti-Fc gammaR scFv on cancer cell surface. *Gene Therapy*. 2001;8:1721–1728.
33. Blochberger TC, Sabatine JM, Lee YC, et al. O-linked glycosylation of rat renal gamma-glutamyltranspeptidase adjacent to its membrane anchor domain. *J Biol Chem*. 1989; 264: 20718–20722.
34. Arribas J, Coodly L, Vollmer P, et al. Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. *J Biol Chem*. 1996;271: 11376–11382.
35. Mullberg J, Rauch CT, Wolfson MF, et al. Further evidence for a common mechanism for shedding of cell surface proteins. *FEBS Lett*. 1997;401:235–238.
36. Kajita M, Itoh Y, Chiba T, et al. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J Cell Biol*. 2001;153:893–904.
37. Wedrychowski A, Kim YW, Chang TW. Immune enhancers composed of polyvalent binding sites of anti-CD3 antibodies. *Biotechnology (NY)*. 1993;11:486–489.
38. Hirsch R, Bluestone JA, DeNenno L, et al. Anti-CD3 F(ab′)2 fragments are immunosuppressive *in vivo* without evoking either the strong humoral response or morbidity associated with whole mAb. *Transplantation*. 1990;49: 1117–1123.
39. Vossen AC, Tibbe GJ, Kroos MJ, et al. Fc receptor binding of anti-CD3 monoclonal antibodies is not essential for immunosuppression, but triggers cytokine-related side effects. *Eur J Immunol*. 1995;25:1492–1496.
40. Anton van der Merwe P, Davis SJ, Shaw AS, et al. Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition. *Semin Immunol*. 2000;12:5–21.
41. van der Merwe PA. The TCR triggering puzzle. *Immunity*. 2001;14:665–668.
42. Seliger B, Cabrera T, Garrido F, et al. HLA class I antigen abnormalities and immune escape by malignant cells. *Semin Cancer Biol*. 2002;12:3–13.
43. Ochsenbein AF, Sierro S, Odermatt B, et al. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature*. 2001;411:1058–1064.
44. Brentjens RJ, Latouche JB, Santos E, et al. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. *Nat Med*. 2003;9:279–286.
45. Surman DR, Dudley ME, Overwijk WW, et al. Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. *J Immunol*. 2000;164:562–565.
46. Bowman L, Grossmann M, Rill D, et al. IL-2 adenovector-transduced autologous tumor cells induce antitumor immune responses in patients with neuroblastoma. *Blood*. 1998;92:1941–1949.
47. Schneeberger A, Koszik F, Schmidt W, et al. The tumorigenicity of IL-2 gene-transfected murine M-3D melanoma cells is determined by the magnitude and quality of the host defense reaction: NK cells play a major role. *J Immunol*. 1999;162:6650–6657.
48. Schoenberger SP, Toes RE, van der Voort EI, et al. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature*. 1998;393:480–483.
49. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature*. 1998;393:474–478.
50. Bourgeois C, Rocha B, Tanchot C. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science*. 2002;297:2060–2063.
51. Huang AY, Golumbek P, Ahmadzadeh M, et al. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science*. 1994;264:961–965.
52. Bai XF, Gao JX, Liu J, et al. On the site and mode of antigen presentation for the initiation of clonal expansion of CD8 T cells specific for a natural tumor antigen. *Cancer Res*. 2001;61:6860–6867.
53. thor Straten P, Becker JC, Guldberg P, et al. *In situ* T cells in melanoma. *Cancer Immunol Immunother*. 1999;48:386–395.
54. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*. 2002;298:850–854.
55. Bloom MB, Perry-Lalley D, Robbins PF, et al. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J Exp Med*. 1997;185: 453–459.
56. Perricone MA, Claussen KA, Smith KA, et al. Immunogene therapy for murine melanoma using recombinant adenoviral vectors expressing melanoma-associated antigens. *Mol Ther*. 2000;1:275–284.
57. Seliger B, Wollscheid U, Momburg F, et al. Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. *Cancer Res*. 2001;61:1095–1099.