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CELL-BASED THERAPY ***RESEARCH ARTICLE***

Activation of lymphocytes by anti-CD3 single-chain antibody dimers expressed on the plasma membrane of tumor cells

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Activation of cytotoxic T cells without MHC restriction was attempted by expressing single-chain antibodies (scFv) against CD3 on the surface of tumor cells. A chimeric protein consisting of a scFv of mAb 145.2C11, the hinge-CH_2-CH_3 region of human IgG_1, and the transmembrane and cytosolic domains of murine CD80 formed disulfide-linked dimers on the plasma membrane of cells and specifically bound lymphocytes. Anti-CD3 scFv dimers expressed on the cell surface induced CD25 (IL-2 receptor α-chain) expression and proliferation of splenocytes. CT26 tumor cells engineered to express surface scFv dimers (CT26/2C11) also induced potent lymphocyte cytotoxicity with or without addition of exogenous IL-2. Splenocytes activated by CT26/2C11 cells also killed wild-type CT26 cells, indicating that activated splenocytes could kill bystander tumor cells. Immunization of BALB/c mice with irradiated CT26/2C11 cells did not protect against a lethal challenge of CT26 cells, suggesting that systemic immunity was not induced. However, the growth of CT26 tumors containing 50% CT26/2C11 cells was significantly retarded compared with CT26 tumors, whereas CT26/2C11 tumors did not grow in syngeneic mice. These results suggest that expression of anti-CD3 scFv dimers on tumors may form the basis for a novel therapeutic strategy for tumors that exhibit defects in antigen processing or presentation. Gene Therapy (2000) **7,** 339–347.

Keywords: *CD3; single chain antibody; gene therapy; immunotherapy; transmembrane; chimeric protein*

Introduction

Cytotoxic T cells can recognize and kill tumor cells that express peptides derived from tumor-associated antigens on their surface in association with MHC class I molecules. The identification of a wide range of tumor-associated antigens from melanoma and other tumor cells[1,2] has generated interest in developing strategies that employ activated $CD8^+$ T cells for selective killing of tumor cells. Many tumors, however, display defects in antigen processing and presentation. Stable expression of MHC class I molecules on the cell surface requires proteolytic generation of peptides by the proteosome in the cytosol and subsequent delivery of cytosolic peptides to the endoplasmic reticulum by the peptide transporters TAP1 and TAP2.[3] Loss or down-regulation of proteosome subunits, TAP-1, TAP-2, β2-microglobulin or MHC class I heavy chain have been documented for a wide range of solid tumors including melanoma[4] and prostate,[5] lung,[6] bladder,[7] renal,[8] colorectal,[9] and breast[10] carcinomas. Defective presentation of peptides by MHC class I molecules can allow tumor escape from immune recognition by $CD8^+$ T cells.[11]

Cytotoxic T cells can be targeted to lyse tumor cells in a non-MHC restricted fashion by engineering chimeric antigen receptors on T cells or by employing bispecific antibodies. Chimeric receptors composed of a single-chain antibody (scFv) fused to the zeta chain of the TCR CD3 complex can be expressed in T cells.[12] Infusion of the genetically modified T cells can allow recognition and lysis of tumor cells that express the surface antigen bound by the scFv.[13–15] Bispecific antibodies with specificity for the CD3 molecule on T cells and tumor cell surface antigens can also redirect activated T cells to lyse tumor cells.[16] Application of these approaches relies on the identification of tumor-associated antigens that are preferentially expressed on the surface of tumor cells. Intravenous administration of bispecific antibodies can also induce systemic cytokine release and toxicity.[17,18]

We have developed an alternative strategy to promote T cell activation and lysis of tumor cells that is independent of the expression of antigens or MHC molecules on tumor cells. Transgenes were constructed to produce chimeric proteins composed of a scFv against the CD3 complex on T cells, a dimerization domain consisting of the hinge-CH_2-CH_3 region of human IgG_1, and the transmembrane domain and cytosolic tail of the murine B7–1 antigen to anchor the chimeric protein on the cell surface. We show that active scFv dimers expressed on the surface of cells can upregulate CD25 and induce the proliferation of naive splenocytes. Culture of syngeneic splenocytes with CT26 transfectants produced strong cytotoxicity against both parental and transfected CT26

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cells. In addition, CT26 scFv transfectants were rejected whereas the growth of mixed tumors containing parental and transfected CT26 cells was retarded. The potent activation of naive splenocytes by scFv dimers expressed on tumor cells indicates that this strategy may be useful for the gene-mediated therapy of tumors that display defects in antigen processing and presentation.

Results

Surface expression of scFv-TM chimeric proteins

The cDNA encoding the 2C11 single-chain antibody (2C11scFv) was generated by RT-PCR from 145.2C11 hybridoma cells. mAb 145.2C11 binds to the epsilon chain of the CD3 complex on murine T cells. p2C11-γ1-PDGFR (Figure 1) was constructed by replacing the single-chain antibody cDNA in pHook-1 with the gene encoding 2C11scFv and inserting the γ1 domain sequence (hinge-CH_2-CH_3 region) of human IgG_1 between the scFv and TM cDNA. This plasmid encodes a chimeric protein composed of the murine immunoglobulin κ chain signal peptide, an HA epitope, the anti-CD3 scFv gene, the γ1 domain, a myc epitope and the transmembrane domain (TM) of the PDGFR (platelet-derived growth factor receptor). p2C11-γ1-B7 encodes a similar chimeric protein except for the substitution of the TM and cytoplasmic tail of murine B7–1 for the PDGFR TM. The PDGFR and B7–1 TM are designed to anchor the 2C11 scFv to the cell surface, whereas the γ1 domain should allow the formation of disulfide-linked dimers.

The ability of chimeric 2C11scFv proteins to be expressed on the surface of cells was examined by flow cytometer analysis. Figure 2a shows that transfection of BHK cells with p2C11-PDGFR resulted in only minimal expression of scFv on the cell surface, whereas high levels of 2C11-B7 were detected after transfection with p2C11-B7. Similarly, much higher levels of 2C11-γ1-B7 were detected on the surface of BHK cells compared with 2C11-γ1-PDGFR (Figure 2b). Quantitation of scFv surface expression in three independent experiments revealed that the mean fluorescence of BHK cells expressing 2C11-B7 or 2C11-γ1-B7 chimeric proteins was significantly greater than chimeric proteins employing the PDGFR TM (Figure 2d). High levels of 2C11-γ1-B7 were also expressed on transfected BALB/3T3 cells (Figure 2c). All subsequent experiments therefore employed p2C11-γ1-B7.

Chimeric scFv form dimers

The expression of 2C11-γ1-B7 chimeric protein was examined by immunoblotting whole cell lysates prepared from BHK cells that were transfected with p2C11-γ1-B7. Immunoblotting with anti-HA mAb against the N-terminal epitope of the chimeric protein showed that BHK cells transfected with either pcDNA3 or p2C11-γ1-B7 displayed a common non-specific band. Only BHK cells transfected with p2C11-γ1-B7, however, displayed a specific band corresponding to the predicted molecular weight of 2C11-γ1-B7 monomer (Figure 3A, lane 1). An additional band that migrated more rapidly is likely due to cleavage of the 2C11-γ1-B7 chimeric protein between the γ1 and B7 TM domains based on the estimated size of the protein. Both the 2C11-γ1-B7 chimeric protein and degradation product contained the γ1 domain as shown by binding of goat anti-human IgG Fc-specific antibody (Figure 3B, lane 1), further localizing the cleavage site to between the γ1 domain and B7 TM. The doublet is unlikely to be due to differences in glycosylation because tunicamycin treatment increased the migration of both bands (results not shown). Electrophoreses of the transfected cells on a non-reducing SDS-PAGE followed by immunoblotting with anti-HA mAb showed that the chimeric protein and its degradation product both efficiently formed disulfide-linked dimers based on their reduced migration rates relative to the common non-specific band (Figure 3c, lane 1).

Surface scFv dimers are active

BHK cells that were transfected with p2C11-γ1-B7 (Figure 4A) but not pcDNA3 (Figure 4C) were able to bind nylon-wool enriched lymphocytes, showing that 2C11-γ1-B7 was active. Over 95% of adherent lymphocytes positively stained for Thy-1.2 (results not shown), demonstrating that 2C11-γ1-B7 bound T cells. Binding of T cells to transfected BHK cells was mediated by specific interactions between 2C11-γ1-B7 on BHK cells and CD3

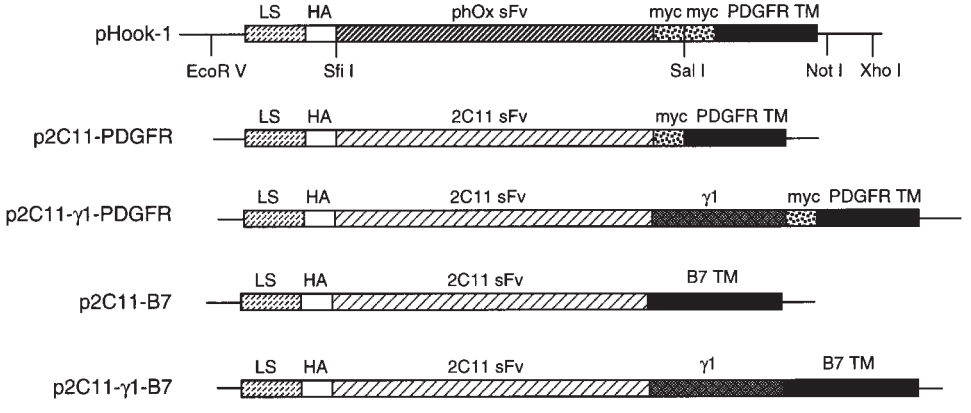

Figure 1 *Diagram of membrane-bound scFv transgenes. The scFv transgenes are based on the pHook-1 vector which codes for a murine immunoglobulin κ chain signal peptide, a 9 amino acid HA epitope, the phOx scFv, two 11 amino acid myc epitopes, and the TM of PDGFR. p2C11-PDGFR contains the 2C11 scFv cDNA in place of the phOx scFv cDNA. The PDGFR TM cDNA was replaced with the murine B7–1 TM and cytosolic tail cDNA in p2C11-B7. The human IgG_1 hinge-CH2-CH3 domain (γ1) cDNA was inserted between the sequences coding for the 2C11 scFv and TM to generate p2C11-γ1-PDGFR and p2C11-γ1-B7. The expression of all the transgenes is under the control of the CMV promoter in pHook-1.*

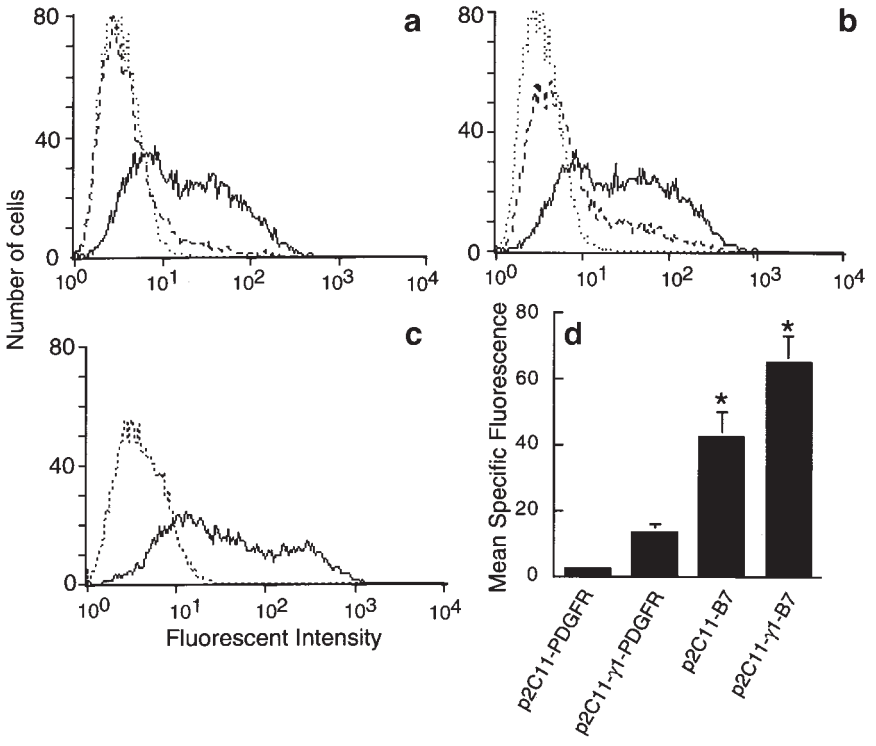

Figure 2 *Surface immunofluorescence of chimeric 2C11 scFv. (a) BHK cells were transfected with pcDNA3 (dotted line), p2C11-PDGFR (dashed line) or p2C11-B7 (solid line). (b) BHK cells were transfected with pcDNA3 (dotted line), p2C11-γ1-PDGFR (dashed line) or p2C11-γ1-B7 (solid line). (c) BALB/3T3 cells were transfected with pcDNA3 (dotted line) or p2C11-γ1-B7 (solid line). After 48 h, cells were stained with mAb 12CA5 against the HA epitope followed by FITC-conjugated goat anti-mouse IgG (Fab')$_2$ before the immunofluorescence of 10^4 viable cells was measured with a FACS caliber flow cytometer. (d) Comparison of mean specific fluorescence of BHK cells transfected with the indicated plasmids. Results represent mean values of three independent experiments. Significant differences between chimeric proteins containing the B7–1 and PDGFR TM domains are indicated: *, $P \leq 0.05$. Bars, s.e.*

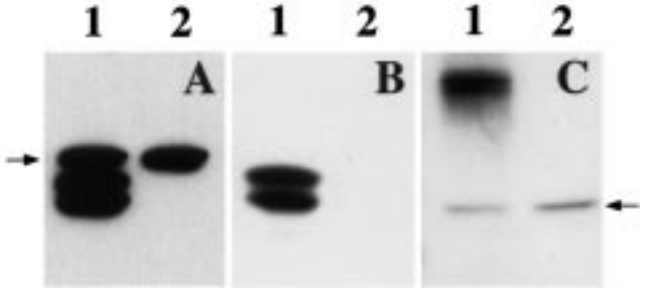

Figure 3 *Immunoblot of the 2C11-γ1-B7 chimeric protein. 5×10^4 BHK cells transfected with p2C11-γ1-B7 (lane 1) or pcDNA3 (lane 2) were lysed and separated on a 8% reduced (A and B) or non-reduced (C) SDS-PAGE. Proteins transferred to nitrocellulose paper were immunoblotted with mAb 12CA5 against the HA epitope (A and C) or HRP-conjugated goat anti-human IgG Fc-specific antibody (B). The position of the cross-reactive protein bound by mAb 12CA5 is indicated by an arrow in A and C.*

on lymphocytes because addition of free mAb 145.2C11 completely blocked binding (Figure 4B). These results show that 2C11-γ1-B7 specifically binds to the CD3 ϵ chain on T cells.

The ability of 2C11-γ1-B7 anchored on the surface of BALB/3T3 cells to up-regulate CD25 (IL-2 receptor α-chain) expression on lymphocytes was examined by flow cytometry. Figure 5 shows that culturing syngeneic splenocytes with BALB/3T3 cells previously transfected with p2C11-γ1-B7 resulted in increased surface expression of the IL-2 receptor compared with naive splenocytes or splenocytes that were cultured with BALB/3T3 cells transfected with pcDNA3.

2C11-γ1-B7 on BALB/3T3 cells also induced the proliferation of splenocytes. BALB/3T3 cells were transfected with p2C11-γ1-B7 or pcDNA3, lethally irradiated, and then cultured with syngeneic splenocytes for 72 h. Figure 6 shows that BALB/3T3 cells transfected with p2C11-γ1-B7 but not pcDNA3 stimulated ^{3}H-thymidine incorporation of splenocytes in a dose-dependent fashion. Significant ($P \leq 0.0005$) incorporation of ^{3}H-thymidine was observed even when only one transfected BALB/3T3 cell was added per 160 splenocytes.

Induction of cytotoxic lymphocytes

CT26 colon carcinoma cells were infected with recombinant retrovirus to produce cell lines that expressed 2C11-γ1-B7 (CT26/2C11) or vector alone (CT26/neo). Flow cytometer analysis of the cells showed that about 41% of the CT26/2C11 cells expressed 2C11scFv dimers on their surface (Figure 7a).

Figure 7b shows that syngeneic splenocytes incubated with CT26/2C11 cells produced significant ($P \leq 0.05$) cytotoxicity against the CT26/2C11 cells compared to splenocytes incubated with CT26/neo cells. IL-2, although not required for cytotoxicity, did significantly ($P \leq 0.05$) enhance killing of CT26/2C11 cells. Activated splenocytes killed over 80% of CT26/2C11 cells even though only about 40% of the cell population expressed 2C11-γ1-B7 on their surface (Figure 7a), suggesting that

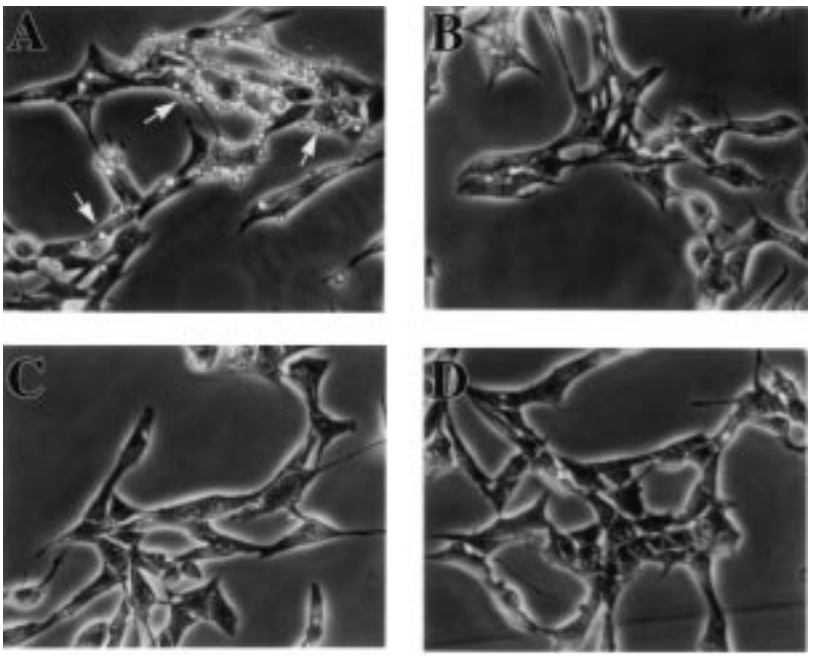

Figure 4 *T cells adhesion assay. BHK cells transfected with p2C11-γ1-B7 (A and B) or pcDNA3 (C and D) were cultured overnight in six-well plates before T cells were added for 3 h at room temperature. 50 μg of mAb 145.2C11 was added to compete for CD3 epsilon on T cells in B and D. Non-adherent cells were removed and the adherent cells were fixed and stained with methylene blue. T cells bound to BHK/2C11 cells (A) are indicated with arrows.*

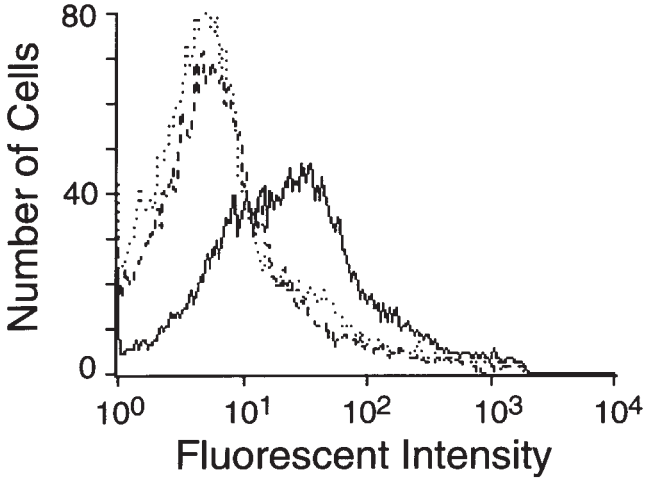

Figure 5 *IL-2 receptor α chain induction. BALB/3T3 cells (10^5) transfected with p2C11-γ1-B7 or pcDNA3 were cultured with 10^6 splenocytes isolated from BALB/c mice for 2 days. Non-adherent cells were collected and analyzed for CD25 expression with rat anti-mouse CD25-FITC conjugate. The surface fluorescence of 10 000 naive splenocytes (dotted lined) or splenocytes cultured with BHK cells transfected with p2C11-γ1-B7 (solid line) or pcDNA3 (dashed line) was measured with a FACS caliber flow cytometer.*

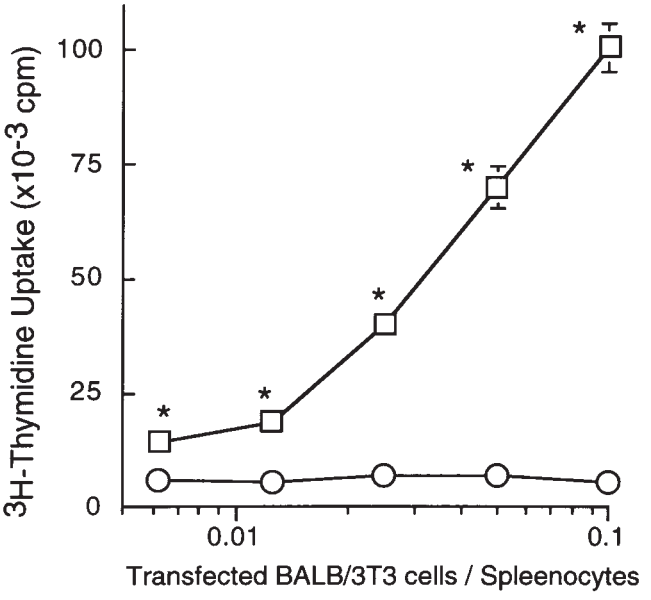

Figure 6 *Splenocyte proliferation. Serial dilutions of lethally irradiated BALB/3T3 cells transfected with pcDNA3 (open circles) or p2C11-γ1-B7 (open squares) were cultured with syngeneic splenocytes for 2 days. Cells were pulsed with 1 μCi ^{3}H-thymidine for 16 h before the nonadherent cells were harvested and radioactivity was measured on a TopCount Microplate Scintillation Counter. Significant differences between ^{3}H-thymidine incorporation of splenocytes cultured with 2C11-γ1-B7 or pcDNA3 transfected cells are indicated (*, $P \leq 0.0005$). Bars, s.e.*

parental CT26 cells were also killed by activated splenocytes. This was directly tested by activating syngeneic splenocytes with CT26/2C11 or CT26/neo cells for 48 h before the splenocytes were transferred to wells containing parental CT26 cells. Splenocytes that were pre-activated with the CT26/2C11 (Figure 8a), but not CT26/neo cells (Figure 8b) exhibited strong cytotoxicity to parental CT26 cells.

In vivo activity

Immunization of BALB/c mice with radiation-killed CT26/2C11 cells 2 weeks before challenge with parental CT26 cells failed to induce protective immunity in

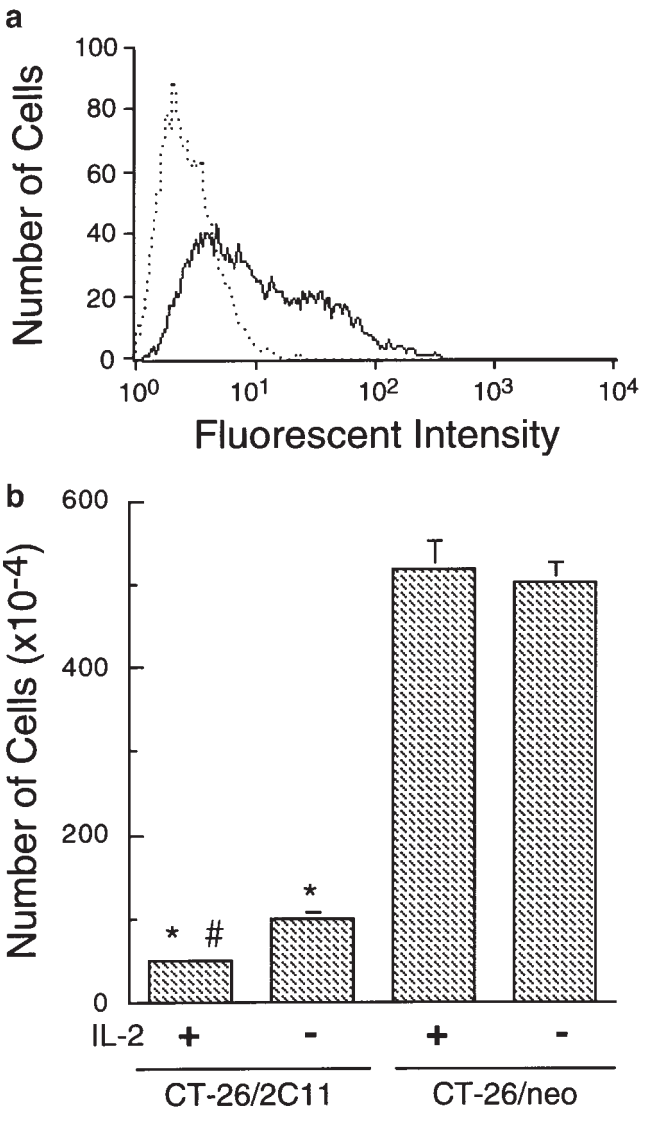

Figure 7 *Induction of splenocyte cytotoxic activity. (a) Surface immunofluorescence of CT26/2C11 cells. CT26/2C11 (solid line) or CT26/neo (dashed line) cells were stained with mAb 12CA5 against the HA epitope and FITC-conjugated goat anti-mouse IgG (Fab')$_2$ before the immunofluorescence of 10^4 viable cells was measured with a FACS caliber flow cytometer. (b) CT26/2C11 or CT26/neo cells (5×10^5) were cultured with or without 200 U/ml human IL-2 and 10^7 naive syngeneic splenocytes for 2 days before adherent cells were harvested and counted under a light microscope. Significant differences between the mean number of CT26/2C11 and CT26/neo cells (*, $P \leqslant 0.005$) or between the mean number of CT26/2C11 cells with or without IL-2 (#, $P \leqslant 0.05$) are indicated. Bars, s.e.*

BALB/c mice (Figure 9a). In contrast, viable CT26/2C11 cells that were enriched for 2C11-γ1-B7 expression by cell sorting were completely rejected in BALB/c mice (Figure 9b). Coinjection of equal numbers of CT26/2C11 and CT26/neo cells resulted in significant suppression of tumor growth compared with CT26/neo tumors in BALB/c mice, demonstrating *in vivo* bystander killing of tumor cells that did not express 2C11-γ1-B7. Flow cytometer analysis of tumor cells recovered from mixed tumors on day 18 failed to detect 2C11-γ1-B7, indicating that CT26/2C11 cells were eliminated from mixed tumors *in vivo* (results not shown).

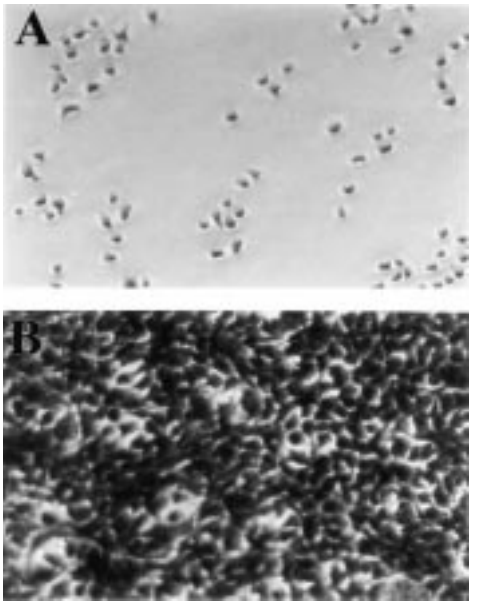

Figure 8 *Cytotoxicity of activated splenocytes to parental CT26 cells. Naive syngeneic splenocytes (5×10^6) were cultured with irradiated CT26/2C11 (A) or CT26/neo (B) cells in 200 U/ml human IL-2 for 2 days. The non-adherent cells (10^7) were collected and incubated with parental CT26 cells (5×10^5 cells) in 20 U/ml human IL-2 for 48 h. Adherent cells were fixed, stained with methylene blue and photographed.*

Discussion

Surface expression of heterologous proteins to elicit specific biological effects is potentially useful for the therapeutic intervention of many diseases. Single-chain antibodies with defined specificity represent a rich source of biologically active molecules for disease treatment. The present study has provided evidence that functional scFv dimers against CD3 can be expressed on the surface of mammalian cells, that surface scFv can activate splenocytes, and that activated splenocytes are cytotoxic to both genetically modified and parental tumor cells.

The PDGFR TM domain present in pHook-1 was originally employed to direct expression of 2C11 scFv monomers to the cell surface. 2C11-PDGFR, however, could not be detected on viable cells by flow cytometry. Chimeric proteins containing alpha fetoprotein and the PDGFR TM were also expressed at low levels on the cell surface,[19] indicating that the PDGFR TM is a poor choice for anchoring proteins to cells. Replacement of the PDGFR TM with the TM and cytosolic tail of murine CD80 (B7–1) dramatically increased surface expression of 2C11 scFv, in agreement with previous studies employing alpha fetoprotein,[19] indicating that this TM is generally applicable for high level expression of heterologous proteins on mammalian cells. Introduction of the γ1 domain further increased surface expression, suggesting that formation of disulfide-linked dimers may enhance scFv stability or transport to the surface.

Immunoblotting of cell extracts revealed that some 2C11-γ1-B7 was likely cleaved between the γ1 and B7–1 TM domains. Glycosylation of membrane receptors is important for their retention on the cell surface.[20,21] Replacement of the highly glycosylated ectodomain of B7–1[22] with the γ1 domain may have allowed proteolytic cleavage of the scFv from the cell surface. Release of sol-

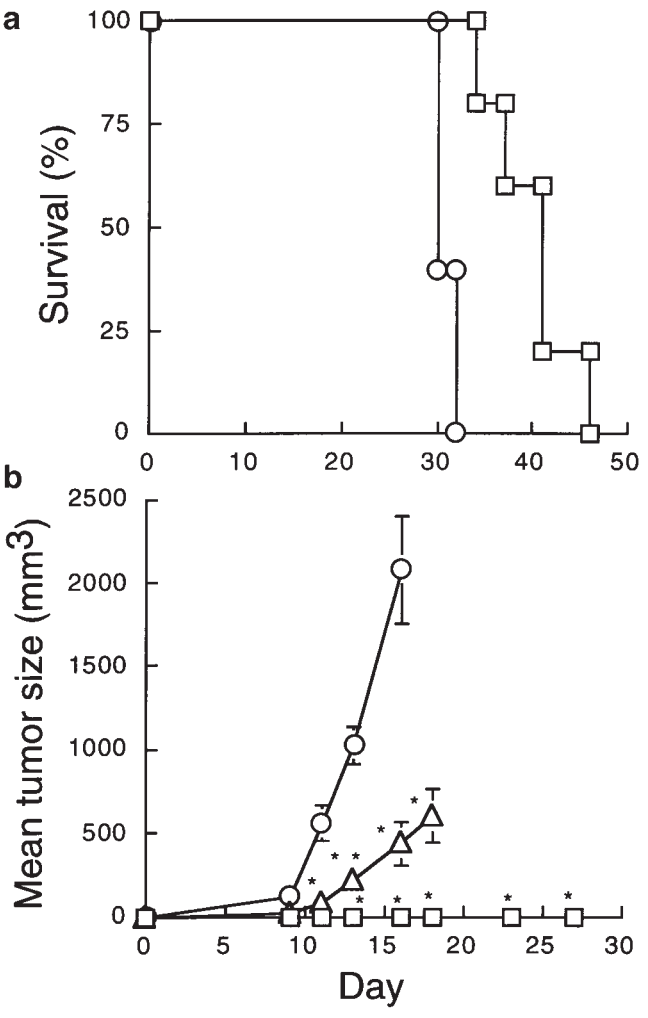

Figure 9 *In vivo activity of 2C11 scFv dimers on tumor cells. (a) Groups of five BALB/c mice were untreated (○) or s.c. injected with 10^6 radiation-killed CT26/2C11 cells (□) 2 weeks before i.v. injection of 5×10^4 viable CT26 cells. (b) Groups of six BALB/c mice were s.c. injected with 5×10^5 viable CT26/neo (○) or CT26/2C11 (□) cells or a mixture of 5×10^5 CT26/neo and 5×10^5 CT26/2C11 cells (△). Significant differences between the mean size of CT26/neo and other tumors are indicated: *, $P \leqslant 0.005$; **, $P \leqslant 0.0005$.*

uble scFv could interfere with the activity of surface scFv by blocking CD3 on T cells. We were unable, however, to detect soluble 2C11 scFv in the culture supernatant of transfected cells (results not shown), arguing that release of soluble scFv was minor. Introduction of additional glycosylation sites into the extracellular spacer or incorporation of the natural B7–1 ectodomain in the 2C11-γ1-B7 chimeric protein may reduce degradation and further improve surface expression.

Several lines of evidence indicate that 2C11scFv dimers on the cell surface can activate resting splenocytes to differentiate into effector cells. First, IL-2 receptor α-chain, a hallmark of lymphocyte activation,[23] increased on splenocytes after culture with cells expressing 2C11-γ1-B7. Second, 2C11-γ1-B7 induced the proliferation of splenocytes in a dose-dependent fashion. Most importantly, CT26/2C11 cells were directly killed by naive splenocytes even without addition of exogenous IL-2. Although activation of splenocytes required the presence of 2C11 scFv dimers on the cell surface, killing of tumor cells was independent of scFv expression because parental CT26 cells were also killed by activated splenocytes. Splenocyte killing of tumor cells was therefore not due to the expression of a foreign protein. The major effector cells are likely T cells because CD3 epsilon-chain expression is limited to T lymphocytes.

T cells typically require two signals for activation, one produced by the interaction of the TCR with antigenic peptide associated with MHC molecules and a second signal generated by the interaction of co-stimulatory molecules such as CD80 or CD86 with CD28 on T cells. Although the scFv domain of 2C11-γ1-B7 can provide the first signal by binding to the CD3 complex on T cells, it is unclear if costimulation was also necessary to activate splenocytes to become effector cells. It is possible that the transfected cells provided costimulatory signals. CD80 (B7–1) and CD86 (B7–2) were not detected on the cell surface of BALB/3T3 or CT26 cells (results not shown), indicating that the transfected cells did not provide co-stimulation through the CD28 receptor.[24] Additional costimulatory molecules such as ICAM-1[25] or CD40L,[26] however, may be present on BALB/3T3 or CT26 cells. Costimulation to T cells may also have been provided by professional APC present in the splenocyte population although costimulation is inefficient when not provided by the same cells that generate signal 1 through the TCR.[27] It is also possible that costimulation may not be necessary for activation of splenocytes by anti-CD3 scFv dimers on the cell surface. Immobilized MHC class I-peptide complexes can activate naive $CD8^+$ T cells to proliferate and secrete IL-2 without APC-derived costimulatory signals.[28] T cells 'count' the number of TCRs engaged by the peptide–MHC complex and become activated when that number reaches about 8000, whereas costimulatory signals lowered the activation threshold to around 1500 TCRs.[29] Although not quantified, the high fluorescent intensity observed in flow cytometry (Figure 2) suggests that the number 2C11 scFv dimers expressed on the cell surface was much greater than the minimal 200–300 MHC–peptide complexes required for T cell activation.[30] Thus, high TCR occupancy and prolonged stimulation by 2C11-γ1-B7 may allow T cell activation without costimulation.

Bispecific antibodies with specificity for CD3 on T cells and tumor-associated antigens on cancer cells have been employed to redirect T cells to tumors, either by coating pre-activated immune effector cells *ex vivo* with the bispecific Ab before infusion into patients[31,32] or by systematic administration of bispecific Ab to patients.[17,18] Activation of T cells by scFv expressed on the surface of tumor cells may differ from bispecific Abs in several important ways. $CD3^+$ cells are immediately coated with antibody after systemic administration of bispecific Abs, causing the Ab-coated lymphocytes to leave the circulation and traffic to lymphoid organs.[17,33] Systemic administration of bispecific antibodies can also induce the acute release of cytokines such as TNF-α, resulting in serious side-effects and toxicity.[17,18] T cell activation by surface scFv, in contrast, is expected to be limited to local tumor-infiltrating lymphocytes (TIL), reducing treatment toxicity. A major limitation of bispecific antibodies is that antibody-coated lymphocytes exhibit only one cytolytic cycle,[34] requiring the administration of excess bispecific antibody or infusion of large numbers of functional T cells with

specific antitumor reactivity into patients for good therapeutic efficacy. Activation of lymphocytes by 2C11-γ1-B7 appears to be efficient as addition of one cell expressing surface 2C11-γ1-B7 to 160 splenocytes induced proliferation of lymphocytes. Finally, in contrast to bispecific antibodies, expression of scFv on tumor cells does not require identification of tumor-associated antigens.

Tumor infiltrating lymphocytes can recognize tumor-associated antigens presented by MHC class I molecules.[35,36] Selective activation of TIL at the tumor site by scFv on tumor cells could allow the development of systemic immunity against disseminated tumors that share common antigens. We were unable, however, to demonstrate the induction of protective immunity after immunization with CT26/2C11 cells. Lack of systemic immunity may indicate that insufficient numbers of tumor-specific T cells are present in the TIL population to generate antitumor immunity or that 2C11-γ1-B7 mediated activation of CTL may be insufficient for generation of memory T cells. Coexpression of scFv and costimulatory molecules such as ICAM-1, CD80, or CD86 on tumor cells may promote more efficient activation of TIL and generation of memory T cells, thereby producing immunity to disseminated tumors.

Tumor cells with defective antigen processing or presentation are refractive to CTL killing because the low levels of MHC-peptide on the cells is inadequate for recognition by CTL. Expression of anti-CD3 scFv on tumor cells may be useful for the treatment of tumors that express low levels of MHC-antigen because activation of T cells by anti-CD3 scFv is not MHC-restricted. The complete rejection of CT26/2C11 tumors demonstrates that potent cytotoxic activity can be generated *in vivo.* Activated lymphocytes also displayed bystander killing of nontransfected tumor cells. For example, the mean size of mixed CT26 and CT26/2C11 tumors was only 76 mm^3 compared with 560 mm^3 for CT26 tumors on day 11. It is likely that the potency of bystander killing may be further enhanced by expressing costimulatory molecules or cytokines (such as IL-2 which significantly enhanced CTL activity after stimulation by CT26/2C11 cells) with anti-CD3 scFv in tumors, allowing tumor regression after transfection of only a subpopulation of the cells.

In summary, our results provide evidence that functional anti-CD3 scFv dimers can be expressed on the surface of tumor cells and that the scFv can activate resting lymphocytes to differentiate into cytotoxic effector cells. These results suggest that expression of anti-CD3 scFv on tumor cells may form the basis of a novel tumor therapy strategy especially suited to tumors with defects in antigen processing or presentation. Expression of scFv with specificity for other molecules[37,38] may be useful to activate a range of effector cells at the tumor site.

Materials and methods

Reagents

mAb S5A8 against 38C13 lymphoma cells[39] was kindly provided by Dr Mi-Hua Tao, Academia Sinica, Taipei, Taiwan. mAb 12CA5 against an epitope of the hemagglutinin protein (HA) of human influenza virus was from Boehringer Mannheim, Mannheim, Germany. Rat anti-mouse CD25-FITC conjugate was purchased from PharMingen, San Diego, CA, USA. Recombinant human IL-2 was from Cetus, San Francisco, CA, USA.

Cell lines and tissue culture

BHK-21 cells were purchased from the American Type Culture Collection, Manassas, VA, USA. BALB/3T3 cells were obtained from the NHRI Cell Bank (Taiwan), Bosc-23 cells[40] were kindly provided by Dr Wen-Chang Lin, Academia Sinica, and CT26 cells[41] were kindly provided by Dr Mi-Hua Tao, Academia Sinica. 145.2C11 hybridoma cells were kindly provided by Dr Jeffrey A Bluestone, Ben May Institute for Cancer Research, Illinois, USA. Cells were cultured in Dulbecco's minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 10% bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2.

Plasmid construction

The variable light (V_L) and heavy (V_H) chain cDNA sequences of mAb 145.2C11 were amplified by RT-PCR from RNA isolated from 145.2C11 hybridoma cells as described.[19] In the first round of PCR, primers P1 (5′-TGC TGG **GGC CCA GCC GGC C**GA CAT CCA GAT GAC CCA GTC TCC ATC-3′) and P2 (5′-ACC GCC GCC CGA AGT ACT GCC CCG TTT GAT TTC CAG CTT GGT GCC AGG-3′) were employed to introduce a *Sfi*I restriction enzyme site at the 5′-end (bold) and half of the linker (underlined) at the 3′ end of the V_L cDNA, respectively. Similarly, primers P3 (5′-AAG TCG AGT GAG GGT AAG GGT GAG GTG CAG CTG GTG GAG TCT GGG GGA-3′) and P4 (5′-TGT TGT **GTC GAC** TGA GGA GAC GGT GAC CAT GGT TCC TTG-3′) were employed to introduce the other half of the linker (underlined) to the 5′ end and a *Sal*I restriction site (bold) at the 3′ end of the amplified V_H cDNA. The V_L and V_H PCR products were assembled in a second round of PCR with primers P1, P4 and a linker primer P5 (5′-GGC AGT ACT TCG GGC GCC GGT AAG TCG AGT GAG GGT AAG GGT-3′) encoding a 14 amino acid residue linker peptide GSTSGAGKSSEGKG.[42] The PCR product was digested with *Sfi*I and *Sal*I restriction enzymes and subcloned into pHook-1 (Invitrogen, Leek, The Netherlands) in place of the phOx scFv cDNA to obtain p2C11-PDGFR. The *Sal*I–*Xho*I fragment encoding a myc epitope and the PDGFR TM in p2C11-PDGFR was replaced with a *Sal*I–*Xho*I fragment from pAFP-B7[19] encoding the murine B7–1 TM and cytosolic tail (Pro237 to the stop codon) to create p2C11-B7. A *Sal*I fragment containing the hinge, CH2 and CH3 domain (γ1) of the human IgG_1 heavy chain from pAFP-γ1-B7[19] was inserted into the unique *Sal*I site in p2C11-PDGFR and p2C11-B7 to produce p2C11-γ1-PDGFR and p2C11-γ1-B7. All transgenes were fully sequenced.

Transfection of transgenes

2.5×10^5 BHK or BALB/3T3 cells per well 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, USA). BHK or BALB/3T3 cells transfected with pcDNA3 or p2C11-γ1-B7 were also selected in 0.5 mg/ml G418 (Calbiochem, San Diego, CA, USA) for 2 weeks to produce the stable transfectant clones BHK/neo, BHK/2C11, BALB/neo and BALB/2C11, respectively.

Flow cytometer analysis

5×10^5 cells were washed and suspended in DMEM containing 0.5% bovine serum and 5 μg/ml mAb 12CA5 or

mAb S5A8 for 60 min at 4°C. Cells were washed and incubated with FITC-conjugated goat anti-mouse IgG $(Fab')_2$ (1:200, Organon Teknika, Durham, NC, USA) for 60 min. Cells were washed and suspended in PBS containing 5 μg/ml propidium iodide before the surface immunofluorescence of 10^4 viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA, USA). Dead cells, identified by red propidium iodide fluorescence, were gated out. Fluorescence intensities were analyzed with Cell Quest Software (Becton Dickinson).

Immunoblotting of chimeric proteins

5×10^4 BHK/neo or BHK/2C11 cells were boiled in SDS-PAGE buffer with or without 2-mercaptoethanol. Proteins were electrophoresed on an 8% SDS-PAGE and transferred to two sheets of nitrocellulose paper (Gelman Sciences, Ann Arbor, MI, USA) in transfer buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.5 mM 2-mercaptoethanol) by capillary diffusion. Blots were blocked with 5% skim milk and incubated with 5 μg/ml mAb 12CA5 or HRP-conjugated goat anti-human Fc-specific antibody (1:1000, Sigma) in 0.1% Tween-20/PBS for 60 min. Blots were extensively washed in the same buffer, incubated with HRP-conjugated goat anti-mouse IgG $(Fab')_2$ (1:2000) for 60 min, washed and developed. Under reducing condition, blots were visualized by ECL detection according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Under non-reducing conditions, blots were visualized with 4-chloro-1-naphthol (Sigma) substrate.

T cell adhesion assay

2×10^5 BHK/neo or BHK/2C11 cells per well were cultured overnight in six-well plates. Splenocytes were isolated from BALB/c mice and cultured in RPMI 1640 medium (Sigma) supplemented with 10% FCS and 20 U/ml human IL-2 overnight. 2×10^7 nylon wool-enriched lymphocytes were added to each well for 3 h at room temperature with or without 50 μg mAb 145.2C11. Non-adherent cells were removed by gently washing the wells five times with medium. Adherent cells were fixed and stained with methylene blue in 50% methanol and examined under a light microscope. Adherent lymphocytes were identified by first staining nylon wool-enriched lymphocytes with FITC-conjugated rat anti-mouse Thy-1.2 mAb (PharMingen, San Diego, CA, USA) before addition to BALB/2C11 cells. Adherent lymphocytes were observed under both fluorescent and light illumination to estimate the fraction of bound T cells.

IL-2 receptor α chain (CD25) induction

BALB/3T3 cells were transfected with p2C11-γ1-B7 or pcDNA3 48 h before 10^5 cells were cultured with 10^6 splenocytes isolated from BALB/c mice in six-well plates for 2 days. Non-adherent cells were collected and incubated with FITC-conjugated rat anti-mouse CD25 (1:500) for 1 h at 4°C. The surface fluorescence of 10 000 viable cells was measured with a FACS caliber flow cytometer.

Splenocyte proliferation

BALB/3T3 cells were transfected with pcDNA3 or p2C11-γ1-B7 48 h before they were lethally irradiated and serially diluted into round-bottom 96-well plates. Splenocytes from BALB/c mice (5×10^5 cells per well in RPMI 1640 supplemented with 10% FBS and 10 mM HEPES) were added to wells for 2 days. 1 μCi ^{3}H-thymidine was added to wells for 16 h, cells were harvested, and the radioactivity was measured on a TopCount Microplate Scintillation Counter (Packard, Meriden, CT, USA).

Generation of CT26 tumor cell line transfectants

The *Eco*RI fragment encoding the 2C11-γ1-B7 transgene was excised from p2C11-γ1-B7 and subcloned into the retroviral vector pBabe-neo (a kind gift from Dr Hartmut Land, Imperial Cancer Research Fund, London, UK) to generate pBabe-2C11-γ1-B7. 10^6 Bosc 23 packaging cells were transfected with 3 μg pBabe-2C11-γ1-B7 or pBabe-neo in 10 μl lipofectamine. After 48 h, 3 ml culture supernatant was incubated with 2×10^5/well CT26 cells in six-well plates for 48 h before adding 0.5 mg/ml G418 for 2 weeks to obtain CT26/neo and CT26/2C11 cells.

Induction of splenocyte cytotoxicity

5×10^5 CT26/2C11 or CT26/neo cells and 10^7 BALB/c splenocytes were cultured with or without 200 U/ml human IL-2 in six-well plates for 2 days. Non-adherent cells were removed and the wells were gently washed with PBS. Adherent cells were harvested with trypsin, stained with Trypan Blue and counted under a light microscope.

Cytotoxicity of activated splenocytes to parental CT26 cells

5×10^5 lethally irradiated CT26/2C11 or CT26/neo cells and 5×10^6 BALB/c splenocytes were cultured with 200 U/ml IL-2 in six-well plates for 2 days. Non-adherent cells (activated splenocytes) were harvested and counted. Parental CT26 cells were plated in six-well plates at 5×10^5 cells per well with 20 U/ml human IL-2 and 10^7 activated splenocytes. After 48 h, the non-adherent cells were removed by washing with PBS. Adherent cells were fixed and stained with 0.5% methylene blue in 50% methanol and examined under a light microscope.

In vivo tumor challenge

Groups of five female BALB/c were untreated or s.c injected with 10^6 lethally irradiated CT26/2C11 cells 2 weeks before mice were i.v. injected with 5×10^4 viable CT26 cells. Mice survival was monitored for 50 days.

In vivo tumor growth

Groups of six female BALB/c mice were s.c. injected with 5×10^5 viable CT26/neo or CT26/2C11 cells or a mixture of 5×10^5 CT26/neo and 5×10^5 CT26/2C11 cells. Tumor dimensions were measured with calipers and tumor volume was estimated as $0.5 \times \text{height} \times \text{width} \times \text{length}$. Mice were killed when tumors reached 2000 mm^3. Some tumors were sterially removed, dissociated with 0.5 mg/ml collagenase, cultured in G418-containing medium for 3 days and then analyzed for 2C11-γ1-B7 expression by flow cytometry with FITC-conjugated goat anti-human IgG Fc-specific antibody as described above.

Statistical analysis

Statistical significance of differences between mean values was estimated with the shareware program Schoolstat (White Ant Occasional Publishing, West Melbourne, Australia) using the independent *t* test for unequal variances.

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References

1 Urban JL, Schreiber H. Tumor antigens. *Annu Rev Immunol* 1992; **10**: 617–644.
2 Wang RF. Tumor antigens discovery: perspectives for cancer therapy. *Mol Med* 1997; **3**: 716–731.
3 Goldberg AL, Rock KL. Proteolysis, proteasomes and antigen presentation. *Nature* 1992; **357**: 375–379.
4 Maeurer MJ *et al.* Tumor escape from immune recognition: loss of HLA-A2 melanoma cell surface expression is associated with a complex rearrangement of the short arm of chromosome 6. *Clin Cancer Res* 1996; **2**: 641–652.
5 Blades RA *et al.* Loss of HLA class I expression in prostate cancer: implications for immunotherapy. *Urology* 1995; **46**: 681–686.
6 Korkolopoulou P *et al.* Loss of antigen-presenting molecules (MHC class I and TAP-1) in lung cancer. *Br J Cancer* 1996; **73**: 148–153.
7 Nouri AM, Hussain RF, Oliver RT. The frequency of major histocompatibility complex antigen abnormalities in urological tumours and their correction by gene transfection or cytokine stimulation. *Cancer Gene Ther* 1994; **1**: 119–123.
8 Luboldt HJ, Kubens BS, Rubben H, Grosse Wilde H. Selective loss of human leukocyte antigen class I allele expression in advanced renal cell carcinoma. *Cancer Res* 1996; **56**: 826–830.
9 Kaklamanis L *et al.* Loss of major histocompatibility complex-encoded transporter associated with antigen presentation (TAP) in colorectal cancer. *Am J Pathol* 1994; **145**: 505–509.
10 Vitale M *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.
11 Restifo NP *et al.* Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J Natl Cancer Inst* 1996; **88**: 100–108.
12 Moritz D, Wels W, Mattern J, Groner B. Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc Natl Acad Sci USA* 1994; **91**: 4318–4322.
13 Altenschmidt U, Moritz D, Groner B. Specific cytotoxic T lymphocytes in gene therapy. *J Mol Med* 1997; **75**: 259–266.
14 Altenschmidt U, Klundt E, Groner B. Adoptive transfer of *in vitro*-targeted, activated T lymphocytes results in total tumor regression. *J Immunol* 1997; **159**: 5509–5515.
15 Alvarez Vallina L, Agha Mohammadi S, Hawkins RE, Russell SJ. Pharmacological control of antigen responsiveness in genetically modified T lymphocytes. *J Immunol* 1997; **159**: 5889–5895.
16 Bolhuis RL, Sturm E, Braakman E. T cell targeting in cancer therapy. *Cancer Immunol Immunother* 1991; **34**: 1–8.
17 Tibben JG *et al.* Pharmacokinetics, biodistribution and biological effects of intravenously administered bispecific monoclonal antibody OC/TR F(ab')2 in ovarian carcinoma patients. *Int J Cancer* 1996; **66**: 477–483.
18 Weiner LM, Clark JI, Ring DB, Alpaugh RK. Clinical development of 2B1, a bispecific murine monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. *J Hematother* 1995; **4**: 453–456.
19 Chou WC *et al.* Expression of chimeric monomer and dimer proteins on the plasma membrane of mammalian cells. *Biotechnol Bioeng* 1999; **65**: 160–169.
20 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.
21 Kozarsky K, Kingsley D, Krieger M. Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. *Proc Natl Acad Sci USA* 1988; **85**: 4335–4339.
22 Freeman GJ *et al.* Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J Exp Med* 1991; **174**: 625–631.
23 Minami Y, Kono T, Miyazaki T, Taniguchi T. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol* 1993; **11**: 245–268.
24 Shahinian A *et al.* Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 1993; **261**: 609–612.
25 Van Seventer GA, Shimizu Y, Horgan KJ, Shaw S. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *J Immunol* 1990; **144**: 4579–4586.
26 von Leoprechting A *et al.* Stimulation of CD40 on immunogenic human malignant melanomas augments their cytotoxic T lymphocyte-mediated lysis and induces apoptosis. *Cancer Res* 1999; **59**: 1287–1294.
27 Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 1999; **283**: 680–682.
28 Goldstein JS *et al.* Purified MHC class I and peptide complexes activate naive $CD8^+$ T cells independently of the CD28/B7 and LFA-1/ICAM-1 costimulatory interactions. *J Immunol* 1998; **160**: 3180–3187.
29 Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 1996; **273**: 104–106.
30 Harding CV, Unanue ER. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T cell stimulation. *Nature* 1990; **346**: 574–576.
31 Kroesen BJ *et al.* Approaches to lung cancer treatment using the CD3 × EGP-2-directed bispecific monoclonal antibody BIS-1. *Cancer Immunol Immunother* 1997; **45**: 203–206.
32 Bolhuis RL *et al.* Adoptive immunotherapy of ovarian carcinoma with bs-MAb-targeted lymphocytes: a multicenter study. *Int J Cancer* 1992; **7** (Suppl): 78–81.
33 Kroesen BJ *et al.* Phase I study of intravenously applied bispecific antibody in renal cell cancer patients receiving subcutaneous interleukin 2. *Br J Cancer* 1994; **70**: 652–661.
34 Blank Voorthuis CJ *et al.* Clustered CD3/TCR complexes do not transduce activation signals after bispecific monoclonal antibody-triggered lysis by cytotoxic T lymphocytes via CD3. *J Immunol* 1993; **151**: 2904–2914.
35 Wang RF *et al.* Recognition of an antigenic peptide derived from tyrosinase-related protein-2 by CTL in the context of HLA-A31 and -A33. *J Immunol* 1998; **160**: 890–897.
36 Jantzer P, Schendel DJ. Human renal cell carcinoma antigen-specific CTLs: antigen-driven selection and long-term persistence *in vivo*. *Cancer Res* 1998; **58**: 3078–3086.
37 Winberg G *et al.* Surface expression of CD28 single chain Fv for costimulation by tumor cells. *Immunol Rev* 1996; **153**: 209–223.
38 Hayden MS *et al.* Costimulation by CD28 sFv expressed on the tumor cell surface or as a soluble bispecific molecule targeted to the L6 carcinoma antigen. *Tissue Ant* 1996; **48**: 242–254.
39 Maloney DG *et al.* Monoclonal anti-idiotype antibodies against the murine B cell lymphoma 38C13: characterization and use as probes for the biology of the tumor *in vivo* and *in vitro*. *Hybridoma* 1985; **4**: 191–209.
40 Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* 1993; **90**: 8392–8396.
41 Belnap LP *et al.* Immunogenicity of chemically induced murine colon cancers. *Cancer Res* 1979; **39**: 1174–1179.
42 Solar I, Gershoni JM. Linker modification introduces useful molecular instability in a single chain antibody. *Protein Eng* 1995; **8**: 717–723.