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DIRECCIÓN DE NUEVAS C**

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Abogado
Alvaro Correa Ordóñez

ASUNTO	Radicación	07-23991
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	
	Folios	1

Como resultado del examen de búsqueda de anterioridades y con fundamento en el artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

SOLICITUD DE SUMINISTRO DE INFORMACIÓN

Se determinó que existe en el estado de la técnica el siguiente documento que eventualmente afectaría los requisitos de patentabilidad de esta solicitud:

& DATABASE Geneseq [Online]
15 March 1999 (1999-03-15), "Anti-5T4
single chain antibody 5T4Sab1."
XP002371117
retrieved from EBI accession no.
GSN:AAW86003
Database accession no. AAW86003

Siendo que la publicación referenciada hace parte de los documentos citados en el examen de búsqueda internacional, para la solicitud aquí estudiada, y no siendo viable la consecución de dicho documento por este despacho, se requiere que el solicitante, proporcione el mismo, **de preferencia en formato digital**

En cumplimiento del artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de tres (3) meses contados a partir de la fecha de notificación de la presente comunicación. Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto (5), del título primero de la circular única N° 10 de 2001.


JOSE LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 5-3-2012

Elaboró: N.Lamprea

Targeting immune effector molecules to human tumor cells through genetic delivery of 5T4-specific scFv fusion proteins

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Although several clinical trials have shown beneficial effects by targeting tumor-associated antigens (TAAs) with monoclonal antibodies, a number of issues, including poor penetration of the tumor mass and human antimouse antibody responses, remain. The use of recombinant single-chain Fv (scFv) fragments has the potential to address these and other issues while allowing the addition of different effector functions. To develop therapeutic strategies that recruit both humoral and cellular arms of the immune response, we have constructed chimeric proteins linking either the human IgG1 Fc domain or the extracellular domain of murine B7-1 to a scFv specific for the oncofetal glycoprotein, 5T4. This TAA is expressed by a wide variety of carcinomas and is associated with metastasis and poorer clinical outcome. We have engineered retroviral constructs that produce fusion proteins able to interact simultaneously with both 5T4-positive cells and with the receptor/ligands of the immune effector moieties. Genetic delivery through a murine leukemia virus vector to 5T4-positive tumor cells results in the secreted scFv fusion protein binding to the cell surface. Furthermore, the scFv-HlgG1 fusion protein is able to direct lysis of 5T4-expressing human tumor cell lines through antibody-dependent cell cytotoxicity, indicating its potential as a gene therapy for human cancers.

Cancer Gene Therapy (2002) 9, 884–896 doi:10.1038/sj.cgt.7700513

Keywords: 5T4 oncofetal antigen; tumor-associated antigens; immunotherapy; scFv fusion proteins; Murine Leukemia Virus; genetic delivery

The development of antibodies as therapeutic agents for the treatment of cancer has recently led to some encouraging results in the clinic.^{1–3} Monoclonal antibodies (mAbs) targeted to tumor-associated antigens (TAAs) have been used alone or linked to a therapeutic moiety such as a radiolabel^{4,5} or a cytotoxic drug, e.g., calicheamicin.⁶ When used on their own, the cytotoxic effects of the mAb may be direct, e.g., by inhibiting growth signaling,⁷ or indirect, such as the induction of apoptosis.^{8–10} Also the recruitment of an immune response by the mAb can lead to cell killing through antibody-dependent cell cytotoxicity (ADCC) and complement fixation as has been seen with the anti-her2 neu antibody, herceptin,¹¹ and the anti-CD20 antibody, rituximab.¹²

There are, however, a number of problems associated with using whole murine antibodies in humans. These include poor targeting specificity,¹³ inefficient penetration of the tumor,¹⁴ human antimouse antibody (HAMA) reactions and general systemic toxicity at the high doses required to deliver sufficient antibody to the site of the tumor.¹⁵ Antibody

engineering now allows these issues to be addressed with the production of smaller recombinant molecules such as single-chain Fv (scFv) antibodies, which are the smallest fragment to contain the original antigen binding site. They consist of the variable heavy (V_H) and variable light (V_L) chain, joined by a flexible linker, and have been shown to penetrate tumors more effectively than whole antibodies or Fab fragments.¹⁴

Some disadvantages of using scFv include decreased stability, short half-life, and, in some cases, decreased affinity to the antigen.^{16,17} One approach to improving the function and circulating half-life is to link immune stimulatory molecules to the anti-TAA scFv. For example, to activate antibody-mediated killing against the target tumor, the constant heavy chain of human immunoglobulins can be added, which will also increase molecular size (and therefore stability) while minimizing immunogenicity. Human IgG1 has a broad specificity for the different Fc receptors present on macrophages, monocytes, and natural killer (NK) cells and thus represents an excellent choice for this approach.¹⁸

To induce a cytotoxic T-cell response, the costimulatory molecules of the B7 glycoprotein family can be used. These important costimulatory molecules are present on antigen-presenting cells and are ligands of the receptors CD28 and CTLA-4 found on lymphocytes.¹⁹ In both cases, delivering

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the immune conjugates as genetic constructs could confer additional advantages. Genetic delivery should circumvent the clinical problems caused by the necessity of repeated injections of high doses of protein by continuously expressing the therapeutic antibody at the tumor sites, resulting in high concentrations where they are needed but low concentrations in the circulation and in healthy tissue.

This could be achieved using viral vectors injected intratumorally, leading to production of the fusion protein, which is then secreted, from the cancer cell. The scFv would then bind back to the target antigen on the cell surface to direct lysis by effector cells such as macrophages, monocytes, and NK cells (Fig 1). Alternatively, the natural tumor-targeting properties of macrophages could be employed, transducing them *ex vivo* and then reintroducing them into the patient. The macrophages would then track to sites of tumor growth where the secreted fusion protein could bind to the target TAA and direct lysis as before. Because there is a hypoxic environment in a tumor mass, an additional level of specificity could be obtained by placing the fusion proteins under control of the hypoxia response element (HRE),²⁰ which would act as tumor-specific regulatory element. To date, expression of fusion proteins from adenoviral vectors has been demonstrated in mouse models with a number of scFvs including those targeted to CEA,²¹ factor VII,²² and also intracellular expression of an anti-p21 ras scFv alone.²³

The oncofetal antigen, 5T4, is a cell surface, leucine-rich glycoprotein,²⁴ originally defined by a mAb raised against glycoproteins isolated from human syncytiotrophoblast microvillus plasma membranes.^{25,26} 5T4 is found on a wide variety of adenocarcinomas,²⁷ including cervical,²⁸ ovarian,²⁹ and those of the GI tract,³⁰ with up to 80% of tumors being antigen-positive. Expression of 5T4 has been shown to be associated with metastasis and is an independent indicator of poor prognosis in a number of cancers, including colorectal³¹ and ovarian.²⁹ Excluding the placenta, the antigen is absent from most normal adult tissues although low levels are found on a few specialized epithelia. This indicates that 5T4 has excellent potential as a target antigen for antibody-mediated cancer immunotherapy and this view is encouraged by recent data obtained with a fusion protein constructed from a Fab of the anti-5T4 mAb and an engineered super antigen (staphylococcal enterotoxin A).³² The purified recombinant product induced, *in vitro*, T-cell-dependent killing of a non small cell lung cancer line expressing 5T4, and intravenous injection of the fusion protein into athymic mice carrying the cells intraperitoneally resulted in a reduction of tumor mass.

A 5T4-specific scFv has been amplified by polymerase chain reaction (PCR) from the hybridoma line producing the mAb³³ and here we report its use in the construction of potentially therapeutic immune conjugates. Genetic constructs have been made to produce chimeric proteins

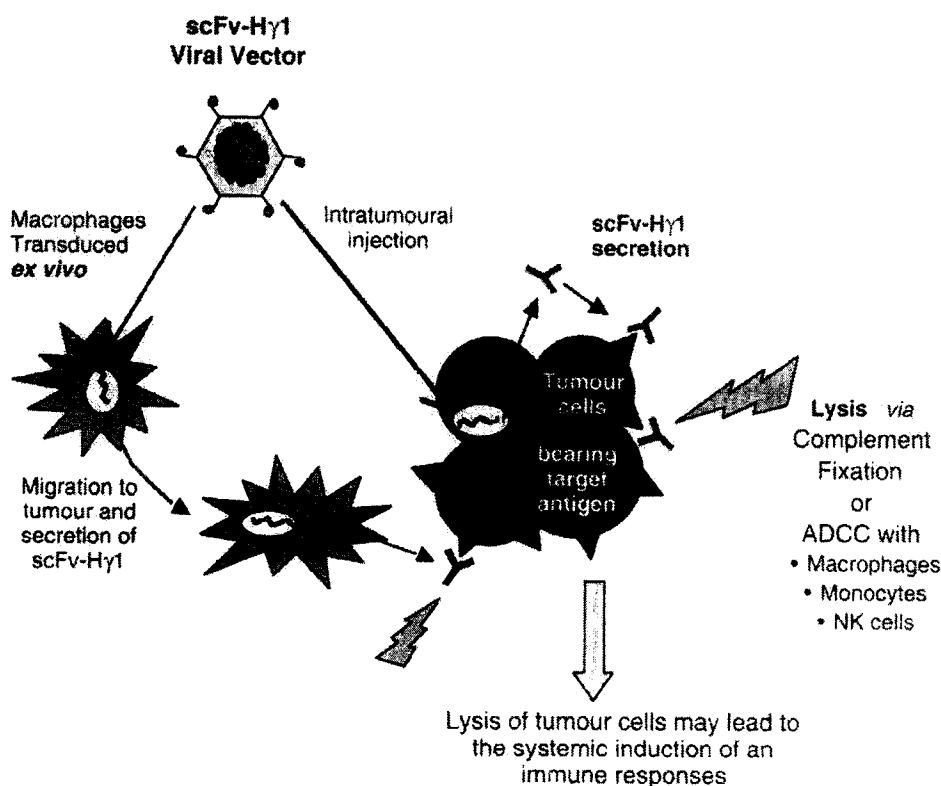


Figure 1 Genetic delivery of 5T4 scFv-Hy1 constructs. Tumour cells transduced by vector particles containing the 5T4 scFv-Hy1 constructs will produce fusion protein, which will then be secreted from the cell. The immune conjugate will then bind back to the 5T4 antigen at the cell surface, directing lysis of both transduced and neighbouring untransduced cells, through monocytes, macrophages, and NK cells and complement fixation. Alternatively, macrophages can be transduced *ex vivo*, and then introduced back into the patient. The tumor tropic properties of these cells will take them to sites of the cancer where secretion of the fusion protein will direct cell lysis. Use of a HRE in the promoter of the construct will limit production to the low oxygen environment of the tumors.

Cancer Chem. Therapy

GCC GCC GCC ACT GCC GCC ACC ACC GCT CCC ACC ACC CCC-3') to produce a double-stranded fragment with *SmaI/SpeI*-compatible ends. This linker was then cloned into *SmaI/SpeI*-digested pBS II to generate the intermediate plasmid pLink. The 735-bp murine B7.1 EC domain (amino acids 1–245) was amplified from the bacterial expression plasmid pLK444-mB7 (kindly supplied by R Germain, NIH, USA), using primers that incorporated a Kozak consensus sequence and a *EcoRI* restriction site at the 5' end and a 3' *SmaI* site (5'-CTC GAA TTC CAC CAT GGC TTG CAA TTG TCA GTT GAT GC-3' and 5'-CTC CCC GGG CTT GCT ATC AGG AGG GTC TTC-3'). The PCR product was digested with *EcoRI* and *SmaI* and inserted into pLink digested with the same enzymes to generate pBS/B7Link. Tagged and untagged versions of the 5T4 scFv were amplified from the plasmid pHEN1-5T4scFv. To generate the untagged construct, a 5' primer (5'-CTC ACT AGT GAG GTC CAG CTT CAG CAG TC-3') containing a *SpeI* restriction site and a 3' primer (5'-CTC GCG GCC GCT TAC CGT TTG ATT TCC AGC TTG GTG CCT CCA CC-3') containing a terminal *NotI* site downstream of the termination codon of the scFv fragment were employed. The resultant product was digested with *SpeI* and *NotI* and inserted into pBS/B7Link digested with the same enzymes. To generate B7-scFv-MH, the scFv was amplified using the same 5' primer and a 3' primer (5'-CTC TCT AGA TCA ATG GTG ATG GTG ATG ATG GTC GAC GGC GCT ATT CAG ATC CTC TTC TGA GAT GAG-3') with flanking *SpeI* and *XbaI* sites and including a Myc-His tag. This PCR product was then digested with *SpeI* and *XbaI* and cloned into pBS/B7Link digested with the same enzymes.

Production of cell lines expressing fusion constructs

The fusion construct cassettes were cloned into the viral expression vector pHIT LXSN.¹⁸ Virus was made in the human kidney cell line 293 T by cotransfecting plasmids containing the MLV *gap-pol* genes (pCIEGPPD) and the VSV G envelope (pRV67) using the triple plasmid HIT system.^{34,35} After chromosomal integration, the fusion protein cassette is placed under transcriptional control of the LTR. Using calcium phosphate, a 10-cm dish of 293 T at ~50% confluence was transfected with 16 µg of each plasmid. After 6 hours, the medium was removed and replaced with 5 mL of fresh medium. The medium was removed 16 hours later, passed through a 0.45-µm filter, and stored in 0.5-mL aliquots at -70°C.

To produce the stable BHK cell lines expressing the fusion protein, cells were plated at 10⁵/well in a 12-well plate in 1 mL of full medium (MEM, 10%FBS). Several hours later, a number of different-sized aliquots (100–1 µL) of viral supernatant were added to the wells.

Approximately 16 hours posttransduction, transduced cells were selected by trypsinisation into 10 mL of fresh culture medium containing 1 mg/mL G418 (Invitrogen, Paisley, Renfrewshire, UK) and plating out in 10-cm dishes. After 7–14 days, growing colonies were ring-cloned and expanded. Positive lines were identified by immunostaining: after fixing the cells with methanol:acetone (1:1), they were washed with PBS then incubated with either goat antihuman IgG-HRP (for the Hy1 construct) or mouse anti-c-myc

mAb followed by rabbit antimouse Ig-HRP (for the Myc-His tagged constructs). Detection used DAB reagent (Vector Laboratories, Peterborough, Cambridgeshire, UK).

Harvesting the fusion proteins

The stable cell lines were grown to ~95% confluence in T150 flasks in EMEM, 10% FBS, and then the medium changed to EMEM containing no serum (20 mL/flask), which was then harvested 3–4 days later. Supernatants from BHK-21 cells transfected with either the LscFv-MH or B7-scFv-MH constructs were filtered through a 0.45-µm membrane, loaded onto a nickel-equilibrated HisTrap column (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), washed with 20 mM (B7scFv) or 40 mM (LscFv) imidazole, and then eluted with 500 mM imidazole buffer. Several protein fractions were pooled based on relative yield and purity as indicated by BioRad (Hemel Hempstead Hertfordshire, UK) protein estimation and silver staining of polyacrylamide gels (QuickSilver; Amersham Biosciences), respectively. Each pool was desalted and then reequilibrated in PBS using Sephadex G-25 based PD-10 buffer exchange columns (Amersham Biosciences).

The scFv and scFv-Hy1 fusion proteins were concentrated and partially purified using centrprep 10 and centricon 10 (Millipore, Watford, Hertfordshire, UK) systems.

Western blot analysis of the fusion proteins

Aliquots of concentrated supernatants and desalted purified protein (10 µL) diluted in 2× Laemmli buffer containing 3-mercaptopethanol were heated at 95°C for 5 minutes and loaded on a precast Novex 10% Tris-glycine gel. Benchmark molecular weight marker (5 µL) from Roche (Lewes, East Sussex, UK) was employed and samples were run in 1× running buffer (2.9 g/L Tris base, 14.4 g/L glycine, 1.0 g/L SDS) at 25 mA for 1.5 hours under reducing conditions. Proteins from the gel were then transferred to Hybond-ECL membrane (Amersham Biosciences) in transfer buffer (1.45 g/L Tris base, 7.2 g/L glycine, 20% methanol), at 25 V (constant) for 2 hours using a Novex XCell II blotting apparatus. The membrane was blocked overnight in PBS containing 0.05% Tween 20 and 5% nonfat dried milk powder. The membrane was incubated with the 1° antibody, rabbit antihuman IgG-HRP (Dako, Ely, Cambridgeshire, UK) at 1/1000, mouse anti-His (Invitrogen) or mouse anti-Myc (Roche), or rat anti-mouse B7 (Becton Dickinson), in the same buffer. After washing in PBS, 0.05% Tween, an HRP-linked 2° antibody was added where appropriate (rabbit antimouse [Dako] 1/1000 or rabbit antirat [Dako]). After washing, ECL reagent mix (0.125 mL/cm²; Amersham Biosciences) was applied to the membrane for 1 minute and then exposed to Kodak X-ray film (Hemel Hempstead, Hertfordshire, UK).

BIAcore analysis

BIAcore analysis was carried out as described by Shaw et al.³³ Briefly, purified 5T4-IgG fusion protein²⁰ was immobilized by amino coupling to the surface of a carboxymethyl-dextran (CM5) biosensor chip, at a level of

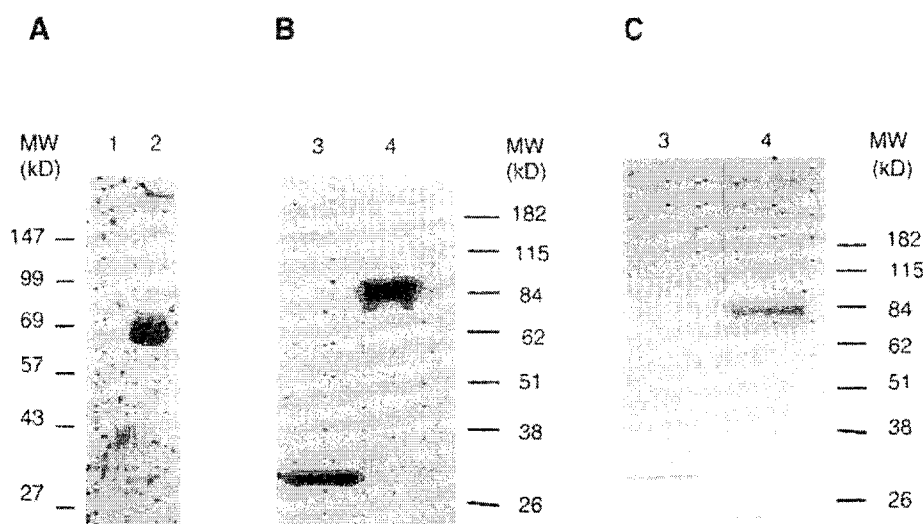


Figure 3 Western blot of cell supernatant and purified fusion proteins. **A:** Probed with goat anti-human IgG-HRP. **Lane 1:** Supernatant obtained from a fusion protein-negative G418-resistant BHK line. **Lane 2:** Supernatant obtained from a stable BHK cell line transfectant expressing the scFv-Hy1 fusion protein (~65 kDa). **B:** Probed with mouse anti-c-myc, followed by rabbit antimouse Ig-HRP. **Lanes 3 and 4:** Nickel column-purified scFv-MH (~32 kDa) and B7-scFv-MH (~85 kDa) fusion proteins respectively. These two samples were also analysed by silver stain (**C**)

500 RU. The surface was activated for 7 minutes with EDC/NHS at a flow rate of 5 $\mu\text{L}/\text{min}$ followed by 10 μL of 5T4-IgG at 50 $\mu\text{g}/\text{mL}$ in 10 mM acetate buffer, pH 4.5. Surplus binding sites were blocked using 1 M ethanolamine for 7 minutes. Kinetic analysis was performed at a flow rate of 30 $\mu\text{L}/\text{min}$. Association was measured over a 3-minute injection, and dissociation for 15 minutes. The antigenic surface was regenerated by a 10- μL pulse of 20 mM HCl, and a baseline reestablished. The mAb was assessed over the concentration range 0.25–6 nM and the scFv constructs from 2.5 to 25 nM.

Immunohistochemical analysis with the scFv fusion proteins

Cryostat sections of normal human placenta were fixed and stained as described previously.³³ Primary antibody, PBS control, scFv-MH or B7-scFv-MH was detected with 9E10 anti-myc followed by rabbit antimouse biotin. Sections were then incubated with HRP-conjugated avidin-biotin complex, developed using DAB, and counterstained with hematoxylin.

FACS analysis of the scFv fusion proteins

Cells expressing 5T4 (PA1, a human ovarian carcinoma line, and A9-5T4, a mouse L-cell line stably transfected with 5T4 under the control of the CMV promoter²⁴) and a control line A9-neo (the mouse L-cell expressing the neomycin gene) were used. Approximately 5×10^5 cells, in a round-bottom 96-well plate (Becton Dickinson), were incubated with either 100 μL of concentrated cell supernatants or purified fusion proteins (between 1 and 100 ng/mL) for 30 minutes on ice. After washing, bound protein was detected using an appropriate FITC-conjugated 2nd antibody. Cells were analysed on a Becton Dickinson *FACScalibur*. In competition experiments, scFv was used as a primary antibody, the

scFv-Hy1 as a second layer, and goat anti-HIgG-FITC as the 3rd antibody.

Analysis to show that the scFv-Hy1 protein produced on transduction with the MLV-based construct is secreted and able to bind back to the cell surface if that cell is expressing 5T4 was also undertaken. A murine colon line, CT26, transfected with 5T4 was transduced with the construct, expanded, and then assayed for binding of the anti-HIgG-FITC antibody. A CT26 line expressing the neomycin gene was used as a negative control.

To demonstrate simultaneous binding of the scFv to its target antigen and B7 to a ligand, B7scFv-MH was used as a 1st antibody followed by the C11A-4 ligand in the form of a fusion protein with mouse IgG at the C-terminus (A cell, Bayport, MN), which was then detected with rabbit anti-mouse Ig-FITC.

To demonstrate that the HlgG constant chain could bind an Fc receptor, the human macrophage line U937 (either untreated or treated overnight with IFN- γ , 1 $\mu\text{g}/\text{mL}$) was incubated with either scFv-Hy1 or scFv and detected using either the anti-HlgG-FITC antibody or an anti-CD64-FITC antibody.

Table 1 BIAcore analysis of B7-scFv-MH and scFv-MH fusion proteins

	K_{on} (1/M s)	K_{d} (1/M)	K_{off} (1/s)	K_{d} (nM)
mAb	5.50×10^7	5.50×10^{10}	1.00×10^{-7}	1.85×10^{-11}
scFv-MH	9.88×10^4	1.30×10^9	7.81×10^{-5}	7.90×10^{-10}
B7-scFv-MH	1.01×10^5	8.27×10^8	1.25×10^{-4}	1.24×10^{-9}

The 5T4-Ig antigen was immobilized on the biosensor chip and the fusion proteins passed over. Proteins were analysed in either duplicate (mAb and scFv-MH) or triplicate (B7-scFv-MH).

ADCC

A9-5T4 and A9-neo cell lines and two other 5T4-expressing lines, EJ (bladder carcinoma) and PA1 (ovarian cancer), were used in chromium release assays. Target cells were incubated with 0.1 mCi (100 μ L) of ^{51} Cr (as Na_2CrO_4 in normal saline at 1 mCi/mL; Amersham Biosciences) at 37°C (5% CO_2) for ~1 hour, washed three times with PBS, then diluted to 3×10^5 /mL. Cells were then added to 96-well plates at 50 μ L/well and incubated at room temperature with concentrated supernatants containing either scFv-H γ 1 or scFv (between 1 and 100 mg/mL, in 50 μ L) for ~10 minutes. Effector cells (peripheral blood mononuclear cells isolated using Ficoll-Paque [Amersham Biosciences] as per manufacturer's instructions either from fresh blood or from buffy coats obtained from blood transfusion service) were then added at various concentrations in 50 μ L, centrifuged, then incubated for 4 hours at 37°C, 5% CO_2 . After incubation, plates were centrifuged and 100 μ L/well taken for scintillation counting.

Percent lysis was calculated as:

$$\% \text{ Lysis} = \frac{\text{Test Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100$$

Results

Construction and expression of the chimeric fusion proteins

The human 5T4-specific scFv has been described previously and we have now produced a number of chimeric gene cassettes encoding this scFv linked to different protein moieties. A control construct, LscFv-MH, was made, comprising an immunoglobulin leader sequence, Igs, fused to the N-terminus of the scFv to allow secretion of the protein from MLV-transduced mammalian cells, and a c-Myc epitope plus 6-His tag added C-terminal to the construct for the purposes of identification and purification. Another control lacking the C-terminal Myc-His tag, LscFv, was also made. Immune enhancer conjugates, comprised of either the EC domain of murine B7-1 (amino acids 1-245, which contain a secretion signal), joined N-terminal through a 15-amino-acid flexible (Gly $_4$ Ser) $_3$ linker to the scFv, either with or without the Myc-His tag (B7-scFv-MH and B7-scFv, respectively) or a C-terminal fusion of the human IgG1 constant heavy chain directly to the LscFv (LscFv-H γ 1) (Fig 2). These cassettes were then inserted into the retroviral genome vector pHIT LXS N , which was then used to prepare high-titer helper-free retroviral particles. These recombinant retroviruses were then used to demonstrate genetic delivery of the constructs in BHK-21 cells, leading to the secretion of



(A) PBS alone



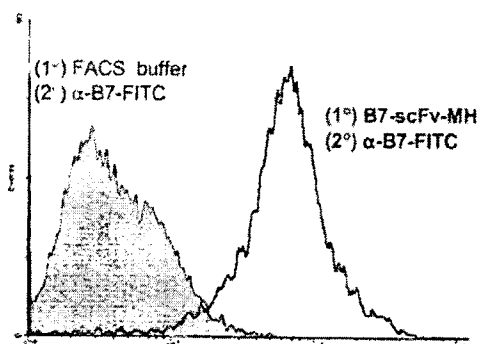
(B) scFv-MH



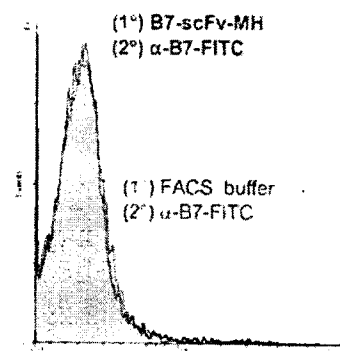
(C) B7-scFv-MH

Figure 4 Immunohistochemistry of human term placenta, incubated with PBS (A), scFv-MH (B) or B7-scFv-MH (C), as the 1st antibody, followed by 9E10 anti-myc as the 2nd layer in all cases. Detection was with rabbit anti-mouse biotin followed by avidin-biotin complex and DAB.

(A) A9-5T4



(B) A9-neo



(C) A9-5T4

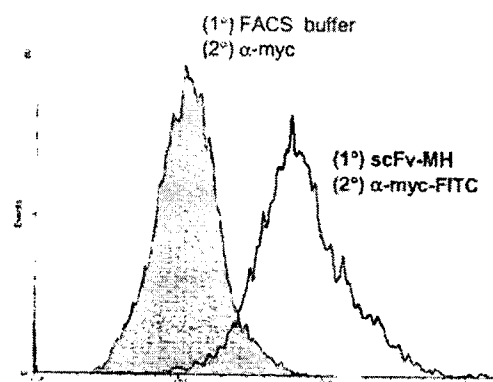


Figure 5 FACS analysis of binding of the B7-scFv fusion protein to the human 5T4 target antigen. A9 5T4 cells (A), or A9-neo cells (B), were incubated with B7-scFv-MH followed by detection with anti-B7-FITC. FACS buffer alone followed by anti-B7-FITC was used as the negative control. The scFv-MH construct was also incubated with A9-5T4 cells (C). Detection used mouse anti-myc mAb followed by FITC-conjugated antimouse IgG. FACS buffer alone, in place of the scFv-MH, was used as the negative control.

active fusion proteins. These cell lines were subsequently cloned to give stable lines from which the fusion proteins could be isolated.

The scFv and scFv-Hyl proteins were concentrated in the media in which they were collected and used in that form for subsequent experiments. Figure 3A shows a Western blot of concentrated supernatant from BHK-21 stable transfectants, demonstrating a specific band identified by the antihuman IgG antiserum.

The scFv-MH and B7-scFv-MH were purified from the harvesting media through the polyhistidine tag using Ni^{2+} affinity chromatography. The expression, harvest, and purification of the scFv-MH antibody were similar to that for the B7-scFv-MH fusion protein, one difference being that 40 mM imidazole buffer was used to wash the immobilized LscFv-MH protein on the Ni^{2+} affinity column during purification, whereas B7-scFv-MH was washed with 20 mM imidazole. The yield of purified soluble protein was between 0.7 and 0.8 mg/L cell supernatant. SDS-PAGE, under reducing conditions, indicated both proteins to be monomers. The scFv-MH migrated as a sharp band, having an apparent molecular mass of approximately 32 kDa (predicted 31 kDa). The B7-scFv-MH migrated as a diffuse band, typical of glycosylated proteins (the murine B7 EC domain is highly charged with seven

potential N-linked glycosylation sites), with an apparent molecular weight of approximately 85 kDa (see Fig. 3). Western blot and silver stain analyses revealed a purity of >90% for both proteins after affinity chromatography and buffer exchange.

Dissociation constant determination of the LscFv-MH and B7-scFv-MH

A biosensor-based assay, using a h5T4 IgG fusion protein³³ as the target antigen, was used to measure the affinity and kinetics of interaction of scFv-MH and B7-scFv-MH (Table 1). The association rate (K_a) for scFv-MH reported previously was approximately 5-fold slower than that of the original mAb to 5T4.³³ The K_a for B7-scFv-MH is very similar to that of the scFv-MH. However, the dissociation (K_d) rate for B7-scFv is approximately 2-fold faster than for the scFv-MH and 13-fold faster than for the original mAb. Overall, B7-scFv-MH has an affinity (K_d) of 1.24×10^{-9} M, which compares favorably with the value of 7.90×10^{-10} M for scFv-MH. The scFv-MH value is approximately 40 times lower than that reported for the 5T4 mAb (1.85×10^{-11} M). These findings indicate that linking the EC domain of murine B7.1 to the high-affinity 5T4-specific scFv does not dramatically alter its binding properties.

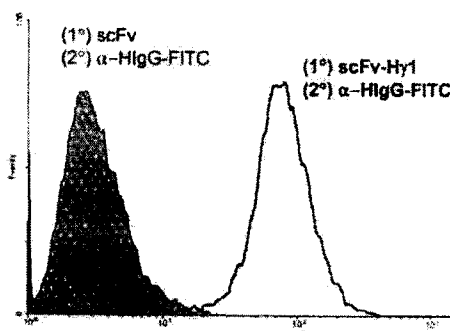
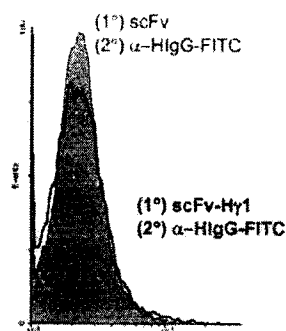
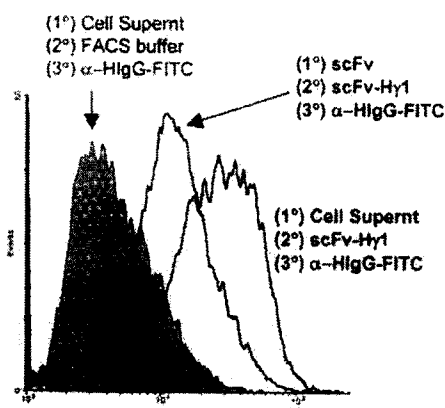
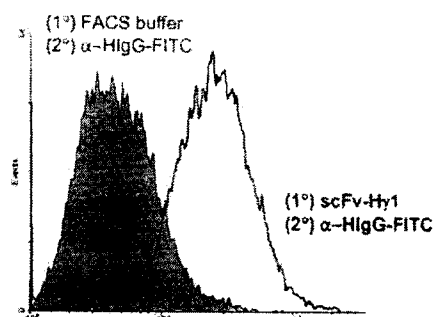
(A) A9-5T4

(B) A9-neo

(C) A9-5T4

(D) PA1


Figure 6 FACS analysis of scFv-Hy1 fusion proteins. In all these experiments, goat anti-HlgG-FITC was used as the final layer for detection. A9-5T4 **(A)** and A9-neo cells **(B)** were incubated with either scFv-Hy1 or scFv cell supernatants. To demonstrate competition between the scFv alone and scFv-Hy1, supernatant from either untransfected BHK cells (as a negative control) or those transfected with the scFv construct, was used as a primary layer. Concentrated scFv-Hy1 supernatant was added subsequently as the second layer **(C)**. Binding of the scFv-Hy1 to a human tumor line expressing native 5T4 was demonstrated by incubating PA1 (ovarian carcinoma) cells with concentrated scFv-Hy1 supernatant **(D)**. FACS buffer alone was used as the negative control.

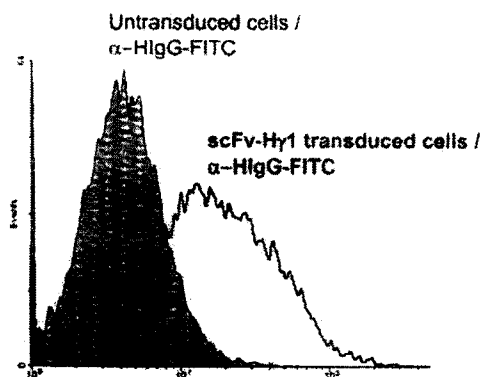
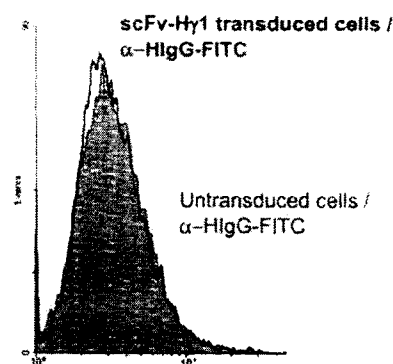
A: CT26-5T4

B: CT26-neo


Figure 7 FACS analysis of cells transduced with the MLV-scFv-Hy1 construct. CT26-5T4 **(A)** or CT26-neo cells **(B)** were transduced with an MLV-based construct expressing scFv-Hy1. Goat anti-HlgG-FITC was used to detect endogenously produced fusion protein that was secreted from the cells and had subsequently bound to the cell surface. Untransduced cells were used as the negative control.

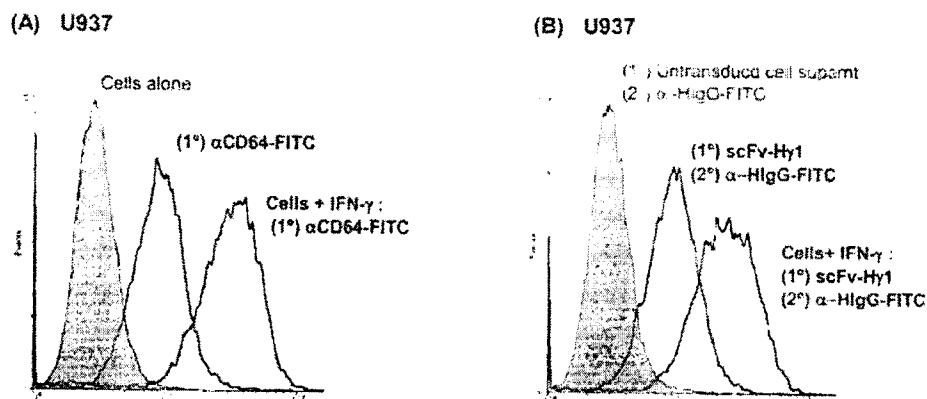


Figure 8 FACS comparison between the binding of anti-CD64 and scFv-Hy1 to U937 cells. The monoblast cell line, U937, treated or untreated overnight with IFN γ , was incubated with either anti-CD64-FITC (A) or scFv-Hy1 (B). The scFv-Hy1 was then detected using anti-HlgG-FITC. The shift in fluorescence was compared to cells alone (no fluorescent antibody added at all).

Immunohistochemical analysis with the scFv fusion proteins

It has been shown previously that, like the parent mAb, the scFv-MH labels the syncytiotrophoblast layer of human placenta.¹³ Figure 4 shows that the B7 scFv-MH likewise binds to human syncytiotrophoblast, indicating that the B7.1 EC domain does not hinder binding to native 5T4 *in situ*. No staining was seen with anti-myc 9E10 antibody alone.

FACS analysis demonstrates binding of the scFv fusion proteins to the 5T4 target antigen

To determine if the scFv fusion proteins are specific for the human 5T4 antigen, FACS analysis was carried out using a mouse fibroblast cell line transfected with the human 5T4 cDNA (A9-5T4) and the same line transfected with the neomycin resistance gene alone (A9-neo). Figure 5, A and B shows that the B7-scFv-MH binds to A9-5T4 cells but not the A9-neo cells, confirming that the scFv is binding to the target antigen and not to some other molecule on the A9 cells. Figure 5C demonstrates binding of the scFv-MH to A9-5T4 cells. The binding of both B7-scFv-MH and scFv-MH can be inhibited by the 5T4 mAb from which the scFv is derived (data not shown). Similarly, the scFv-Hy1 construct demonstrates at least 1 log shift in fluorescence activity in the 5T4-positive cells compared to the control scFv construct, when detected with rabbit anti-HlgG-FITC (Fig 6A). Again, no shift occurs with A9-neo cells (Fig 6B). Competition FACS analysis demonstrated that the concentrated supernatant from the cells transfected with the scFv construct did indeed contain scFv protein because this supernatant was able to block the binding of the scFv-Hy1 to the 5T4 antigen on the A9-5T4 cells (Fig 6C). The scFv-Hy1 was also shown to bind to the human ovarian carcinoma line (PA1), which expresses native 5T4 at the cell surface (Fig 6D).

In addition, it was demonstrated that the production of the fusion protein from an MLV-based construct within the cell leads to secretion of the protein from the cell and binding to the cell surface, if the transduced cell expressed the 5T4 antigen. Figure 7 shows FACS analysis of a murine colon

cancer cell line, CT26, expressing human 5T4, compared to CT26 only expressing neomycin, after transduction with the MLV-LscFvHy1 construct. Using the anti-HlgG-FITC antibody, HlgG is detected at the surface of the CT26-5T4 cells but not the CT26-neo cells. If the cells are permeabilized, then HlgG is detected in the CT26-neo transductants (data not shown), indicating that the lack of detection of HlgG at the cell surface is not due to a lack of production of the protein within the cell. This also indicates that the scFv-Hy1 protein is binding to the 5T4 on the cell surface of the CT26-5T4 cells rather than being trapped in the membrane or binding nonspecifically to the cells. Thus, genetic delivery of these immunocjugates *in vivo* has the potential to result in binding of active protein to the surface of any tumor cell expressing 5T4 (both

A9-5T4

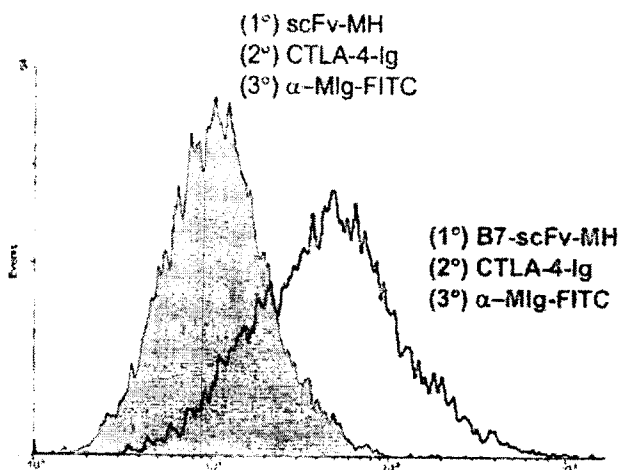


Figure 9 FACS analysis of B7-scFv binding to both 5T4 and CTLA-4. To demonstrate simultaneous binding of the B7 extracellular domain to a ligand, CTLA-4, while the scFv is bound to its target antigen, A9-5T4 cells were incubated with either scFv-MH (as a negative control) or B7-scFv-MH, followed by CTLA-4 in the form of a fusion protein with mouse Ig at the C-terminus. This was then detected using FITC-conjugated anti-mouse Ig.

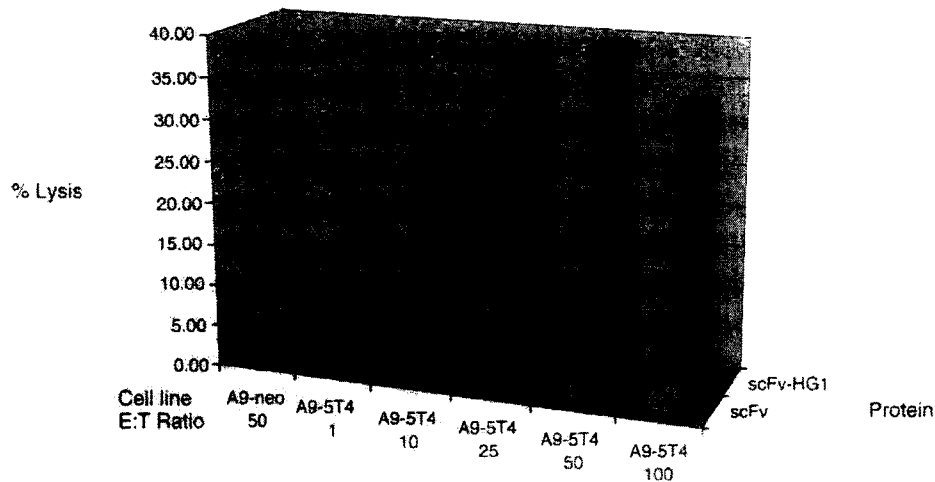
transduced and nontransduced) and hence direct ADCC of the tumor (see Fig 1).

Increased binding of scFv-Hγ1 to U937 cells is observed after treatment with IFN-γ

The human IgG1 constant heavy chain is able to bind a number of Fc receptors, including CD64, the high-affinity Fc receptor (FcRγ1) that is present on the human monoblast cell line U937. Treatment of this cell line with IFN-γ up-regulates FcRγ1 but not FcRγII (CD32), which is also present.³⁶ To determine if the scFv-Hγ1 fusion protein is

capable of binding to the surface of U937 cells, FACS analysis, with or without prior IFN-γ treatment, was carried out. Figure 8A shows binding of an anti-CD64-FITC antibody to U937 cells. Treatment with IFN-γ gives rise to a further shift to the right, as expected. Incubation of U937 cells with the scFv-Hγ1 fusion protein results in binding of the protein to the cell surface as detected by the anti-human IgG-FITC antibody and this, too, shifts to the right with IFN-γ treatment (Fig 8B). The similarity of the profiles between the anti-human IgG and the anti-CD64 antibody suggests that the fusion protein is binding to the Fc receptor.

(A) A9—5T4 transfectants with increasing E:T ratio



(B) Human Tumour lines with E:T of 30:1

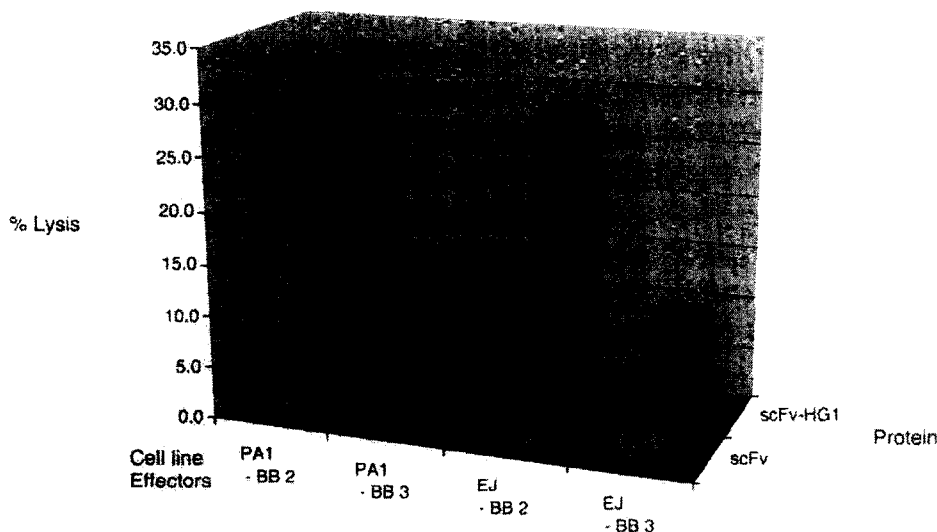


Figure 10 Antibody-dependent cell cytotoxicity of 5T4-expressing cells. **A:** A9-5T4 and A9-neo cells were labeled with ⁵¹Cr and then incubated with either scFv or scFv-Hγ1. Increasing amounts of peripheral blood lymphocytes were used as the effector cells and lysis allowed to proceed for 4 hours. Chromium released into the supernatant was measured by scintillation counting to assess the extent of lysis achieved. **B:** EJ (human bladder carcinoma line) and PA1 (human ovarian carcinoma line) cells were used as targets and peripheral blood lymphocytes from two different donors (BB2 and BB3) were used at a ratio of 30:1.

B7 scFv-MH is able to bind simultaneously to cells expressing 5T4 and to the ligand CTLA-4

B7.1 binds to its counterreceptors CD28 and CTLA-4 on the surface of T cells. To show that the B7.1 domain of the recombinant B7-scFv-MH is able to bind a ligand while the scFv is bound to the 5T4 antigen on the cell surface, a three-layer FACS was performed (Fig 9). Cells were incubated with B7-scFv-MH followed by the CTLA-4 ligand in the form of a fusion protein with mouse IgG at the C-terminus. Bound CTLA-4 was then detected using rabbit antimouse IgG-FITC. The LscFv-MH was used in place of B7-scFv-MH as the negative control and a clear shift in fluorescence indicates that the B7.1 fusion protein can simultaneously bind 5T4 expressed at the cell surface and the CTLA-4 ligand. FACS analysis with B7-scFv lacking the Myc-His tag demonstrated that the tag had no effect on binding (data not shown).

The scFv-Hy1 fusion protein is able to direct ADCC lysis of 5T4-positive human tumor cells

Figure 10A shows a chromium release assay that demonstrates that the scFv-Hy1 fusion protein can direct cytotoxicity against cells expressing the 5T4 antigen at the cell surface. There is increasing lysis of the cells with increasing protein concentration and also with an increasing effector-to-target (E/T) ratio. This is clearly 5T4-specific when the A9-5T4 cells are compared with the negative control A9-neo. In addition, the results using the scFv alone clearly indicate that the lysis is directed by the Fc portion of the fusion protein. Interestingly, a low level of lysis does occur with the scFv alone, but this is a maximum of 4% compared to 40% with the scFv-Hy1. ADCC could also be directed against the human tumor cell lines EJ and PA1 that express 5T4 under control of its native promoter (Fig 10B), demonstrating that the levels of 5T4 occurring naturally on human tumor lines are sufficient to mediate lysis.

Discussion

The successful treatment of cancers with therapeutic antibodies or immunoconjugates will require recruitment of an effective immune response, specific to the malignant cells. Whole mAbs and recombinant antibodies are now being tested in the clinic and immune responses have been measured. However, a number of issues need to be addressed to maximize the efficacy of such treatments; these include delivery to the cancer cells, penetration of the tumor mass, affinity for the target antigen, stability, clearance from the blood stream, and eliciting the best possible response from the immune system. With this in mind, we have made genetic constructs in viral vectors that produce chimeric proteins, joining an scFv specific for the 5T4 cell surface glycoprotein to the immune enhancers B7.1 and human IgG1 constant heavy chain, Hy1. Transduction of a mammalian cell line, BHK-21, with these constructs leads to the production and secretion of functional immune conjugates. Transduction of the murine colon cancer line CT26, engineered to express human 5T4, with the scFv-Hy1-MLV-based viral vector

leads to the protein being secreted and binding back to the target antigen at the cell surface. This illustrates that genetic delivery of such therapeutic constructs can produce active fusion proteins capable of binding TAAs on cancers and, therefore, has the potential to stimulate lysis of the tumor cells *in vivo*, through immune effector cells. This should include both transduced and nontransduced cells bearing the target antigen. Others have successfully achieved ADCC of human carcinoma cells transduced with MLV-based recombinant viruses expressing heavy and light chains of an anticolon cancer TAA mAb.¹ Importantly, both transduced and nontransduced cells were sensitized to ADCC, demonstrating that the antibody made in one cell can spread to adjacent, nontransduced cells that express the target antigen. However, this required sequential transduction of two separate viruses, and therefore development of a single virus expressing an scFv, would be advantageous, particularly in a clinical setting.

The binding affinities for the Myc-His tagged scFv (scFv-MH) control and the B7.1-scFv have been determined. The binding of the scFv-MH has been previously reported³¹ and although this is approximately 40-fold lower than the parent mAb, the value of 7.9×10^{-10} M compares favorably with the binding affinities of both mAbs and scFvs in the literature such as those against FpCAM.^{32,39} The binding affinity of B7-scFv-MH is less than 2-fold lower than the scFv alone, demonstrating that the addition of a bulky molecule such as B7.1 has little effect on the binding of the fusion protein to the target antigen. These data, coupled with the fact that the fusion protein is able to simultaneously bind the B7 ligand, CTLA-4, and 5T4 at the cell surface, through the scFv, indicate the potential for this approach in tumor immunotherapy. Others have shown that the B7/CD28 costimulatory pathway can be exploited to enhance specific immune responses against tumors and generate antitumor immunity. This has been achieved in murine models using the expression of B7.1 in a variety of tumor cell lines⁴⁰⁻⁴² and in the context of coexpression with TAAs.^{43,44} Also an anti-CFA scFv linked to B7.1 has been demonstrated to redirect a cytotoxic response to CEA expressing tumor cells in combination with an anti-CEA anti-CD3 diabody.⁴⁵

The scFv-Hy1 fusion protein has been shown to bind the target antigen through the scFv. It is also able to bind to cells expressing the high-affinity Fc receptor, CD64, and can direct ADCC against cells expressing 5T4. Using murine cells expressing high levels of human 5T4, specificity for the target antigen and increasing lysis with increasing E/T ratio were demonstrated. The killing of human tumor cell lines expressing native 5T4 (EJ, bladder carcinoma; PA1, ovarian carcinoma; HT29, colon cancer) indicates that the levels of 5T4 found in human cancers should allow directed lysis of tumor cells *in vivo*. The hope that such constructs could prove effective for immunotherapy of cancers expressing the 5T4 target antigen is encouraged by the results of a number of clinical trials which indicate that the efficacy achieved with whole antibody therapy is mediated by ADCC⁴⁶ including trials of mAb 17-1A, in which patients with above median pretreatment ADCC activity had significantly longer survival rates.⁴⁶

HAMA reactions are well documented in clinical trials using murine antibodies^{3,4,47} leading to toxicity and also a more rapid clearance of the anti-TAA antibody.⁴⁸ Interestingly, some reports have indicated that HAMA has little adverse effect on the overall clinical outcome of the mAb therapy;^{49,50} however, reduction in toxicity will be of benefit as would increased persistence of the immune conjugate. Our use of a human IgG should limit immune reaction against the fusion protein⁵¹ and although the scFv is murine, techniques to humanize this part of the construct such as the use of human phage libraries⁵² could be used.

In addition, genetic delivery of the immune conjugates will take full advantage of the relatively small size of the molecules for good tumor penetration and should also be able to circumvent the potential disadvantages of rapid clearance from the blood plasma. When antibodies are delivered systemically as proteins, they need to persist for long enough to reach the tumor sites. However, production of the antibody from a genetic construct at the sites of malignant disease will reduce the requirement for persistence in the blood stream and subsequent clearance of the antibody from the blood should help to minimize any toxicity including HAMA.

The concept of using 5T4 as a target antigen has been reinforced by the results obtained in experiments using a purified 5T4-specific Fab-Super Antigen fusion protein,³² i.e., induction of *in vitro* killing of a non small cell lung cancer cell line and reduction in tumor mass obtained on administration of the protein to nude mice bearing tumors derived from that cell line. Here we have shown that transduction of mammalian cells with MLV-based genetic constructs produces anti-5T4 scFv immune conjugates that have good affinity for the target antigen. Moreover, when cells expressing 5T4 at the cell surface are transduced, the fusion protein is secreted and binds back to the 5T4 antigen. We are now developing both lentiviral and adenoviral constructs for *in vivo* delivery to tumor cells and also to transduce macrophages *ex vivo*. The intrinsic tumor-targeting properties of the macrophages would target the constructs to both the primary tumor and distant metastases (see Fig 1) and inclusion of a Hypoxic Response Element (HRE), should limit the expression of the fusion proteins to the sites of tumor mass.²⁰ Having targeted the cancer cells with the scFv constructs, it is envisaged that the presence of immune enhancer molecules on the surface of tumor cells may induce further 5T4 bystander immune responses.

Having shown, *in vitro*, that the scFv-Hyl protein can direct lysis against 5T4-expressing tumor cells, we will assess the effectiveness of this and the B7-scFv fusion protein in mouse models. Such models are under development and will provide important information regarding the *in vivo* efficacy of our immune conjugates. At present, taking into account the specificity of expression of the 5T4 antigen and its potential as an immunotherapeutic target and also the encouraging results emerging from a number of clinical trials, we are optimistic that the *in vitro* data we have obtained on the functionality of these constructs can be successfully transferred to mouse models and ultimately the clinic.

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