

Redirecting adenoviruses to tumour cells using therapeutic antibodies:

Generation of a versatile human bispecific adaptor

Snezana Vasiljevic, Emma V. Beale, Camille Bonomelli, Iona S. Easthope, Laura K. Pritchard,

Gemma E. Seabright, Alessandro T. Caputo, Christopher N. Scanlan¹, Martin Dalziel²,

Max Crispin²

Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South

Parks Road, Oxford OX1 3QU, United Kingdom.

¹ This article is dedicated to Chris Scanlan who passed away on the 4th May 2013 after a short battle with cancer.

² To whom correspondence should be addressed, Martin Dalziel, E-mail: martin.dalziel@bioch.ox.ac.uk Tel: +44(0)1865275381 or Max Crispin, Email: max.crispin@bioch.ox.ac.uk, Tel: +44(0)1865275381

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Abstract

Effective use of adenovirus-5 (Ad5) in cancer therapy is heavily dependent on the degree to which the virus's natural tropism can be subverted to one that favours tumour cells. This is normally achieved through either engineering of the viral fiber knob or the use of bispecific adaptors that display both adenovirus and tumour antigen receptors. One of the main limitations of these strategies is the need to tailor each engineering event to any given tumour antigen. Here, we explore bispecific adaptors that can utilise established anti-cancer therapeutic antibodies. Conjugates containing bacterially derived antibody binding motifs are efficient at retargeting virus to antibody targets. Here, we develop a humanized strategy whereby we synthesise a re-targeting adaptor based on a chimeric Ad5 ligand/antibody receptor construct. This adaptor acts as a molecular bridge analogous to therapeutic antibody mediated cross-linking of cytotoxic effector and tumour cells during immunotherapy. As a proof of principle, we demonstrate how this adaptor allows efficient viral recognition and entry into carcinoma cells through the therapeutic monoclonal antibodies Herceptin/trastuzumab and bavituximab. We show that targeting can be augmented by use of contemporary antibody enhancement strategies such as the selective elimination of competing serum IgG using "receptor refocusing" enzymes and we envisage that further improvements are achievable by enhancing the affinities between the adaptor and its ligands. Humanized bispecific adaptors offer the promise of a versatile retargeting technology that can exploit both clinically approved adenovirus and therapeutic antibodies.

Introduction

Adenoviruses are increasingly being exploited as a means of therapeutic intervention in human disease (1) and adenoviral therapy is now a realistic prospect for the treatment of many forms of cancer (2). The anti-tumour activity of adenoviral therapy relies on coupling their innate host-cell lytic ability of the virus with an effective means of altering viral tropism away from its natural targets to those associated with tumour cells.

Adenoviruses offer several significant advantages to tumour cell therapy. They can be replicated to very high titre, transduce a wide variety of cell types, lytically kill their host cell as part of their natural infective cycle, do not integrate into host genomes and possess a viral genome capable of accepting a significant degree of molecular engineering. This latter trait has led to a variety of innovative developments including oncolytic strains that selectively kill tumour cells such as the p53null specific strains H101 and Onyx-015 that have reached phase III clinical trials (3).

Adenoviruses have a broad range of natural targets. Therefore, selectively modifying viral tropism, also known as transductional retargeting, is clearly a key step in the use of adenoviruses in killing any tumour cell *in vivo*. Adenoviruses must be engineered in such a manner that they ignore their natural ligand in favour of a marker presented by tumour cells. A second consideration is that prolonged adenoviral exposure in humans results in not only their effective hepatic clearance, with accompanying liver toxicity, but also immunogenic toxicity as a result of neutralising antibody production. This latter issue can be overcome by engineering out the dominant antigenic determinants of the virus such as the generation of the so-called 'gutless' series of Ad5 (4) or masking them by direct modifications such as PEGylation (5).

Engineering targeting molecules directly into the viral fiber knob has shown promise in altering tropism in favour of tumour cells. These insertions together with selective

mutations to the fiber knob designed to ablate natural receptor recognition have been shown to work against several tumour-associated antigens (TAA). However, a significant drawback is the need to genetically re-engineer the virus for each TAA targeted. Moreover, given the necessity for correct cytoplasmic folding of viral capsid proteins prior to nuclear transport and virion assembly, many preferred TAA ligands, particularly those dependent on disulphide bonds and glycosylation, are unsuitable for this methodology (6). Nevertheless, relatively large high affinity peptide ligands, including affibody antibody mimics (7), can be incorporated into the fiber H-loop that successfully re-target adenoviruses.

The main alternative to recombinant viral capsids is the use of chimeric bispecific adaptor proteins that directly link viral binding to an appropriate retargeting entity. These adaptors fuse either an anti-fiber knob antibody or natural receptor fragment with a second TAA binding domain, such as antibody fragments (8-11) or peptide ligands (12-14). An interesting variant on this theme is the effective use of bifunctional PEG in linking Ad5 with TAA antibody (15, 16). The use of such adaptors, whether chemical or protein, does not impact upon viral infective ability as binding is a sufficiently distinct mechanism from internalization (17). Moreover, the lack of recombination within the viral genome ensures viral replicative fitness. Several such adaptors have been described based on Ad5's high affinity recognition of its natural primary receptor CAR (coxsackievirus and adenovirus receptor) (18, 19). For example, Ad5 binding to trimeric CAR fused to a cluster of anti-HER2 scFv antibody fragments effectively retargets Ad5 to HER2 expressing breast carcinoma cells (9).

Several groups have reported success coupling Ad5 to fragments of protein A, an immunomodulatory virulence protein that generically binds IgG Fc, either engineered directly into the fiber shaft (20-22) or as part of a CAR fusion adaptor (23). These strategies fulfil the criteria of a universal monoclonal antibody Ad5 adaptor that effectively refocuses Ad5 to the TAA dictated by the monoclonal specificity. However, a potential impediment to

the use of such constructs in humans is that protein A, being bacterially derived, is immunogenic and individuals with prior exposure will have developed immunity. Nevertheless, minimising the amount of protein A to just the critical 33 amino acid Ig binding domain Z33 may circumvent this potential pitfall (21, 22, 24).

We now describe the logical next step in making a fully human bispecific adenoviral adaptor that can be used with any IgG based therapeutic monoclonal antibody (Figure 1). We have fused CAR with the IgG Fc binding receptor FcγRIIIa (CD16a). Docking of this adaptor with two test therapeutic antibodies, the clinically licensed anti-HER2 monoclonal Herceptin and bavituximab a current phase III clinical trial anti-phosphatidylserine monoclonal (25), allow effective Ad5 recognition and internalization into carcinoma cells expressing the relevant antigen. Finally, we also demonstrate a useful synergy between this methodology and a means of clearing non-specific serum antibody Fc receptor engagement using the enzyme, endoglycosidase S (EndoS).

MATERIALS & METHODS

Adenovirus strain

All experiments were performed using E1A/E1bΔ replication incompetent Gateway® adapted adenoviral-5 vector (pAd/CMV/V5-DEST) (Invitrogen/Life technologies) recombined with pENTR/D. pTurbo FP650 gene encoding near-infrared fluorescent protein from the pTurbo FP650-C a mammalian expression vector (#FP731, emission wavelength 635nm) was cloned into directional TOPO vector pENTR/D-TOPO (Invitrogen/Life technologies). The pENTR/D-TOPO is designed to facilitate cloning of blunt end PCR products and generate the entry clone into Gateway System. The entry of desired clone containing the FP650 gene is performed by LR recombination reaction between the pENTR/D-TOPO and pAd/CMV/V5-DEST. The *Pac I*-digested vector is used to transfect 293A cells (genetically modified to carry

Ad5 sequences necessary for replication) to produce an adenovirus stock. After amplification in 293A cells the adenovirus stock was titred and frozen at -80°C in 200 µl aliquots at 2×10^8 particles/ml.

CAR-FcγRIIIa construct cloning

CAR cDNA was obtained from Source Bioscience (ORFeomeID 100008823/OCAA69 A07) and FcγRIIIa (Genebank accession: BC033678, CD16a) cDNA was isolated as previously described (26). Chimeric construct was made using overlapping PCR fusion cloning (AmpliTaq Gold 360 Master Mix, Life Technologies) with CAR as the 5' (N-terminus) section and FcγRIIIa 3' (C-term) then inserted AgeI/KpnI into pHLSEC. Relevant primers used were CARrev3: CAGCCTTTGGGAGCGCACCTGAAG and RIIIAfwr3: CCTTCAGGTGCGCTCCCAAAGGCTG for PCR fusion junction amplification; AgeICARFWR: CACACCGGTATCACTACTCCTGAAGAGATG and CARrev1: GATCTTCAGTCCGCGCACCTGAAGG for external chimeric cDNA final cloning and insertion into pHLSec. The amino acid sequence was confirmed as follows:

ITTP EEMIEKAKGETAYLPCKFTLSPEDQGPLDIEWLISPADNQKVDQVILYSGDKIYDDYYPDLK
GRVHFTSNDLKSGDASINVTNLQLSDIGTYQCKVKKAPGVANKKIHLVVLVKPSGALPKAVVFLEPQWYR
VLEKDSVTLKCGAYSPEDNSTQWFHNESLISSQASSYFIDAATVDDSGEYRCQTNLSTLSDPVQLEVHIG
WLLLQAPRWVFKEEDPIHLRCHSWKNTALHKVTYLQNGKGRKYFHHNSDFYIPKATLKDSGSYFCRGLV
GSKNVSSETVNITITQGLSVSTISSFFPPGYQGTHHHHHH

The fusion protein was expressed in HEK293T cells. Cells were grown to 90% confluence and transiently transfected with polyethyleneimine (PEI) using a transfection mix with DNA and PEI in ratio of 1:1.5 (27). Following transfection, cells were grown in DMEM/1% fetal bovine serum at 37°C and 5% CO₂ for 5 days. Protein was purified from cell supernatant by immobilized metal affinity chromatography using chelating sepharose fast

flow Ni²⁺-agarose beads (GE Healthcare, UK) followed by size exclusion chromatography using a Superdex S75 (16/600) column equilibrated in phosphate buffered saline (PBS).

AD5 knob isolation

Ad5 knob cDNA was PCR amplified from construct pAd/CMV/V5-DEST (knobF1: CCGAATTCATGGGTGCCATTACAGTAGGAA; knobR1: CCAAGCTTAATAAGAACCCGTTACATACT). The resulting 588nt product encodes 196 amino acids beginning (M)GAITVGN, the start of the 22nd repeating motif of the shaft of the fiber protein, through to SYIAQE of the carboxy terminus (28). This sequence was then inserted into mammalian expression vector pET23 using EcoRI/HindIII and the resulting construct, named Knob-pET23, subsequently transformed into competent BL21 (DE3) *E. coli* cells. Verified clones were grown to OD₆₀₀ 0.6 and protein expression induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 5 hours of induction, cells were harvested at 10,000 g, 4°C for 20 min. Pellets were frozen overnight at -20°C, and run on SDS-PAGE and subsequently western blotted onto PVDF. Pellets were then resuspended in 50ml buffer of 20mM K₂PO₄ (pH 8.0) and 200 mM NaCl and sonicated for 3 x 1 min with 1 min in between. They were then spun for 30 mins, 13,000g at 4°C followed by immobilized metal affinity chromatography using chelating sepharose fast flow Ni²⁺-agarose beads (GE Healthcare, UK).

Monoclonal antibodies

Herceptin (trastuzumab), a humanized IgG1 monoclonal against HER2/erbB2 (29) was obtained from Genentech/Roche, USA (MTA: OR-214253 and OR-211350). IgG1 b12 monoclonal against HIV gp120 CD4 binding domain has been described elsewhere (30). DNA corresponding to the heavy and light chains of bavituximab, a chimeric (mouse/human)

monoclonal against phosphatidylserine, was synthesised and cloned by AmpliTaq PCR using heavy chain primer pair BaviHfwd: GTAGCTGAAACCGGTGAAGTGCAG and BaviHrev: GTGGTGCTTGGTACCCTTGCCGGG and light chain primer pair BaviLfwd: GTAGCTGAAACCGGTGACATCCAG and BaviLrev GTGGTGCTTGGTACCACACTCGCC. These cDNA were then cloned AgeI/KpnI into pHLSec and transiently transfected into HEK-293T 1:1 (mass) heavy to light chain. Protein was purified from culture supernatant using Ni²⁺ Sepharose 6 Fast Flow column (HisTrap FF, GE Healthcare). Expression and purification was monitored by SDS-PAGE and Western blot using Protein A-HRP.

ELISA verification of CAR-FcγRIIIa construct

The ability of CAR-FcγRIIIa to bind both IgG Fc knob was tested as follows. Recombinant CAR-FcγRIIIa, CAR, or FcγRIIIa alone were coated in a titration from 0.165 to 10 µg/ml onto high-binding microtiter plates (3690, Corning, NY, U.S.A.) overnight at 4 °C. Coated plates were washed with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) in PBS. Recombinant IgG1 b12 (10 µg/ml) expressed from HEK 293T cells (26) was allowed to bind for 1.5 h at room temperature. Plates were washed five times with PBS containing 0.05% Tween and binding was detected using peroxidase-conjugated goat anti-human IgG (Fab'-specific; Pierce). The substrate 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific, U.S.A.) was used for development according to the manufacturer's directions and was stopped by the addition of 2 M H₂SO₄. Absorbance was measured at 450 nm.

HER2 positive breast cancer cell lines

SKBr3 cells that constitutively expressed high levels of HER2 were obtained from Cancer Research UK. MCF-7^{HER2Tet}, a transgenic breast carcinoma cell line based on MCF-7 that can be induced to transiently express HER2 from a stably transfected transgene under the

control of a doxycycline repressed promoter, were provided by Dr Joaquin Arribas, Vall d'Hebron Institute of Oncology, Barcelona, Spain. HER2 induction in this line is as previously described (31), briefly, in order to effectively wash off all doxycycline, cells were washed three times, trypsinised (5 ml), diluted to 30 ml and spun at 1,000g. Cell resuspension was in 5 ml medium followed by dilution to 30 ml, and this process repeated three times.

HER2 FACs

Cell surface HER2 expression was quantitated by FACs using Herceptin. Cells were washed three times in PBS/0.1% BSA, gently scraped and spun at 800g for 5 min. Cells were then incubated with Herceptin (8 µg/ml) on ice for two hours, then washed three times and subsequently incubated with a secondary antibody (mouse anti-human FITC (Sigma F0767) at 1/20 dilution) again on ice, for one hour, washed and spun three times as before. Cells were then diluted to 500 µl in PBS/0.1%BSA/0.1%Na Azide with 2 µg/ml propidium iodide, then run through a FACSCalibur (BD Biosciences) and analyzed using CellQuest Pro software.

EndoS treatment

EndoS was cloned and purified as previously described (32) using a plasmid containing an N-terminally glutathione S-transferase (GST) tagged construct of the full-length codon-optimized EndoS (33). For release of *N*-linked glycans by EndoS, reactions were performed in PBS at a molar ratio of 1:10 EndoS to substrate. The reaction was allowed to proceed for 24 hours. Release of the total or remaining pool of *N*-linked glycans was carried out in solution using protein *N*-glycosidase F (PNGase F; New England Biolabs). Untreated and EndoS-treated IgG were resolved by SDS-PAGE and stained with Coomassie Blue. Following destaining, bands corresponding to IgG heavy chain were excised and washed extensively with acetonitrile and water. Gel bands were then incubated with PNGase F at 5000 U/ml for 16 hours, following manufacturer's instructions. Released glycans were eluted from the gel

with water and dried. They were subsequently labelled with 2-aminobenzoic acid (2-AA) and purified using Spe-ed Amide-2 cartridges, as previously described (34).

Deglycosylation of both serum IgG and Herceptin used in cell culture studies was carried out using an EndoS column where EndoS was conjugated to CNBr activated Sepharose™ beads (Sigma). This was done to avoid carry-over of residual EndoS when treating cells with EndoS treated antibodies. Briefly, Sepharose™ was swelled using 0.1 M HCl and then rinsed with water and finally binding buffer (0.1 M NaHCO₃, 0.5 M NaCl) immediately prior to addition of purified EndoS. EndoS was incubated with the resin for 2 hours at room temperature with constant mixing. Excess ligand was drained before washing the column with binding buffer followed by blocking buffer (0.1 M Tris pH 8.0). The column was then incubated with blocking buffer for 2 hours at room temperature. Five cycles of alternating washes of 0.1 M acetic acid pH 4.0, 0.5 M NaCl and 0.1 M Tris pH 8.0, 0.5 M NaCl, were used to block unreacted groups. The column was then equilibrated into PBS. Serum IgG or Herceptin was added to the column to give an EndoS:antibody ratio of approximately 1:10 which was then incubated for 1 hour at 37 °C with constant mixing. EndoS treated antibodies were eluted from the column by gravity flow.

Normal phase HPLC analysis of N-linked glycans

Fluorescently labelled glycans were separated by normal phase-HPLC using a 4.6 mm × 250 mm XBridge BEH Amide Column (3.5 µm particle size) (Waters, Elstree, UK). The following gradient was run at a flow rate of 1 ml/min: time = 0 min ($t=0$): 35% A, 65% B; $t=6$: 35% A, 65% B; $t=46$: 46% A, 54% B; $t=48$: 80% A, 20% B; $t=50$: 80% A, 20% B; $t=52$: 35% A, 65% B; $t=75$: 35% A, 65% B, where solvent A was 50 mM ammonium formate, pH 4.4, and solvent B

was acetonitrile. Fluorescence was measured using an excitation wavelength of 360 nm and a detection wavelength of 425 nm.

Etoposide mediated induction of cell surface PS

Etoposide was obtained from Sigma-Aldrich (E1383) and dissolved in DMSO at 30 mg/ml. SKBr3 cells were incubated in normal growth media with etoposide (25-100 mM) for 20 hours and washed three times in normal growth media before further experiments. A mock treated control using the same volume of DMSO alone was included for each appropriate sample. Cells were either used for Ad5/CAR-Fc γ RIIIa/bavituximab infection using the same parameters as Herceptin or stained for cell surface PS expression by Annexin-V^{FITC} (Dead cell apoptosis kit, Invitrogen V13242) using the protocol provided.

Viral transduction of cell lines

Chimeric CAR-FcR γ IIIa protein (1 μ M) was bound to Ad5-turboFP650 (1×10^7 particles) and relevant monoclonal antibody (1 μ g) in 1ml of serum free medium for one hour at room temperature. Each reaction complex was then added to washed cells (serum free medium) in a single well of a six well plate and incubated for one hour in CO₂ humidified incubator at 37°C. Cells were then aspirated and fed with normal media and left overnight. 48 hours post-induction, cells fluorescence was visualized using a fluorescent microscope (Nikon TE2000-U/ACT-1 software).

RESULTS

Cloning and expression of CAR-Fc γ RIIIa.

Expression and purification of CAR-FcγRIIIa by immobilized metal affinity chromatography and size exclusion chromatography generated a primary protein species of the correct molecular weight when stained with Coomassie on SDS-PAGE (Figure 2). The ability of this chimeric protein to efficiently bind both Ad5 knob attachment spike and antibody Fc (IgG1 b12 monoclonal) via its CAR and FcγRIIIa domains respectively was confirmed by ELISA (Figure 2C).

Directed Ad5 transduction of HER2 positive breast carcinoma cells.

SKBr3 breast carcinoma cells express high amounts of cell surface HER2, the target of the therapeutic monoclonal antibody Herceptin (Figure 3A). Chimeric CAR-FcγRIIIa protein was bound to Ad5-turboFP650 and either Herceptin or the control anti-gp120 monoclonal IgG1 b12. When these complexes were introduced to HER2 expressing breast carcinoma cell line SKBr3, significant Ad5 cellular transduction was only observed when the CAR-FcγRIIIa adaptor was used in combination with Herceptin (Figure 3B). In contrast, the anti-gp120 monoclonal b12 failed to illicit any significant transduction using these same cells (Figure 3B). The failure of Ad5 alone to gain entry to SKBr3 cells is consistent with their reported low CAR expression (35, 36). Given that SKBr3 cells do express very high amounts of HER2 receptor (~2 million/cell), and coupled with their very low CAR expression represent a potentially artificially skewed signal over noise system, we decided to verify these observations using a cell line where HER2 expression can be manipulated experimentally.

Ad5 transductional dependence on Herceptin-HER2 binding was tested using the conditional HER2 expressing cell line MCF-7^{HER2Tet}. Whilst under doxycycline repression, this cell line expresses small amounts of HER2 (Figure 4A). It is unclear whether this basal expression of HER2 represents leaky repression or endogenous expression. Consistent with these observations, little or no Ad5 transduction could be observed with Herceptin (Figure 4B). In contrast, cells extensively washed and grown for 48 hours in the absence of

doxycycline express HER2 (Figure 4B) at levels comparable with SKBr3 cells (although the broad base of the FACs signal suggests a very heterogeneous population), and high levels of Ad5 transduction could be achieved using Herceptin (Figure 4B). Like SKBr3 cells, the failure of Ad5 to gain entry to MCF-7 is likely due to the lack of endogenous CAR (36).

EndoS overcomes the inhibition of Ad5 transduction by serum IgG. The specific activity of EndoS toward both Herceptin and commercial serum derived polyclonal IgG was confirmed by HPLC (Figure 5A and 5B respectively). In both cases, EndoS removed all glycan variants with the notable exception of some glycans containing either bisecting *N*-acetylglucosamine (GlcNAc) and/or disialylated termini found in serum IgG. These IgG glycans are more resistant to EndoS, as previously been reported (32). In contrast with serum IgG, Herceptin showed no detectable sialylated structures. Further, Herceptin also lacks bisecting GlcNAc glycans typical of serum IgG i.e. neutral bisecting GlcNAc (at 19 min), monosialylated bisecting GlcNAc at 29 min, disialylated bisecting GlcNAc at 34 min. However, a single peak is observed at around 16 min, consistent with a bisecting G0F structure. However, we interpret this species as a $\text{Man}_5\text{GlcNAc}_2$ structure for the following reasons, (1) $\text{Man}_5\text{GlcNAc}_2$ also is detected here (superimposition of the traces reveals that these 16 min peaks are slightly different; (2) It is α -mannosidase sensitive but β -galactosidase resistant (data not shown); (3) It is EndoS sensitive, unlike the bisected agalactosylated and fucosylated biantennary glycan which is partially resistant (Figure 5A); and (4) Significant levels of $\text{Man}_5\text{GlcNAc}_2$ structures are expected with Herceptin as previously published (37).

EndoS deglycosylation of IgG has been reported to impede Fc engagement with several activatory Fc γ receptors (38). Therefore, we sought to confirm the dependence upon Herceptin-Fc γ R1IIa binding of Ad5 entrance into SKBr3 cells using EndoS. As predicted, Herceptin treated with EndoS was no longer capable of mediated Ad5 entry into SKBr3 cells (Figure 5C, panels 1-2). Given that EndoS had no effect on Herceptin's ability to bind HER2

using FACS, we conclude that EndoS is likely preventing Herceptin-FcγRIIIa engagement, consistent with previous studies (38, 39).

EndoS has been proposed as a means of enhancing antigen-bound monoclonal antibody signalling *in vivo* via FcγR by prior clearing of irrelevant serum IgG that has engaged effector cell FcγR in preference to the therapeutic monoclonal being used (40). Serum IgG also has the potential to impede therapeutic antibody mediated Ad5 targeting by actively competing for occupancy of the FcγR component of the chimeric adaptor. We demonstrate that serum derived polyclonal IgG does indeed inhibit Herceptin mediated Ad5 entry into SKBr3 cells and that EndoS pre-treatment of serum IgG effectively abolishes this effect (Figure 5C, panels 1-4).

Directed Ad5 transduction of PS positive breast carcinoma cells.

To demonstrate that the chimeric adaptor is able to accommodate any therapeutic monoclonal antibody and retain the functional link between Ad5 infective ability and antibody antigen specificity, we applied our re-targeting system to a second monoclonal, bavituximab, developed by Peregrine Pharmaceuticals. This chimeric mouse/human IgG1 monoclonal antibody target cells with elevated PS such as in solid tumour blood vessels (25, 41) and is currently undergoing phase III non-small cell lung cancer clinical trials (42). PS is normally expressed on the cytosolic side of the plasma membrane. However, upon pathophysiologic stress PS is relocated to the outer membrane as a signal for macrophage engulfment. Various solid tumours express significant amounts of PS in response to hypoxia, particularly on their blood vessels, leading to the development of bavituximab as a potential anti-vascular therapy in cancer treatment.

We used etoposide, a topoisomerase II inhibitor currently in use as a cytotoxic anti-cancer chemotherapeutic agent, to induce cell surface PS through pathophysiological stress in SKBr3 cells. PS expression was detected using Annexin-V^{FITC}, a routine monitor of PS

induction during cellular apoptosis. Treatment of SKBr3 cells with 25-100 μ M etoposide for 20 hours was sufficient to increase Annexin V staining in treated cells relative to mock treated controls (Figure 6A, top row and Figure 6B, second row). Elevated Annexin V staining, indicative of external membrane PS expression was concomitant with significant cell growth arrest (Figure 6B, top row), a known effect of etoposide.

When etoposide treated/Annexin-V positive cells were incubated with bavituximab mixed with CAR and Ad5, a strong fluorescent signal was seen relative to mock treated cells (Figure 6A, bottom row panel; Figure 6B, bottom row). That this signal was not as homogeneous as that seen with Herceptin is explicable by the significant loss of viable cells upon etoposide treatment (Figure 6B, top panel).

DISCUSSION

We have demonstrated a human bispecific adaptor protein that can successfully refocus adenovirus toward TAAs using therapeutic antibodies. The inherent versatility of this system means that it is in principle capable of use with any IgG based therapeutic antibody. Monoclonal antibodies represent one of the largest and fastest growing pools of established pharmaceutical agents against TAAs, an expansion driven by, amongst other things, engineering affinity/effector function improvements, new TAA identification, therapeutic effector conjugation and the generation bispecific antibodies geared towards tumour therapy. The ability of our adaptor to tap into this diverse resource without the need for the development of individual adenovirus components could potentially broaden the applicability of adenoviruses to cancer therapy, particularly multiple TAA personalised therapy. Moreover, being based on human sequence, this adaptor should minimise immunogenicity issues potentially associated with similar technological entities associated with bacterially derived sequences. However, possible chimeric protein neoantigenicity cannot be ruled out at this stage.

Whole animal imaging with fluorescently modified adenoviral constructs is a key technique in monitoring adenoviral delivery to the site of tumour growth *in vivo*. The coupling of our Ad5 system to Turbo650 fluorescence should be a significant advantage in monitoring the performance of the bispecific adaptor with relevant *in vivo* models (43). Given that fluorescence is a consequence of viral replication, this system is ideal for monitoring both tumour targeting as well as viral escape. One potential limitation could be serum half-life. However, although the adaptor is below the threshold for kidney filtration its half-life may well be extended by interaction with the immunoglobulin pool.

Several issues also remain unaddressed before this system can be tested *in vivo*. Although Ad5 knob-CAR association is of reasonably high affinity, endogenous multimeric CAR mediated viral escape might still be possible *in vivo*. One solution is the use of specific

knob mutations that prevent CAR binding coupled with combinatorial library mediated evolution of CAR to match. Recent work has shown that bispecific DARPIn based adaptors can be engineered by ribosome display to bind with very high affinity to an adenoviral strain with precise mutations that ablate its natural tropism (44). Further, when used in a trimeric modular form, DARPIn-adenoviral particles remain fully bound for longer than 10 days with no deleterious impact upon viral target cell entry (45). Whilst these systems can be fused with DARPins against tumour antigens for highly efficient delivery, they do have the ease-of-use drawback entailed in recloning for each novel TAA being targeted. Therefore, our system, fused with elements of the DARPIn technology, or similar should allow for the generation of a stable Ad5-universal adaptor association *in vivo*.

A similar argument can be made for Fc-FcγR disassociation and loss of monoclonal mediated targeting. For convenience we used a relatively weak FcγR and switching to a higher affinity variant such as FcγRI may mitigate the problem of dissociation *in vivo*. There is scope to employ combinatorial libraries to enhance this interaction still further. Moreover, given that our system is antibody based, it is therefore amenable to contemporary strategies designed to enhance *in vivo* antibody efficacy, including overcoming competitive serum IgG. We have shown that IgG inactivating bacterial immune evasion enzymes, in this case EndoS may be useful in this context. Although, given the shared serum IgG/therapeutic monoclonal EndoS sensitivity, differential treatment timing would obviously be required. A further factor that could limit efficacy is the Ad5-adaptor disassociation as Ad5-adaptor can theoretically re-associate with the monoclonal either free or bound to its target, especially if the monoclonal is given in excess. Effective adenoviral therapy requires not only efficient delivery and entry of the virus to the site of the tumour itself, but ideally an immediate refocusing of newly synthesised viral particles liberated upon tumour cell lysis to perpetuate viral dissemination throughout the tumour. Assuming further antibody/adaptor delivery,

clearance of serum IgG competition by EndoS (or similar) would aid this process by allowing new virion particles to re-engage free or pre-bound therapeutic anti-TAA antibodies.

The use of IgG-deactivating bacterial enzymes may not be considered practicable for two reasons: (1) it would erode the patient's immune system and (2) immunogenicity of EndoS may have safety implications. These concerns are valid, however, we note that the use of a similar IgG-degrading bacterial enzyme, IdeS, has passed Phase I clinical safety trials (46). The immunogenicity concerns could be side stepped if EndoS/IdeS is only used once as a primer to boost the initial efficacy of the therapy.

Targeted oncolytic adenoviruses have gained significant scientific and medical acceptance as a credible treatment of several forms of human cancer. This in turn is driving a significant body of research geared toward improving their therapeutic efficacy through maximising the efficiency of several critical parameters including antigenicity, targeting and tumour dissemination. The development of various technologies, including our human adaptor antibody mediated system, aimed at providing flexible TAA targeting coupled with simplicity of use will go some way to facilitating the versatility of adenoviruses as a widespread immunotherapeutic agent.

FIGURE LEGENDS

Figure 1. Theory and construction of a chimeric CAR-FcγRIIIa Ad5 adaptor. Cartoon of proposed adaptor mechanism showing Ad5 bound to a monoclonal antibody via an intermediate CAR-FcγRIIIa adaptor protein. Colours used to identify specific components are Ad5: purple, CAR: blue, FcγRIIIa: orange and monoclonal antibody: green. The zoom box displays a putative molecular model generated using the deposited crystal structures of Ad12 knob-CAR D1 (PDB 1KAC) and the IgG1 Fc-FcγRIIIA (PDB accession codes 1KAC and 1T83, respectively) depicted using the same colour scheme. The model highlights the key binding events mediated by this adaptor, specifically Ad5 knob/CAR and FcγRIIIa/IgG Fc.

Figure 2. Expression of a chimeric CAR-FcγRIIIa Ad5 adaptor. **A.** Purification of CAR-FcγRIIIa from HEK293T cell medium by size-exclusion column following immobilised metal affinity chromatography. **B.** Peaks from size-exclusion chromatography analysed by SDS-PAGE analysis. **C.** ELISA of IgG1 b12 binding to three plated test proteins. ELISA error bars represent standard deviation and curves are present to aid visualisation.

Figure 3. CAR-FcγRIIIa adaptor allows Herceptin mediated Ad5 entry into HER2 positive breast carcinoma cell line SKBr3. **A.** Confirmation that the monoclonal antibody Herceptin recognises cell surface HER2 on the breast carcinoma cell line SKBr3 as detected by FACs analysis. Broken black trace: secondary alone, red trace: Herceptin. **B.** Series of fluorescent microscopy images showing TurboFP650 expression, indicative of cellular Ad5 genome transcription, in SKBr3 cells transduced with different combinations of Ad5, adaptor and monoclonal antibody. Construct combinations are indicated to for each panel. Ad5 alone (1×10^7) or in combination with CAR-FcγRIIIa (10 μ M) gave no detectable signal 48 hours post

infection. Upon introduction of monoclonal antibodies b12 and Herceptin (both 1 µg/ml), a strong fluorescent signal was only observed with Herceptin.

Figure 4. CAR-FcγRIIIa adaptor allows Herceptin mediated Ad5 entry into breast carcinoma cell line MCF-7 cells only when transiently expressing HER2. **A.** CMV promoter driven expression of HER2 cDNA upon removal of doxycycline repression in MCF-7^{HER2Tet} cells was confirmed using FACS quantitation of Herceptin binding. Grey dashed traces: secondary alone (left peak is doxycycline exposed cells, right peak is doxycycline exposed cells), green trace: Herceptin staining of doxycycline exposed cells, red trace: Herceptin staining of doxycycline negative cells. Relative Herceptin signal over secondary alone for both data pairs is illustrated by green (Dox⁺) and red (Dox⁻) lines above the traces. **B.** A series of fluorescent microscopy images showing TurboFP650 expression 48 hours after infection of MCF-7 cells (grown in the presence or absence of doxycycline) with Ad5/CAR-FcγRIIIa alone or in combination with Herceptin (1 µg/ml).

Figure 5. EndoS enhances Herceptin FcγRIIIa binding in the face of non-specific polyclonal serum antibody competition. The bacterial endoglycosidase EndoS is highly specific for IgG N-glycan removal and has been shown to modulate antibody function, particularly FcγR engagement. EndoS cleavage of Herceptin glycans (**A**) and human polyclonal serum IgG (**B**) as shown by PNGaseF released fluorescently labelled glycan HPLC (black trace: untreated, blue trace: EndoS treated). Peaks were assigned using negative ion ESI-MS/MS and are labelled using Oxford glycan nomenclature (26, 47) with the colour scheme of the Consortium for Functional Glycomics as previously implemented (26, 48). Only neutral biantennary N-glycans were found on Herceptin, including a small amount of partially

processed Man₅ GlcNAc₃ structures, consistent with previous reports (37). EndoS effectively removed all of these glycans from Herceptin. Human serum polyclonal IgG also yielded N-glycan species predicted from multiple past studies, i.e. primarily neutral diantennary with small amounts of mono-sialylated and even less disialylated structures present. All sialylated structures are depicted as α 2,6 linked (49). EndoS was also effective at removing all of these glycans with only two minor exceptions. N-glycans terminating in disialylated monosaccharides appear fully resistant, and those containing bisecting GlcNAc partly so, as previously reported (32). EndoS removal of Herceptin glycans ablates this antibody's ability to mediate Ad5 entry into SKBr3 cells. **C.** Panels 1 and 2, consistent with both the need for Fc binding to CAR-Fc γ RIIIa in this assay and the ability to EndoS to impair IgG Fc γ R binding (38). Panels 3-4 show that pre-incubation of the Herceptin-complex with human serum polyclonal IgG significantly impedes Ad5 entry into SKBr3 cells, most likely due to competition between Herceptin and serum IgG for the Fc γ RIIIa. Finally, in Panels 5 and 6, Herceptin mediated Ad5 entry into SKBr3 cells can be restored upon EndoS treatment of serum polyclonal IgG.

Figure 6. CAR-Fc γ RIIIa adaptor allows bavituximab mediated Ad5 entry into cells expressing cell surface phosphatidylserine. Evidence that a second therapeutic monoclonal bavituximab, will upon induction of its preferred antigen phosphatidylserine (PS), mediate Ad5 infection of tumour cells. Series of microscopy images showing TurboFP650 fluorescence, indicative of cellular Ad5 genome expression, in SKBr3 cells induced to express PS by pre-treatment with etoposide and transduced with different combinations of Ad5, adaptor and the therapeutic monoclonal bavituximab. **(A).** Ad5 entry into etoposide (24 hours, 100 μ M) induced PS, as detected by Annexin V (panels 1 and 2), expressing SKBr3 cells is dependent on the presence of bavituximab (panels 5 and 6). **(B).** Series of etoposide treatment indicate that bavituximab mediated Ad5 fluorescence is likely under-represented

due to the inhibition of cell proliferation by etoposide (top row). This might explain why this phenomenon is only partially dose dependent with respect to PS induction by different etoposide concentrations (Annexin V: second row, bavituximab mediated Ad5 infection fluorescence, third row).

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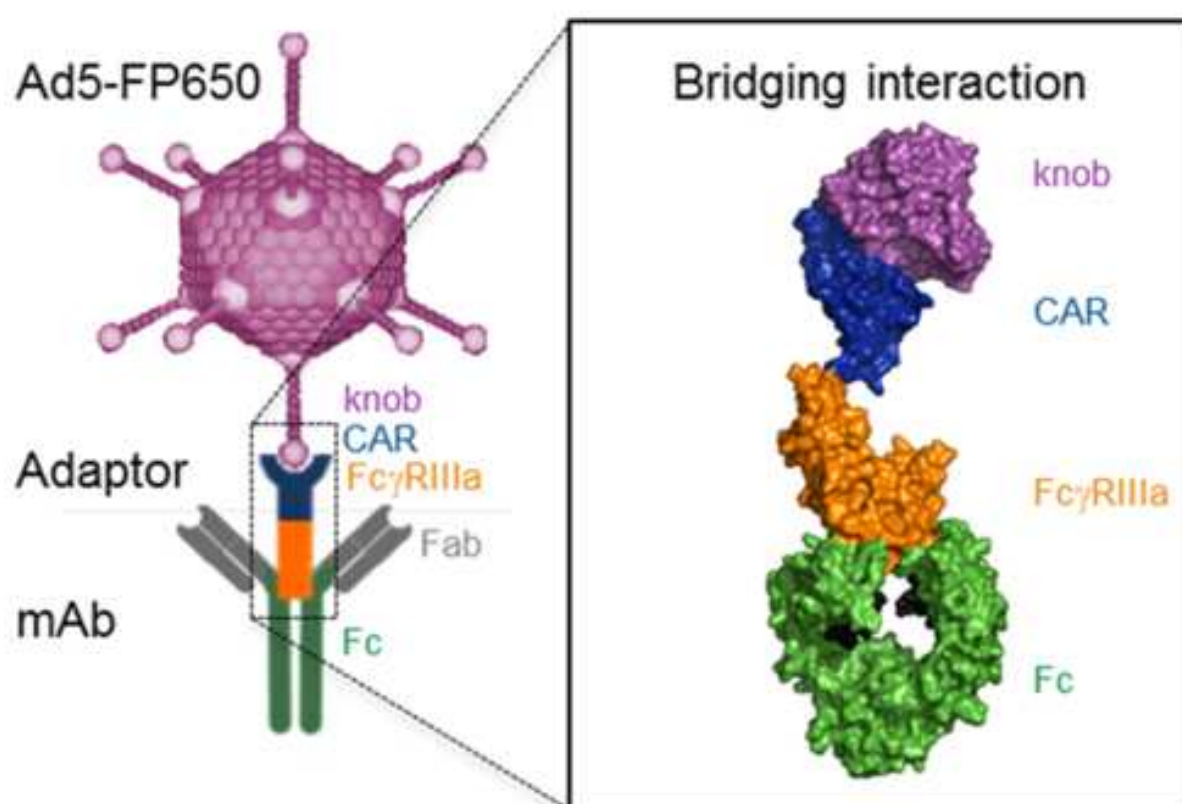
FOOTNOTES

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Abbreviations: Ad5, Adenovirus-5; CAR, coxsackievirus and adenovirus receptor; DMEM, Dulbecco's Modified Eagle's Medium; Dox, doxycycline; Endo S, endoglycosidase S; FBS, fetal bovine serum; Fuc, fucose; Glc, glucose; GlcNAc, N-acetylglucosamine; GST, glutathione S-transferase; HEK, human embryonic kidney; IgG, immunoglobulin G; IPTG, isopropyl β -D-1-thiogalactopyranoside; Man, mannose; PAGE, polyacrylamide gel

electrophoresis; PBS, phosphate-buffered saline pH 7.4; PEI, polyethyleneimine; PS, phosphatidylserine; P/S. penicillin/streptomycin; PNGase F, peptide *N*-glycosidase F; SA sialic acid; SDS, sodium dodecyl sulfate; TAA, tumour associated antigen.

Figure
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Figure

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