Mesenchymal stem cells expressing TRAIL lead to tumour growth inhibition in an experimental lung cancer model

Andrea Mohr [a, #], Mark Lyons [b, #], Laura Deedigan [a], Tina Harte [b], Georgina Shaw [b], Linda Howard [b], Frank Barry [b], Tim O'Brien [b, c], Ralf Zwacka [a, b] *

[a] Molecular Therapeutics Group, National Centre for Biomedical Engineering Science,
National University of Ireland, Galway, Ireland
[b] Regenerative Medicine Institute (REMEDI), National Centre for Biomedical Engineering Science,
National University of Ireland, Galway, Ireland
[c] Department of Medicine, National University of Ireland, Galway, Ireland

Abstract

Lung cancer is a major public health problem in the western world, and gene therapy strategies to tackle this disease systemically are often impaired by inefficient delivery of the vector to the tumour tissue. Some of the main factors inhibiting systemic delivery are found in the blood stream in the form of red and white blood cells (WBCs) and serum components. Mesenchymal stem cells (MSCs) have been shown to home to tumour sites and could potentially act as a shield and vehicle for a tumouricidal gene therapy vector. Here, we describe the ability of an adenoviral vector expressing TRAIL (Ad.TR) to transduce MSCs and show the apoptosis-inducing activity of these TRAIL-carrying MSCs on A549 lung carcinoma cells. Intriguingly, using MSCs transduced with Ad.enhanced-green-fluorescent-protein (EGFP) we could show transfer of viral DNA to cocultured A549 cells resulting in transgenic protein production in these cells, which was not inhibited by exposure of MSCs to human serum containing high levels of adenovirus neutralizing antibodies. Furthermore, Ad.TR-transduced MSCs were shown not to induce T-cell proliferation, which may have resulted in cytotoxic T-cell-mediated apoptosis induction in the Ad.TR-transduced MSCs. Apoptosis was also induced in A549 cells by Ad.TR-transduced MSCs in the presence of physiological concentrations of WBC, erythrocytes and sera from human donors that inhibit or neutralize adenovirus alone. Moreover, we could show tumour growth reduction with TRAIL-loaded MSCs in an A549 xenograft mouse model. This is the first study that demonstrates the potential therapeutic utility of Ad.TR-transduced MSCs in cancer cells and the stability of this vector in the context of the blood environment.

Keywords: mesenchymal stem cells ● TRAIL ● lung cancer ● apoptosis ● adenovirus

Introduction

Current treatments for most metastatic cancers have poor outcomes due to the dispersed nature of the disease, toxicity of the therapeutic agent and inaccessibility of some of the tumour sites. The development of a successful treatment for metastatic cancer has long been one of the most elusive goals of cancer gene therapy. While many systems have been developed that induce high levels of apoptosis in cancer tissue, selectively delivering the therapeutic transgene to widely disseminated metastatic sites *via* the blood stream has often proven unachievable. The hurdles that a therapeutic vector faces in the blood stream are manifold and include the innate and adaptive immune response, potential adsorption to erythrocytes and clearance by specialized cells in the liver and spleen.

Recently, mesenchymal stem cells (MSCs) have begun to show promise as a potential delivery vector for a number of reasons. These cells may be transduced to high levels with adenovirus and lentivirus [1]. They have immune privilege as they do not express the costimulatory molecules B7-1, B7-2, CD40 and CD40 ligand [2–4]. This property of MSCs may be important in protecting the cells from immune responses against the vector or transgene that they are carrying. These cells also secret prostaglandin E2, transforming growth factor β and hepatocyte growth factor, which

[#]Both authors contributed equally.
*Correspondence to: Dr. Ralf ZWACKA,
NCBES, National University of Ireland,
Galway, University Road, Galway, Ireland.
Tel.: +353-91-495166
Fax: +353-91-495547
E-mail: ralf.zwacka@nuigalway.ie

regulate the immune response of T cells to novel antigens thereby decreasing the chances of a cytotoxic T-cell response to the transduced cells [2, 5]. MSCs have also been shown to migrate towards tumours in response to chemokines such as stromal cell-derived factor (SDF)-1, hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) produced by tumour cells [6, 7]. MSCs are also capable of extravasating towards tumours when introduced into the organism *via* the local blood stream [8]. It appears that tumour growth requires formation of supportive mesenchymal stroma and that the tumour stroma formation is similar to tissue remodelling during wound healing that is characterized by high proliferation of mesenchymal cells. MSCs have greater potential and capacity to contribute to the population of stromal cells than fully differentiated fibroblasts. This allows the development of therapeutic strategies that are based on the local production of tumouricidal biological agents by gene-manipulated MSCs [9].

In order to use MSCs as transgene delivery vehicles they would ideally be transduced with an agent that will infect a high percentage of MSCs and express high levels of transgene. Adenovirus type 5 (Ad5) has undergone extensive evaluation as a gene therapy vector due to its ease of production to high titre, high levels of transgene expression and the potential to produce the virus to good manufacturing practice (GMP) standards [10]. Adenovirus also has a good safety profile and has been used in a wide range of clinical trials though high doses of the virus can induce potentially lethal immune responses [11, 12]. Also, in the context of systemic delivery adenovirus may be hampered by innate and adaptive immune responses [13, 14]. Up to 96% of Western healthy adults have antibodies against Ad5 with approximately 55% of these being neutralizing [15]. Repeat administration of the virus into donors results in high levels of vector-specific antibodies, which impair transgene delivery [16]. Furthermore, destruction of transgenic protein producing cells by the immune system has been shown to have hampered many of the adenovirus-based gene therapy strategies in the past [17]. Adenovirus is bound and sequestered by erythrocytes at titres up to those that may be safely administered to a human patient [18]. These issues have been addressed by other workers by modifying the surface epitopes of the virus capsid or masking the virus with synthetic polymers [19–22]. However, these technologies, while promising, have not yet been shown to be capable of bypassing all of the body's immune defences. Given the particular properties of MSCs it is envisaged that these cells may act as cellular vectors and transgenic protein factories without inducing an immune response. MSCs have previously been shown to be effective anti-cancer agents when transduced with adenoviruses expressing a range of transgenes including interferon (IFN)-β and interleukin (IL)-12 as well as oncolytic adenovirus [8, 23, 24].

Many of the current therapeutic modalities for the treatment of cancer are limited by their toxicity to healthy tissue. Consequently, tumour-specific apoptosis-inducing agents are being sought and one potentially promising candidate that fulfils this criterion is tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Healthy tissue has been shown to express both the active and decoy receptors for TRAIL and is resistant to TRAIL-induced apoptosis, interestingly however, many cancer cell lines have been shown to be sensitive to TRAIL-induced apoptosis [25, 26]. Recombinant TRAIL protein and an agonistic antibody to TRAIL-R2 have been shown to be safe for administration to humans at doses consistent with those shown to be effective in xenograft models [27, 28]. Gene therapy approaches utilizing TRAIL have also been shown to be effective in experimental tumour models, and murine embryonic stem cells expressing TRAIL have been demonstrated to be effective in the treatment of malignant glioma *in vitro* [29–32].

While many tests have been performed on the suitability of MSCs as vectors in animal models there are no published data examining the stability and efficacy of these adenovirus-transduced MSCs in the context of human blood from donors that have pre-existing anti-adenovirus immunity. In this paper we describe the ability of TRAIL expressing, adenovirus transduced, MSCs to block tumour growth in a mouse model and to induce apoptosis in a relatively TRAIL refractory epithelial lung cancer cell line (A549 cells). We demonstrate that adenovirus-transduced MSCs do not induce peripheral blood mononuclear cells (PBMCs) proliferation. We also describe how these cells are capable of inducing apoptosis in A549 cells in the presence of neutralizing antibodies, white blood cells (WBCs) or erythrocytes at physiological concentrations, which normally block or inhibit adenovirus-mediated transgene delivery.

Materials and methods

Reagents

All reagents were supplied by Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. Recombinant human TRAIL was supplied by R&D Systems (Minneapolis, MN, USA). The CD105 antibody and isotype control were from BD Bioscience (San Jose, CA, USA). The pan-caspase inhibitor zVAD was bought from Santa Cruz (Santa Cruz, CA, USA).

Adenovirus preparations

Three Ad5-based vectors were used in these studies, Ad.EGFP, Ad.BGal, and Ad.TR each deleted of E1 and E3 and with a reporter gene (EGFP, β-galactosidase), or TNF-related apoptosis-inducing factor (TRAIL) inserted in E1 under the control of the cytomegalovirus (CMV) promoter. All viruses were grown on 293 cells and purified by double banding on CsCl gradients. Virus concentration was determined by plaque assay.

Isolation and expansion of human MSCs and cell culture

MSCs were obtained through the Regenerative Medicine Institute at the National University of Ireland, Galway. Bone marrow aspirates were obtained from the iliac crest of normal donors; all procedures were performed with informed consent and approved by the Clinical Research Ethical Committee at University College Hospital, Galway. MSCs were

isolated and expanded in culture as described previously by direct plating [33]. Aspirates were washed with medium (Dulbecco's Modified Eagle Medium (DMEM)-low glucose containing 1% antibiotic) and centrifuged; the precipitated cells were suspended in medium with 10% selected foetal bovine serum (FBS, Hyclone, South Logan, UT, USA) and plated at a final density of approximately 3.0×10^5 cells/cm^2. Cells were seeded in T-175 flasks and maintained at 37°C with 95% humidity and 5% CO$_2$ in the same medium. After 5 days, red blood cells were washed off with phosphate-buffered saline and fresh medium added. Colonies of adherent cells formed within 9 days. At the end of primary culture, adherent colonies were detached by treatment with 0.25% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) and hMSCs cryopreserved in 10% Dimethylsulfoxide (DMSO)/90% FBS until used. MSCs were cultured in DMEM (low glucose) (Sigma) 10% FBS (Hyclone), 1% Pen/Strep (= MSC medium). The ability of cells to differentiate into osteocytes and adipocytes was confirmed before use. Osteogenesis was induced in the presence of dexamethasone (100 nmol/l), ascorbic acid (50 µmol/l), and ß-glycerophosphate (10 nmol/l). Calcium deposition was detected using Von Kossa staining. Adipogenesis was induced in the presence of methylisobutylxanthine (0.5 mmol/l), dexamethasone (1 µmol/l), insulin (10 µg/ml) and indomethacin (200 µmol/l). Accumulation of lipid deposits was detected using Oil Red O (32). A549 cells were obtained from ATCC and grown in Roswell Park Memorial Institute (RPMI) 10% FBS, 2 mM L-glutamine, 1% Pen/Strep. HCT116 cells were obtained from ATCC and grown in McCoy's 10% FBS, 1% Pen/Strep. Human primary fibroblast were obtained from the Department of Dermatology, University of Ulm and grown in DMEM 10% FBS, 1% Pen/Strep.

Transduction of MSCs

MSCs were plated at a density of 10^5 cells per well in a 6-well plate (Sarstedt, Nuembrecht, Germany) and left to adhere overnight. Cells were untransduced or transduced with virus at 100 pfu/cell (unless otherwise stated) and the plates were spun at $600 \times g$ for 90 min. at 37°C. The virus was left on the cells overnight and then washed off.

MSC-mediated apoptosis induction

A549 cells were plated in 24-well plates at 10^5 cells per well and left overnight to adhere. MSCs or adenovirus-transduced MSCs were then added at a concentration of 10^4 cells per well (unless otherwise stated). Forty-eight hours after the addition of MSCs, the cells were trypsinized. The supernatant, wash fraction and trypsin/cell suspension were centrifuged at $400 \times g$ for 5 min. The supernatant was removed and the cell pellet was re-suspended in Nicoletti assay buffer containing propidium iodide [34]. Cells were assayed for DNA fragmentation after 24–48 hrs by flow cytometry.

Blood cell preparation

Erythrocytes were prepared by collecting blood by venepuncture of the cephalic vein from normal donors after obtaining consent. The procedure received ethical approval from the NUI Galway research ethics committee. Blood was collected into syringes containing heparin anti-coagulant buffer. The blood was centrifuged at ($1350 \times g$) for 10 min. and the plasma was removed. The cell pellet was then washed four times with PBS. The pellet was then re-suspended in MSC medium at physiological concentration (5×10^9 cells/ml). WBC were prepared by pelleting 1 ml of whole blood as previously described. The pellet was re-suspended in 5 ml H$_2$O for 20 sec. and immediately afterwards 1 ml of $6\times$ saline was added. The WBC were pelleted, the supernatant was discarded and the cells were re-suspended in RPMI 1640 10% FBS, 2 mM L-glutamine, 1% Pen/Strep, 10 mM HEPES, 50 µM β-mercaptoethanol (PBMC medium). PBMCs were prepared by taking blood and diluting it 1:1 in RPMI. This mixture was then layered onto half its volume of Ficoll Hypaque Premium (GE Healthcare, Bucks, UK). The preparation was then spun at $800 \times g$ for 30 min. The PBMC layer was removed using a pastette and diluted in 10 ml PBS and centrifuged at $600 \times g$ for 5 min. The supernatant was discarded and the cell pellet was washed in 10 ml PBS. The cells were then re-suspended in PBMC medium. Serum was prepared by taking blood by venepuncture without anti-coagulant. The blood was left to clot and then agitated with a pipette until liquid. This suspension was then centrifuged at ($1350 \times g$) for 10 min. and the serum fraction was removed. The serum was then heat inactivated at 56°C for 30 min.

Stimulation of T cells

PBMCs were prepared as described above. PBMCs were then stained with CFSE dye (Vybrant CFDA SE Cell Tracer kit; Molecular Probes, Invitrogen, Carlsbad, CA, USA) by suspending the cells in 5 µM CFSE in PBS for 10 min. at 37°C. The reaction was stopped by adding 5 vols. ice cold PBMC medium and the cells were washed three times. The cells were then left overnight to facilitate removal of excess dye before being used for assays. 10^5 CFSE-stained PBMCs were added to each well of a 96-well round bottom plate. Pre-prepared untransduced or Ad-transduced MSCs were added to these wells at a concentration of 10^4 MSCs per well to assess their ability to induce T-cell proliferation. As a control PBMCs were also stimulated by the addition of 2.5 µl CD3/CD28 beads (Dynal, Invitrogen, CA, USA). Proliferation was determined after 5 days by flow cytometry, gating on the lymphocyte population.

Western blot analysis

Cells were harvested by trypsinization and lysed in cell lysis buffer containing 50 mM Tris pH 7.4, 10% glycerol, 0.5% NP40, 150 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM KCl and Complete Mini Protease Inhibitors (Roche, Basel, Switzerland). Twenty-five µg protein was loaded per track. Cell lysates were subjected to 10% SDS-PAGE. Protein detection was performed by diluting anti-human Trail Ab (Peprotech, Rocky Hill, NJ, USA) 1:200 or anti-CuZnSOD Ab (The Binding Site, Birmingham, UK) 1:2000 in TBS 0.1% Tween, 5% Marvel milk powder (Premier Brands, Spalding, UK). The secondary anti-rabbit IgG antibody (TRAIL) and anti-sheep IgG antibody (CuZnSOD) conjugated to horse-radish-peroxidase (Santa Cruz) were added at a dilution of 1:5000. For the E1A Western blot a mouse-anti-E1A antibody (BD Bioscience, Franklin Lakes, NJ, USA) diluted 1:500 and an antimouse IgG antibody conjugated to horse-radish-peroxidase (Santa Cruz) diluted 1:2000 were used. Detection was performed using ECL Western blot chemiluminescence reagent (GE Healthcare) and a Fluorochem imaging system.

Quantitative polymerase chain reaction

DNA samples were prepared for Q-PCR by extraction of cell lysates with a QIamp DNA Minikit (Qiagen, Hilden, Germany). Real-time PCR was

carried out using the ABI Prism 7000 sequence detector (Applied Biosystems, Warrington, UK). PCR primers for the Ad5 knob–turn region (KT) of the fibre gene were designed using Primer Express software (Applied Biosystems). These were KTf (forward primer TGGCTGTTAAAG-GCAGTTTGG), KTr (reverse primer GCACTCCATTTTCGTCAAATCTT). A final reaction volume of 25 μl consisted of 12.5 μl SYBR Green Jump Start *Taq* Ready Mix, 2.5 μl of each primer (10 μM) and 5 μl of sample. The standard curve consisted of 10-fold serial dilutions of virus in 100 μl of PBS mixed with 100 μl lysis buffer (Promega, Southampton, UK) containing the relevant number and type of cells. All samples were DNA extracted and amplified in triplicate under the following conditions: 50°C for a 2-min. hold, 95°C for a 10-min. hold, and then 40 cycles of 95°C for 15 sec. and 60°C for 1 min. The results were analysed with the Sequence Detection System software (Applied Biosystems).

Neutralization assay

The neutralization properties of the sera were determined by incubating 10^7 pfu Ad.EGFP in serum diluted 1:10 or 1:100, final volume 500 μl, for 30 min. at 37°C. The suspension was then added to A549 cells that had been seeded the previous day at 10^5 cells per 24-well plate well. The samples were assayed for EGFP expression after 48 hrs by flow cytometry.

Cell tracker green (CTG) staining

1.2×10^6 A549 cells were re-suspended in RPMI containing 25 μM Cell Tracker Green (Molecular Probes, Invitrogen) and incubated at 37°C for 30 min. Cells were pelleted and the supernatant discarded. The cells were then washed in 1 ml RPMI 10% FCS, 2 mM L-glutamine, 1% Pen/Strep. In assays employing CTG, cells were plated at 10^5 cells per well of a 24-well plate 6 hrs before the assay was started to minimize the effects of dye dilution due to the fast growth rate of the cells.

Animal studies

Ten-week-old female Balb/c nu/nu mice (B&K Universal, Hull, UK) were injected with 5×10^6 A549 cells in 200 μl PBS. After 1 week and a tumour size of ca. 100 mm^3, the tumours were directly injected with 1×10^6 MSC that had been transduced with Ad.TR (100 pfu/cell) and Ad.EGFP (100 pfu/cell), respectively, 24 hrs earlier. The transduced MSCs were extensively washed, trypsinized and washed again before their *in vivo* use. The growth of the tumours was then followed over 3 weeks. Three different diameters of each tumour were measured once a week. The tumour volume was calculated using the formula: 3,14158/ 6*(d1*d2*d3). Five animals per group were analysed. Two additional animals with A549 xenografts were injected with untransduced MSCs. Their tumour growth was indistinguishable from those treated with MSC.EGFP (data not shown). The animal studies were performed according to national laws and covered by license from the Irish government.

Statistical analysis

Experimental values are expressed as mean value ± standard deviation. For significance analyses, Student's t-tests were used and $P < 0.05$ (*) was considered significant and $P < 0.001$ (**) as highly significant.

Results

Adenovirus expressing TRAIL induces apoptosis in TRAIL resistant A549 cells

In order to determine the potential of adenovirus expressing TRAIL (Ad.TR), of which a schematic view is depicted in Fig. 1A, as a cancer cell apoptosis inducing agent in A549 lung carcinoma cells we compared this virus to recombinant TRAIL protein (rTR). A549 cells are traditionally classed as TRAIL resistant. At doses of 10 ng/ml rTR apoptosis levels were only approximately 4 percentage points (%) above background equivalent to cells infected with Ad.TR at 1 pfu/cell. In contrast, A549 cells exposed to Ad.TR at 100 pfu/cell demonstrated levels of apoptosis of approximately 45% above background after 48 hrs. The toxicity of the virus appeared to be transgene specific as the levels of apoptosis in cells exposed to adenovirus expressing β-galactosidase (Ad.BGal) at similar pfu were only approximately 3.5% above background (Fig. 1B). Thus, membrane- bound TRAIL has a significantly higher tumour cell-killing activity than rTR, which led us to test MSCs that have been shown to infiltrate growing tumours as a potential cellular carrier of full-length TRAIL.

MSCs express transgenic TRAIL protein and induce apoptosis in A549 cells

In order to determine whether MSCs could be used as a potential vehicle for adenovirus-expressed TRAIL, MSCs were transduced with Ad.EGFP (MSC.EGFP) and Ad.TR (MSC.TRAIL) at 100 pfu/cell and compared to untransduced MSCs. The images in Fig. 2A, which were taken 48 hrs after transduction, demonstrate that adenoviral transduction did not change the overall morphology of the cells and transduction with Ad.EGFP gave rise to green fluorescent signals in almost all cells (Fig. 2A). Further quantification of the EGFP signal by fluorescence activated cell sorting (FACS) analysis revealed that 99% of all cells were expressing the transgene (Fig. 2B). Subsequently, MSCs transduced with Ad.BGal and Ad.TR at 100 pfu/cells were harvested and protein lysates prepared 48 hrs after transduction. In Western analysis, only cells transduced with Ad.TR demonstrated detectable levels of the TRAIL protein (Fig. 2C). Next, we tested whether MSCs would be resistant to the effects of the expressed TRAIL and compared it to the effects of rTR. We found MSCs to be completely resistant to rTR as well as the adenoviral vector-encoded full-length membrane bound form on the surface, whereas HCT116 cells, our positive controls, are driven into apoptosis by rTR (Fig. 2D). To evaluate whether MSCs transduced with Ad.TR were capable of inducing apoptosis in A549 cells, coculture experiments were performed. MSCs were seeded at 10^5 cells per well and transduced with adenovirus. After 24 hrs MSCs were trypsinized and counted. 10^4 of Ad-transduced or untransduced MSCs (*i.e.* 1 MSC per 10 seeded A549 cells) were then added to each well containing A549 cells. After 48 hrs, the
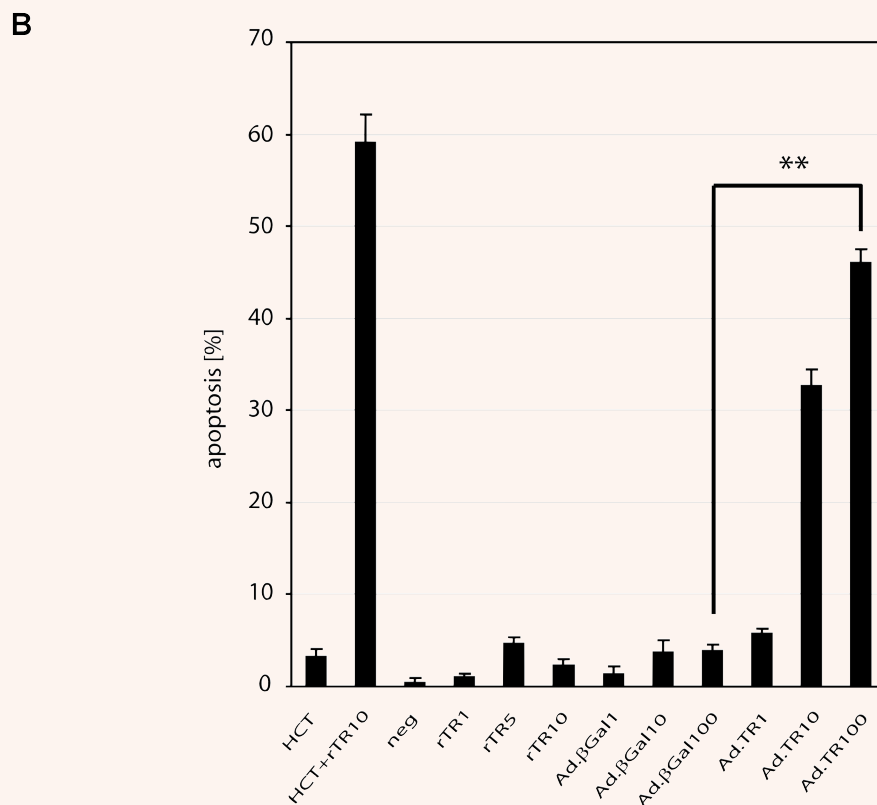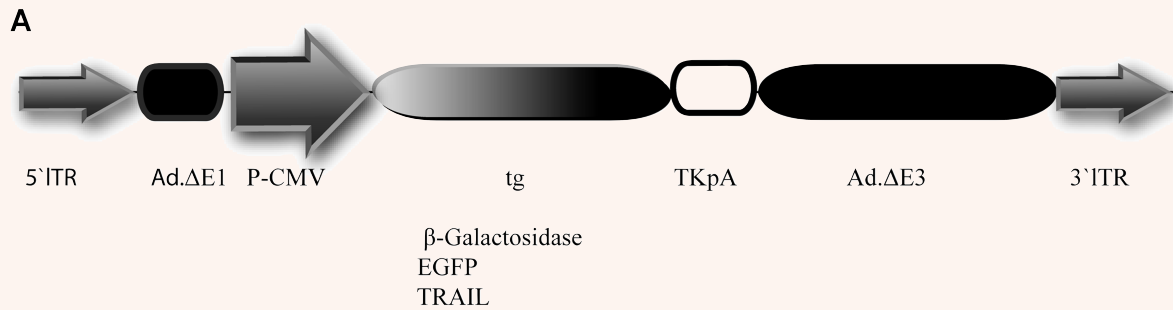
Fig. 1 Adenovirally expressed TRAIL induces higher levels of apoptosis than recombinant TRAIL in A549 cells. (**A**) Replication-defective adenoviral vectors with deletions of the E1 and E3 regions (Ad.ΔE1 and Ad.ΔE3) were used in our study. They expressed TRAIL (Ad.TR), β-galactosidase (Ad.BGal) or EGFP (Ad.EGFP) from the cytomegalovirus (CMV) promoter (P-CMV). Other elements are the 5′ and 3′ inverted-terminal-repeats (ITR) and the thymidine kinase polyA-addition signal (TKpA). (**B**) A549 cells were seeded at 10^5 cells per well overnight. Adenovirus (expressing TRAIL or β-galactosidase, Ad.TR and Ad.BGal, respectively) was added at 1, 10 or 100 pfu/cell or recombinant TRAIL (rTR) was added at 1, 5 or 10 ng/ml. As positive control for the effects of rTR human colorectal cancer HCT116 cells were treated with 10 ng/ml rTR. Cells were assayed for apoptosis after 48 hrs by Nicoletti hypodiploidity assay. Samples were analysed by flow cytometry. Numbers represent mean values of five samples ± standard deviation. $^{**}P < 0.001$.
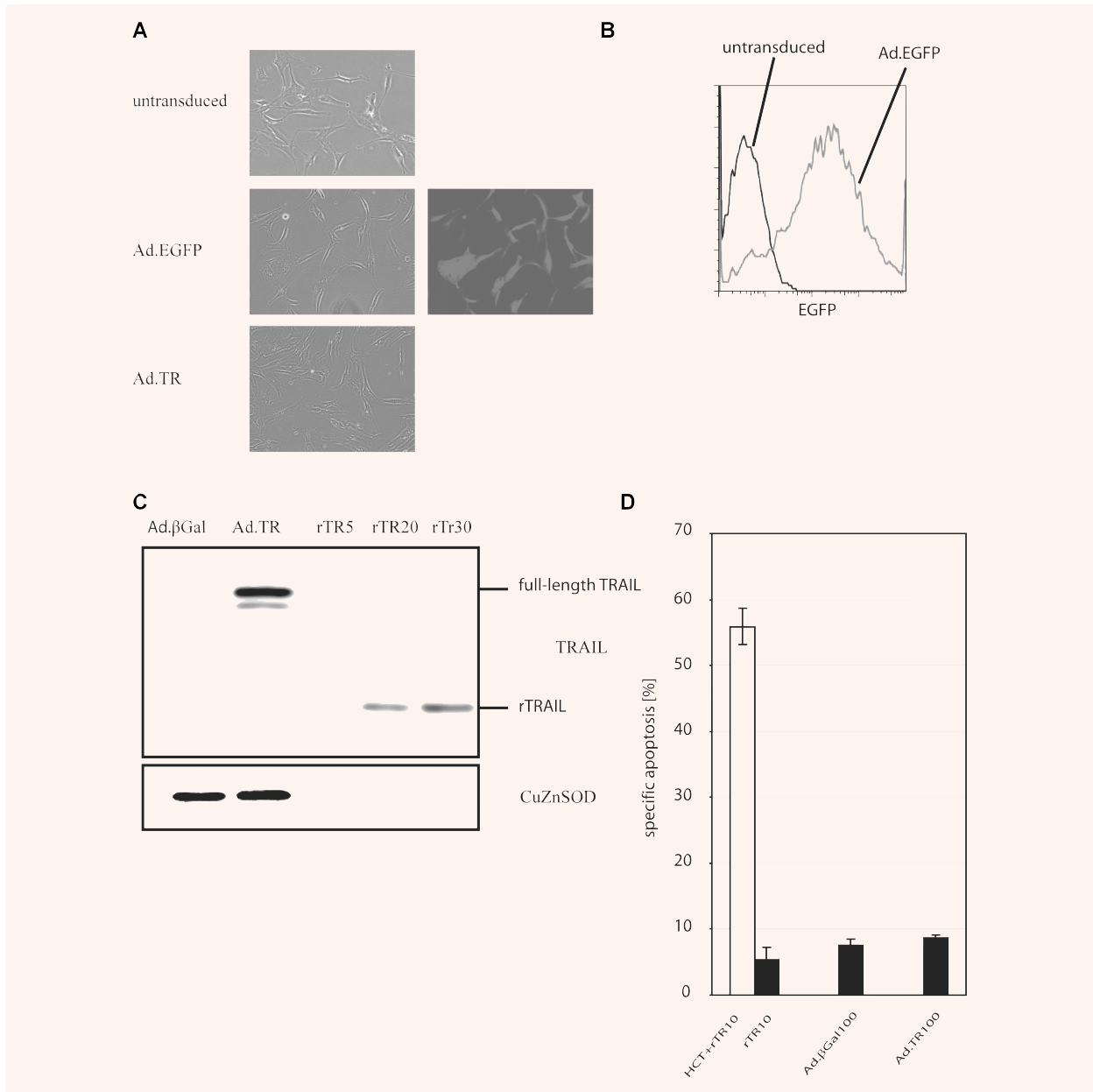
Fig. 2 Transgene expression after adenovirus transduction of MSCs. (**A**) MSCs were transduced with Ad.EGFP and Ad.TR at 100 pfu and were analysed by fluorescent and phase contrast microscopy 48 hrs later. The images show that green fluorescent expression was established in Ad.EGFP-transduced cells. Transduction with Ad.EGFP and Ad.TR did not alter the morphology of MSCs as compared to untransduced cells. (**B**) EGFP transgene expression was also quantified by FACS analysis. MSCs were transduced with Ad.EGFP at 100 pfu/cell. Almost all cells were transduced after 48 hrs. (**C**) Cell lysates from MSCs transduced with Ad.BGal or Ad.TR were probed for TRAIL expression by Western blotting and compared to signals from rTR that was loaded at 5, 20 and 30 ng on the same gel. The Ad.TR-encoded full-length membrane-bound form runs at 30 kD, whereas the soluble rTR, representing only the ectodomain of TRAIL, runs at 20 kD. A CuZnSOD Western blot serves as loading control for the protein lysates from Ad.BGal and Ad.TR-transduced cells (**D**) Neither treatment with rTR nor transduction with Ad.TRAIL causes apoptosis in MSCs. MSCs were either treated with rTR at concentration of 10 ng or transduced with Ad.TRAIL (100 pfu/cells) or Ad.BGal (100 pfu/cells). Specific apoptosis was measured 48 hrs later by Nicoletti hypodiploidity assay. HCT116 cells (white bar) treated with rTR serve as positive control.

wells were assayed for apoptosis. In wells containing Ad.TR-transduced MSCs levels of apoptosis were approximately 27% above background with no appreciable difference between the levels of apoptosis in untreated wells or wells containing untransduced or Ad.BGal-transduced MSCs (Fig. 3A). In order to test that apoptosis was indeed caused by TRAIL and not some other properties of the Ad.TR-transduced MSCs, we cotreated with neutralizing anti-TRAIL antibody and the pan-caspase inhibitor zVAD. Both cotreatments were able to significantly reduce or even block the MSC.TRAIL-induced apoptosis in A549 cells (Fig. 3A). Due to the differences in the growth rates of both cell types (MSC doubling time approximately 2.3 days, A549 doubling time approximately 1.2 days) it was desirable to determine the relative numbers of each cell type in the coculture system. A549 cells and MSCs were prepared using enzyme-free cell dissociation buffer (Specialty Media, NJ, USA) and stained for CD105 (endoglin), a marker for endothelial cells and bone marrow-derived stromal cells. A549 cells were only approximately 0.4% positive for this marker above isotype controls, whereas MSCs were approximately 98% positive for this marker above isotype controls. MSCs, untransduced and transduced with Ad.Bgal were cocultured with A549 cells as previously described and wells were assayed for CD105 expressing cells after 48 hrs. The levels of CD105 cells in wells containing MSCs untransduced or Ad.BGal transduced were similar and were only approximately 1% above the levels observed in wells containing A549 cells alone. This suggests that MSCs untransduced or transduced with adenovirus represented only approximately 1% of the final cell population in the assay system being employed here and that the measured apoptosis stemmed almost exclusively from A549 cells (Fig. 3B). In order to assess whether lower concentrations of MSCs per well could be employed, MSCs transduced with Ad.TR were cocultured with A549 cells seeded as before. 10^4 (1:10) and 3.3 $\times 10^3$ (1:30) MSC cells were added per well. Cells were assayed for apoptosis after 48 hrs. There was a trend towards lower levels of apoptosis in wells cultured at a 1:30 ratio than at the 1:10 ratio, however, this was not significant (Fig. 3C). In order to check that MSC.TRAIL did not have detrimental effects on normal, non-tumour cells we cocultured human primary fibroblasts with MSCs loaded with TRAIL. Figure 3D shows that MSCs loaded with TRAIL did not induce apoptosis in fibroblasts indicating that the use of TRAIL-expressing MSCs is principally safe.

Given the surprisingly high-apoptosis induction activity, we addressed whether the effect was solely caused by TRAIL-displayed on MSCs or whether other mechanisms such as virus transfer from MSC to target cells (A549) might also account for the observed cell-killing effect. To this end, we mixed Ad.EGFP-transduced MSCs with A549 cells and measured the green fluorescence in A549 cells 48 hrs later.

MSCs transduced with Ad.EGFP transfer virus and transgene expression to neighbouring cells

When performing coculture experiments using MSCs that had been transduced with Ad.EGFP, we noticed that higher numbers of cells were EGFP positive in this assay system than could be accounted for by MSCs alone. Therefore, to determine whether the MSCs were delivering Ad.EGFP to A549 cells, MSC cells were untransduced or transduced with Ad.EGFP at 10, 100 or 1000 pfu/cell. After 48 hrs, MSCs were prepared for addition to wells containing A549 cells as previously described, however, prior to being added the final MSC cell suspension was centrifuged and the supernatant was aspirated. The cell pellet was then washed and re-suspended in the equivalent volume of medium to the aspirated supernatant. 10^4 untransduced and Ad.EGFP-transduced MSCs or an equivalent volume of medium from the supernatant samples was then added to wells containing A549 cells. Cells were assayed for EGFP expression by flow cytometry after 48 hrs. The wells with MSCs infected at 1000 pfu/cell contained approximately 47% positive EGFP cells, while the wells to which MSCs infected at 100 and 10 pfu/cell contained approximately 14% and 1% EGFP positive cells, respectively. In the wells to which the supernatants had been added only those from the 1000 pfu/cell infected MSCs had levels of EGFP positive A549 cells above background of approximately 0.7%. This suggests that MSCs can transfer virus to A549 cells and that the EGFP positive A549 cells were not the result of free virus being carried over in the medium supernatant (Fig. 4A). To assess whether the EGFP positive cells were the result of virus genome transfer resulting in production of novel transgenic protein or were due to the transfer of EGFP protein between MSC cells and A549 cells, the following experiment was performed. MSCs were untransduced or transduced with Ad.BGal (100 pfu/cell) and harvested after 48 hrs. 10^4 untransduced or Ad.BGal-transduced MSCs were added to wells that had been seeded the previous day with 10^5 293 cells (which is a producer cell line for adenovirus). After 24 hrs, cells were harvested and DNA was extracted. Numbers of viral genomes were quantified by quantitative PCR. Wells containing MSCs transduced with adenovirus and cocultured with 293 cells had approximately 100-fold higher numbers of adenoviral genomes than adenovirus-transduced MSCs cultured alone indicating that MSCs transferred adenovirus over to 293 cells, which replicated the virus resulting in higher genome numbers (Fig. 4B). Untransduced MSCs cultured with 293 cells did not contain any detectable adenoviral genomes (Fig. 4B). All adenoviral vectors were found negative for replication competent adenovirus (RCA) by PCR analyses prior to their use ruling out replication inside the MSCs as a source for the viral vectors that 're-transduced' (or 'post-transduced') the A549 and 293 cells in our experiments. Furthermore, we examined MSCs as well as MSCs transduced with Ad.EGFP for E1A expression by Western blot to test whether virus could be replicated in MSCs. However, in contrast to our positive control, 293 cells, we could not detect E1A protein in MSCs (Fig. 4C). Taken together, our data demonstrate that our adenoviral vectors are RCA-free and that they do not replicate inside MSCs. Thus, MSCs are able to convey the genetic information carried by adenoviral vectors onto target tumour cells by an unknown mechanism, thereby potentially increasing the efficacy of therapeutic transgenes such as TRAIL beyond the expression on the surface of MSCs alone. Next, we investigated whether
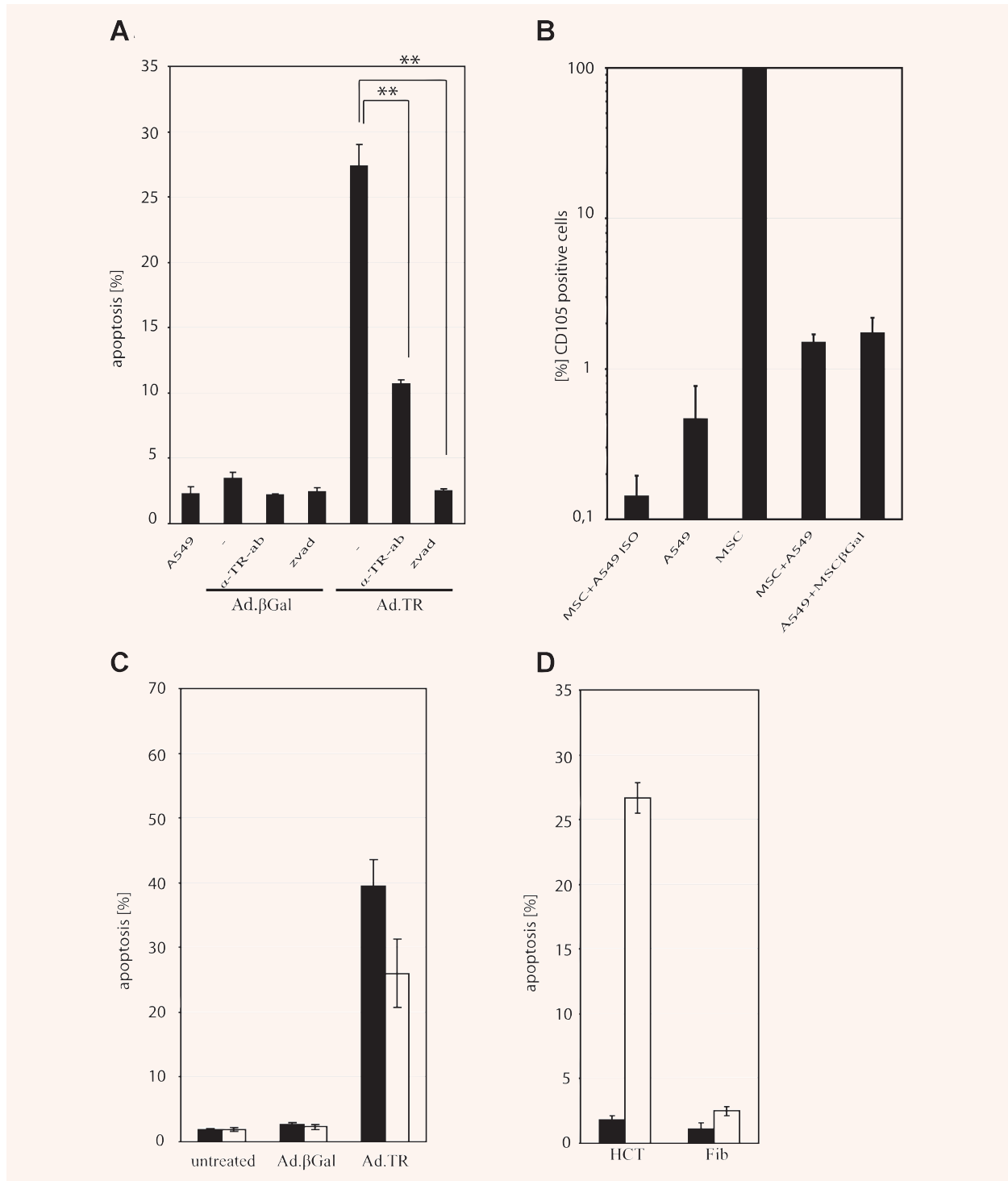
Fig. 3 MSCs transduced with Ad.TR can induce apoptosis in A549 cells. (**A**) MSCs were transduced with Ad.BGal (100 pfu/cell) or Ad.TR (100 pfu/cell). After 48 hrs MSCs were trypsinized and 10^4 of these cells were added to wells that had been seeded the day before with A549 cells at 10^5 cells per well. After 48 hrs, wells were trypsinized and the complete mixed cell population was measured by Nicoletti apoptosis assay. A549 cells

and MSCs cultured alone as well as A549 cells mixed with MSCs transduced with Ad.BGal showed only background apoptosis of below 5%. In contrast, in A549 mixed with MSCs transduced with Ad.TR apoptosis rates of almost 30% could be measured. This apoptosis could be inhibited by TRAIL neutralizing antibodies (α-TR-ab) and the pan-caspase inhibitor zVAD. Numbers represent mean values of three samples ± standard deviation. **$P < 0.001$. (**B**) MSCs cocultured with A549 cells represent approximately 1% of the final cell population. 10^4 untransduced or Ad.BGal transduced (100 pfu/cell) MSC cells were added to wells that were unseeded or had been seeded the previous day with 10^5 A549 cells. After 48 hrs, cells were detached from wells using trypsin free buffer (Specialty Media, NJ, USA). Cells were stained with anti-CD105 and analysed by flow cytometry. The iso-type control for a 1:10 MSC:A549 mix (MSC+A549 ISO) was, as expected, negative for CD105, whereas 98% of all cells in a pure MSC culture (MSC) were CD105 positive. A549 cells only (A549) showed a weak CD105 signal in 0.5% of all cells. In 1:10 mixes with MSCs (MSC+A549) or MSCs transduced with Ad.BGal (A549+MSCβGal) the CD105 signal increased to about 1.5% 48 hrs after mixing and coculturing. Please note that graph is depicted in log-scale. (**C**) The experiment was performed as described above (Fig. 3A), however, untransduced and transduced (Ad.BGal and Ad.TR) MSC cells were added at 10^4 (1:10) (black bars) and 3.3×10^3 (1:30) (white bars) cells per well. After 48 hrs, cells were analysed by Nicoletti apoptosis assay proving that even at a lower ratio against the target cancer cells, MSCs expressing TRAIL can trigger significant levels of apoptosis. Numbers represent mean values of 12 samples ± standard deviation. **$P < 0.001$. (**D**) MSCs were transduced with Ad.EGFP (100 pfu/cell) (black bars) or Ad.TR (100 pfu/cell) (white bars). After 48 hrs, MSCs were harvested and 10^4 of these cells were mixed with 10^5 pre-seeded primary human fibroblasts (Fib). After 48 hrs, apoptosis was determined by Nicoletti apoptosis assay. HCT116 cells (HCT) served as positive controls in this experiment.
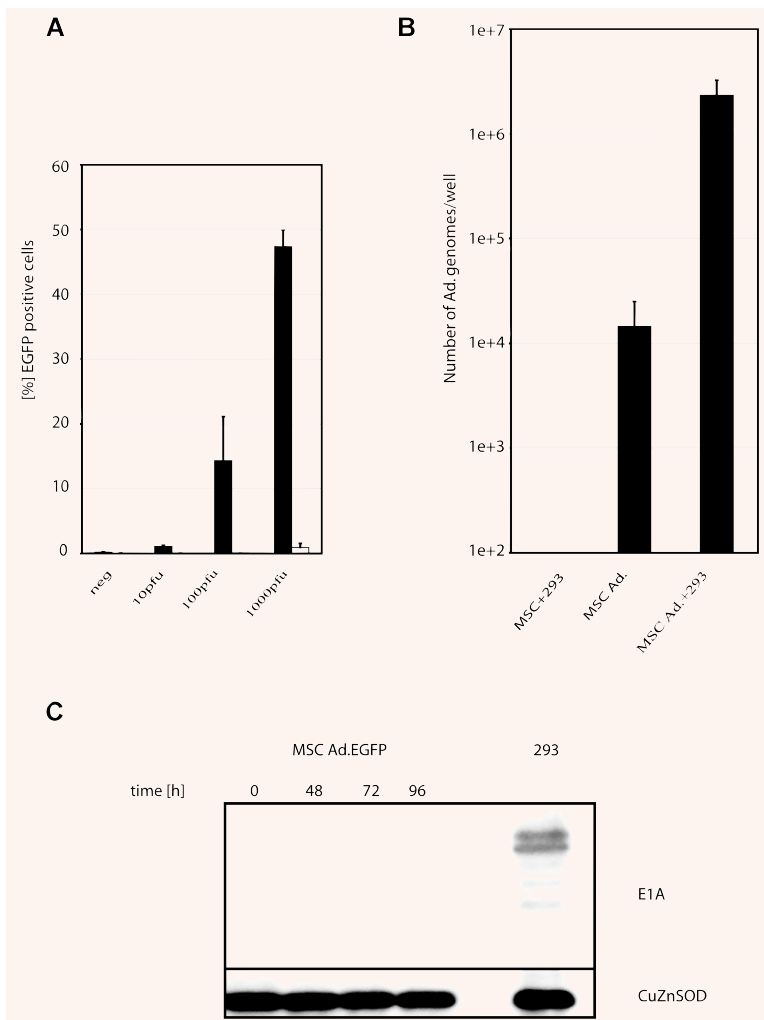

Fig. 4 Ad.EGFP-transduced MSCs transfer adenoviral-encoded transgene expression onto A549 cells. (**A**) MSC cells were transduced with Ad.EGFP at 10, 100 or 1000 pfu/cell. After 48 hrs MSCs were trypsinized and prepared as previously described. Before addition to A549 seeded wells the Ad.EGFP-transduced MSC cell suspension was centrifuged. The supernatant was removed from the cell pellet and also added to A549 cells (white bars, which are hardly visible except for 1000 pfu). This resulted in almost no transduction, indicating that no virus was left in the supernatant medium or produced in the MSCs. The MSCs themselves were re-suspended in a volume of medium equivalent to that of the aspirated supernatant. 10^4 Ad.EGFP-transduced MSCs (black bars) were added to wells seeded with A549 cells. Samples were assayed for EGFP expression by flow cytometry after a further 48 hrs. (**B**) Adenovirus-transduced MSC cocultured with 293 cells results in novel virus genome production. MSCs were transduced with Ad.BGal (100 pfu/cell) or left untreated. After 48 hrs, untransduced (MSC+293) and Ad-transduced MSCs (MSC Ad.+293) were cocultured with 293 cells that had been seeded the previous day at 10^5 cells/well or Ad.BGal-transduced MSCs were replated into wells containing no 293 cells (MSC Ad.). After 24 hrs, cells were harvested and DNA was extracted. Cells were analysed for viral DNA content by quantitative PCR using primers specific for the knob turn region of the virus and the SYBR Green quantitative PCR system. Samples were run on an ABI Prism 7000 sequence detector. Please note that the graph is depicted in log-scale. (**C**) In order to rule out that our MSCs expressed adenoviral E1A and could facilitate virus replication, we performed a Western blot on protein lysates from MSCs, MSCs transduced with Ad.EGFP (0, 48, 72 and 96 hrs after transduction) and 293 cells as positive control. Only 293 cells showed a signal for E1A. A CuZnSOD Western blot was carried out as loading control.

Ad.TR-loaded MSCs would be recognised and inhibited by immune-effector cells.

Ad.TR-transduced MSCs do not stimulate T-cell proliferation

In order to evaluate whether MSCs transduced with adenoviral vectors (and in particular Ad.TR) were more immunogenic and would stimulate a T-cell response, MSCs untransduced and transduced with Ad.BGal or Ad.TR were cocultured with carboxyfluorescein diacetate succinimidyl ester (CFSE) stained PBMCs from a donor with high levels of anti-adenovirus antibodies. In order to assess whether any lack of T-cell proliferation was due to factors released by MSCs, the same experiments were performed using fibroblasts instead of MSCs. Neither Ad.BGal transduced nor Ad.TR-transduced MSCs (or fibroblasts) induced a T-cell proliferation response significantly above background (Fig. 5). When PBMCs were stimulated with CD3/CD28 expressing beads over 90% proliferation was observed serving as positive control.

The presence of white blood cells or erythrocytes does not inhibit Ad.TR-transduced MSCs from inducing apoptosis in A549 cells

In order to determine whether MSCs transduced with Ad.TR could induce apoptosis in A549 cells in an environment approximating that of the blood stream, WBCs and washed blood cells (whole blood that was washed and pelleted four times in excess PBS, cellular content approximately 99.9% erythrocytes) were prepared. These cells were overlaid onto A549 cells that had been stained with Cell Tracker Green (Molecular Probes) and seeded at 10^5 cells per well. MSCs were untransduced or transduced with Ad.BGal (100 pfu/cell) or Ad.TR (100 pfu/cell) and harvested after 48 hrs. These cells were then added to medium, washed blood or WBC containing wells at 10^4 cells per well. The wells were harvested after 48 hrs and the medium and WBC containing samples were prepared for Nicoletti assay. The wells containing washed blood cells were harvested and the red blood cells were lysed by exposure to a hypotonic solution before being prepared for Nicoletti assay. MSCs transduced with Ad.TR-induced levels of apoptosis in A549 cells significantly above background both in the presence of washed blood cells (Fig. 6A) and WBCs (Fig. 6B). The background levels of apoptosis were increased in wells containing washed blood cells and WBCs compared to those of A549s cultured in medium alone. However, under both conditions (presence of washed blood cells or WBCs) there were increases in apoptosis induction in A549 cells exposed to MSCs transduced with Ad.TR. In samples containing washed blood cells the difference between Ad.BGal and Ad.TR-transduced MSCs was approximately 13% (Fig. 6A). In samples containing WBCs, the difference between controls and Ad.TR-transduced MSCs was approximately 35% (Fig. 6B).
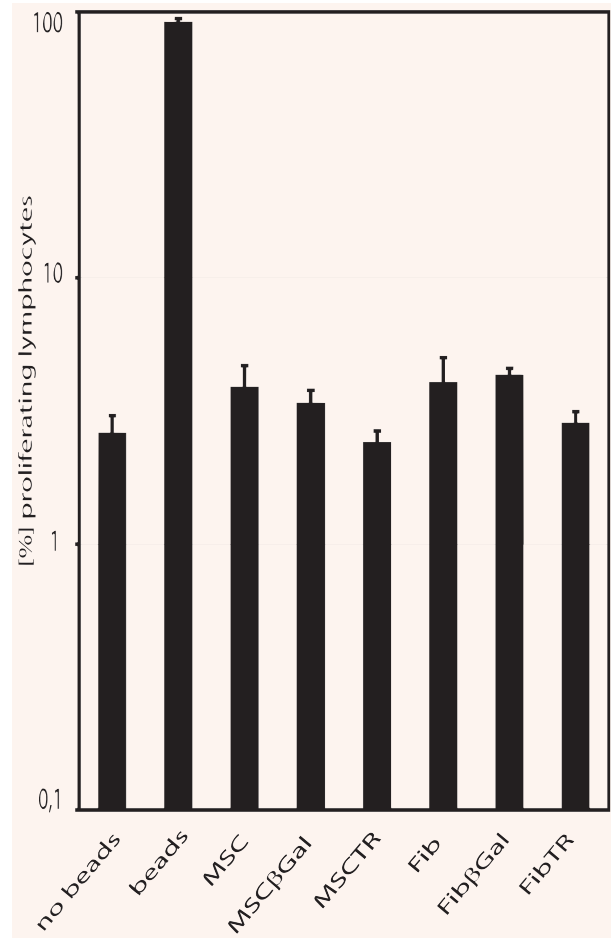

Fig. 5 Transduction of MSCs with adenovirus does not induce T-cell proliferation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient from blood samples taken from a healthy donor. PBMCs were then stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye and left overnight in PBMC culture medium. MSCs and fibroblasts were untransduced or transduced with Ad.BGal (100 pfu/cell) or Ad.TR (100 pfu/cell) (MSC, MSCβGal, MSCTR and Fib, FibβGal, FibTR) and harvested after 48 hrs. 10^4 MSCs were cocultured with 10^5 PBMCs for 5 days. As a positive control, PBMCs were also stimulated by the addition of 2.5 μl CD3/CD28 beads (Dynal, Invitrogen). Cells were then collected and analysed by flow cytometry for cell proliferation, gating on the lymphocyte population. Please, note that graph is depicted in log-scale.

The presence of serum, containing anti-adenovirus antibodies, does not block MSC-mediated Ad.EGFP transfer or TRAIL-induced apoptosis induction

Next, we wanted to know whether adenovirus-neutralizing antibodies could interfere with the transfer of adenoviral vector and
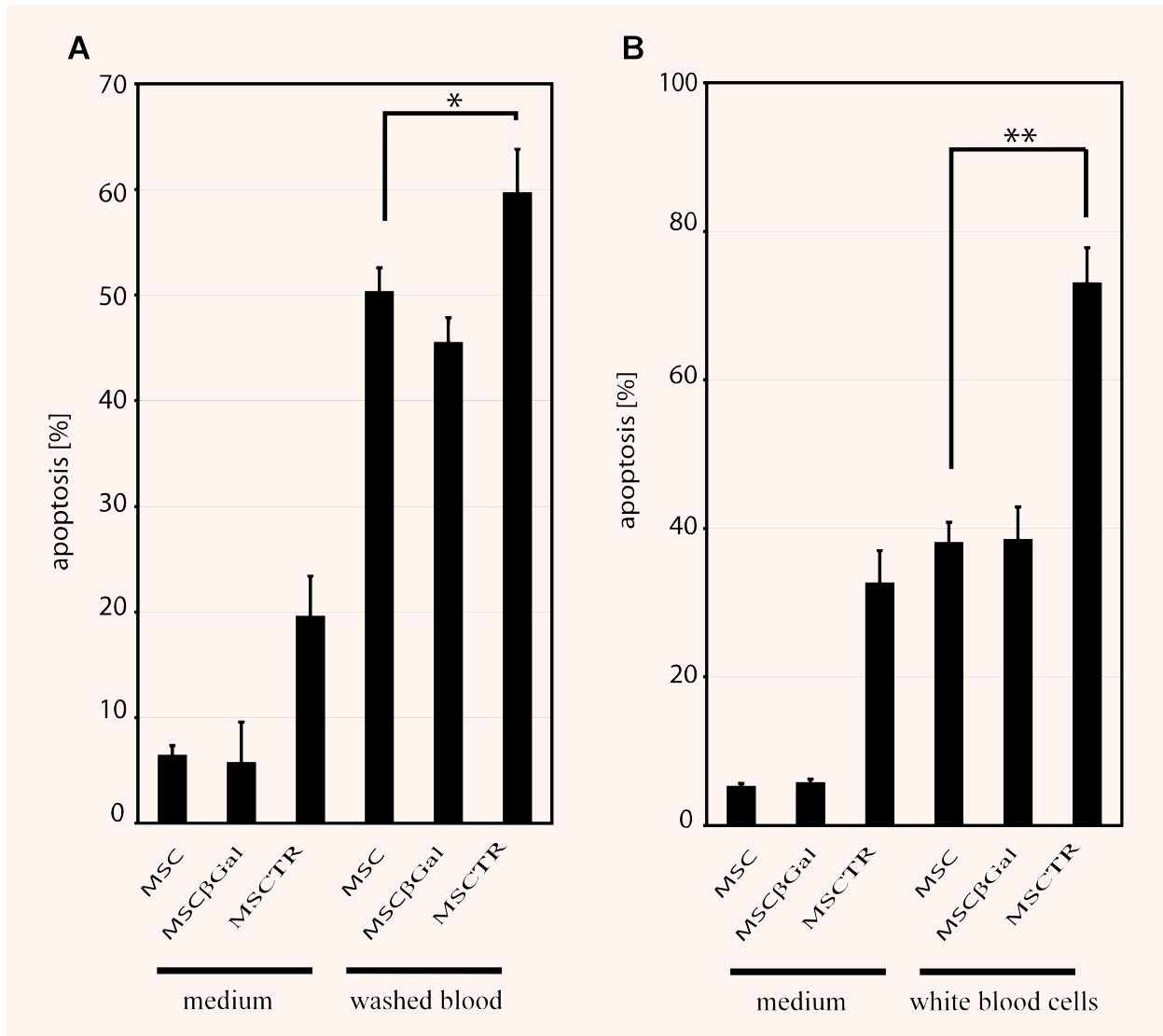
Fig. 6 Ad.TR-transduced MSCs induce apoptosis in A549 cells in the presence of washed blood (erythrocytes) and white blood cells at physiological concentrations. MSCs were untransduced or transduced with Ad.BGal (100 pfu/cell) or Ad.TR (100 pfu/cell) (MSC, MSCβGal, MSCTR) and harvested after 48 hrs. A549 cells were stained with cell tracker green and seeded into wells at 10^5 cells per well. Once these cells had adhered to the tissue culture wells (**A**) white blood cells were added at a concentration of 3×10^6 cells/ml or (**B**) washed blood cells were added at approximately 5×10^9 cells/ml. 10^4 untransduced or Ad-transduced MSC cells were added to each well. After 48 hrs, washed blood cells (**A**) or white blood cells (**B**) and medium alone containing wells were harvested and prepared for Nicoletti assay. The wells containing washed blood cells were harvested and the red blood cells were lysed by exposure to a hypotonic solution before these samples were prepared for Nicoletti assay. All samples were assayed by flow cytometry gating on the cell tracker green positive population alone. Overall, the level of apoptosis in white blood cell and washed blood exposed wells was increased, however, samples exposed to Ad.TR-transduced MSCs were consistently higher than background or controls. Numbers represent mean values of three experiments ± standard deviation. *$P < 0.05$; **$P < 0.001$.

transgene information to target cells as described in Fig. 4 or could block the apoptosis-inducing activity of MSCs loaded with Ad.TR. First, we tested the sera of 18 donors for the presence and activity of anti-adenovirus antibodies and identified a num-

ber of sera with high and low adenovirus-neutralizing antibody levels. In Fig. 7A, one serum with high adenovirus-neutralizing antibody levels (Serum A) and one with low adenovirus-neutralizing activity (Serum B) are shown. Pre-incubation of Ad.EGFP
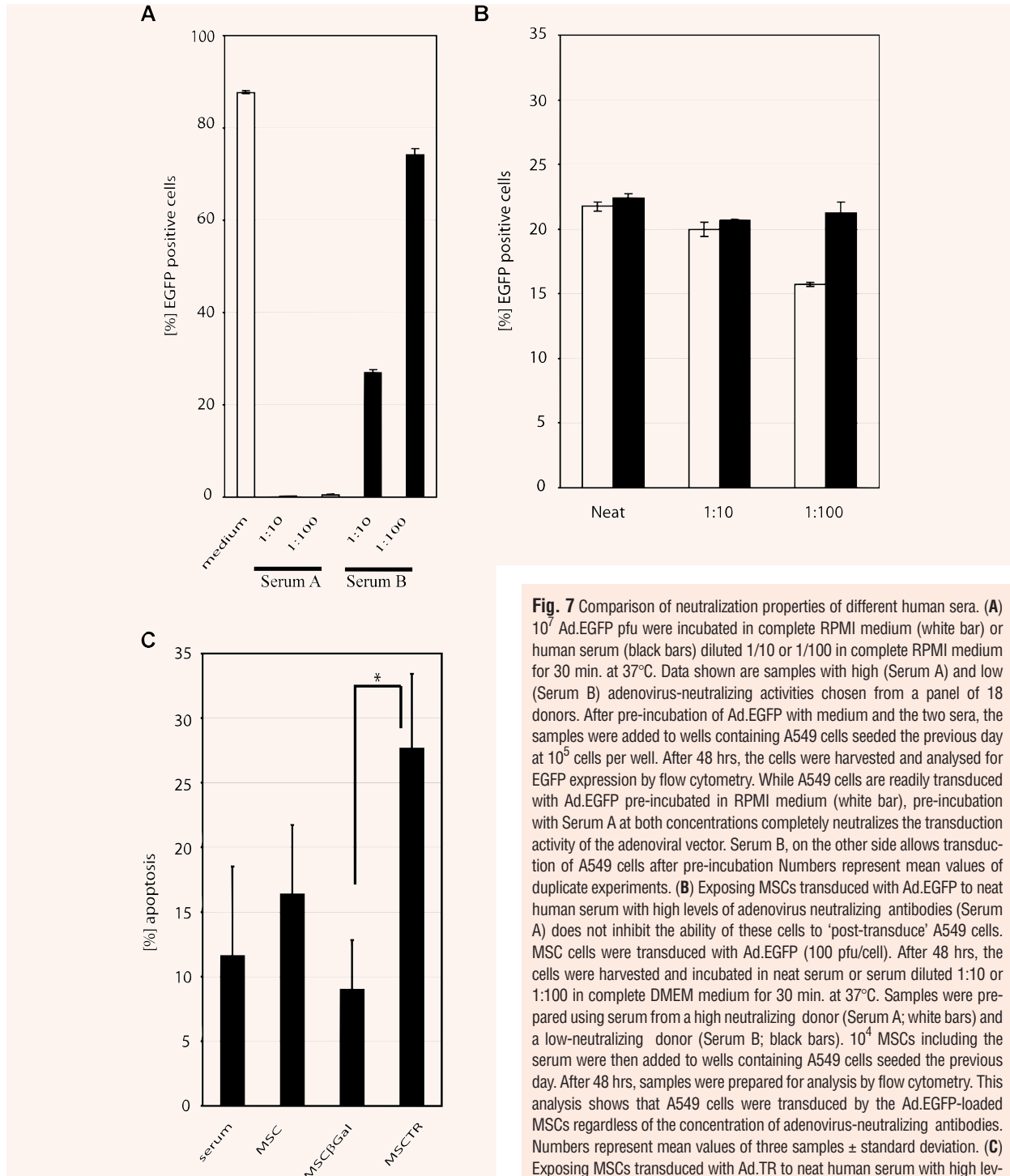
Fig. 7 Comparison of neutralization properties of different human sera. (**A**) 10^7 Ad.EGFP pfu were incubated in complete RPMI medium (white bar) or human serum (black bars) diluted 1/10 or 1/100 in complete RPMI medium for 30 min. at 37°C. Data shown are samples with high (Serum A) and low (Serum B) adenovirus-neutralizing activities chosen from a panel of 18 donors. After pre-incubation of Ad.EGFP with medium and the two sera, the samples were added to wells containing A549 cells seeded the previous day at 10^5 cells per well. After 48 hrs, the cells were harvested and analysed for EGFP expression by flow cytometry. While A549 cells are readily transduced with Ad.EGFP pre-incubated in RPMI medium (white bar), pre-incubation with Serum A at both concentrations completely neutralizes the transduction activity of the adenoviral vector. Serum B, on the other side allows transduction of A549 cells after pre-incubation Numbers represent mean values of duplicate experiments. (**B**) Exposing MSCs transduced with Ad.EGFP to neat human serum with high levels of adenovirus neutralizing antibodies (Serum A) does not inhibit the ability of these cells to 'post-transduce' A549 cells. MSC cells were transduced with Ad.EGFP (100 pfu/cell). After 48 hrs, the cells were harvested and incubated in neat serum or serum diluted 1:10 or 1:100 in complete DMEM medium for 30 min. at 37°C. Samples were prepared using serum from a high neutralizing donor (Serum A; white bars) and a low-neutralizing donor (Serum B; black bars). 10^4 MSCs including the serum were then added to wells containing A549 cells seeded the previous day. After 48 hrs, samples were prepared for analysis by flow cytometry. This analysis shows that A549 cells were transduced by the Ad.EGFP-loaded MSCs regardless of the concentration of adenovirus-neutralizing antibodies. Numbers represent mean values of three samples ± standard deviation. (**C**) Exposing MSCs transduced with Ad.TR to neat human serum with high levels of adenovirus neutralizing antibodies does not inhibit the ability of these cells to induce apoptosis in A549 cells. MSC cells were untransduced (MSC) or transduced with Ad.BGal (MSCβGal) or Ad.TR (MSCTR) with 100 pfu/cell. A549 cells that were treated with serum only are shown on the left (serum). After 24 hrs, the cells were harvested and incubated in neat serum for 30 min. at 37°C. The cell suspension (10^4 MSC cells/well) and serum were then added to wells containing A549 cells seeded the previous day. After 48 hrs, samples were prepared for Nicoletti assay and analysed by flow cytometry. Numbers represent mean values of duplicate samples ± standard deviation. *$P < 0.05$.
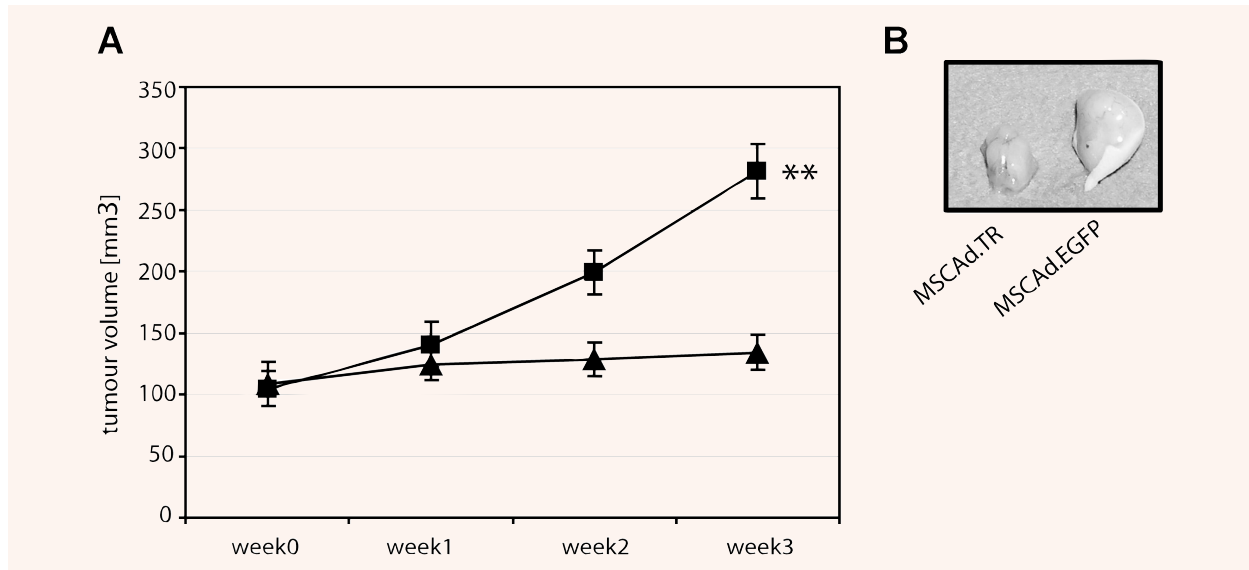
Fig. 8 MSCs transduced with Ad.TR give rise to tumour growth reduction *in vivo*. (**A**) Immune-deficient mice were injected with 5×10^6 A549 cells into the right flank. After 1 week when tumours were approximately 100 mm³ in size, 1×10^6 MSCs either transduced with Ad.TR or Ad.EGFP were directly injected into the tumour. The tumour growth was followed over 3 weeks. This is depicted in the graph. Numbers represent mean values of five animals in each group ± standard deviation. **P < 0.001 (**B**) Representative tumours of two animals are shown after dissection clearly showing the smaller size in the animal from the group treated with Ad.TR-transduced MSCs.

in Serum A could block transduction of A549 cell, which are otherwise readily transduced as shown in the medium control on the left (Fig. 7A). Serum B on the other hand had some inhibitory effect on the transduction efficiency at a dilution of 1:10, which is probably due to the non-specific blocking effect of serum proteins at this high concentration, but almost completely lost this inhibitory effect at a 1:100 dilution (Fig. 7A).

Having identified high and low adenovirus-neutralizing antibody containing sera, we set out to ascertain whether MSCs transduced with adenovirus could 'post-transduce' (see Fig. 4) A549 cells in the presence of human serum. Ad.EGFP-transduced MSCs (100 pfu/cell) were again exposed to sera from two donors, one with high adenovirus-neutralizing antibody levels (Serum A) and one low adenovirus-neutralizing antibody levels (Serum B). The sera were used undiluted (neat), or at 1:10 and 1:100 dilutions and the cells were incubated in the serum preparations for 30 min. at 37°C. The cell suspensions were then overlaid onto A549 cells. After 48 hrs, the cells were harvested and analysed for EGFP expression by flow cytometry (Fig. 7B). The results from both donors were almost identical. This means exposure to high- or low-neutralizing sera did not decrease the efficiency of MSC-mediated transduction of A549 cells with Ad.EGFP. Hence, the piggy-backing of adenovirus by MSCs cannot be inhibited by neutralizing antibodies and this effect might be able to increase the effectiveness of MSC gene therapy strategies. However, further and stringent tests have to be performed in the future to confirm the existence and usefulness of this effect *in vivo*.

In order to assess whether serum could block MSC.TRAIL-mediated apoptosis induction in A549 cells, MSCs were untransduced or transduced with Ad.TR or Ad.BGal and incubated with neat serum for 30 min. at 37°C. MSCs transduced with Ad.TR induced approximately 18% higher levels of apoptosis in A549 cells than MSCs transduced with Ad.BGal (Fig. 7C).

MSCs transduced with Ad.TR inhibits the growth of A549 xenografts in mice

We next wanted to know whether MSCs transduced with TRAIL would also have anti-tumour activity *in vivo*. Therefore, we injected 5×10^6 A549 cells into nu/nu mice. When the resulting tumours had reached an average volume of 100 mm³ after 1 week, 1×10^6 MSC.TRAIL cells in 100 μl PBS were injected into the tumour mass. As controls tumours were injected with MSC.EGFP cells. Figure 8A shows the growth curve of the tumours over a period of 3 weeks following MSC.TRAIL and MSC.EGFP injections. During this period, MSC.EGFP-treated tumours grew to about three times the size compared to the beginning of the MSC-injections, whereas MSC.TRAIL-treated tumours only grew by a factor of 1.25, almost completely arresting the tumour growth. Figure 8B shows representative tumours after the conclusion of the experiment. In conclusion, MSCs expressing TRAIL possess tumouricidal activity *in vivo*.

Discussion

In this paper, we demonstrate the potential of MSC- borne, virally expressed TRAIL to induce apoptosis in A549 cells in the context of the blood environment. A549 cells are traditionally described as a TRAIL resistant cell line [35] and only low levels of apoptosis were observed in cells treated with up to 10 ng/ml rTR. However, transduction of A549 cells with Ad.TR resulted in high levels of apoptosis in these cells in agreement with data described by Seol *et al.* [34]. Though the ability of Ad.TR to overcome TRAIL resistance in a range of cell lines has been explored no clear mechanism has been elucidated [36–38]. While administration of TRAIL protein or TRAIL receptor agonistic antibody may have potential for cancer therapy [26, 27], it lacks many of the potential benefits that may be gained by using a gene therapy strategy for the production of the protein. Nakamizo *et al.* [8] demonstrated the increased efficacy of IFN-β expression from MSCs over intravenous injection of IFN-β protein. In a similar manner production of TRAIL from cells localized to the tumour may enhance the effects of the therapy. MSCs have been shown to have very low levels of active TRAIL receptors and doses of up to 50 ng/ml rTR were shown not to induce apoptosis in MSCs (manuscript in preparation).

Ad.TR-transduced MSCs were capable of seeding in A549 cell monolayers and could effectively induce apoptosis in the latter. It has been shown that cell-to-cell contact is required to mediate a TRAIL bystander effect [37]. In this experimental system, approximately 1% of the final cell population were MSCs and these cells were responsible for inducing apoptosis in approximately 27% of the final cell population after 48 hrs. Interestingly, Ad.EGFP-transduced MSCs appeared to transfer the virus to A549 cells. An important consequence of this result may be that transduced MSCs mediate a bystander effect, in which they transfer virus to neighbouring cells resulting in adenoviral transgenic protein production by these cells. In the case of TRAIL, this second round of protein production may substantially increase the tumour cell-killing effect. However, this remains to be addressed in detailed studies in the future. While some evidence argues for carry-over of virus attached to the surface of MSCs, other alternative or additional mechanisms might account for this bystander effect. For example membrane-derived microvesicles containing mRNA and/or protein have been described as a way to transfer genetic information or their products from one cell to another [39–41]. Such a mechanism could explain how MSCs transfer EGFP (mRNA and/or protein) to the A549 target cells in our experiments.

The treatment of metastatic cancer ideally would be achieved through the introduction of the therapeutic vector into the blood stream from where it would actively seek out tumour sites that may be otherwise inaccessible or undetectable by the clinician. However, many previous adenovirus-based strategies have been hampered by the innate and adaptive immune response [12–14, 17]. A cytotoxic T-cell response against adenoviral antigens displayed on the surface of the transduced MSCs may result in the destruction of the cells before they can be therapeutically effective. Addition of adenovirus-transduced MSCs to PBMCs did not induce an immune response and Ad.TR-transduced MSCs and fibroblasts had lower levels of proliferation than untransduced or Ad.BGal-transduced controls. This is potentially due to the ability of virally expressed TRAIL ligand to induce apoptosis in proliferating T cells. Other factors that may be responsible for blocking MSC delivery of therapeutic agents to the tumour site include WBCs and erythrocytes. Erythrocytes have been shown to bind adenovirus and at physiological concentrations will inhibit transduction of an A549 cell monolayer by over 200-fold [17]. Importantly, the presence of these cells at physiological concentrations had very little effect on Ad.TR-transduced-MSC-mediated apoptosis induction in A549 cells. In the blood stream, serum components such as antibodies may neutralize viral vectors. However, even at serum concentrations 10-fold in excess of those required to neutralize high MOIs (100 pfu/cell) of adenovirus, no inhibition of MSC-mediated adenovirus transfer was observed. Also, MSCs transduced with Ad.TR could also effectively induce apoptosis in A549 target cells.

The ability of MSCs to piggy-back adenovirus, while shielding it from neutralizing antibodies, to neighbouring cells is an interesting and potentially important finding in the drive to develop a systemically administered gene therapy vehicle. Furthermore, the capacity of MSCs transduced with adenovirus expressing TRAIL for inducing apoptosis in A549 lung epithelial cancer cells in the presence of serum, WBCs and erythrocytes supports the potential of these cells to become an exciting new delivery vector for the targeted treatment of cancer and lung cancer in particular, as MSCs preferentially home to the lung [42].

Acknowledgements

References

1. **McMahon JM, Conroy S, Lyons M, Greiser U, O'shea C, Strappe P, Howard L, Murphy M, Barry F, O'Brien T.** Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors. *Stem Cells Dev.* 2006; 15: 87–96.

2. **Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM.** Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood.* 2002; 99: 3838–43.

3. **Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, Mosca JD.** Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci.* 2003; 10: 228–41.

4. **Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC.** Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation.* 2003; 75: 389–97.

5. **Aggarwal S, Pittenger MF.** Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood.* 2005; 105: 1815–22.

6. **Schichor C, Birnbaum T, Etminan N, Schnell O, Grau S, Miebach S, Aboody K, Padovan C, Straube A, Tonn JC, Goldbrunner R.** Vascular endothelial growth factor A contributes to glioma-induced migration of human marrow stromal cells (hMSC). *Exp Neurol.* 2006; 199: 301–10.

7. **Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, Ratajczak MZ, Janowska-Wieczorek A.** Migration of bone marrow and cord blood mesenchymal stem cells *in vitro* is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells.* 2006; 24: 1254–64.

8. **Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, Chen J, Hentschel S, Vecil G, Dembinski J, Andreeff M, Lang FF.** Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res.* 2005; 65: 3307–18.

9. **Hall B, Dembinski J, Sasser AK, Studeny M, Andreeff M, Marini F.** Mesenchymal stem cells in cancer: tumor-associated fibroblasts and cell-based delivery vehicles. *Int J Hematol.* 2007; 86: 8–16.

10. **Lusky M.** Good manufacturing practice production of adenoviral vectors for clinical trials. *Hum Gene Ther.* 2005; 16: 281–91.

11. **Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML.** Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab.* 2003; 80: 148–58.

12. **Reid T, Warren R, Kirn D.** Intravascular adenoviral agents in cancer patients: lessons from clinical trials. *Cancer Gene Ther.* 2002; 9: 979–86.

13. **Bramson JL, Hitt M, Gauldie J, Graham FL.** Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. *Gene Ther.* 1997; 4: 1069–76.

14. **Worgall S, Wolff G, Falck-Pedersen E, Crystal RG.** Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. *Hum Gene Ther.* 1997; 8: 37–44.

15. **Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J.** Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.* 1999; 6: 1574–83.

16. **Nunes FA, Furth EE, Wilson JM, Raper SE.** Gene transfer into the liver of nonhuman primates with E1-deleted recombinant adenoviral vectors: safety of readministration. *Hum Gene Ther.* 1999; 10: 2515–26.

17. **Yang Y, Xiang Z, Ertl HC, Wilson JM.** Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes *in vivo. Proc Natl Acad Sci USA.* 1995; 92: 7257–61.

18. **Lyons M, Onion D, Green NK, Aslan K, Rajaratnam R, Bazan-Peregrino M, Phipps S, Hale S, Mautner V, Seymour LW, Fisher KD.** Adenovirus Type 5 Interactions with Human Blood Cells May Compromise Systemic Delivery. *Mol Ther.* 2006; 14: 118–28.

19. **Bangari DS, Mittal SK.** Current strategies and future directions for eluding adenoviral vector immunity. *Curr Gene Ther.* 2006; 6: 215–26.

20. **Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW.** Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther.* 2001; 8: 341–8.

21. **Myhre S, Henning P, Granio O, Tylo AS, Nygren PA, Olofsson S, Boulanger P, Lindholm L, Hong SS.** Decreased immune reactivity towards a knobless, affibody-targeted adenovirus type 5 vector. *Gene Ther.* 2006; 14: 376–81.

22. **Roberts DM, Nanda A, Havenga MJ, Abbink P, Lynch DM, Ewald BA, Liu J, Thorner AR, Swanson PE, Gorgone DA, Lifton MA, Lemckert AA, Holterman L, Chen B, Dilraj A, Carville A, Mansfield KG, Goudsmit J, Barouch DH.** Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature.* 2006; 441: 239–43.

23. **Chen XC, Wang R, Zhao X, Wei YQ, Hu M, Wang YS, Zhang XW, Zhang R, Zhang L, Yao B, Wang L, Jia YQ, Zeng TT, Yang JL, Tian L, Kan B, Lin XJ, Lei S, Deng HX, WenYJ, Mao YQ, Li J.** Prophylaxis against carcinogenesis in three kinds of unestablished tumor models *via* IL12-gene-engineered MSCs. *Carcinogenesis.* 2006; 27: 2434–41.

24. **Komarova S, Kawakami Y, Stoff-Khalili MA, Curiel DT, Pereboeva L.** Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther.* 2006; 5: 755–66.

25. **Schneider P, Bodmer JL, Thome M, Hofmann K, Holler N, Tschopp J.** Characterization of two receptors for TRAIL. *FEBS Lett.* 1997; 416: 329–34.

26. **Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P, Ashkenazi A.** Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science.* 1997; 277: 818–21.

27. **Ling J, Herbst RS, Mendelson DS, Eckhardt SG, O'Dwyer P, Ebbinghaus S, Osborne R, Cheu M, Lieberman G, Lum BL.** Apo2L/TRAIL pharmacokinetics in a phase 1a trial in advanced cancer and lymphoma. *J Clin Oncol Annual Meeting Proceedings Part I.* 2006; 24: 3047.

28. **Patnaik A, Wakelee H, Mita M, Fitzgerald A, Hill M, Fox N, Howard T, Ullrich S, Tolcher A, Sikic B.** HGS-ETR2–A fully human monoclonal antibody to TRAIL-R2: Results of a phase I trial in patients with advanced solid tumors. *J Clin Oncol Annual Meeting Proceedings Part I.* 2006; 24: 3012.

29. **Griffith TS, Anderson RD, Davidson BL, Williams RD, Ratliff TL.** Adenoviral-mediated transfer of the TNF related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. *J Immunol.* 2000; 165: 2886–94.

30. **Mohr A, Henderson G, Dudus L, Herr I, Kuerschner T, Debatin KM, Weiher H, Fisher KJ, Zwacka RM.** AAV-encoded expression of TRAIL in experimental human colorectal cancer leads to tumor regression. *Gene Ther.* 2004; 11: 534–43.

31. **Wenger T, Mattern J, Haas TL, Sprick MR, Walczak H, Debatin KM, Buchler MW, Herr I.** Apoptosis mediated by lentiviral TRAIL transfer involves transduction-dependent and -independent effects. *Cancer Gene Ther.* 2007; 14: 316–26.

32. **Germano IM, Uzzaman M, Benveniste RJ, Zaurova M, Keller G.** Apoptosis in human glioblastoma cells produced using embryonic stem cell-derived astrocytes expressing tumor necrosis factor-related apoptosis-inducing ligand. *J Neurosurg.* 2006; 105: 88–95.

33. **Barry FP, Murphy JM.** Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol.* 2004; 36: 568–84.

34. **Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C.** A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods.* 1991; 139: 271–9.

35. **Seol JY, Park KH, Hwang CI, Park WY, Yoo CG, Kim YW, Han SK, Shim YS, Lee CT.** Adenovirus-TRAIL can overcome TRAIL resistance and induce a bystander effect. *Cancer Gene Ther.* 2003; 10: 540–8.

36. **Voelkel-Johnson C, King DL, Norris JS.** Resistance of prostate cancer cells to soluble TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) can be overcome by doxorubicin or adenoviral delivery of full-length TRAIL. *Cancer Gene Ther.* 2002; 9: 164–72.

37. **Zhang L, Gu J, Lin T, Huang X, Roth JA, Fang B.** Mechanisms involved in development of resistance to adenovirus-mediated proapoptotic gene therapy in DLD1 human colon cancer cell line. *Gene Ther.* 2002; 9: 1262–70.

38. **Huang X, Lin T, Gu J, Zhang L, Roth JA, Liu J, Fang B.** Cell to cell contact required for bystander effect of the TNF-related apoptosis-inducing ligand (TRAIL) gene. *Int J Oncol.* 2003; 22: 1241–5.

39. **Ratajczyak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczyak MZ.** Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia.* 2006; 20: 847–56.

40. **Aliotta JM, Sanchez-Guijo FM, Dooner GJ, Johnson KW, Dooner MS, Greer KA, Greer D, Pimentel J, Kolankiewicz LM, Puente N, Faradyan S, Ferland P, Bearer EL, Passero MA, Adedi M, Colvin GA, Quesenberry PJ.** Alteration of Marrow Cell Gene Expression, Protein Production and Engraftment into Lung by Lung-derived Microvesicles: A Novel Mechanism for Phenotype Modulation. *Stem Cells.* 2007; 25: 2245–56.

41. **Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B, Camussi G.** Endothelial progenitor cell-derived microvesicles activate an angiogenic program in endothelial cells by an horizontal transfer of mRNA. *Blood.* 2007; 110: 2440–8.

42. **Hakkarainen T, Särkioja M, Lehenkari P, Miettinen S, Ylikomi T, Suuronen R, Desmond RA, Kanerva A, Hemminki A.** Human mesenchymal stem cells lack tumor tropism but enhance the antitumor activity of oncolytic adenoviruses in orthotopic lung and breast tumors. *Hum Gene Ther.* 2007; 18: 627–41.