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Use of Macrophages to Target Therapeutic Adenovirus to Human Prostate Tumors

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Abstract

New therapies are required to target hypoxic areas of tumors as these sites are highly resistant to conventional cancer therapies. Monocytes continuously extravasate from the bloodstream into tumors where they differentiate into macrophages and accumulate in hypoxic areas, thereby opening up the possibility of using these cells as vehicles to deliver gene therapy to these otherwise inaccessible sites. We describe a new cell-based method which selectively targets an oncolytic adenovirus to hypoxic areas of prostate tumors. In this approach, macrophages were co-transduced with a hypoxia-regulated E1A/B construct and an E1A-dependent oncolytic adenovirus, whose proliferation is restricted to prostate tumor cells using prostate-specific promoter elements from the TARP, PSA and PMSA genes. When such co-transduced cells reach an area of extreme hypoxia, the E1A/B proteins are expressed, thereby activating replication of the adenovirus. The virus is subsequently released by the host macrophage and infects neighbouring tumor cells. Following systemic injection into mice bearing subcutaneous or orthotopic prostate tumors, co-transduced macrophages migrated into hypoxic tumor areas, upregulated E1A protein and released multiple copies of adenovirus. The virus then infected neighboring cells but only proliferated and was cytotoxic in prostate tumor cells, resulting in the marked inhibition of tumor growth and reduction of pulmonary metastases. This novel delivery system employs three levels of tumor specificity; the natural 'homing' of macrophages to hypoxic tumor areas, hypoxia-induced proliferation of the therapeutic adenovirus in host macrophages, and targeted replication of oncolytic virus in prostate tumor cells.

Introduction

In recent years, two crucial components of anti-cancer gene therapies have emerged - the need to both develop effective methods for delivering the 'therapeutic' gene to the tumor site and restrict the expression of genes to the tumor site alone (to avoid extra-tumoral gene expression and concomitant side effects). Transcriptional control, exploiting promoter elements from the prostate specific antigen (PSA) gene, have been used to confine therapeutic gene expression to prostate epithelial cells (1). The presence of multiple areas of extremely low oxygen tension (hypoxia) in tumors, including prostate carcinomas (1), has also been exploited to target therapeutic gene expression. In this case, the therapeutic gene is placed under the control of hypoxia-regulated promoter elements so it is only expressed in hypoxic cells (2-4).

An array of viral, synthetic and cell-based vectors have been developed to transport such therapeutic gene constructs from the bloodstream into tumors but most have demonstrated poor practical efficacy (5). Only large doses ($>10^{12}$ particles) of virus injected directly into tumors resulted in widespread infection of tumor cells. Intravenous injection is considerably less efficient, as most virus fails to penetrate the tumor and ultimately sequestered by the liver (6, 7). New strategies are urgently required to deliver therapeutic genes to sites deep within primary and metastatic tumors.

Recently, central inflammatory cells: macrophages - and their precursors in the bloodstream, monocytes - have attracted considerable attention as gene delivery vehicles as they are continually recruited into tumors and migrate into avascular, hypoxic/necrotic areas (8-10). There, they up-regulate expression of hypoxia-activated transcription factors (HIFs 1α and 2 α) which then bind to hypoxic response elements (HRE) within the promoters of multiple tumor-promoting and adaptive genes to activate their expression (8, 9). This prompted us to propose

that macrophages could be exploited to deliver HIF-regulated therapeutic genes to otherwise inaccessible areas in tumors. Initially, we showed that human macrophages adenovirally-infected with a HIF-regulated reporter or a therapeutic gene and then co-cultured with human tumor spheroids *in vitro*, migrated into the hypoxic centre of these structures and expressed the exogenous gene (11). In the current study, we show for the first time that macrophages can be engineered to silently transport to, and then produce high titres of therapeutic adenovirus specifically in hypoxic areas of experimental prostate tumors. An extra level of (prostate) tumor targeting was achieved by placing either the exogenous gene in the virus and/or the replication of the virus itself under the control of promoter sequences from such prostate-specific genes (12).

Material and Methods

Immunolabelling of hypoxia and macrophages in human prostate tumors

Consenting patients with localized prostate adenocarcinoma received 0.5gm/m^2 pimonidazole (PIMO) i.v. 16-24 h before radical prostatectomy (Mount Vernon Hospital, London). Serial sections (4µM) were immunostained with CD68 (1:100, Dako, Ely, UK) and anti-PIMO (Hypoxyprobe Store, Burlington, Mass, USA) and visualized with DAB or Vector Red (Vector Laboratories, UK), respectively. To determine the distribution of TAM relative to blood vessels, 30 cases of adenocarcinoma of the prostate were selected at random from the 1997–1999 archives (Royal Hallamshire Hospital, Sheffield, UK). Sections were double-immunostained with Factor VIII (FVIII) (Dako, UK) and CD68 and visualized using Vector Red. Sections were scanned at low power (x40 – x100) to identify areas of macrophage and vascular density – the 'hotspots'. The mean densities of macrophages (MɸI) and blood vessels (MVD) within macrophage and vascular hotspots were determined using the Chalkley point array method (7).

Cell lines

HEK 293, A549 (human lung carcinoma) and LNCaP (human prostate carcinoma) were purchased from the ECACC. HEK 293s are purchased yearly to obtain low passage cells for viral transduction. Tumor cell lines are routinely tested for authenticity by micro-satellite genotyping at the ECACC.

Isolation of human monocytes and generation of monocyte-derived macrophages (MDMs)

Macrophages were prepared from mononuclear cells isolated from buffy coats (Blood Transfusion Service, Sheffield, UK) (9).

Plasmid construction and adenoviruses

The techniques for constructing HRE-regulated E1A/B plasmids and adenoviruses (CMV-AdV5-GFP (driven by a CMV promoter) and PSA-AdV5-GFP (driven by the prostate-specific promoter, PSA), are outlined in the supplementary methods. Optimal transduction of HEK 293 cells with adenovirus (CMV-AdV5-GFP) was achieved with an MOI of 5 PFU/cell as measured by flow cytometry for GFP expression (Supplementary **Fig. S1**).

Co-transduction of primary MDMs

To prevent undesirable viral recombination events, the HRE-regulated E1A/B gene constructs were transferred into macrophages by plasmid transfection rather than co-infection with a second viral vector. MDMs that had been cultured for 3 days were infected with an MOI of 100 PFU/cell. For co-transduction, macrophages (2 x 10^6) were incubated overnight with the virus and then transfected with 5µg pcDNA3.1(+)-HRE-E1A/B (HRE-E1A/B) construct or the pcDNA3.1(+)-No-HRE-E1A/B (No-HRE-E1A/B) using the Amaxa Macrophage Nucleofection Kit (Amaxa Biosystems, Cologne, Germany).

Protein detection by 1-D SDS-PAGE

SDS page was performed on MDMs lysed in SDS buffer all techniques and antibodies used were described by us recently (13).

Complementation system

Clarified supernatants from co-transduced MDM cultured in normoxic or hypoxic conditions were applied to 293 cells at increasing dilutions. Plaque formation and GFP development was monitored daily in an end-point dilution assay. Viral particles (vp) were calculated by measuring the optical density (OD) at 260 nm after particle lysis in PBS/0.5% sodium dodecyl sulfate (SDS) for 15 min at 37°C. Titres were calculated using OD260 x 1.1 x 10^{12}vp/ml (14, 15).

Infiltration of primary human macrophages into tumor spheroids *in vitro*.

Tumor spheroids were generated using LNCaP and A549 cells by seeding 5 x 10^3 cells into each well of a 2% agarose-coated 96-well tissue culture plate. After 7–10 days, each well contained a 700-800μm spheroid, to which 3 x 10^4 co-transduced macrophages were added. These experiments were then repeated in the presence of anti-adenoviral antibodies obtained from pooled human serum (as outlined in detail in the supplemental methods).

Macrophage trafficking to hypoxic prostate tumor xenografts

Male CD1 athymic mice (aged 6-8 weeks; Harlan laboratories, Bicester, UK) were injected subcutaneously with 2 x 10^6 human prostate cancer PC3 cells. Mice were injected with 3 x 10^6 either; co-transduced (CMV-AdV5-GFP or PSA-AdV5-GFP and HRE-E1A/B plasmid) macrophages, singly-transduced (CMV-AdV5-GFP) macrophages, untransduced macrophages or PBS alone through the tail vein. After 48h, mice were injected with 60 mg/kg PIMO for 1 hour before sacrifice. For flow cytometry tumor, liver, lung, kidney and spleen were made into cell suspensions and incubated with phycoerythrin (PE)-conjugated anti-CD14 (Serotec Ltd., Oxford, U.K.). For microscopic studies, 7 uM frozen sections were stained with human anti-CD68, rabbit anti-PIMO and rabbit anti-GFP (1:100). PE-anti-mouse (1:250; Dako), Alexa-647-anti-rabbit

(1:250) and Alexa-488-anti-rabbit (1:250) secondary antibodies, were incubated with tumor sections for 30 min and then DAPI (30nM) stained. Prolong Gold Anti-fade used to preserve fluorescence (all reagents from Molecular Probes Inc, Eugene, OR). Images were captured using a Zeiss LSM 510 confocal microscope (magnification x400).

For the therapeutic studies, the replication competent adenovirus Ad[I/PPT-E1A] replaced the CMV-AdV5-GFP and we used the LNCaP xenograft model (both subcutaneous and orthotopic) as Ad[I/PPT-E1A] has been shown to be highly lytic in this cell line (16). [NB. macrophage trafficking studies performed in the presence of human serum are detailed in the supplementary methods].

(i) Subcutaneous model

LNCaPs were mixed 1:1 with matrigel (BD Biosciences) and 2×10^6 injected subcutaneously into the hind flank region. When the tumors reached 4mm in diameter, mice were injected via tail vein with 100ul PBS containing either 3 million singly-transduced human MDMs (infected with the oncolytic virus Ad[I/PPT-E1A] at MOI 100), or co-transduced macrophages (Ad[I/PPT-E1A] at MOI 100 and HRE-E1A/B), 5×10^{10} Ad[I/PPT-E1A] only or PBS alone. Tissues including tumors, kidney, liver, lungs and spleen, were paraffin wax embedded. Human and mouse macrophage populations were identified in sections by staining with antibodies to human CD68, and murine F4/80 (AbD Serotec) all sections were H & E stained.

(ii) Orthotopic model

One million cells were mixed 1:1 in Matrigel and injected into the dorsolateral prostate. Tumor take was monitored by ultrasound using Vevo 770 High resolution imaging platform (Visual Sonics, Ontario, Canada) and mice were treated as above. Thirty days after treatment, animals

were sacrificed and tumors/organs were collected, sectioned and stained by H&E. Whole tumor sections were scanned on Aperio slide scanner (Aperio slide scanner, Vista, CA) to facilitate tumor diameter measurements.

MTS assay

Singly-transduced (Ad[I/PPT-E1A]) or co-transduced macrophages (Ad[I/PPT-E1A] and HRE-E1A/B) were incubated for 16 hours at 37°C in humidified Heto multi-gas incubators (0.5% oxygen). Medium was collected and centrifuged at 10,000 g for 5 minutes to remove cell debris, and then applied to LNCaPs at different dilutions (Neat, 10^2, 10^3, 10^4, 10^5). LNCaPs were also transduced with Ad[I/PPT-E1A] (50pfu/cell) as a positive control. Cell viability was analyzed on day 6 after transduction using the MTS Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

In vitro tubule formation assay

Conditioned medium was collected from LNCaP spheroids or spheroids infiltrated with untreated MDMs or Ad[I/PPT-E1A] co-transduced MDMs after 48h of culture. Serum starved human umbilical vein endothelial cells (HUVECs; 8000cell/well) were resuspended in conditioned medium and seeded onto growth factor-reduced Matrigel (BD Biosciences)- coated wells. After 12h, tubule formation was measured using ImageJ software (National Institute of Health, Bethesda, MD).

Statistical analysis

Statistical comparison was performed using the GraphPad prism programme (GraphPad software, San Diego, CA). Non-parametric statistical tests were used to analyse the data and the logrank test was used to compare tumor survival curves using Kaplan-Meier. A value of $P = 0.05$ was the limit of statistical significance.

Results

TAMs accumulate in hypoxic areas of human prostate carcinoma.

TAMs can be detected in both well-oxygenated (**Fig. 1A**) and hypoxic (**Fig. 1B**) areas of human prostate tumors. Poorly vascularised (hypoxic) areas contained higher numbers of TAMs (**Fig. 1Ci**) than highly vascularised ones (**Fig. 1Cii**).

E1A complementation and replication of adenovirus in macrophages under hypoxic conditions.

The E1A complementation system in co-transduced MDMs in the presence of tumor hypoxia is illustrated in **Fig. 2A**. To test this, MDMs were infected with the adenovirus vector CMV-Ad5-GFP at increasing MOIs (0-100). The efficiency of infection (i.e. GFP expression with this vector) was routinely found to be $75 \pm 9.5\%$ (n=5) and reached its peak after 72 h of infection with minimal cytotoxicity compared to uninfected macrophages (Supplementary **Fig. 1A& 1B**).

Co-transduced macrophages, exposed to hypoxia (0.1% O_2) for 16h, up-regulated HIF-1α, HIF-2α, and (hypoxia regulated) E1A proteins compared to normoxic conditions (**Fig. 2B**). Furthermore, when supernatants from co-transduced MDMs were titrated in HEK 293 cells, only supernatants from hypoxic MDMs (**Fig. 2Ci**) resulted in production of detectable infectious virus. Average yields of 5001 ± 1495 PFU/infected macrophage (n=5) were confirmed in the plaque assays, by both limiting dilution (**Fig. 2Ci**) and flow cytometry even when supernatants were diluted to 1 in 1 x 10^6 (**Fig. 2D**). No infectious adenovirus was detected under normoxia (**Fig. 2Cii and D**) or in cells transfected with the No-HRE-E1A/B construct (**Fig. 2C i, ii & D**) indicating that the hypoxic trigger for viral replication was tightly regulated.

Hypoxic activation of adenovirus production by macrophages infiltrating prostate tumor spheroids *in vitro*.

Inner areas of tumor spheroids are severely hypoxic due to limited diffusion of oxygen from the surrounding culture fluid (11, 17). Lung (A549) and prostate (LNCaP) tumor spheroids were used to assess the prostate-specificity of the macrophage-based viral delivery system. The inner, peri-necrotic regions of both sets of spheroids were confirmed as hypoxic (**Fig. 3A**). MDMs co-transduced with the HRE-E1A/B plasmid and a CMV-AdV5-GFP were seen to infiltrate both LNCaP and A549 spheroids equally (**Fig. 3B**). Co-transduced MDMs released sufficient adenovirus to cause widespread CMV-driven GFP expression throughout A549 and LNCaP spheroids (normoxic and hypoxic areas) within 72h of infiltration (**Fig. 3C**).

When MDMs co-transduced with an adenovirus where GFP expression was regulated by the PSA promoter (PSA-AdV5-GFP) and HRE-E1A/B infiltrated LNCaP and A549 spheroids widespread expression of PSA-driven GFP was only seen LNCaPs (**Fig. 3D & E**).

Macrophage delivery of adenovirus to hypoxic areas of human prostate tumors.

(i)   Delivery of a GFP-expressing adenovirus to prostate tumor xenografts in vivo.

Initially, we confirmed the relevance of our prostate tumor xenograft models to human prostate tumors by showing a similar level of hypoxia in prostate xenografts and primary human prostate carcinomas (**Supplementary Fig. 2S**).

We then co-transduced MDMs with CMV-Ad5-GFP and HRE-E1A/B and administered these via tail vein injection to mice bearing subcutaneous human prostate (PC3) tumors. Control mice received PBS alone, untransduced MDMs or singly-transduced MDMs (no HRE-E1A/B). Triple immuno-fluorescence microscopy on frozen human tumors labelled with antibodies

against PIMO (white), human CD68 (red) and GFP (green) revealed that human MDMs had migrated into hypoxic/peri-necrotic areas of the tumor (**Fig. 4A**). No human CD68+ cells were detected in tumors from mice injected with PBS alone and no GFP was detected in mice injected with untransduced MDMs (data not shown). In mice receiving singly transduced MDMs, GFP expression was largely restricted to CD68+ human macrophages (**Fig. 4A;** upper panel). This contrasted with the abundance of GFP+ cells in hypoxic epithelial cells and macrophages in tumors treated with co-transduced MDMs (**Fig. 4A;** lower panel). Therefore, hypoxia present in prostate tumor xenografts triggered viral replication within MDMs and viral spread from MDMs resulted in the infection of neighbouring tumor cells.

In contrast to the hypoxic tumors, the frequency of human MDMs in the liver and lung was low (< 1 cell/field/view in a total of 10 fields) and no MDMs were detected in the kidney or spleen (data not shown). These immunohistochemical data were supported by flow cytometry. Similar levels of human MDM infiltration into tumors (2-5%) was seen in all mice injected with MDMs (**Fig. 4B**). This equates to approx $442,000 \pm 25,000$ human MDMs per LNCaP xenograft (i.e. 2.21% of the 3×10^6 MDMs injected), as determined using the regression equation described by Meyskins et al (1984)(18) [No. of cells/tumor = 2.40 (tumor diameter)$^{2.378}$ / (cell diameter)$^{2.804}$]. In contrast, mice injected with PBS showed negligible levels of GFP (1.5% $\pm$ 0.4) - whereas in mice injected with singly transduced MDMs, this increased to 5.6% $\pm$ 1.4 of cells. However, in tumors from mice injected with co-transduced MDMs GFP expression was significantly higher (21% $\pm$ 5.4 of cells) (**Fig. 4B;** right panel). These data were reproduced when mice bearing LNCaP xenografts were injected with macrophages co-transduced with Ad5-PSA-GFP and HRE-E1AB. The presence of human macrophages and adenovirus in these tumors was confirmed using antibodies to human CD68 and E1A (data not shown).

(ii) Use of macrophages to target oncolytic adenovirus, Ad[I/PPT-E1A] to prostate tumors

(a) Immunophenotype of MDMs co-transduced to express the Ad[I/PPT-E1A].

The oncolytic adenovirus, Ad[I/PPT-E1A], specifically replicates in prostate tumor cells (12, 16), since E1A gene expression is controlled by a recombinant regulatory sequence designated PPT, consisting of a PSA enhancer, PSMA enhancer and a T-cell receptor γ-chain alternate reading frame protein promoter (12) However, the therapeutic efficacy of Ad[I/PPT-E1A] has only been seen following intra-tumoral administration into mice bearing prostate tumors (12, 16, 19). Systemic delivery of such adenoviruses is limited by liver sequestration, complement, pre-immune IgM and neutralising antibodies (20).

The impact of co-transduction on the phenotype of macrophages was investigated before injection *in vivo*. Flow cytometric analysis of co-transduced MDMs revealed no significant alteration in the expression of CD11b, CD14, CD68 or classic anti-inflammatory markers such as, CD36, MHC-class II and mannose receptor when compared to untreated MDMs or singly transduced MDMs (data not shown). However, an increase in cell surface expression of toll-like receptors (TLRs) 2 and 4 was evident (**Supplementary Fig. 3A**). TLRs mediate adenoviral regulation and transgene expression in human peripheral blood mononuclear cells following infection, activating their expression of proinflammatory cytokines (21, 22). This was confirmed when co-transduced MDMs were found to release elevated levels of various pro-inflammatory cytokines (**supplementary Fig.3B**).

(b) Effect of MDMs co-transduced with HRE-E1A/B and Ad[I/PPT-E1A] on tumor spheroids.

Infiltration of LNCaP spheroids by co-transduced MDMs triggered sufficient adenoviral production to cause widespread cell death after 48h (**Fig. 5A & B**). The ability of hypoxic, co-transduced MDMs to kill monolayers of LNCaPs was confirmed using the MTS assay (**Fig. 5C**). No cell death was evident for monolayers of A549 cells infected in parallel MTS assays (data not shown).

As macrophages in tumors can be pro-angiogenic (9), we investigated whether this might be the case with our co-transduced MDMs, by taking media conditioned by LNCaP spheroids alone or following infiltration by co-transduced MDMs and adding this to human umbilical vein endothelial cells (HUVECs). Co-transduced MDMs failed to induce tubule formation (**Supplementary Fig. 4A & B**). Interestingly, the presence of co-transduced MDMs significantly ($p = 0.035$) reduced rather than increased the release of the pro-angiogenic cytokine, VEGF, by LNCaP spheroids. It also increased release of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8 (**Supplementary Fig. 4C**) while leaving IL-10, IL-12p70 or GMCSF levels in the media unaffected (data not shown).

(c) Effect of MDMs co-transduced with HRE-E1A/B and Ad[I/PPT-E1A] on subcutaneous and orthotopic LNCaP xenografts *in vivo*.

Mice bearing subcutaneous LNCaP xenografts were injected with 100ul PBS containing either 3 x 10^6 singly-transduced MDMs, co-transduced MDMs or 5 x 10^{10} Ad[I/PPT-E1A] particles. As early as day 5 after treatment initiation, a single injection of co-transduced MDMs resulted in 50% reduction in tumor size, and by day 15, 2 mice were tumor-free. Even after 50 days, and a single treatment, the 3 remaining mice in the cotransduced macrophage group had tumors <20% of their original volume (**Fig. 6A**), whereas a single injection of Ad[I/PPT-E1A] alone initially

delayed tumor growth for up to 30 days, but tumors then regrew and were 50% greater than their starting volume. Inhibition of tumor growth between these two groups was significant ($p=$ 0.001). By contrast, mice that received PBS or singly-transduced MDMs had to be killed early due to large tumors. To confirm that tumor regression correlated with the cytolytic ability of Ad[I/PPT-E1A], immunohistochemistry with an anti-E1A antibody detected E1A protein expression in the tumors of mice receiving both co-transduced MDMs and Ad[I/PPT-E1A] but not in tumors treated with PBS or singly-transduced MDMs (data not shown). E1A expression was also only detected in the livers of mice directly injected with Ad[I/PPT-E1A] alone (data not shown).

Survival data are summarized in **Fig. 6A** (right panel). Tumor-bearing mice that received co-transduced MDMs survived longer than the other treatment groups. **Fig. 6B** shows representative images of tumors from each group of mice. Tumors from mice receiving co-transduced MDMs were necrotic and contained few viable cells (**Fig. 6B**). Interestingly, the tumors from mice treated with Ad[I/PPT-E1A] or co-transduced MDM were considerably paler and less vascularised (**Fig. 6B**). In the case of Ad[I/PPT-E1A] injected mice, this could have resulted from the reduction in blood vessels on the under surface of tumor and/or the marked leukocyte infiltration that reportedly occurs with oncolytic adenoviruses (20). Indeed, tumors in the Ad[I/PPT-E1A] injected group had significantly more murine F480+ cells (64±13%) compared to the other groups (**Fig. 6B**). By contrast, mice receiving co-transduced MDMs had low F4/80+ cell infiltration - most likely because these tumors were very small and mainly necrotic/acellular. This implies that the anti-angiogenic effect of co-transduced MDMs seen *in vitro* also occurred in LNCaP tumors *in vivo*.

Tumor growth in mice bearing orthotopic LNCaPs was significantly inhibited when mice were

injected with Ad[I/PPT-E1A] alone or co-transduced macrophages (**Fig. 6C**), the latter having a more marked effect. Significantly more F480+ TAMs (71%±17) were detected in mice injected with Ad[I/PPT-E1A] than those injected with PBS alone, singly-transduced MDMs or co-transduced MDMs (data not shown). In both tumor models considerably more necrosis was detected in tumors from mice injected with co-transduced MDMs (**Fig. 7A&B**). Notably, although lung metastases were visible in mice in control groups (**Fig. 7A,B ii, iii**), these were absent in the mice treated with co-transduced MDMs.

(iii) Macrophages protect Adenovirus from adenoviral neutralizing antibodies in human sera

In humans, pre-existing humoral immunity to adenovirus can interfere with the systemic delivery of adenovirally-based therapies. We hypothesised that our macrophage delivery system would protect adenoviruses against these pre-existing anti-adenoviral neutralising antibodies (NAb). Indeed, the presence of high titer NAb in cultures of co-transduced MDMs (CMV-AdV5-GFP and HRE-E1A/B) did not prevent E1A complementation and replication under artificially induced hypoxic conditions (data not shown) or in hypoxic tumor spheroids as seen by the widespread expression of GFP (**Supplementary Fig.5A- upper panel**). By contrast, the same concentration of NAb significantly neutralized the transduction of tumor spheroids by adenovirus (CMV-AdV5-GFP) alone (**Supplementary Fig. 5A; upper panel**). Delivery of oncolytic Ad[I/PPT-E1A] by co-transduced MDM was also not effected by NAb and replication of the oncolytic virus resulted in reduced spheroid size comparable to that of untreated co-transduced MDMs (no NAb) (**Supplementary Fig. 5A; lower panel**). Prior to injecting co-transduced MDM or adenovirus alone, tumor-bearing mice were passively immunized with this high titer NAb and the presence of circulating NAb was confirmed 3h post injection

(**Supplementary Fig. 5b**). Mice treated with co-transduced MDMs armed with CMV-AdV5-GFP or Ad[I/PPT-E1A] resulted in far superior viral delivery and replication within the tumors compared to virus administered on its own and this was unaffected by NAb (**Supplementary Fig. 5B**).

Discussion

Adenoviruses in which HIFs regulate gene expression have been used to target hypoxic tumor cells in human tumor xenografts and in clinical trials but these had to be injected directly into primary tumors and at very high viral titres of adenovirus. Moreover, this approach fails to target metastatic tumors growing at distant sites (23-26).

Our finding that macrophages accumulate in hypoxic areas of human prostate tumors and human prostate tumor xenografts prompted us to develop a means of using these cells to deliver therapeutic adenovirus to these sites - and via a systemic route permitting the targeting of primary tumors and their metastases. Since hypoxia also exists in diseased tissues other than tumors (27) and mild hypoxia can exist in healthy tissues (28), we added a further degree of tumor targeting by placing the exogenous gene (eg. GFP) in the virus - or the further replication of the virus itself when released by macrophages - under the control of prostate-specific promoters.

We found hypoxia-induced replication and release of adenovirus by infiltrating co-transduced macrophages resulted in the widespread infection of tumor cells and expression of the PSA-driven GFP reporter gene construct in the adenovirus. Interestingly, infiltration of tumor spheroids or tumor xenografts with relatively small numbers of co-transduced human macrophages resulted in widespread dissemination of the virus (due to the amplification and release of thousands of viral copies by each hypoxic macrophage).

We then used macrophages to systemically deliver an oncolytic adenovirus (Ad[I/PPT-E1A]). This virus previously demonstrated therapeutic potential but only when injected directly into prostate tumors (16, 19). Studies using different oncolytic viruses in various tumor models have shown a heterogeneous and incomplete dissemination of virus (29-31) since adenoviruses

interact with human blood cells following systemic delivery. This could be responsible for reducing the therapeutic effect (32). The compromised vascular supply within necrotic areas, the distorted functional properties of tumor vessels, and the elevated tumor interstitial fluid pressure also contribute to an unequal viral distribution within tumors (30).

Mice bearing both subcutaneous and orthotopic tumors exhibited remarkable tumor regression following a single injection of co-transduced MDMs and showed no signs of regrowth. Viral delivery by macrophages therefore resulted in a lasting anti-tumor effect with negligible metastatic frequency. Moreover, we required at least 1000-fold fewer adenoviral particles in this system, compared to animals administered with virus alone. We have therefore exceeded previous benchmarks by greatly reducing the amount of virus tumor-bearing mice receive, circumventing the need for systemic intratumoral administration. We are aware however, that our *in vivo* studies were carried out in immunocompromised mice. This choice of animal model was because human Ad replication is known to be negligible in rodent cells so syngeneic murine tumor models could not be used (33, 34). Although host immunity against such co-transduced, 'therapeutic' autologous macrophages may be activated in a fully immunocompetent host, the fact that the adenovirus is in a latent phase during trafficking of macrophages to hypoxic tumor sites, suggests that expression of viral antigens is likely to be extremely low. The oncolytic adenovirus used in this study has been tested extensively against numerous human tissues as part of a clinical assessment of its safety in possible clinical trials. Even at high multiplicities of infection this virus showed no ability to replicate in primary cells from such healthy human tissues as endothelium, urothelium, lungs, liver etc (Maitland NJ and co-workers unpublished observations). Moreover, neutralizing antibodies from human sera administered to human prostate tumor-bearing mice, had no impact on the activity of this

oncolytic virus when delivered systemically via our macrophage system suggesting that patients

with preexisting adenovirus immunity would be suitable candidates for i.v. administration of

such viruses (35). The application of a cell-mediated viral delivery system like the one described

in this study may also prevent adenovirus neutralization from taking place by offering protection

to the virus from humoral immunity (36, 37).

In summary, we describe a novel system whereby the natural ability of macrophages to

harbor and support viral replication was used as 'silent carriers' to trigger oncolytic viral

production in hypoxic areas of tumors, resulting in intratumoral spread and a lasting therapeutic

effect.

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Figure Legends:

Figure 1. Macrophages are present in hypoxic areas of human prostate carcinoma. Sections were dual stained for CD68/FVIII to identify the areas of greatest intra-tumoral macrophage and vascular density – the 'hotspots'. Mean MVD and mean MφD were assessed microscopically using a 25-point Chalkley array eyepiece graticule (x250 magnification; field area $0.189mm^2$). Representative bright-field images of CD68 positive macrophages (brown; arrows) co-stained (red) for pimonidazole. (A) A non-hypoxic tumor area. (B) A hypoxic (PIMO positive) area. Bar = 50μM. (C) MφD were highest in areas of poor vascularity ($P < 0.001$) (i), whereas MVD was significantly ($P < 0.001$) higher in areas of low macrophage counts (ii).

Figure 2. Use of macrophages to deliver tissue-specific therapeutic adenovirus to tumors. (A) (i) Macrophages were infected with a replication-deficient adenovirus (Ad) carrying a therapeutic gene controlled by prostate-specific promoters and then (ii) transfected with a hypoxia-driven E1A/B plasmid. (iii) Migration of these co-transduced macrophages into hypoxic areas of tumors activates the HRE-driven expression of E1A/B allowing viral replication and release. (iv) The Ad then infects prostate cancer cells in which the PSA promoters activate the expression of the therapeutic gene. Cells in which the PSA promoter is inactive do not transcribe the therapeutic gene and are not affected. Immunoblots showed that MDM co-transduced with CMV-Ad5-GFP and HRE-E1A/B expressed HIF-1α, HIF-2α and E1A protein following exposure to hypoxia (B). Supernatants from these cells transduced HEK 293s to express GFP and form plaques, (C) in hypoxia (i) but not normoxia (ii). Combined data from 4 independent experiments showed this effect was statistically significant (P<0.05) (D).

Figure 3. Hypoxia induces adenovirus production in co-transduced macrophages infiltrating human tumor spheroids. LNCaP and A549 tumor spheroids were co-cultured individually with 3 x 10^4 MDMs 24h post CMV-Ad5-GFP and HRE-E1A/B transduction. A549 and LNCaP spheroids were (A) PIMO stained (N=necrotic centre and pink=hypoxia) and then (B) stained with anti-human CD68. (C) Fluorescent microscopy of the infiltrated spheroids revealed GFP expression. However, in spheroids infiltrated with MDM co-transduced with PSA-AdV5-GFP & HRE-E1A/B GFP was detected in only LNCaPs (D) and (E). Bars in panels A and B = 200μM.

Figure 4. Co-transduced MDMs release GFP-expressing adenovirus in hypoxic prostate tumors. Forty eight hours after systemic injection of human untransduced, singly-transduced or co-transduced MDMs into mice bearing LNCaP xenografts, tumor xenografts were removed, sectioned for immunoflourescence or enzymatically dispersed for flow cytometry. Frozen sections were labelled with antibodies against PIMO (white), human CD68 (red) and GFP (green). White arrows=hypoxic macrophages (A). Single cell suspensions were stained with anti-human CD14PE or propidium iodide (PI) and analyzed by flow cytometry (B). Representative fluorescent histograms from the same treatment groups (C). Data average±SD, n=5 mice/group.

Figure 5. MDMs co-transduced to express Ad[I/PPT-E1A] efficiently lyse tumor cells in LNCaP spheroids *in vitro*. Human prostate tumor (LNCaP) spheroids were co-cultured with 3 x 10^4 MDMs 24h co-transduced with Ad[I/PPT-E1A] and HRE-E1A/B. Parallel sets of spheroids were also infiltrated with singly-transduced MDMs or Ad[I/PPT-E1A] (50 pfu/cell). (A) Representative dot plots of enzymatically-dispersed spheroids (B) Light microscopy images of

whole, individual spheroids (C) Loss of LNCaP tumor cell viability was also confirmed using the

MTS assay. The data are averages for triplicate samples. Bar in b = 200μM.

**Figure 6.   Systemic delivery of MDMs co-transduced with HRE-E1A/B plasmid and
Ad[I/PPT-E1A] markedly slows the growth of both subcutaneous and orthotopic LNCaP
xenografts.** (A, B) *Subcutaneous LNCaP tumor model***:** (A) Only injections of co-transduced
MDMs and Ad[I/PPT-E1A] significantly reduced tumor volume compared to PBS treatment
(*p*=0.003 and *p*=0.01, respectively) and singly-transduced MDM (*p*=0.0001 and *p*=0.0012,
respectively). Survival data for all four groups was plotted as a Kaplan–Meier survival curve (a-
right panel). (B) Photographs of the tumors following treatment (*top row*). Representative (x 20)
images for necrosis (middle-panel) and murine macrophage infiltration (F480) (bottom panel)
are presented. (C) *Orthotopic LNCaP tumor model*: Representative images of H&E tumor
sections and analysis of tumor volumes (panel c, bottom). Data shown are means ± SEMs.

**Figure 7. Increased primary tumor necrosis and reduced pulmonary metastases following
systemic delivery of co-transduced MDMs expressing HRE-driven Ad[I/PPT-E1A].** Male
nude mice bearing either: (A) subcutaneous or (B) orthotopic LNCaP xenografts were injected
i.v. with PBS, or 3 million either singly-transduced MDMs or co-transduced MDMs or 5 x 10^{10}
Ad[I/PPT-E1A] viral particles. (i) After 50 days (s.c tumors) or 30 days (orthotopic tumors)
tumors and lungs removed and processed for H&E. (ii) Representative appearance of metastatic
tumors ('M') growing in the lungs of mice injected with Ad[I/PPT-E1A] (x20 magnification;
H&E stained tissue). (iii) Number of metastatic foci (> 5 tumor cells) in lungs from all 4
treatment groups. Data are the means ± SEMs (5 mice per group were used in the s.c model and

3 mice per group in the orthotopic model. These experiments were repeated twice). Bar = 200μM.
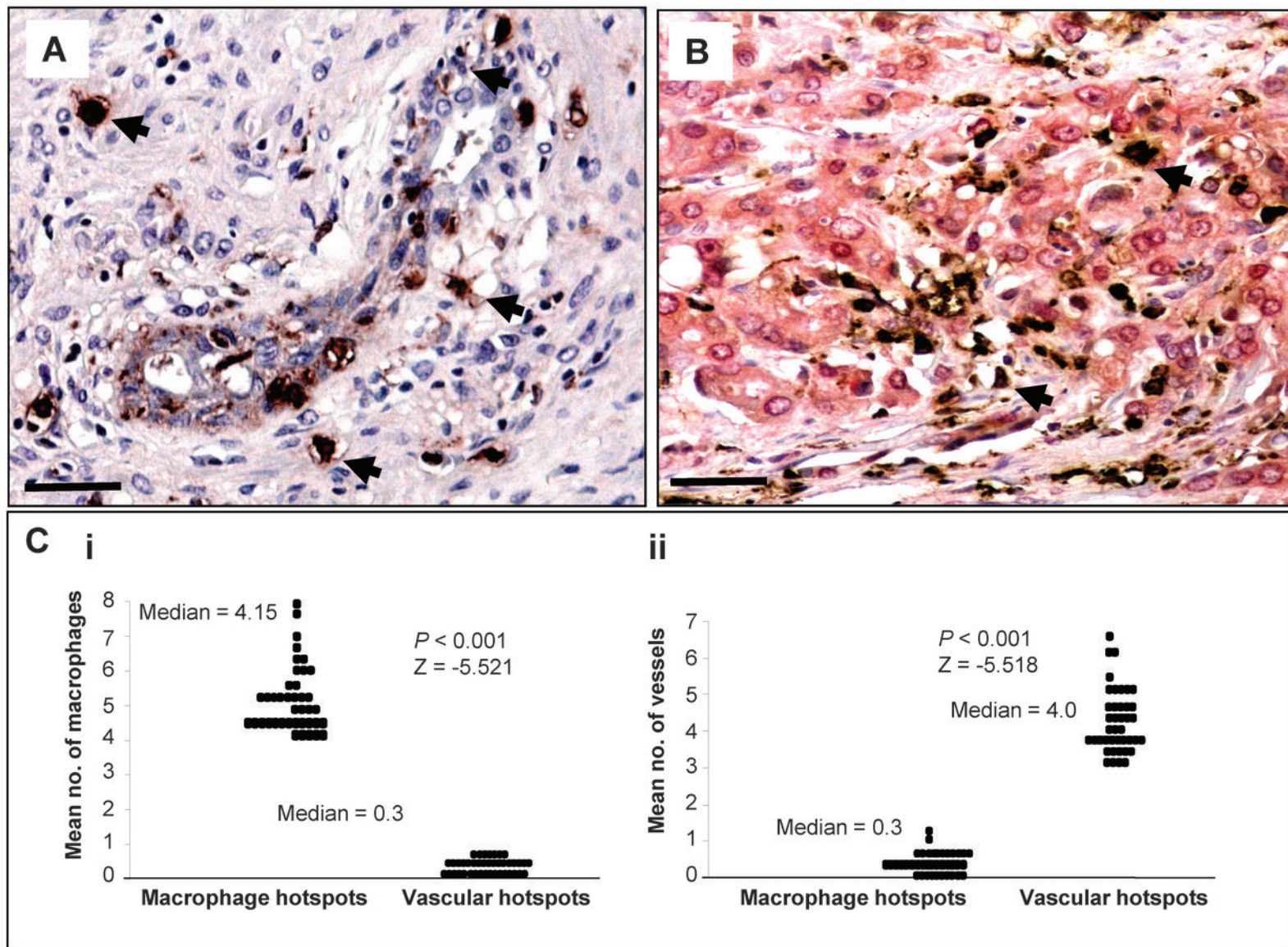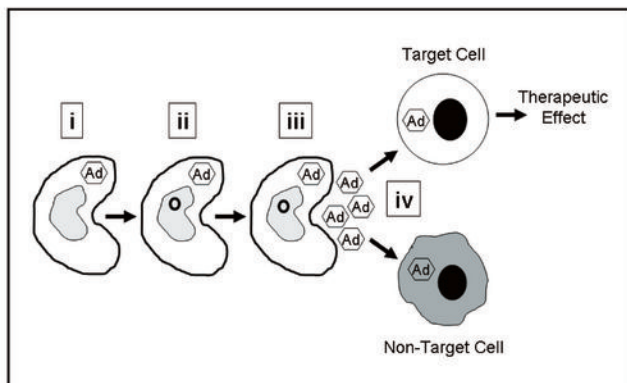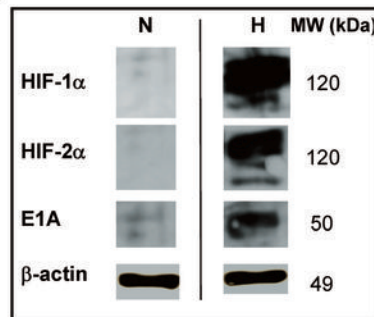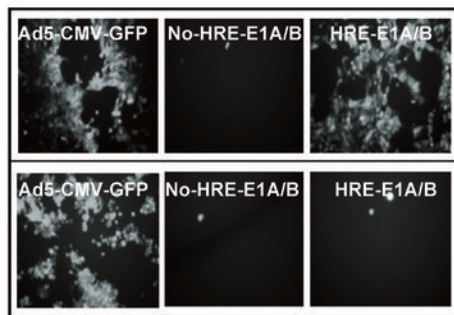
Figure 1 Lewis

Figure 2 Lewis

A

i → ii → iii

Target Cell

Therapeutic Effect

iv

Non-Target Cell

B

	N	H	MW (kDa)
HIF-1α			120
HIF-2α			120
E1A			50
β-actin			49

C

i — Ad5-CMV-GFP | No-HRE-E1A/B | HRE-E1A/B

ii — Ad5-CMV-GFP | No-HRE-E1A/B | HRE-E1A/B

D

% GFP+ Cells

Normoxia

Hypoxia

Macrophage dilution →

10^1 10^2 10^3 10^4 10^5 10^6
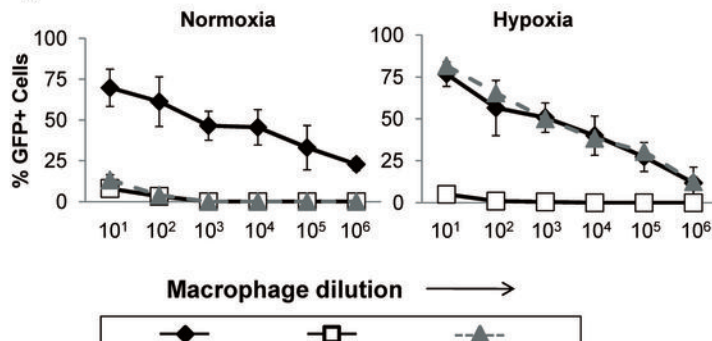
Figure 3 Lewis
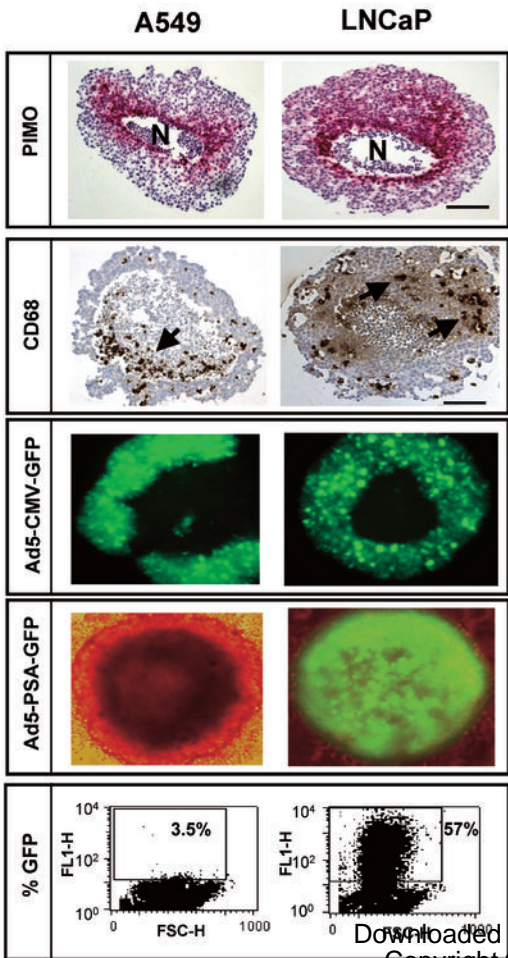
Figure 4 Lewis
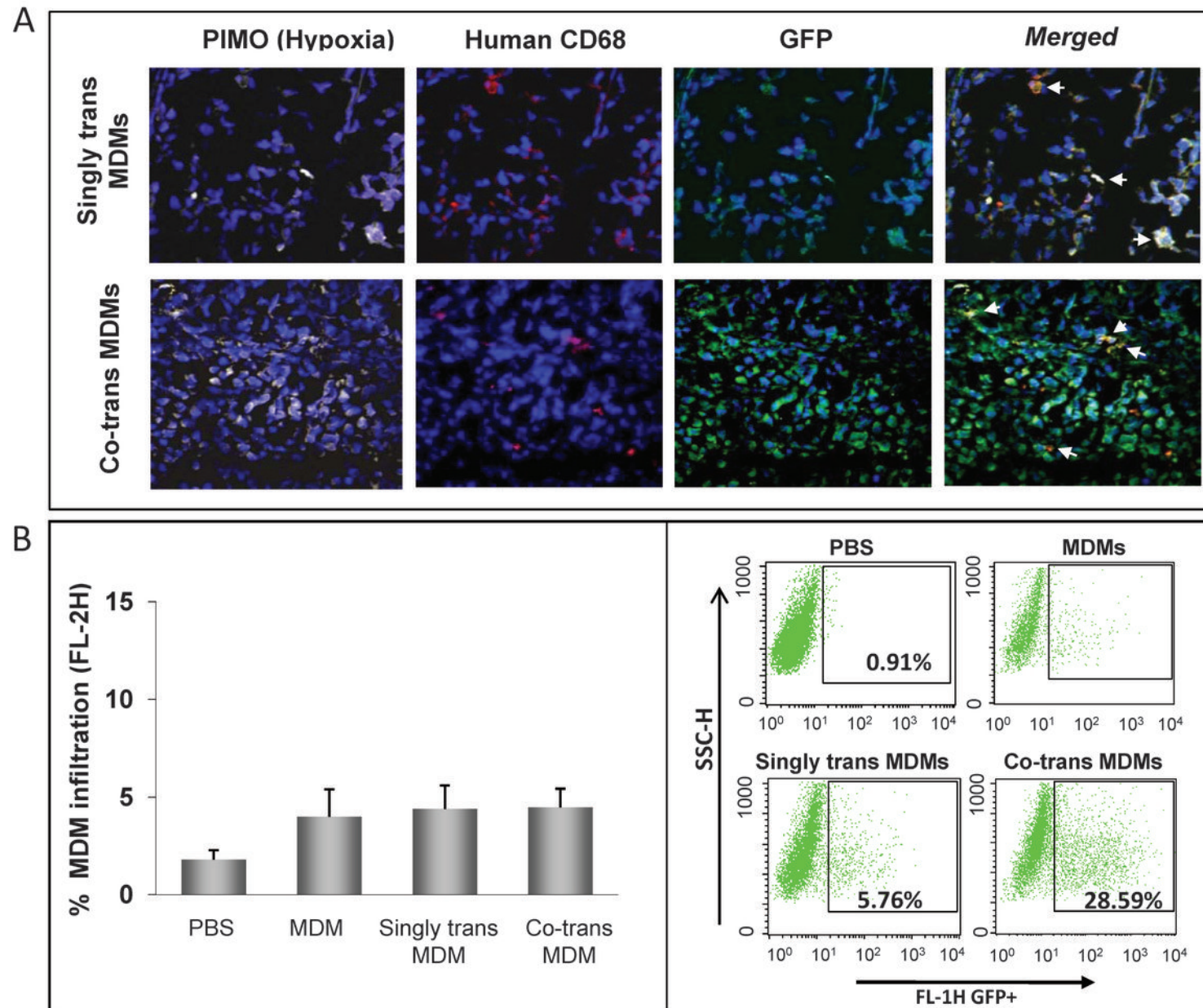
Figure 5. Lewis

A

B

C

Figure 6 Lewis

A

Tumor Volume MM³ vs Days; Percent survival vs Days
- PBS
- Ad[I/PPT-E1A]
- Singly trans MDM
- Co-trans MDM

(p=0.001)

B

	PBS	Singly-trans MDM	Ad[I/PPTE1A]	Co-trans MDM
2 cm	709 mm3	636 mm3	316 mm3	42 mm3
H&E				
F480				

C

PBS | Singly-trans MDMs
Ad[I/PPT-E1A] | Co-trans MDMs

Tumour volume (mm³)
- PBS
- Singly-trans MDMs
- AdPPT1E1A
- Co-trans MDMs

p=0.03

Figure 7. Lewis