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Pseudomonas aeruginosa exotoxin T induces potent cytotoxicity against a variety of murine and human cancer cell lines

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In patients with malignancy, the major barrier to achieving complete response is emergence of resistance to current chemotherapeutic agents. One of the major mechanisms by which tumour cells become resistant to therapies is by altering cellular drug targets through mutations and/or deletions. Resistance by this mechanism is achieved more easily if the drug has limited cellular targets and/or processes. We hypothesized that as *Pseudomonas aeruginosa* exotoxin T (ExoT) targets six proteins that are required for cancer cell survival and proliferation, it is highly unlikely for cancer cells to develop resistance to this toxin. We assessed ExoT's cytotoxicity against multiple invasive and highly resistant tumour cell lines in order to evaluate its potential as a chemotherapeutic agent. Our data demonstrated that ExoT induced potent cytotoxicity in all tumour cell lines that we examined. Collectively, our data highlighted the potential of ExoT as a possible chemotherapeutic candidate for the treatment of cancer.

INTRODUCTION

Resistance to cancer chemotherapeutic drugs is a frequent cause of cancer treatment failure (Gonzalez-Angulo *et al.*, 2007) and highlights the need for novel therapies. Tumour cells can utilize a number of mechanisms to develop resistance to cancer drugs (Gottesman, 2002; Calcagno & Ambudkar, 2010). One of the main mechanisms by which resistance to therapy develops in cancer is by modifications in the cellular drug target through mutations, deletions and/or gene amplification (Gottesman, 2002; Calcagno & Ambudkar, 2010). Development of drug resistance by this mechanism is achieved more easily if the therapy has limited cellular targets and/or processes. For instance, mutations in the epidermal growth factor receptor (EGFR) gene have been identified in specimens from patients with non-small-cell lung cancer treated with anilinoquinazoline EGFR inhibitors (Kobayashi *et al.*, 2005). Similarly, resistance to the Abl tyrosine kinase inhibitor STI-571 in chronic myeloid leukaemia patients was shown to occur through either *bcr–abl* gene amplification or a single C→T nucleotide change within the *abl* locus at nt 944 (Gorre *et al.*, 2001).

Bacterial toxins have been, and continue to be, evaluated in various clinical trials as potential cancer therapeutics (Kawakami *et al.*, 2006; Kreitman, 2006, 2009). For example, *Pseudomonas* exotoxin A and diphtheria toxin are the most common bacterial toxins that have been or are currently under clinical evaluation against a variety of haematologic malignancies and solid tumours with promising results (reviewed by Becker & Benhar, 2012). Although these new recombinant immunotoxins have shown high potency in killing tumour cells with high specificity, they too share the limitation of targeting a single cellular substrate, eEF-2 (Jørgensen *et al.*, 2008). In fact, mutations in eEF-2 have been shown to emerge easily, conferring resistance to these toxins in cancer cells (Foley *et al.*, 1995; Jørgensen *et al.*, 2008; Wei *et al.*, 2012).

Pseudomonas aeruginosa exotoxin T (ExoT) is different from exotoxin A and diphtheria toxin in that instead of a single putative target (e.g. eEF-2), it has at least six cellular proteins (Krall *et al.*, 2000; Kazmierczak & Engel, 2002; Sun

Abbreviations: EGFR, epidermal growth factor receptor; ExoT, exotoxin T; LDH, lactate dehydrogenase; PI, propidium iodide; T3SS, type III secretion system.

Two supplementary figures and five supplementary movies are available with the online Supplementary Material.

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& Barbieri, 2003; Garrity-Ryan *et al.*, 2004), which play important roles in survival, proliferation, metastasis and angiogenesis in cancer. ExoT's substrates include the small GTPase proteins, Ras homologue gene family, member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homologue (Cdc42); which serve many important functions in cancer, such as regulation of actin cytoskeletal dynamics, activation of protein kinases, cell cycle progression, cytokinesis, metastasis and cellular survival (del Peso *et al.*, 1997; Gómez *et al.*, 1997; Bagrodia *et al.*, 1998; Murga *et al.*, 2002; Feng *et al.*, 2010); the C10 regulator of kinases (CrkI, CrkII adaptor proteins), which are important for the formation and maintenance of cellular focal adhesions and cytokinesis (Cho & Klemke, 2000; Lamorte *et al.*, 2002; Rodrigues *et al.*, 2005; Heasman & Ridley, 2008); and the glycolytic enzyme phosphoglycerate kinase 1 (PGK1), which is important for angiogenesis in cancer (Lay *et al.*, 2000; Hwang *et al.*, 2006). Therefore, development of resistance in cancer cells to ExoT-induced cytotoxicity is highly unlikely.

We have shown previously that ExoT intoxication results in potent cell death in human cervical adenocarcinoma, HeLa cells (Shafikhani *et al.*, 2008). In this report, we investigated ExoT's cytotoxicity against a number of invasive and highly resistant skin, breast, lung and ovarian tumour cell lines in order to evaluate ExoT's potential as a possible candidate for chemotherapy. We found that *in vitro*, ExoT was capable of causing potent cytotoxicity in all cell lines studied. We also found that ExoT was sufficient to induce cytotoxicity and to reduce tumour establishment and growth of B16 melanoma *in vivo*. Collectively, our data suggested that *P. aeruginosa* ExoT may be an attractive novel candidate as a cancer drug.

METHODS

Transformed and non-transformed cell lines. Tumour cell lines MCF-7 [human metastatic breast adenocarcinoma (Soule *et al.*, 1973)], MDA-MB-231 [triple-negative human metastatic breast adenocarcinoma (Cailleau *et al.*, 1974)], EMT6 [murine breast carcinoma (Rockwell *et al.*, 1972)], 4T1 [murine metastatic breast cancer (Aslakson & Miller, 1992)], MCA-205 [murine-derived fibrosarcoma cell line (Korrer & Routes, 2014)], B16 murine melanoma [WT BRAF (Wellbrock *et al.*, 2008)], A375 human melanoma [with BRAF(V600E) mutation (Alcazar *et al.*, 2011)], Calu-3 [human lung adenocarcinoma (Fogh *et al.*, 1977)], LLC1 [murine lung carcinoma (Bertram & Janik, 1980)], SK-OV-3 [human ovarian adenocarcinoma (Fogh *et al.*, 1977)] and HeLa [human cervical adenocarcinoma (Scherer *et al.*, 1953)] were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) with phenol red supplemented with 10 % FBS, 1 % L-glutamine (Gibco) and 1 % penicillin/streptomycin (Gibco). The non-transformed cell types used in these studies were mouse embryonic fibroblasts, isolated as described previously (Park *et al.*, 2006), and peripheral blood mononuclear cells, cultured as described above. Cells were treated with bacteria when they were ~80–90 % confluent and transfected when they were ~60–70 % confluent (see below).

Plasmids. Expression vectors pIRES2-ExoT-EGFP (pExoT-GFP) and the empty expression vector (pGFP) were used in experiments involving transient transfection, and were described previously (Shafikhani & Engel, 2006).

Cytotoxicity assessment by time-lapse immunofluorescent video microscopy. ExoT was delivered into cancer cells either by *P. aeruginosa* bacteria or by transient transfection using pIRES2 mammalian expression vector and cytotoxicity was assessed as described previously (Shafikhani *et al.*, 2008; Wood *et al.*, 2013). Briefly, bacteria were grown overnight in LB media, adjusted to OD_{600} 0.05, corresponding to m.o.i.~10. The bacterial strains used in these studies were an ExoT-expressing ExoU-deleted PA103 strain (ΔU) or its isogenic ExoT-defective type III secretion system (T3SS) mutant strain [PA103 pscJ-gent^R (pscJ)]. These strains were described previously (Shafikhani & Engel, 2006; Shafikhani *et al.*, 2008; Mahmood *et al.*, 2013). Cancer cells, infected with ExoT-expressing and ExoT-defective *P. aeruginosa*, were placed under 5 % CO_2 at 37 °C in an incubation chamber (Pecon) fitted to an AxioVert Z1 (Zeiss) microscope using AxioVision version 4.2 software. Time-lapse video microscopy was performed at ×100 magnification (unless otherwise specified) and images were acquired at 15 min intervals, as described previously (Shafikhani & Engel, 2006; Shafikhani *et al.*, 2008; Wood *et al.*, 2013). Cytotoxicity associated with ExoT was assessed by the uptake of propidium iodide (PI) impermeant dye using time-lapse video microscopy as previously described (Wood *et al.*, 2013). Briefly, the number of red pixels (PI) for each video frame was measured by thresholding the red fluorescence channel using ImageJ version 1.48 (http://imagej.nih.gov/ij/). For transfection experiments, cells were grown as described above and transfected with Effectene (Qiagen) as per the manufacturer's protocol 1 h prior to the addition of PI and the start of time-lapse video microscopy. Time-lapse videos were assessed for cytotoxicity by determining the percentage of transfected GFP that became PI-positive, as described previously (Shafikhani & Engel, 2006; Shafikhani *et al.*, 2008; Wood *et al.*, 2013).

Cytotoxicity assessment by flow cytometry. Transformed cells were transfected with Effectene, whilst non-transformed cells were transfected using an electroporation kit (Lonza). At 24 h after transient transfection, cytotoxicity was also assessed using Fixable Viability Dye (eBiosciences) stain to determine the per cent cytotoxicity in transfected cells and the data were analysed by flow cytometry (FACSCanto II; BD Biosciences) as per the manufacturer's protocol. After 24 h, peripheral blood mononuclear cells were additionally stained with anti-CD3 antibody (eBiosciences) in order to specifically gate on the expression of ExoT in T-lymphocytes alone.

Cytotoxicity assessment by lactate dehydrogenase (LDH) release assay. LDH release was measured using an LDH Cytotoxicity Detection kit (Clontech). In a 24-well plate, cancer cells were seeded at 8×10^4 cells per well and cultured in 1 ml DMEM (Gibco) media without phenol red, sodium pyruvate and penicillin/streptomycin antibiotics, and supplemented with 1 % FBS. To further assess the cytotoxic effect ExoT had on cancer, tumour cell lines were infected with either ExoT-expressing ExoU-deleted PA103 strain (ΔU) or its isogenic ExoT-defective T3SS mutant strain (pscJ) at m.o.i.~10 (Shafikhani & Engel, 2006; Shafikhani *et al.*, 2008; Mahmood *et al.*, 2013). To measure the amount of LDH release, the suspensions of uninfected and infected cells were collected and centrifuged at 2.5 ***g*** for 10 min to remove bacteria. Uninfected and infected samples, along with LDH-high control samples treated with 1 % Triton X-100 and LDH-low control samples with media alone, were added into a 96-well plate and treated with LDH detection reagents as outlined in the LDH Cytotoxicity Detection kit user manual. A_{490} was measured using a Thermo Scientific Multiskan Spectrum spectrophotometer and SkanIt software. Per cent LDH release was calculated by substituting the appropriate absorbance values into the following formula:

$$\text{LDH release}\,(\%) = \frac{\text{triplicate absorbance} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

Animal experiments. All mouse experiments were approved by the Rush University Medical Center Institutional Animal Care and Use Committee. Female C57BL/6 mice (aged 6–8 weeks) were obtained from Harlan Laboratories. B16 tumours were established by injecting mice subcutaneously with 1×10^6 cells in 200 µl sterile PBS. Tumour size was determined as the product of the two longest dimensions as measured by callipers. For *in vivo* assessment of ExoT cytotoxicity, tumours were generated as above and allowed to grow until they reached 50 mm^2. At this time, the tumours were injected with 250 ng plasmid DNA (Shafikhani *et al.*, 2008) packaged in Lipofectamine transfection reagent (Invitrogen) to facilitate uptake. At 24 h after transfection, the mice were injected (intravenously) with 200 µl SR-FLIVO (ImmunoChemistry Technologies) cellular death dye and then euthanized after 1 h. Tumours were assessed by flow cytometry or fixed and mounted with VectaShield (Vector Laboratories) containing DAPI. For tumour growth studies, B16 cells were transfected with plasmid DNA, as described previously (Shafikhani *et al.*, 2008), for 15 h prior to injection. Cell viability was assessed using Trypan blue dye staining and transfection efficiency was determined by identifying GFP expression by flow cytometry. ExoT-GFP- or GFP-transfected B16 cancer cells (1×10^5) were injected into the right flank of C57BL/6 mice. Tumour surface area was measured daily with the use of callipers. Animals were euthanized using CO_2 asphyxiation at the indicated time, or when tumour size reached >150 mm^2 or animals were moribund.

Statistical analysis. All studies were performed in triplicate or as indicated. Statistical significance was determined by Student's two-tailed *t*-tests using Prism 6 (GraphPad). $P \leqslant 0.05$ was considered significant.

RESULTS

P. aeruginosa ExoT induces potent cytotoxicity in a variety of murine and human tumour cell lines

ExoT has previously been shown to induce potent cytotoxicity in HeLa cells (Shafikhani *et al.*, 2008). As ExoT targets six cellular proteins (Shafikhani & Engel, 2006; Shafikhani *et al.*, 2008) that are essential for cancer cell survival and proliferation (del Peso *et al.*, 1997; Gómez *et al.*, 1997; Cho & Klemke, 2000; Lay *et al.*, 2000; Lamorte *et al.*, 2002; Murga *et al.*, 2002; García *et al.*, 2006; Hwang *et al.*, 2006; Heasman & Ridley, 2008; Zhu *et al.*, 2008; He *et al.*, 2010), we hypothesized that resistance to ExoT-induced cytotoxicity would be highly unlikely and therefore it may be able to induce cytotoxicity in a wide range of tumour cell lines.

To gain insight into the efficacy of the ExoT-induced cytotoxicity, we first used *P. aeruginosa* to deliver ExoT into a number of highly resistant cancer cell lines, including B16, HeLa, EMT6, 4T1, MDA-MB-231, SK-OV-3, MCA-205 and Calu-3 (see Methods).

Tumour cells were treated with either an ExoT-expressing *P. aeruginosa* PA103 strain ΔU or ExoT-deficient *P. aeruginosa* PA103 strain pscJ, or left untreated (see Methods). We assessed ExoT-mediated cytotoxicity by measuring the extracellular release of the cytoplasmic protein LDH into the culture media. Treatment with the ExoT-expressing *P. aeruginosa* strain (ΔU) resulted in significantly higher LDH release in all the tumour cells, as early as 10 h post-infection (Fig. 1).

In order to home in on the kinetics of ExoT-induced cytotoxicity in these tumour cell lines, we assessed cytotoxicity at 15 min intervals using PI impermeant nuclear dye uptake as a marker for cell death. PI uptake and subsequent PI fluorescence is an established and irreversible marker for cell death (Shafikhani *et al.*, 2008; Kroemer *et al.*, 2009; Wood *et al.*, 2013). ExoT-expressing *P. aeruginosa* was able to induce cytotoxicity in all the tumour cell lines we tested within 15–25 h post-infection (Fig. 2, Movies S1, S2 and S3, available in the online Supplementary Material; only the representative movie frames of B16 are shown in Fig. 2b).

ExoT is sufficient to induce cytotoxicity in tumour cells

Although LDH release is routinely used as a measure of cytotoxicity (Korzeniewski & Callewaert, 1983; Decker & Lohmann-Matthes, 1988; Arechabala *et al.*, 1999), injured but viable cells can also release LDH as a result of damage to their membrane (Reddy *et al.*, 2001). Moreover, LDH release could potentially underestimate cytotoxicity in apoptotic cells, because activated caspases, which are proteases that mediate apoptosis (Fiers *et al.*, 1999; Lavrik *et al.*, 2005), could degrade LDH peptides, thus reducing the amount of LDH released into the medium. ExoT is known to induce apoptosis (Shafikhani *et al.*, 2008). In addition, the use of bacteria to deliver ExoT into tumour cells could introduce additional bacterial factors, which may contribute and/or synergize the ExoT-mediated killing of tumour cells. In fact, treatment with the ExoT-defective pscJ strain resulted in increased LDH release and cytotoxicity in some tumour cell lines, albeit at significantly lower levels than the LDH release or the cytotoxicity observed in the presence of the ExoT-expressing ΔU strain (Figs 1 and 2).

To determine if ExoT was sufficient to cause cytotoxicity in the aforementioned tumour cell lines and to account for the ExoT-independent cytotoxic effects of *P. aeruginosa* on tumour cells, we transfected the tumour cell lines with an expression vector harbouring either the *exoT* gene, C-terminally fused to *gfp* (pExoT-GFP), or the *gfp* empty vector control (pGFP) and assessed cytotoxicity by time-lapse fluorescence video microscopy, using PI uptake as a marker for cell death. This technique allowed us to quantify ExoT's cytotoxic effect in tumour cells on a per cell basis. Some cancer cell lines were poorly transfectable and thus were not included in these analyses. However, in those tumour cell lines that we were able to transfect, ExoT expression resulted in significantly more killing than the GFP control vector, indicating that ExoT was sufficient to cause cytotoxicity in these cancer cells (Fig. 3, Movies S4 and S5; only selected frames from B16 melanoma cells are shown in Fig. 3b).

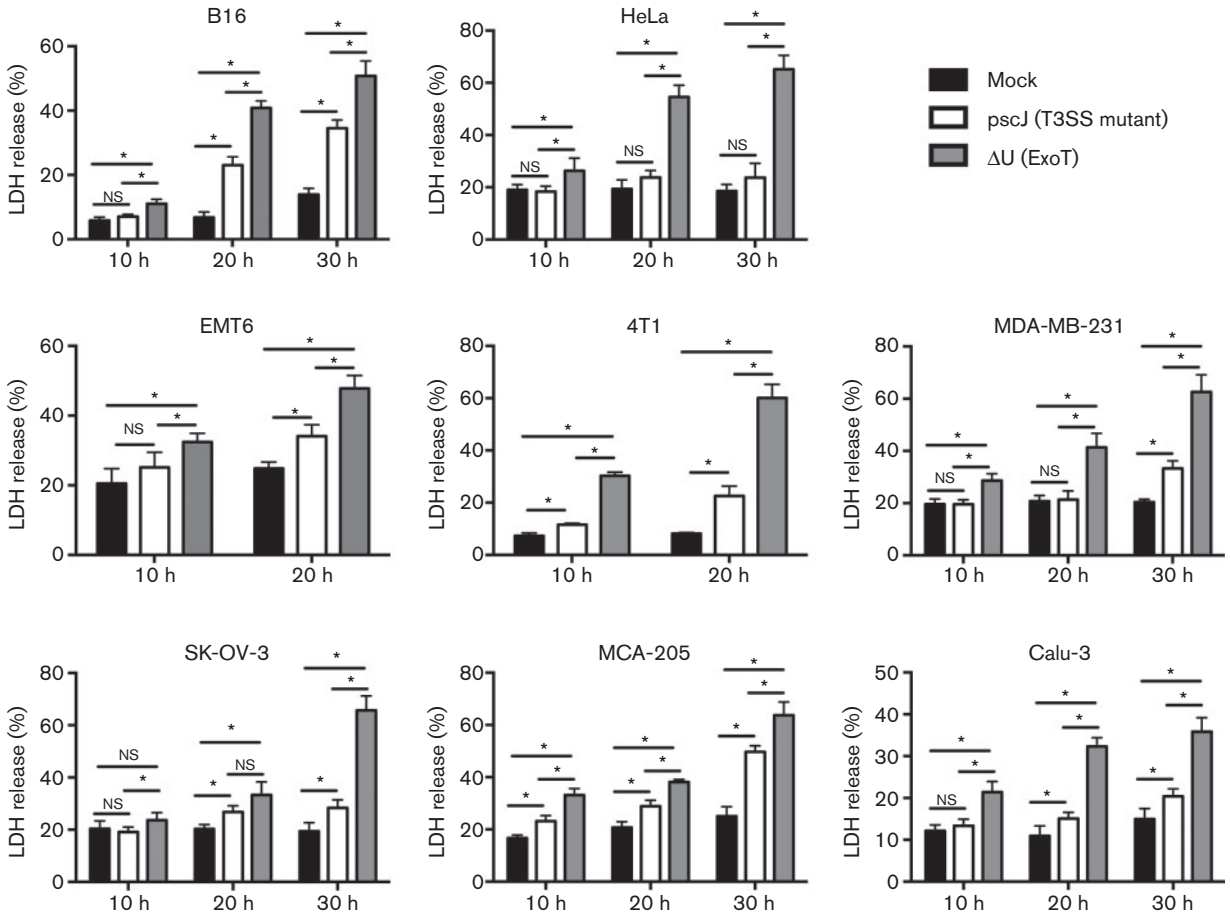

Fig. 1. Infection with ExoT-expressing *P. aeruginosa* can cause cytotoxicity in a number of cancer cell lines. The indicated cancer cells were either infected with ExoT-expressing (ΔU) or ExoT-defective T3SS mutant (pscJ) *P. aeruginosa* isogenic strains at m.o.i.~10, or cultured in media alone. Media were collected at 10 h intervals over a 30 h period and the amount of LDH released into media was analysed using the LDH release assay. The per cent LDH release was determined by the ratio of experimental LDH release to the total LDH release in the presence of Triton X-100. Analysis of LDH release of infected cancer cells, especially at later time points, indicated that infection with ExoT-expressing ΔU resulted in significantly more cytotoxicity compared with cells treated with the pscJ strain or left uninfected. *$P<0.05$, $n=6$, Student's *t*-test.

We further corroborated the cytotoxic effects of ExoT in transfected tumour cells by flow cytometry using a Viability Dye (eBiosciences), which, instead of using PI staining as a marker for cell death, works by binding to free amino acids in the cytosol (Perfetto *et al.*, 2010). As expected, cells intoxicated with ExoT exhibited significantly higher cytotoxicities as than the GFP control at 24 h post-transfection (Fig. 4). Depending on the techniques, there were some variations in the ExoT-induced cytotoxicities in these cell lines (compare Figs 1, 2 and 3). This is likely due to sensitivity of the techniques and/or the limitation of the use of peptides (i.e. LDH or Viability Dye) as markers for cell death in apoptotic cells as discussed above. Nevertheless, these data indicate that ExoT is sufficient to induce potent cytotoxicity in all tumour cell lines we tested.

Most cancer chemotherapeutics exert cytotoxicity in normal non-transformed cells, leading to well-chronicled side-effects associated with cancer therapy. To gain insight into potential side-effects of ExoT-based therapy, we transfected mouse embryonic fibroblasts and human T-lymphocytes with an ExoT expression vector as described above. Not surprisingly, ExoT also induced comparable cytotoxicity in these cell lines (Fig. S1). We were unable to evaluate ExoT's cytotoxicity in human or mouse primary epithelial cell lines due to low transfection efficiencies as well as their sensitivity to bacterial infection as ExoT-deficient bacteria also caused massive cytotoxicity in these cell lines. Nevertheless, these results highlighted the need to target this toxin directly into tumour cells (see Discussion for possible approaches).

ExoT is sufficient to induce cytotoxicity *in vivo*

We next assessed the ability of ExoT to induce cytotoxicity *in vivo*, using the B16 melanoma tumour model (Overwijk

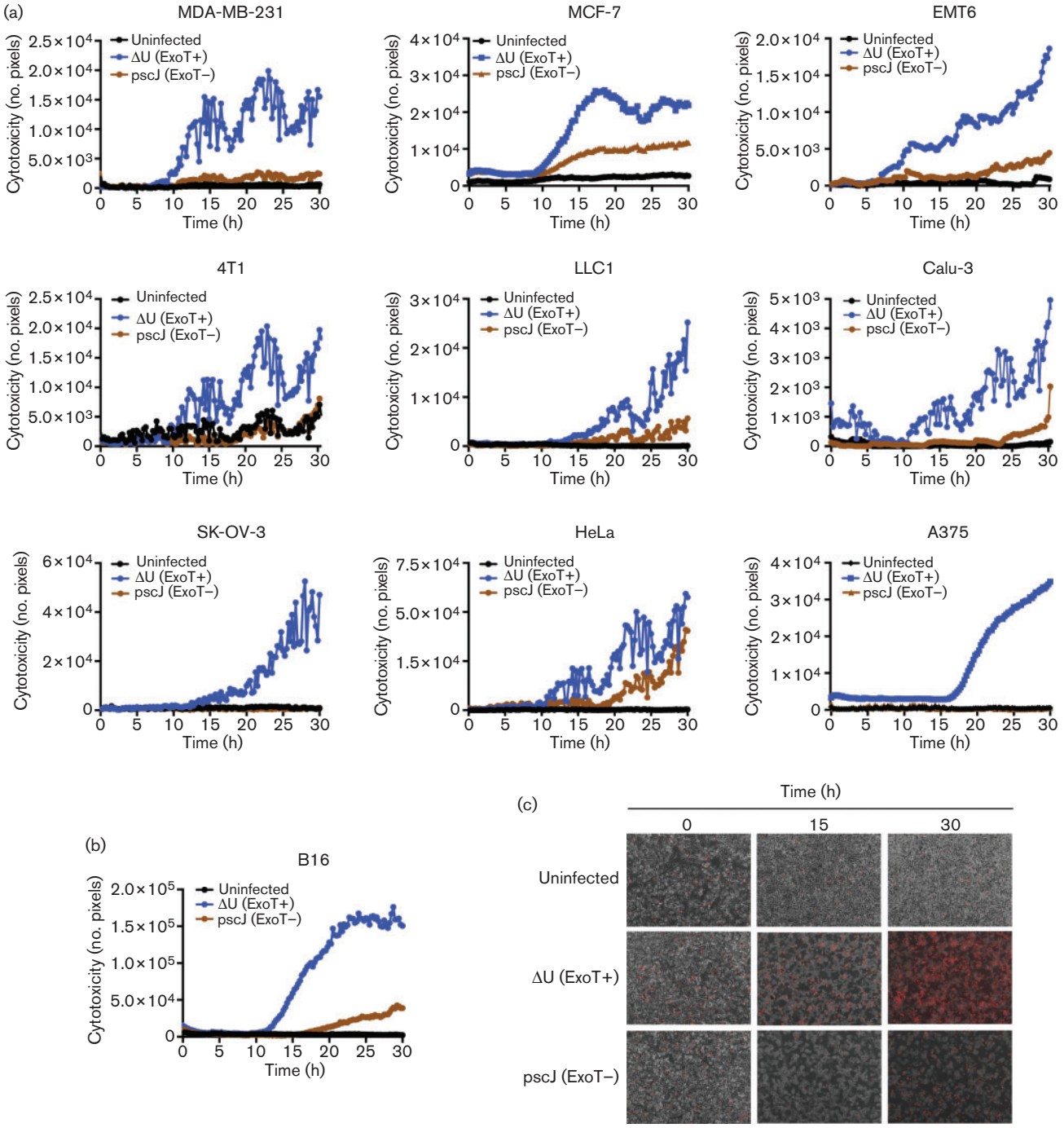

Fig. 2. Kinetics of ExoT-induced cytotoxicity in cancer cell lines. (a) The indicated cancer cell lines were treated with ExoT-expressing (ΔU) or ExoT-defective (pscJ) *P. aeruginosa* strains at m.o.i.~10, or left untreated. Cells were observed by time-lapse fluorescence video microscopy in the presence of PI (×100 magnification, 15 min intervals). Cytotoxicity was assessed by total PI uptake at 15 min intervals (cytotoxicity is shown as the mean of three fields of view). (b) Cytotoxicity analysis of B16 melanoma cells as assessed by PI staining (red). (c) Representative frames from B16 time-lapse video microscopy.

& Restifo, 2001). We transplanted B16 melanoma cells subcutaneously in the flanks of C57BL/6 mice. Once the tumours reached 50 mm^2, the expression vector expressing ExoT-GFP was packaged within a lipid-based transfection reagent to facilitate its uptake and injected directly into the lesion. After 24 h, the animals were injected systemically (intravenously) with SR-FLIVO, a marker for apoptotic cell death *in vivo* (Riol-Blanco *et al.*, 2009), and the tumours

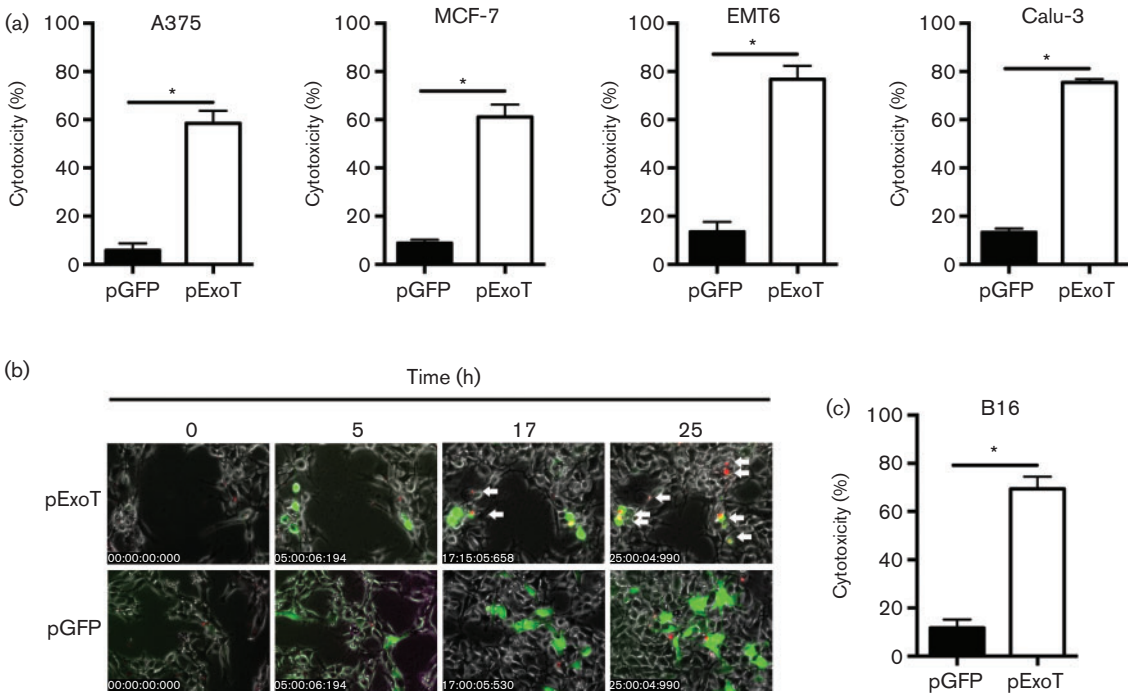

Fig. 3. ExoT is sufficient to cause cytotoxicity in cancer cells *in vitro*. Indicated cancer cells were transfected with pGFP vector control or pExoT-GFP and cytotoxicity was assessed by time-lapse fluorescence video microscopy in the presence of PI. (a) Tabulated data for A375, MCF-7, EMT6 and Calu-3. (b) Representative frames of B16 transfected with pExoT-GFP or pGFP. (c) Tabulated data for B16. *$P<0.0001$; $n=3$ independent experiments each with ~100 events counted; Student's *t*-test.

were analysed by fluorescence microscopy. The data indicated that transfection with ExoT resulted in cell death in B16 cells *in vivo* as indicated by co-localization of ExoT-GFP (green) and SR-FLIVO (red) (Fig. S2).

As *in situ* transfection efficiencies into the tumours by this method were extremely low (~3 %), ExoT failed to control overall tumour growth (data not shown). We modified our experimental approach to enhance transfection efficiencies in order to better evaluate the ability of ExoT to affect tumour establishment and growth *in vivo*. B16 cells were transfected with either pExoT-GFP or pGFP vector control *ex vivo* prior to subcutaneous injection in the mice. This approach increased the transfection efficiencies to ~20 %. Next, pExoT-GFP- or pGFP-transfected B16 tumour cells were transplanted into mice. Despite these low transfection efficiencies, the mean size of the ExoT-transfected B16 tumours was significantly less than pGFP-transfected B16 tumours (Fig. 5a, $n=5$ per group, $P<0.05$; mean tumour size was analysed up to the first tumour end point). Tumour measurements for individual animals showed that tumours transfected with pExoT-GFP reached their end point significantly slower than pGFP tumours. Collectively, these data indicated that ExoT was sufficient to induce cytotoxicity in B16 tumour cells and delay tumour growth *in vivo*.

DISCUSSION

Bacteria have been and continue to be used to deliver toxins into tumours (Dang *et al.*, 2001; Van Mellaert *et al.*, 2006; Patyar *et al.*, 2010; Karbach *et al.*, 2012). This study is the first evaluation of ExoT as a potential candidate for cancer therapy. Our results demonstrate that ExoT is capable of causing potent cytotoxicity in all murine and human tumour cell lines that we have examined thus far (Figs 1, 2, 3 and 4, Movies S2 and S5, and data not shown). ExoT-induced cytotoxicity occurred across a wide range of tumour cell lines, including breast, lung, cervical and even melanoma that is largely refractory to current cytotoxic drugs in the clinical setting (Atkins *et al.*, 2008; Flaherty *et al.*, 2012; Homet & Ribas, 2013).

Our *in vivo* results are encouraging in that they demonstrate that ExoT can significantly slow B16 melanoma tumour growth *in vivo* (Fig. 5). Low transfection efficiency is likely a major reason why these tumours eventually grew out and reached their end point. Nevertheless, the ExoT-mediated reduction in tumour growth *in vivo* highlights the potential of ExoT in cancer therapy.

The primary challenge concerning the formulation of ExoT as a potential cancer therapy is the development of means to safely deliver this toxin and specifically target it to

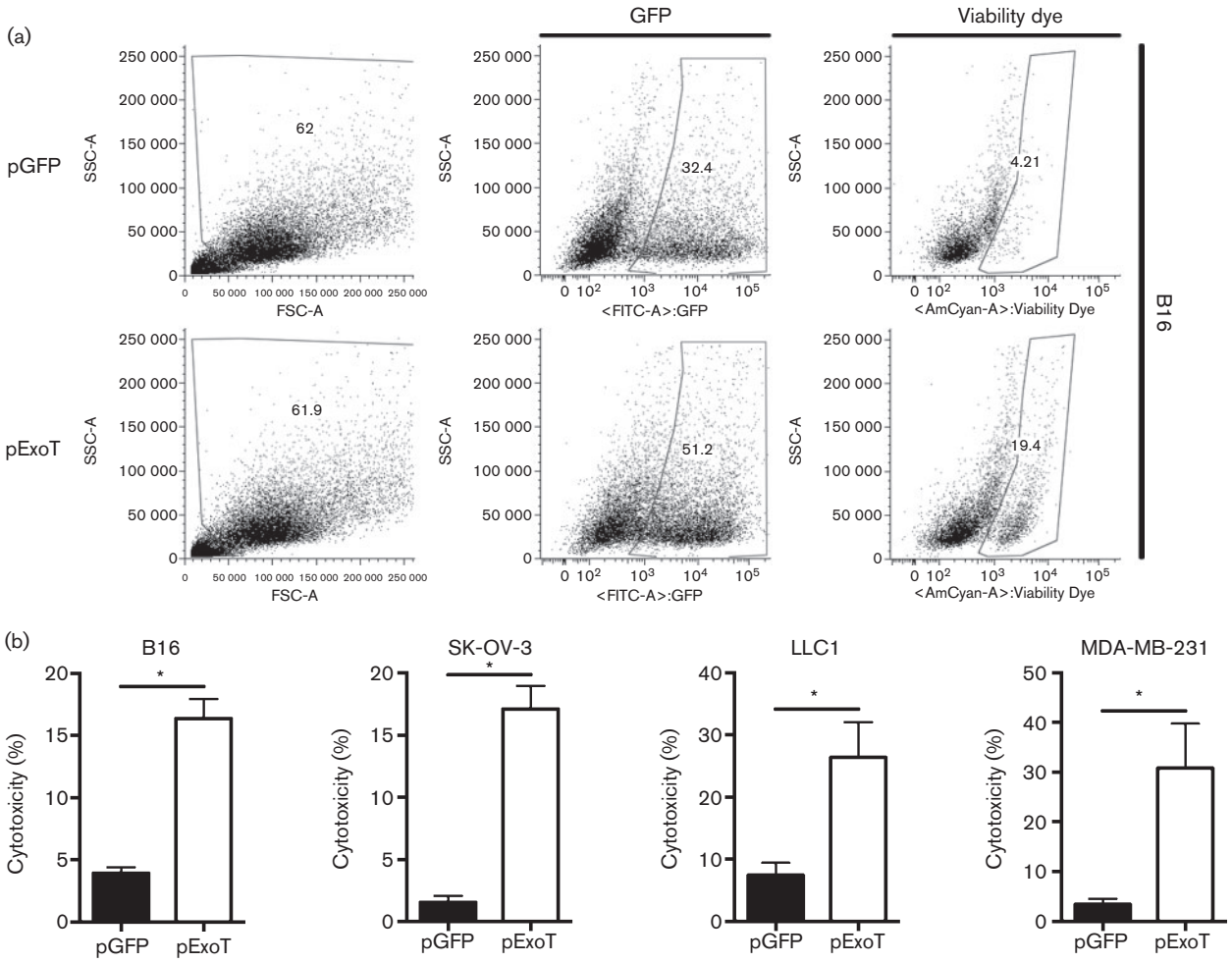

Fig. 4. Assessment of ExoT-induced cytotoxicity in cancer by Viability Dye. Cancer cells were transfected with pGFP vector control or pExoT-GFP. At 17 h after transfection, cells were stained with Viability Dye and analysed by flow cytometry. (a) Representative FACS plots for B16 show ExoT was sufficient to cause cytotoxicity in transfected cells. SSC, side scatter; FSC, forward scatter. (b) FACS analysis of transfected B16 cells indicated that pExoT-GFP resulted in significantly more cytotoxicity compared with pGFP-transfected cells. *$P<0.0001$; $n=7$; Student's two-tailed *t*-test.

tumours *in vivo*. There are multiple approaches that have been or are currently being investigated in several clinical trials that may accomplish this task. One approach is conjugation with tumour-specific antibodies (reviewed by Becker & Benhar, 2012). Conjugating ExoT to tumour-specific antibodies or the immunoglobulin variable fragment regions can enhance the delivery of the toxin (e.g. intravenously) as well as improve the specificity of ExoT to target the tumour. Indeed, engineered toxins combined to a receptor ligand or the variable domain of a mAb can differentiate between normal and malignant cells by binding to tumour-associated cell surface receptors (e.g. CD22, CD25, claudin-4, glycoprotein-NMB and mesothelin) (Chaudhary *et al.*, 1987, 1989; Pastan *et al.*, 2004).

Another approach is the use of recombinant viral delivery systems, such as vaccinia virus, with inherent tropism toward tumour cells (Baguley, 2010). A viral-based delivery platform has a number of unique biological properties that make it ideally suited for delivery and amplification of transgenes within tumours. These include intravenous stability and the ability to spread to distant tissues (Vanderplasschen *et al.*, 1998), preferential accumulation in solid tumours where neovasculature shows increased permeability (Kirn & Thorne, 2009), tropism toward cancerous cells (Park *et al.*, 2008; Breitbach *et al.*, 2011), and only minor side-effects in cancer patients even at extremely high infection titres (10^7–10^9 p.f.u.) (Park *et al.*, 2008; Breitbach *et al.*, 2011).

Another potential translational approach is the use of image-guided ultrasound-based microbubble technology to deliver and activate ExoT selectively in the tumour microenvironment. Gene therapy using this technology has gained significant momentum in recent years (Smith *et al.*, 2011; Chen *et al.*, 2012; Sirsi & Borden, 2012; Smith & Land, 2012).

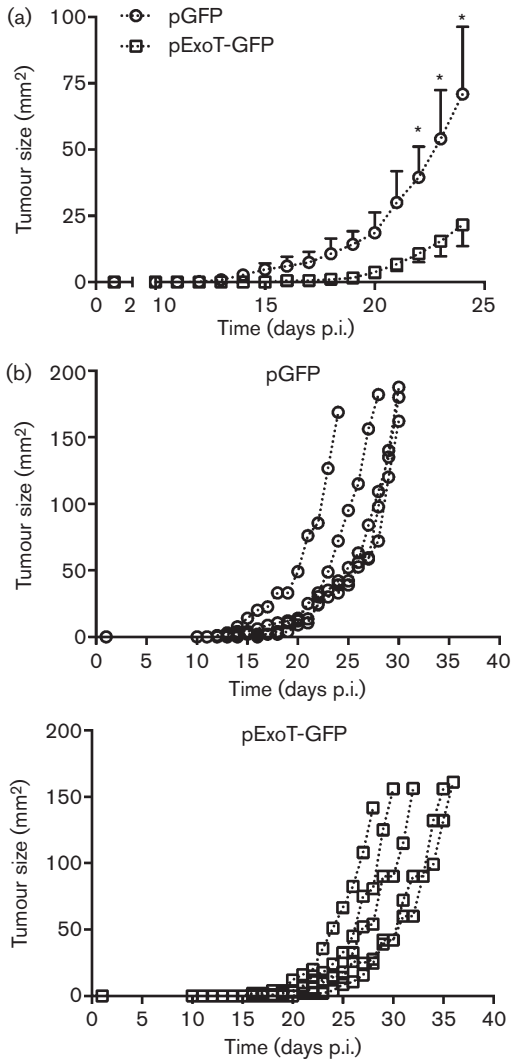

Fig. 5. ExoT slows B16 tumour burden *in vivo*. (a) B16 tumour cells were transfected with pGFP or pExoT *ex vivo*. At 15 h after transfection, 1×10^5 pGFP- or pExoT-GFP-transfected B16 tumour cells were transferred subcutaneously by injection in the flank of C57BL/6 mice. Tumour size was measured daily by use of callipers until tumours reached 150 mm^2. Tumour sizes are shown as mean ± SEM from the time of injection to the first animal death. $n=5$ mice per group, $^*P<0.05$. (b) Individual tumour growth curves for the experiment performed in (a).

In summary, we propose that *P. aeruginosa* ExoT possesses attractive anti-cancer properties that make it an attractive cancer drug candidate. ExoT's attractive anti-cancer properties include: (1) its ability to induce potent cytotoxicity in all cancer cell lines, as we demonstrated in this report; (2) its anti-proliferative effect in cancer (Shafikhani & Engel, 2006); (3) its ability to inhibit cell migration (Garrity-Ryan *et al.*, 2004), thus potentially being able to interfere with metastasis; and (4) the low probability of tumour resistance to ExoT due to its multiple cellular targets.

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