In vivo comparison of local versus systemic delivery of immunostimulating siRNA in HPV-driven tumours

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Abstract

Small interfering RNAs (siRNAs) to inhibit oncogene expression and also activate innate immune responses via Toll-like receptor (TLR) recognition have been shown to be beneficial as anti-cancer therapy in certain cancer models. In this study, we investigated the effects of local versus systemic delivery of such immune-stimulating Dicer-substrate siRNAs (IS-DsiRNAs) on a human papillomavirus (HPV)-driven tumour model. Localized siRNA delivery using intratumour (I.T.) injection of siRNA was able to increase siRNA delivery to the tumour compared to intravenous (I.V.) delivery and potently activated innate immune responses. However, I.V. injection remained the more effective delivery route for reducing tumour growth. While IS-DsiRNAs activated innate immune cells and required IFNα for full effect on tumour growth, we found that potent silencing siRNA acting independently of IFNα were overall more effective at inhibiting TC-1 tumour growth. Other published work utilizing IS-siRNAs have been done on tumour models with low levels of MHC-class 1, a target of natural killer cells that are potently activated by IS-siRNA. As TC-1 cells used in our study express high levels of MHC-class I, the addition of the immunostimulatory motifs may not be as beneficial in this particular tumour model. Our data suggests that selection of siRNA profile and delivery method based on tumour environment is crucial to developing siRNA-based therapies.

Introduction

RNA interference (RNAi) is a technology to silence gene expression by using small complementary RNA fragments to specifically bind coding RNA and induce RNA degradation [1, 2]. This ability to inhibit gene expression makes it an ideal anti-cancer therapy where a known oncogene drives the malignancy, such as cervical cancer where HPV genes E6 and E7 are involved in the majority of cases [3, 4]. Silencing HPV oncogenes E6/E7 with RNAi has been shown to drive HPV-infected cells into senescence, apoptosis, or both; subsequently causing reduced tumour growth *in vivo* [5-8]. While the focus of most studies has been the gene silencing ability of siRNA, certain sequences or modifications of siRNA are recognized as foreign by the innate immune system and induce an immune response [9-11]. Liposome-based delivery vectors are taken into endosomes and detected by TLRs, particularly TLRs 3, 7, and 8 [12-14]. SiRNA recognition by TLR7 triggers activation of the myeloid differentiation primary response gene 88 (MyD88)- interferon regulatory factor 7 (IRF7) innate activation pathway, ultimately leading to the secretion of type 1 interferons (IFNs), including interferon-α (IFNα) [14-16]. MyD88 also triggers nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation, activating protein-1 (AP-1) which functions in the regulation of IFNα [17]. While all cells have the ability to produce IFNα, it is believed phagocytic cells such as macrophages and dendritic cells (DC) are the key players in the innate immune response to IS-siRNA [18, 19]. When these cells are activated by siRNAs, they release large quantities of cytokines and type I IFNs, which can act directly on tumours or virally infected cells as well as activate immune cells such as natural killer (NK) cells, T cells and B cells [20].

While the activation of the innate immune system has been seen as an undesired side effect of siRNAs, many groups have explored the use of immunostimulatory siRNAs (IS-siRNA) as a potential cancer therapeutic [18, 21]. These IS-siRNAs act by both silencing oncogenes and activating the innate immune system, as certain cytokines, such as IFNα, are known to act as anti-cancer agents [22-24]. While siRNAs are detected by TLR7 in a sequence-dependent manner [9, 14]; several modifications can also be made to increase or decrease detection by the innate immune system. Poeck *et al* was the first to demonstrate the addition of a 5'-triphosphate on the siRNA resulted in recognition by RIG-I, subsequently inducing the release of IFNs and significantly increasing the efficacy of a Bcl-2 silencing siRNA at treating B16F10 melanoma tumours [21]. Soon after, Kortylewski *et al* showed that synthetic siRNAs conjugated with CpG oligonucleotides were able to silence targeted gene and activate tumour-associated immune cells through recognition of siRNA by TLR9 [18]. Conversely, modifications such as the addition of a 2'-O methyl (2'O-Me) group to the backbone strand are able to reduce immune stimulation by siRNAs [25]. We have also been able to show that 25/27 mer Dicer substrate siRNAs (DsiRNAs) [26, 27] directed against the HPV oncogenes E6/E7 are able to induce IFNα in a sequence-specific manner. We demonstrated that IS-DsiRNA had improved treatment of a HPV driven tumour model compared to the same DsiRNA modified with the 2'-Ome backbone with no IS ability [15].

While both *in vitro* and small animal studies of RNAi as a cancer therapy have shown promise, clinical success with the technology has not been as impressive. One of the treatment prerequisites is that any drug administered must be delivered to the targeted site effectively with minimal toxicity. While systemic delivery by I.V. injection is the most common route of siRNA delivery, local delivery of siRNA is thought to improve siRNA efficacy. Injecting siRNA I.T. has successfully been used to treat tumours in a number of model systems, in a manner that depended on the RNAi effect [28-30].

In this study, we investigated whether I.T. delivery of IS-DsiRNAs can improve siRNA efficacy in a cervical cancer model. We observed that I.T. delivery of IS-DsiRNAs greatly improves delivery of siRNAs to tumours compared to I.V. and potently activated innate immune responses. However, I.V. delivery showed a better control of overall tumour growth. Further, blocking IFNα receptor resulted in a reduced efficiency by IS-DsiRNA to control tumour growth, while potent siRNA acting independently of IFNα were unaffected, and generally more effective at inhibiting TC-1 tumour growth overall. Our results provide the insight into the design and preferred delivery method to be used in RNAi-based cancer therapeutics.

Methods

Preparation of siRNA and PEGylated liposomes

DsiRNAs IDT-4, IDT-4m7, IDT-12, and scrambled (SC) SCm7 first described in Khairuddin *et al* [15] were obtained from Integrated DNA technologies (IDT) (Iowa, USA), while BP1Mod2 first described in Judge *et al* [9] was purchased from Sigma Aldrich (Victoria, Australia). All siRNAs were reconstituted in DEPC water and stored as per manufacturer's instructions with sequences and siRNA profile listed in Table 1. PEGylated liposome containing siRNAs were prepared using the Hydration of Freeze-Dried Matrix method as previously described [31]. Liposomes made for each experiment were tested for consistent size and quality by measuring particle dispersion with a zetasizer (Malvern Instruments). Where biodistribution was studied, prepared liposomes were stained with 155 ng 1,1-dioctadecyl-3,3,3,tetramethylindotricarbocyanine iodide (DiR) (Invitrogen, Carlsbad, CA) for 2 hrs at RT prior to injection.

Cell line culture

The TC-1 cell line was derived from primary lung epithelial cells of C57BL/6 mice immortalized with the retrovirus vector LXSN16E6E7, co-transformed with the pVEJB plasmid carrying the activated human c-Ha-ras oncogene [32], were obtained with permission from TC Wu. Cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gilbco Invitrogen, Mount Waverly, Australia) supplemented with 10% heat inactivated fetal calf serum (FCS) (LONZA, USA), 100 units/mL Penicillin G, 100µg/ml of Streptomycin sulphate and 0.29mg/ml of L-glutamine (P/S/G) (Gibco Invitrogen, Mount Waverley, Australia). Cells were passaged thrice weekly with Trypsin-EDTA

(Gibco, Invitrogen) used to detach cells for sub-culturing.

Mice and animal models of HPV driven cancer

C57BL/6 female mice aged between 6 to 12 weeks were obtained from commercial suppliers (Animal Resources Centre Perth, Australia or Herston Medical Research Centre, Queensland, Australia) and housed under standard rodent PC2 conditions. TC-1 tumours were established by subcutaneous (S.C.) injection of $1x10^6$ TC-1 cells into the neck scruff of female C57BL/6 mice. Mice were monitored thrice weekly with tumour size measured using calipers and size determined by multiplying tumour width by tumour height. All animal work was approved by The University of Queensland, Animal Ethics Committee prior to commencement.

In vivo delivery of siRNAs

For systemic delivery of siRNAs, liposome-encapsulated siRNAs were injected I.V. into the lateral tail vein of mice at a dose of 40µg/mouse in 300 µL to achieve ~2mg/kg siRNA in an average 20 grams female C57BL/6 mouse. For local delivery of siRNAs liposome-encapsulated siRNAs were injected I.T. at a dose of 10 µg/mouse delivered in two injections of 15 µL. Liposomes for I.T. injections were concentrated prior to injection by centrifugation at 16 000 *x g* for 20 minutes using an ultra-0.5 centrifugal filter unit (Millipore, Australia).

In vivo blocking of IFNα uptake

To inhibit IFNα uptake, mice were injected with 0.5mg of anti-IFNα receptor

(MAR1) antibody [33] or an isotype control intraperitoneally (kindly donated by Prof Paul Hertzog of Monash Institute of Medical Research, Clayton) on days 4 and 7 relative to tumour challenge. Mice were treated with siRNAs on days 5, 8, and 12 post S.C. injection.

Isolation of single cell suspensions

As TC-1 tumours were injected into the neck scruff of mice, pooled auxiliary and brachial lymph nodes were deemed as the tumour draining lymph nodes (TDLNs). All organs were harvested in PBS containing 2.5% FCS and stored on ice before cell extraction. Single cell suspensions from TDLN and spleen were obtained by passing tissue through a 70µm cell strainer before samples were washed and resuspended in PBS 2.5 %FCS. Spleens were additionally treated with 2ml of ACK lysing buffer (Invitrogen, USA) for 2 minutes before being washed with PBS 2.5% FCS to lyse red blood cells.

Single cell suspensions were obtained from tumours by cutting samples into $2mm^3$ squares with a scalpel and incubated for 30 minutes at 37°C with RPMI 1640 media (Invitrogen) containing 1mg/ml Collagenase D (Roche) and 100µg/ml DNAse (Roche) and 5% FCS at 37°C. Tumours were gently agitated using the back end of a 5ml syringe and returned to the incubator for another 30 minutes. Samples were then agitated again and the suspension passed through a 70µm cell strainer and washed twice with cold PBS 2.5% FCS.

Flow Cytometry

Single cell suspensions were resuspended to 1-10x10^7 cells per ml and 100 µL added to 5ml tubes (BD biosciences). FC receptors were blocked by addition of 10 µL 2.4G2 hybridoma supernatant (anti-CD16/CD32) before samples stained with antibodies CD3-PE, CD4-PE, CD8-PerCPcy5.5, CD11b-PerCPcy5.5, CD11c-FITC, CD45.1-PE, CD69-FITC, NK1.1-APC, Gr1-FITC (Biolegend) and incubated on ice for 30 minutes. Samples were then washed once with PBS, 0.1% BSA, 2mM EDTA before being analysed on a Gallios flow cytometer (Beckmann Coulter).

<u>In vitro</u> transfection of TC-1 cells with siRNA

TC-1 cells were transfected using cationic liposomes with 40nM of respective siRNAs according to the protocol as described previously[15]. Briefly, TC-1 cells were seeded at a density of 100,000 cells per well a day prior to transfection. On the day of transfection, siRNAs were diluted in 3µL dH_20 and brought up to a final volume of 100µL in OptiMEM I reduced serum media (Invitrogen). Cationic liposomes were diluted in 5% dextrose/dH_20 to achieve a liposome to siRNA N:P ratio of 4:1 in a final volume of 100µL. The liposome was added to the siRNA mixture and left to incubate at room temperature for 20 min. Liposome/siRNA was mixed with 800µL Opti-MEM I before being added to the cells and incubated for 4hrs at 37°C in a 5% CO_2 atmosphere. After 4hrs, the transfection mix was removed, cells washed with 1 x PBS, and supplemented with complete DMEM overnight before collected for downstream applications. Transfected cells were collected using trypsin-EDTA to detach cells.

RNA extraction and quantification

Whole cell RNA was isolated from cell line or tumour samples using Trizol (Invitrogen, USA) as described by the manufacturer's protocol. Where cell lines were used, approximately 1×10^6 trypsinised cells were resuspended in 1 ml Trizol and incubated overnight at -20°C before RNA extraction. Where tumour samples were tested, tumours were initially cut into pieces and snap frozen in liquid nitrogen. Frozen tumor sections were then ground into a fine powder using a mortal and pestle and added to 1ml of TRIzol. Samples were vortexed well and stored at -20°C overnight. Samples were then thawed, and centrifuged at 12000g for 10 minutes at 4°C and supernatant used for RNA extraction. cDNA was synthesized using Omniscript Reverse Transcriptase Kit (Qiagen, USA) using polyDT primers (Invitrogen) as per manufacturer's instructions. Real-time PCR analysis was performed using GoTaq® qPCR Master Mix from Promega (USA), following the manufacturer's instructions with primers described in Supplementary Table 1. Real-time PCR was performed on the Rotogene (QIAGEN/Corbett Research) using the Corbett Research robotics system (72-well gene disk, QIAGEN/Corbett) under normal cycling conditions.

Senescence-associated β-galactosidase assay

Cells transfected *in vitro* with 40 nM DsiRNAs for 4 hours had media replaced and left in culture for 10 days before cells were fixed with 2% paraformaldehyde/PBS and stained with X-gal solution (pH 6.0) as previously described [34]. Cells were then left overnight at 37°C, 5% CO_2, washed thrice in PBS, left to dry and number of senescent cells, indicated by the cells having

turned blue were counted under light microscope. 5 field views under 10x magnification were randomly chosen per each well and number of blue cells were counted, average count was taken as 1 data point per well.

Colony forming assay

Cells transfected *in vitro* with DsiRNAs as previously described were re-plated at a density of 100 cells/well and left in culture for up to 14 days whereby distinct macroscopic colonies were visible. Cells were then fixed and stained using crystal violet stain and visible colonies were counted using cell counter.

Statistical analyses

Statistical significance of differences was determined by unpaired two-tailed student *t*-test and one-way, multivariate ANOVA under Newman-Keuls test, using the GraphPad Prism 5.0 software (GraphPad Software, Inc). Differences with P values $p < 0.05$ were considered to be statistically significant.

Results

I.T. injection improves liposome delivery to tumours compared to I.V.

Previous work in our laboratory was able to successfully inhibit HPV-driven tumour growth following I.V. injection of PEGylated liposomes containing siRNAs [15]. While I.V. injection is a common mode of drug delivery, we wished to see how the efficacy might improve if we delivered siRNA directly to the tumour. As we were delivering siRNA directly to the tumour rather than systemically, the dose of siRNA was reduced accordingly. A dose of 10 μg/mouse was decided based on an initial titration of BP1mod2 to determine siRNA dose required to achieve maximal immune activation of T- and NK-cells (Supplementary Figure S1). We also wanted to determine the amount of siRNA delivered to the tumours by each injection route, for this we determined uptake of DiR labeled liposomes, previously demonstrated to be an excellent surrogate marker of siRNA delivery [31, 35]. Mice were injected with TC-1 cells and tumours allowed to establish for 10-12 days before siRNAs were injected either I.T. or I.V. with DiR-labeled liposomes. After 20 hrs, uptake in the tumour, tumour draining lymph nodes (TDLN), and spleen of mice was determined using flow cytometry. A representative gating strategy to determine DiR uptake in CD11b+ macrophages or CD11b+, GR1+ myeloid suppressive cells within these cells are presented (Figure 1a). Shown in Figure 1b, I.T. delivery significantly increased uptake of liposomes within the tumour while decreased uptake in the spleen when compared to I.V. injection despite the reduced siRNA dose used. Uptake was predominately within dendritic cells (CD11c+) and macrophages (CD11b+), however, there was increased uptake within CD4+ T-cells (Figure 1 c, d). While uptake within

these phagocytic cells was similar in the tumour following I.T. or I.V. delivery, spleen uptake was far lower with I.T. delivery, consistent with initial findings that I.T. injection delivers siRNA more specifically to the tumour.

I.T. delivery of IS-DsiRNA activates local and systemic immune responses

While I.T. delivery of siRNA increased tumor uptake compared to I.V., we wanted to test the effects of I.T. siRNA injection on both localized and systemic immune responses. Mice were injected with TC-1 cells and tumours allowed to establish for 10-12 days before siRNAs were injected I.T. After 20 hrs, mice were euthanized, spleen and TDLN removed, and the activation status of NK cells (Figure 2a and c) and T cells (Figure 2b and d) was determined by measuring CD69 expression on CD3+ or NK1.1+, CD3- cells respectively. Injection of the IS-DsiRNA IDT-4 was able to potently increase activation of both NK cells (Figure 2a) and T-cells (Figure 2b) within the TDLN. In addition to activation in the TDLNs, strong NK and T cell activation was observed in the spleen of mice, suggesting that local delivery of siRNA was able to also increase systemic immune responses (Figure 2 c, d). Activation of T and NK cells by IDT-4 was dependent on packaging in liposomes, as no activation was seen when siRNAs were injected naked in saline (Figure 2 e, f). Induction of CD69 was not observed with the modified homologue IDT-4m7 or a negative control DsiRNA SCm7 in TDLN and spleen (Figure 2). I.V. injection of mice with 40μg DsiRNA also caused potent NK activation with IDT-4, while IDT-4m7 or SCm7 again had no impact on the immune system (Supplementary Figure S2a, b). This data demonstrates I.T. injection of IS-DsiRNA activated both strong local and systemic immune

response in mice.

Control of TC-1 tumour growth by I.T. injection of E6/E7 silencing DsiRNA

Having established I.T. injection increases DsiRNA delivery to tumours and can activate immune responses; we tested the efficacy of I.T. delivery at inhibiting TC-1 tumour growth compared to I.V. delivery. Mice with established TC-1 tumours were given 3 doses of siRNA delivered I.T. (10μg siRNAs) or I.V. (40μg siRNAs) on days 3, 6 and 10 relative to tumour challenge before mice were euthanized, tumours measured by calipers, tumours removed, and weighed on day 14. The DsiRNAs used for the purpose of our experiment have the following profile: IDT-4 (IS-DsiRNA, targeting HPV16 E6/E7), IDT-4m7 (2'OMe modified homologue of IDT-4 to eliminate all IS ability), IDT-12 (non-IS-DsiRNA, targeting HPV16 E6/E7), and Scm7 (scrambled control, modified with 2'OMe, non-IS-DsiRNA) (Table 1). Both IDT-4 and its non-stimulatory version, IDT-4m7, appeared to reduce tumour growth compared to tumours treated with the control siRNA Scm7, when siRNAs were delivered I.T., however, these reductions were not statistically significant (Figure 3a). Conversely, we observed significant reduction in tumour size with tumours treated I.V. with DsiRNAs compared to controls (Figure 3b), with IDT-4 showing slightly better control of tumour growth compared to its homologue, IDT-4m7. For both these experiments, we also included treatment with a non-IS-DsiRNA against HPV E6/E7, IDT-12, as control for immune activation regulating tumour growth. Interestingly, tumours treated I.T. with IDT-12 showed no reduction in tumour growth when delivered I.T. (Figure 3a) but significantly reduces tumour growth when delivered I.V. (Figure 3b). This data

suggest that I.V. delivery of DsiRNAs has a greater anti-tumour effect than the same siRNAs delivered by I.T. injection.

With IDT-4m7 shown to be non-immunostimulatory, it appeared that silencing ability, not immune stimulation was the defining factor for the treatment of TC-1 tumours *in vivo*. In order to test this theory, mice with established TC-1 tumours were treated by a single I.T. injection of 10 µg DsiRNA. Following 24 hrs, tumours were removed, RNA extracted and E6/E7 expression determined by qRT-PCR. To normalise data, human-k-ras, a gene introduced as part of the transformation process in making TC-1 cells, was used as a housekeeping gene to minimize result skewing by mouse cells infiltrating the tumour site. As shown in Supplementary Figure S3a, despite reduction in tumour growth shown in previous experiments when DsiRNAs were delivered I.V., we were unable to observe HPV 16 E6/E7 silencing in tumours. This was despite all DsiRNAs showing potent E7 silencing ability *in vitro* (Supplementary Figure S3b), and the ability to reduce colony formation and induce senescence in TC-1 cells (Supplementary Figure S4). With all siRNA showing similar silencing ability, the IS ability of IDT-4 did not have an additive effect on *in vivo* tumour growth, with no increased impact on tumour growth compared to IDT-12.

IS-DsiRNA effect on tumour growth is reduced with IFNα blockade

Our results suggested that I.V. delivery of DsiRNAs is better than I.T. delivery at reducing HPV-driven tumours *in vivo*. We therefore investigated the more clinically relevant I.V. administration in greater detail. To further investigate

the importance of immune activation in reducing HPV-driven tumours, we treated mice with siRNA while simultaneously blocking the IFNα receptor with a functionally blocking antibody [33]. Shown in Figure 4a, tumour sizes on day 10 were reduced with the IS-siRNA IDT-4, however this effect was significantly reduced when IFNαR was blocked. By day 14 the tumour inhibition caused by IDT-4 was lost when compared to mice treated with the control scrambled siRNA (Figure 4 b, c). While IFNαR blockade appeared to increase tumour size in all groups, this effect was only significant in mice treated with IDT-4, suggesting immune activation was the main factor for the IS-DsiRNA IDT-4. Conversely, the non-IS-DsiRNA IDT-12 had a much greater effect on tumour growth with highly significant tumour inhibition at all time points tested and impact on tumours unaffected by IFNα blockade. This data suggests that while IS-DsiRNA is inhibiting TC-1 growth through IFNα, greater benefit can be achieved with potent silencing DsiRNA that does not require IFNα to inhibit tumour growth.

Discussion

Liposomal-based delivery vectors remain one of the most effective at delivering siRNAs *in vivo*. We have compared the effects of either I.T. or I.V. delivery of liposome encapsulated IS-DsiRNA on the growth of the cervical cancer TC-1 tumours. I.T. delivery was tested to see if we could increase tumour delivery and reduce activation of the systemic immune system. Indeed, I.T. delivery increased tumour uptake of liposomes around 2-fold compared to I.V., achieved with injecting 4 times less siRNAs. I.T. delivery also reduced siRNA escape from the tumour site, with significantly reduced uptake in the spleen compared to I.V. delivery. The IS-DsiRNA IDT-4 has previously shown to be a potent IS-DsiRNA and again was found to stimulate local innate immune responses. Using CD69 expression as a marker of siRNA activation of TLR7 [36], both NK and T-cells within the TDLN expressed high levels of CD69 following I.T. delivery of IDT-4, while the addition of a 2'OMe group to this DsiRNA's backbone completely ablated the response. Interestingly while I.T. delivery drastically reduced systemic uptake of siRNA, IDT-4 also induced NK and T-cell activation in the spleen to a similar level as in the TDLN, suggesting a localized injection of siRNA is sufficient for systemic immune activation. Liposome encapsulation was required for immune activation with naked siRNA having no impact on the immune system. Despite increasing siRNA delivery to tumours, overall we found that I.T. delivery did not improve the therapeutic effect of any of the siRNAs against established TC-1 tumours compared to I.V. delivery.

While the DsiRNAs used were shown to efficiently knockdown HPV16 E7 expression *in vitro*, we observed no significant reduction of E7 expression in

tumours treated with DsiRNAs delivered using PEGylated liposomes. We know that the PEGylated liposome delivery system can cause *in vivo* gene knockdown in TC-1 tumours, however to demonstrate that siRNA against GFP and a TC-1 GFP tumour line were used [37]. This was done as TC-1 tumour cells undergo apoptosis following treatment with E6/E7 siRNA [6]. HPV expressing cells successfully treated with siRNA would be cleared and impact on our ability to detect gene silencing within the tumour mass. Thus a more sensitive, albeit laborious methods, such as 3'-RACE PCR [38] or Amplification of Papillomavirus gene Transcripts assay [39] to detect cleaved HPV16 E7 mRNA may be required to observe *in vivo* gene silencing.

Also examined in this study was the tumour reducing potency of the TLR7-activating DsiRNA IDT-4 compared to IDT-4m7, the same siRNA with a 2'-OMe modification to eliminate TLR7 activation, as well as to the non-modified, non IS-DsiRNA IDT-12. Overall we found that the addition of TLR7 immune activating sequences had only a minor increase in potency compared to the modified IDT-4m7, and a decrease in potency compared to IDT-12 through the I.V. delivery route. The impact of IDT-4 on TC-1 tumour growth was also neutralized by blocking IFNα via a receptor blocking antibody [33]. While IFNα blockade significantly reduced the potency of IDT-4, no significant effect on the potency of IDT-12 was observed, demonstrating IDT-12 effects on tumour growth were independent of IFNα. This result was consistent with our previously published finding that IDT-4 could not reduce TC-1 tumour growth in mice lacking the key innate immune signaling gene MyD88 [15, 40]. Thus while IDT-4 impacted tumour growth predominately through immune activation, we postulate that the IS-effects of IDT-4 was only transient with the effect

weakening after day 10. While IDT-12 being a potent silencer was able to reduce tumour growth and maintain greater tumour control for longer, following cessation of siRNA administration.

The most prominent examples of immune modulating siRNA both used the B16F10 tumour model as targets for IS-sIRNA [21, 41]. The B16F10 tumour line differs from the TC-1 model in that it expresses low levels of MHC class I and is a well-established target for NK cells [42, 43]. In another study [36], TLR7 stimulating RNA oligonucleotides were tested in a tumour model and NK cells found to be the critical cell for inducing anti-tumour effects. Using the RMA cell line they observed TLR7 activation caused very potent tumour inhibition against the MHC class I low RMA-S clone, while MHC class I high RMA cells were unaffected by therapy. In addition, HPV E6 and E7 genes have been shown to perturb key modulators of the IFN response, IRF3 and IRF9, respectively [44, 45], potentially reducing the direct effect of IFNα on tumour growth. While we found NK cells activated by IDT-4, the role of NK cells in TC-1 clearance has been shown to be only minor [46]. Thus while our choice of cell line may not have been optimal for a good responder to IS-siRNA therapy, it has demonstrated that low MHC class 1 expression may be a critical factor in designing IS-siRNA for clinical use. With the majority of human malignancies maintaining MHC class 1 expression [47] results generated with the B16 model may not represent results able to be achieved with the majority of human cancers.

Overall we have found both I.T. and I.V. delivery of DsiRNA can inhibit the growth of TC-1 tumours *in vivo*. The addition of IS motifs activated innate

immune responses however did not improve therapy compared to a non IS-DsiRNA. Taken together this suggests that RNAi play a more important role at reducing tumour growth than immunostimulation in our model of cervical cancer. SiRNA delivery to tumours was increased with I.T. injection compared to I.V. However, the impact on tumour growth was less compared to I.V. delivery, leading us to the conclusion that the more clinically relevant I.V. delivery route is optimal in our model. While IS-DsiRNA was not as beneficial in our model compared to other studies, a few alternative approaches may improve its effectiveness against cervical cancer. Using immune activating modifications like 3'-tri phosphate or CpG conjugation to the most potent silencing DsiRNA may be a more logical approach to designing an effective therapy, rather than relying on motif dependent TLR7 activation of some DsiRNAs, which appears to give an overall less potent activation compared to these "professional" TLR agonists. Another approach would be to add the E7 CD8 CTL peptide E7 49-57 (RAHYNIVTF) [48] to the liposome preparation to be injected into mice. TLR7-activating RNA oligonucleotides have been shown to be an effective T-cell adjuvant [49] and may generate an effective anti-tumour T-cell response that would be more beneficial against a MHC class I expressing tumour. Overall, our results show the importance of characterizing tumours to achieve the greatest outcome from immune modulating therapies.

CONFLICT OF INTEREST

The authors would like to declare no conflict of interest.

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List of Figures

HPV16 targeting) was used as a control. Tumour measurements were determined on day 14 in mice treated by I.T. injection **(a)** and I.V. injection **(b)**. Each graph represents the mean ± s.e.m of 5 individual animals from a single experiment, representative of 2 independent experiments. *p≤0.05* represented by * *p≤0.01* by ** as determined by ANOVA with Newman-Keuls post test analysis.

Figure 4. Effect of IS-siRNA on TC-1 tumour growth requires IFNα

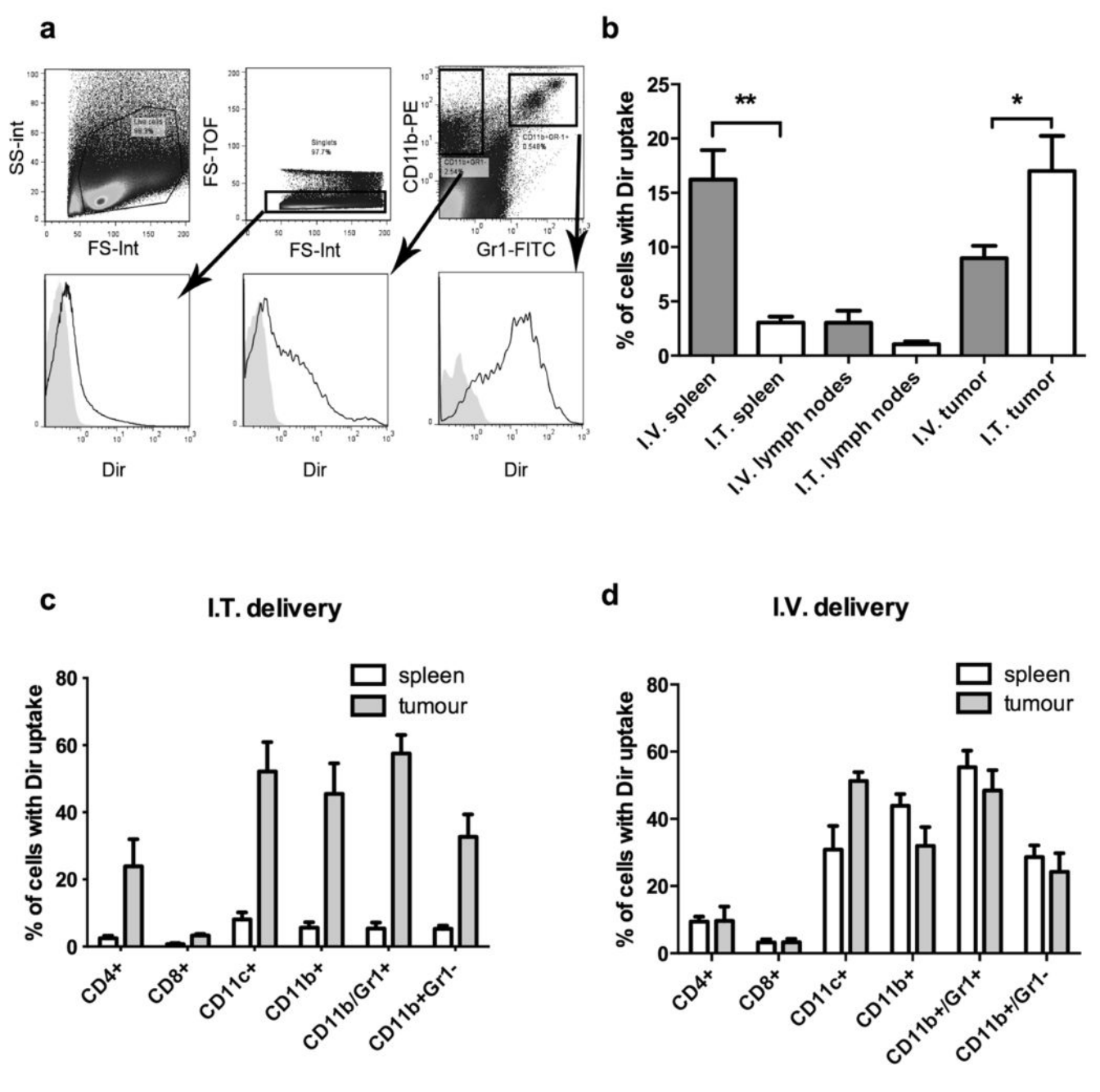
Mice were S.C. injected with $1x10^6$ TC-1 cells per mouse on day 0. Liposome encapsulated siRNAs IDT-4, IDT-12 or scrambled as a negative control were injected I.V. on days 5, 8, and 12 post S.C. injection. To determine impact of IFNα on siRNA function, mice were injected I.P. with the anti-IFNαR antibody (MAR1) or isotype control on days 4 and 7 post initial S.C. injection. Tumour sizes were measured with calipers on day 10 **(a)** and day 14 **(b)** and tumour weights determined on day 15 **(c).** Data represents mean ± s.e.m from 10 individual mice, pooled from 2 independent experiments with *p≤0.05* represented by * *p≤0.01* by ** and *p<0.001* by *** as determined by ANOVA with Newman-Keuls post test analysis.

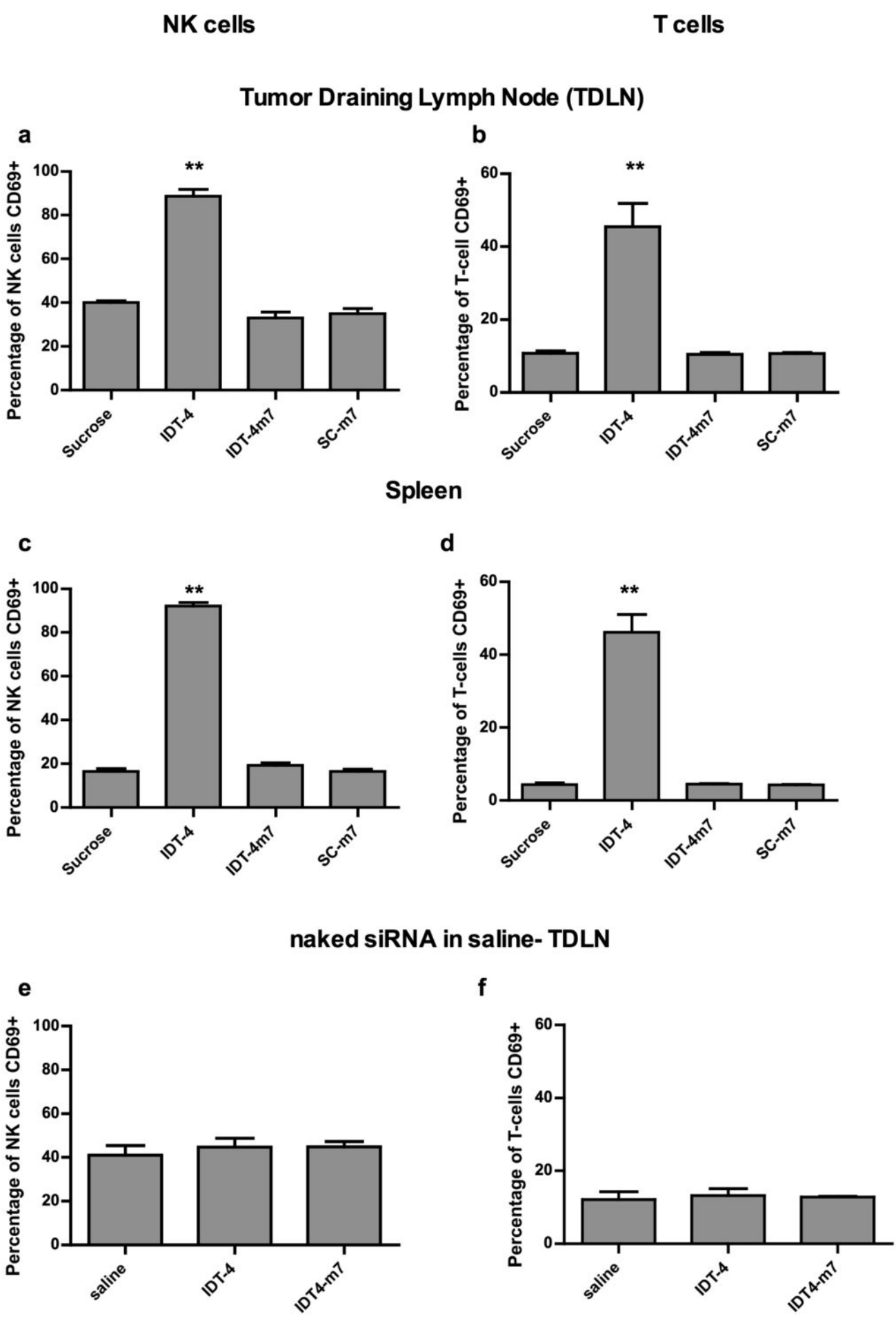
List of Tables

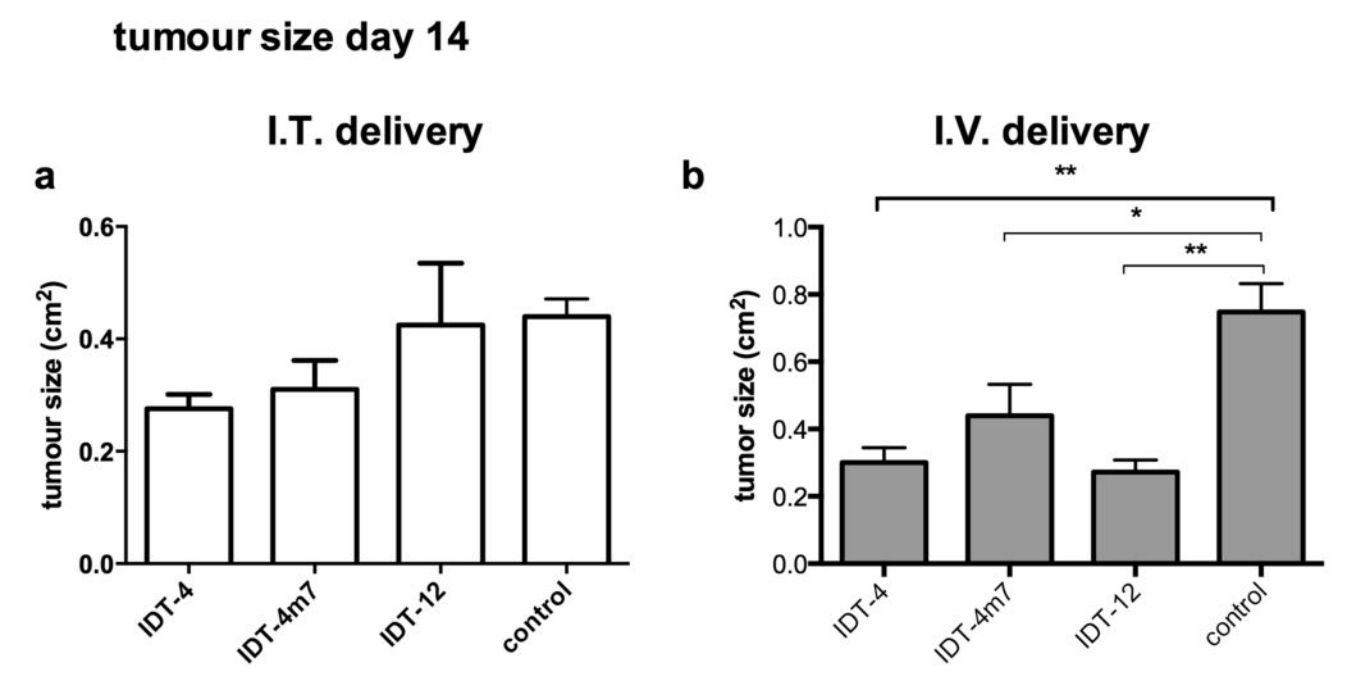
Table 1

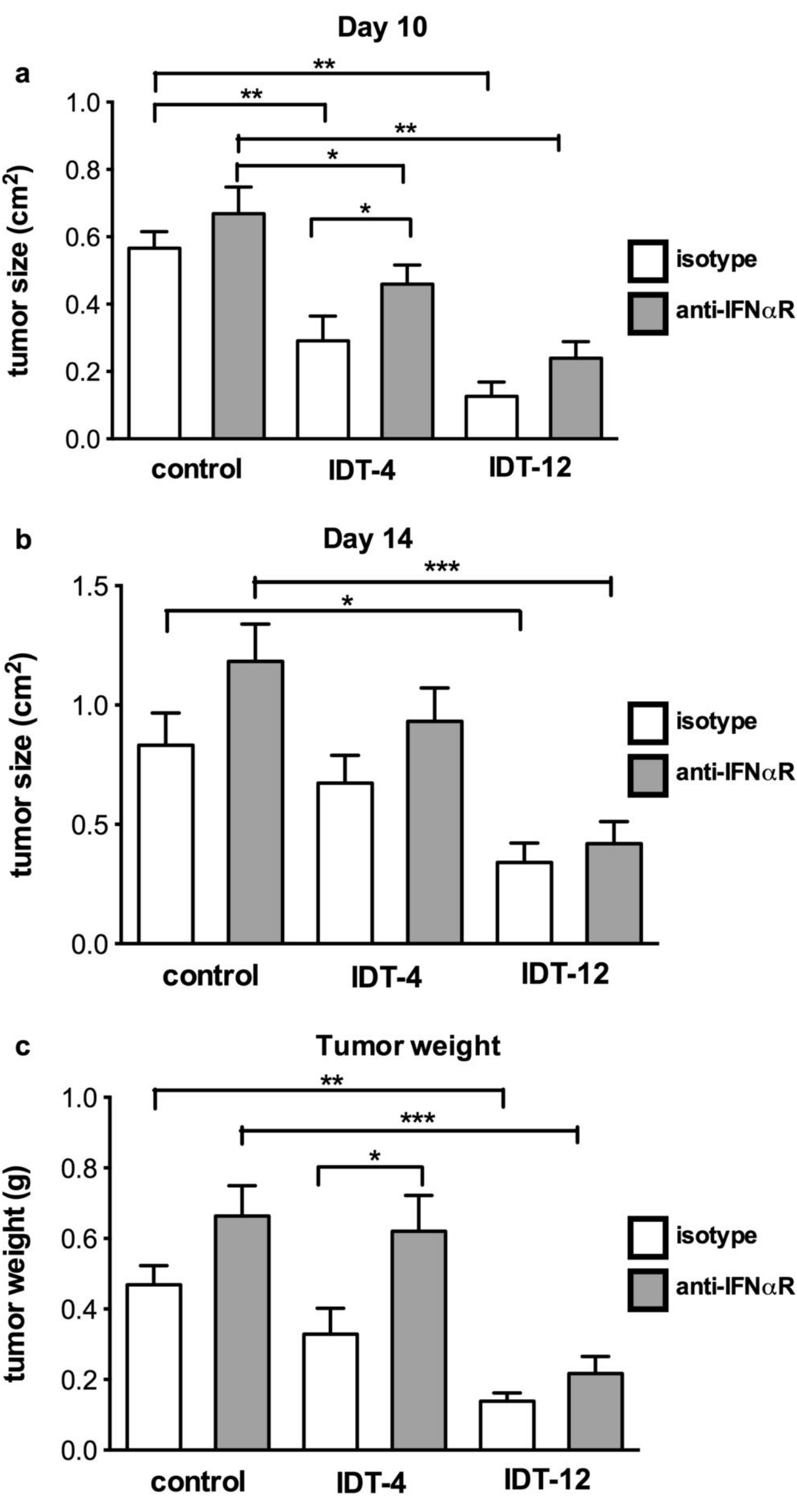
siRNA	siRNA sequence	HPV 16 E7 binding site	Properties
IDT-4	5′-ACAAAGCACACACGUAGACAUUCgt-3′ 5′-ACGAAUGUCUACGUGUGUGCUUUGUAC-3′	position 686-713 of bicistronically transcribed HPV16 E6/E7	- DsiRNA - Targeting E6/E7 - Immunostimulatory
IDT-4m7	5′-ACAAAGCACACACGUAGACAUUCgt-3′ 5′-ACGAAUGUCU**A**C**G**UGUGUGC**U**U**U**G**UAC**-3′	as above, modified bases with methyl groups (underlined and bold)	- DsiRNA - Targeting E6/E7 -Non-immunostimulatory(m7 modified)
IDT-12	5′-ACCGGACAGAGCCCAUUACAAUAtt-3′ 5′-AAUAUUGUAAUGGGCUCUGUCCGGUCC-3′	position 617-644 of the bicistronically transcribed HPV16 E6/E7	- DsiRNA - Targeting E6/E7 -Non-immunostimulatory
scrambled	5′-CGUUAAUCGCGUAUAAUACGCGUat-3′ 5′-AUACGCGUAUUAUACGCGAUUAACGAC-3′	Not applicable (N/A)	- DsiRNA - Non-targeting -Non-immunostimulatory
SCm7	5′-CGUUAAUCGCGUAUAAUACGCGUat-3′ 5′-AUACGCGUAU**U**A**U**ACGCGAU**U**A**A**C**GAC**-3′	N/A	
BP1Mod2	5′-CAGCUUUGUGUGAGCGUAUUU-3′ 5′-AUACGCUCACACAAAGCUGUU-3′	N/A	- Non-targeting - Immunostimulatory

Table 1: SiRNA sequences and profile. Bases underlined and bold are methylated; bases represented in small letters are DNA. A schematic representation of the siRNA binding sites on HPV16 E7 gene is provided as supplementary Figure S5.


a
SS-int
FS-Int
FS-TOF
FS-Int
CD11b-PE
Gr1-FITC
Dir
Dir
Dir
b
% of cells with Dir uptake
**
*
I.V. spleen
I.T. spleen
I.V. lymph nodes
I.T. lymph nodes
I.V. tumor
I.T. tumor
c
I.T. delivery
% of cells with Dir uptake
spleen
tumour
CD4+
CD8+
CD11c+
CD11b+
CD11b/Gr1+
CD11b+Gr1-
d
I.V. delivery
% of cells with Dir uptake
spleen
tumour
CD4+
CD8+
CD11c+
CD11b+
CD11b+/Gr1+
CD11b+/Gr1-


NK cells
T cells
Tumor Draining Lymph Node (TDLN)
a
b
Percentage of NK cells CD69+
Percentage of T-cell CD69+
**
Sucrose
IDT-4
IDT-4m7
SC-m7
Spleen
c
d
Percentage of T-cells CD69+
naked siRNA in saline- TDLN
e
f
saline
IDT4-m7


tumour size day 14
I.T. delivery
a
tumour size (cm2)
0.6
0.4
0.2
0.0
IDT-4
IDT-4m7
IDT-12
control
I.V. delivery
b
**
*
**
tumor size (cm2)
1.0
0.8
0.6
0.4
0.2
0.0
IDT-4
IDT-4m7
IDT-12
control


Day 10
a
**
**
**
*
*
1.0
0.8
0.6
0.4
0.2
0.0
tumor size (cm2)
isotype
anti-IFNαR
control
IDT-4
IDT-12
b
Day 14
***
*
1.5
1.0
0.5
0.0
tumor size (cm2)
isotype
anti-IFNαR
control
IDT-4
IDT-12
c
Tumor weight
**
***
*
1.0
0.8
0.6
0.4
0.2
0.0
tumor weight (g)
isotype
anti-IFNαR
control
IDT-4
IDT-12