SiRNA-induced immunostimulation through TLR7 promotes anti-tumoral activity against HPV-driven tumors *in vivo*.

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Short running title: siRNA-induced immunostimulation and HPV-driven tumors

ABSTRACT

Oncogene-specific down-regulation mediated by RNA interference (RNAi) is a promising avenue for cancer therapy. In addition to specific gene silencing, *in vivo* RNAi treatment with short interfering RNAs (siRNAs) can initiate immune activation through innate immune receptors including Toll-like receptors, (TLRs) 7 and 8. Two recent studies have shown that activation of innate immunity by addition of tri-phosphate motifs to oncogene-specific siRNAs, or by co-treatment with CpG oligos, can potentiate siRNA anti-tumor effects. To date, there are no reports on applying such approach against Human Papillomavirus(HPV)-driven cancers. Here, we characterized the anti-tumor effects of non-modified siRNAs that can target a specific oncogene and/or recruit the innate immune system against HPV-driven tumors. Following the characterization of silencing efficacy and TLR7 immunostimulatory potential of 15 siRNAs targeting the HPV type 16 E6/E7 oncogenes, we identified a bifunctional siRNA sequence that displayed both potent gene silencing and active immunostimulation effect. *In vivo* systemic administration of this siRNA resulted in reduced growth of established TC-1 tumors in C57BL/6 mice. Ablation of TLR7 recruitment via 2'O-methyl modification of the oligo backbone reduced these anti-tumor effects. Further, a highly immunostimulatory, but non-HPV targeting siRNA was also able to exert anti-tumoral effects although for less prolonged time compared to the bifunctional siRNA. Collectively, our work demonstrates for the first time that siRNA-induced immunostimulation can have anti-tumoral effects against HPV-driven tumors *in vivo*, even independent of gene silencing efficacy.

Keywords: bifunctional siRNA, dicer-substrate siRNA, HPV16 E6/E7, immunostimulation, RNAi, TLR7

INTRODUCTION

Cancer formation is the result of an accumulation of numerous, ongoing genetic and epigenetic changes, and it is this complexity that allows cancer cells to escape monotherapy. Consequently, much effort has gone into developing a single-molecule with multiple functions as an anti-cancer strategy. SiRNA-driven RNA interference has received positive attention in this regard [1-3], as in addition to anti-tumor effects through oncogene silencing, there is a growing body of evidence suggesting that innate immune activation can increase the anti-tumor effects of siRNA [4, 5]. Hence, this approach offers great promise as an anti-cancer therapy, either alone or as a co-treatment.

SiRNAs were originally thought to escape innate immune recruitment, however they are now known to be potent activators of several innate immune sensors including TLR7. In both human and mouse, TLR7 senses both siRNAs and single-stranded RNAs in a sequence-specific manner [6-8], which leads to the production of several pro-inflammatory cytokines, such as IFNα [6, 8]. IFNα has anti-proliferative abilities and is widely used in the treatment of a range of cancers including renal cell carcinoma, melanoma and a range of hemopoetic malignancies (as reviewed in *Mitchell* [9]). IFNα is also used in the treatment of genital warts and cervical intraepithelial lesion (CIN) caused by HPV infection [10-12]. Moreover, evidence suggests that induction of IFNα by TLRs is immunomodulatory by activating natural killer (NK) cells, macrophages and dendritic cells (DC) [13, 14]. IFNα-dependent DC maturation subsequently promotes maturation of antigen-presenting cells (APC) [15], accelerating tumor cells' clearance by regulating T-cell response [16]. Hence, induction of Type I IFN such as IFNα links the innate and adaptive immune responses beneficial as an anti-tumor strategy.

SiRNA itself has potent anti-tumor activity due to the direct down-modulation of target genes such as HPV E6 and E7 [1, 17, 18] and such control of tumors is known to occur in the absence of measurable innate immune activation [3]. Due to the plasticity of cancer cells, it is thought that developing 'bifunctional' siRNAs that can both silence target genes and activate innate immune responses would elicit a more potent anti-cancer response which is less susceptible to therapeutic escape. Evidence of siRNA bifunctionality yielding an improved anti-tumor effect was recently demonstrated by Poeck *et al* [4], where innate immune activation relied on the recruitment of RIG-I by 5'-triphosphate-siRNA. Using a slightly different approach, Kortylewski *et al* have shown that combining STAT3 siRNA with CpG oligo DNA, a potent TLR9 agonist, yielded potent anti-tumor effects via activation of tumor-resident macrophages and restoration of normal immune function[5]. Despite this progress, anti-tumor strategies using siRNA to both silence oncogenes and recruit TLR7 are still ill-defined. To date there is no knowledge of whether siRNA bifunctionality could be exploited to treat HPV-driven tumors.

In this work we investigated the use of unmodified siRNAs in combination with innate immune recruitment for the treatment of tumors driven by HPV where cancer survival is entirely reliant on the expression of E6/E7 oncogenes [19]. It has been previously shown that the loss of E6/E7 expression in various cervical cancer cell lines via RNAi is a potent inducer of apoptosis *in vitro* [17, 18, 20, 21]. We studied the contribution of immunostimulatory properties of siRNAs on E6/E7 driven tumor development. By showing that siRNA-induced immunostimulation via TLR7 has a significant anti-tumor effect, even in the absence of specific gene silencing, our *in vivo* findings

show for the first time that bifunctionality improves overall siRNA anti-tumor effects.

RESULTS

Selection of siRNAs against HPV16 E6/E7

In this study we investigated whether bifunctional siRNAs, those able to silence target genes and stimulate TLRs, were more potent anti-cancer agents than non-stimulating siRNAs. We synthesized 15 dicer-substrate siRNAs (D-siRNAs) spanning the entire HPV16 E6/E7 mRNA (Supplementary Table 1). These D-siRNAs were 25 and 27bp for the sense and antisense strand respectively and were designed using the parameters previously outlined, which result in increased silencing efficacy through enhanced strand selection and RNA-induced silencing complex (RISC) loading [22, 23]. We tested these D-siRNAs on HPV16 E6/E7 mRNA in order to assess their silencing ability. As E6 and E7 are expressed from the same mRNA[19], RNAi targeting affects both genes. Caski cells (an HPV 16-positive human cervical cancer cell line) were transfected with 40nM D-siRNAs for 4 hours, collected at 20 hours after transfection, and E6/E7 levels were measured by quantitative real-time PCR (qRT-PCR) normalized against β-actin. Most D-siRNAs were able to down-regulate HPV16 E6/E7 mRNA, with five (IDT-3, -4, -12, -13, and -15) exhibiting potent knockdown (Figure 1a). These five D-siRNAs were further tested for their knockdown ability using a similar protocol in the murine lung epithelial cell line, TC-1, which also expresses HPV16 E6/E7 [24]. As TC-1 cells are syngeneic with C57BL/6 mice, this allows us to test treatments *in vivo* using mouse models with a fully functional immune system. Reproducing the results from the assay on Caski cells, all 5 siRNAs showed significant gene knockdown in TC-1 cells, however at a slightly lower efficacy (Figure 1b and Supplementary Figure S1a).

Immunostimulatory profiles of D-siRNAs are sequence-dependent

It has been previously shown that engagement of TLR7 by siRNAs results in the production of IFNα from pDCs [6, 7], and that TLR7 sensing of RNA is conserved between human and mouse [8, 25, 26]. To test the ability of these siRNAs to engage TLR7/8 and induce an innate immune response, we first measured TLR7 engagement by observing the release of IFNα from primary human PBMCs following endosomal uptake of siRNAs (Figure 1c). PBMCs were collected from healthy male donors and stimulated with 90nM siRNA, delivered via DOTAP, for 16 hours before supernatants were collected and IFNα detected by ELISA. We observed a wide range of IFNα induction following siRNA stimulation of these cells (Figure 1c). Amongst the D-siRNAs previously identified as having potent silencing efficacy, IDT-4 stimulated the highest level of IFNα release; IDT-15 gave an intermediate response, and IDTs-3, -12, and -13 induced low IFNα productions. From these RNAi and immunostimulatory profiles, IDT-4 was identified as a “bifunctional” D-siRNA for its ability to recruit RNAi and facilitate TLR7 activation.

Modification to D-siRNA removes TLR7 activation but retains RNAi.

In order to delineate between the relative contribution of RNAi and that of innate immunity in the putative anti-tumoral effects of IDT-4, we synthesized a modified version of the siRNA, IDT-4m7, with 2’-O-methyl (2'OMe) modifications to the guide strand (Supplementary Table 2). Addition of 2’-O-methyl groups to siRNA has been shown to abolish TLR7 recruitment [3, 27]. To test the ability of these siRNAs to activate TLR7 in mouse, we stimulated mouse bone marrow macrophages (BMMs) with each siRNA and measured the induction of TNFα. We utilized mouse BMMs as it has

been reported that ssRNAs/siRNAs delivered using DOTAP-based agents relies exclusively on TLR7 in mouse cells, due to lack of mouse TLR8 response to RNA agonists [6, 8]. We observed that while IDT-4 maintained highest immunostimulation in levels of TNFα, 2'OMe modification of IDT-4 ablated TLR-activating ability, giving background levels of TNFα (Figure 2a). To confirm TLR7 recruitment by these siRNAs, we measured the levels of TNFα in immortalized $TLR7^{-/-}$ and $TLR7^{+/+}$ BMMs [28]. In $TLR7^{-/-}$ cells, IDT-4-driven cytokine production levels were similar to those obtained with IDT-4m7 (Figure 2b). However, immortalized $TLR7^{+/+}$ BMMs maintained similar immunostimulation profiles as seen in mouse BMMs (Figure 2c), with IDT-4 producing a greater immunostimulatory effect than IDT-4m7. In order to test whether modification altered RNAi ability, we then quantified the mRNA knockdown level of IDT-4m7 using qRT-PCR (Figure 2d) following transfection of TC-1 cells as described above. We observed that IDT-4m7 retained RNAi ability and was able to significantly knockdown HPV16 E6/E7, albeit with a 13% reduction of efficacy as compared to IDT-4 (Figure 2d).

Induction of IFNα is transient *in vivo*

To confirm whether the activation of TLR7 by D-siRNAs observed *in vitro* would also occur *in vivo*, we measured IFNα levels in serum collected from mice at 4, 8, 24, and 48 hours post I.V. injection of siRNA (40μg/mouse) complexed in liposomes. The method for siRNA-encapsulated liposomes preparation was previously established in our laboratory [29]. All siRNAs tested induced IFNα at 4 hours to varying degrees, while the empty liposomes used as our vector for systemic delivery did not (Figure 2e). The level of IFNα induction peaked at 4 hours and then decreased over time, suggesting a transient response. In agreement with our previous *in vitro* results, IDT-

4 induced the highest levels of IFNα, with cytokine levels remaining high at 8 hours. We also tested BP1 Mod2 siRNA, previously shown to be a strong IFNα inducer *in vivo* [7]. Although BP1 Mod2 induced IFNα to lower levels compared to IDT-4, IFNα was still detectable at 24 hours post-injection. The scrambled siRNA control also induced a low level of IFNα at 4 and 8 hours, but at significantly reduced levels compared to all other siRNAs. Induction of IFNα was not detectable at 48 hours post-injection in all siRNAs, showing that IFNα induction *in vivo* by siRNAs was not sustained.

TLR7 activation by siRNAs leads to activation of immune response cells

Immunomodulation by TLRs has been shown to activate dendritic cells (DCs) [30, 31], natural killer (NK) cells [32] and T cells [33], which are important mediators for immune clearance of tumor cells. Prior to using our siRNAs against HPV-driven tumors *in vivo*, we sought to observe activation of these immune cells consistent with TLR7 activation by IDT-4 as opposed to IDT-4m7. We stimulated splenocytes from C57BL/6 mice with siRNAs IDT-4 and IDT-4m7 complexed in the liposomes [29] to be used for *in vivo* I.V. injection, and measured expression of CD86 in DCs, and CD69 in NK and T-cells. CD86 expression following IDT-4 stimulation was doubled that of IDT-4m7, and similar to the expression of synthetic TLR7 agonist Imiquimod (Figure 3a). Similar results were observed for CD69 activation in NK and T-cells (Figure 3b and 3c). Together, these results provide further evidence for activation of TLR7 by IDT-4 complexed with our liposomes and leading to presentation of DCs and activation of NK cells and T-cells.

Pre-treatment with siRNAs impacts tumor formation

Having established the silencing efficacy and innate immune potential of IDT-4, we next tested whether this siRNA could impact tumor formation. IDT-12 was included in this study as this D-siRNA has similar E6/E7 knockdown ability to IDT-4 but lower immunostimulatory potency (Figure 1c and data not shown), while scrambled D-siRNA was used as a negative control. TC-1 cells stably expressing luciferase (TC-1-luci) were treated with 40nM siRNAs delivered via liposomes for 4 hours at which time 1×10^6 cells were subcutaneously injected into each C57BL/6 mouse. Tumors were allowed to form over 14 days, and caliper measurements were carried out every two days from the day tumors could be detected from palpation, until the end of the experiment. On day 14, mice were injected with D-luciferin and the tumors were imaged by luminescence. Tumors were also collected and weighed at day 14. Tumors treated with oncogene-specific siRNAs (IDTs-4, -12) appeared to be substantially smaller than tumors treated with scrambled D-siRNA (Figure 4a). IDT-4 appeared highly effective at impairing tumor development compared to the non-targeting scrambled D-siRNA (Figures 4a, b). Although both target-specific siRNAs significantly reduced tumor growth compared to scrambled siRNA, there was no significant difference between the two target specific siRNAs, IDT-4 and IDT-12 (Figure 4c), despite the bifunctional profile of IDT-4. However, this experiment did not measure the contribution of the innate immune response as TC-1 cells were pre-treated with the siRNAs before inoculation into the mouse, thus macrophage uptake of siRNAs as well as exposure of siRNAs to circulating and infiltrated tumor phagocytes did not occur.

Systemic injection of siRNA results in significant tumor reduction

To test for anti-tumor activity by systemically delivered siRNAs, tumors were

established in mice by subcutaneous injection of 1×10^6 TC-1 cells on day 0 followed by systemic RNAi treatment of 40μg D-siRNA per dose (~2mg/kg) via I.V. tail vein injection on days 5, 8, and 12. This protocol was performed in parallel to our recently published studies on liposomal delivery *in vivo,* which showed substantial siRNA uptake by target cells using this delivery method [34]. To investigate the contribution of innate immune activation in the anti-tumor effects, we compared the effects of IDT-4 versus IDT-4m7, which had shown no immunostimulatory effect (Figure 5a, b). Scrambled-m7 was used as a negative control as it had neither silencing nor immunostimulatory effects (Figure 2a, d). Tumors were measured by caliper on day 10 (Figure 5a) and day 14 (Figure 5b). A significant reduction in tumor size was observed on day 10 in mice treated with IDT-4 ($*P < 0.05$) compared to control. Meanwhile, treatment with IDT-4m7 yielded a 28% reduction in tumor size as compared to control on day 10, albeit not-significant according to student's *t*-test (Figure 5a). A similar result was obtained on day 14 (Figure 5b), where tumors were observed to be more significantly reduced in groups treated with oncogene-specific siRNAs compared to control. This profile is consistent with that obtained in a similar independent experiment using only half the dose of siRNAs at 20μg siRNA/dose (~1mg/kg) (Supplementary Figure S2). Although both IDT-4 and IDT-4m7 each demonstrated significant anti-tumoral effects compared to the control siRNA, the stronger anti-tumoral effect of IDT-4 (Figures 5a, b; Supplementary Figure S3a) suggested that its immunostimulatory activity played a significant role in its anti-tumor ability.

To provide further *in vivo* evidence that siRNA-induced TLR7 activation could have an anti-tumoral effect, we tested two siRNAs that are non-specific to HPV and which

vary widely in their immunostimulatory activity. These were BP1Mod2, which is strongly immunostimulatory, and βgal 924 which has no immunostimulatory activity[7] (Figure 5c, d). In accordance with the increased anti-tumoral effect of IDT-4 versus IDT-4m7, at day 10, we found that the immunostimulatory siRNA BP1 Mod2 exhibited a significant anti-tumor effect (Figure 5c) while the non-targeting and non-immunostimulatory siRNA, βgal 924, had no impact on tumor development. However, this effect was transient as the tumor sizes in BP1 Mod2 returned to control levels at day 14 (Figure 5d). This result could be attributed to the transient immunostimulation observed previously (Figure 2e). To examine the possibility that anti-tumor effects could have been caused by non-specific loss of E6/E7 expression, we examined E7 protein levels following siRNA treatment and showed that neither BP1Mod2 nor βgal 924 siRNAs altered the levels of HPV16 E7 protein (Figure 5e, Supplementary Figure S1b). This confirmed that the observed transient anti-tumor effects of BP1Mod2 seen in Figure 5c were due to innate immune activation alone.

Finally, to directly establish the role of innate immunity in the anti-tumoral effects observed at day 10, we examined the impact of IDT-4 and BP1 Mod2 siRNAs in MyD88 deficient mice[35], where TLR7/8 siRNA-induced immunostimulation is ablated (Figure 5f). Both siRNAs lost significant anti-tumor activity when delivered into MyD88$^{-/-}$ mice. These preliminary results suggest further the important contribution of immunostimulation in the anti-tumor effects previously seen with these sequences. Overall, our data highlights the anti-tumor ability of siRNA-induced immunostimulation via TLR7, even in the absence of specific gene-silencing.

DISCUSSION

Recent reports have highlighted the ability of siRNAs to induce innate immune responses via TLR activation [25, 36]. Knockdown of an oncogene coupled with an activation of the tumor immune milieu provides the basis for an elegant co-therapy for cancer [4]. Here we show that activation of the innate immune response by siRNAs has significant anti-tumor effects against HPV-driven tumors, even in the absence of a specific gene target. Although the most prolonged siRNA-induced immunostimulation in our system reached 24 hours, impact on tumor growth was observed up to 3 days following the last siRNA injection (i.e. on day 10). On the other hand, oncogene-specific siRNA with immunostimulatory ability gave long term anti-tumor control with anti-tumor effect observed on day 10 and sustained on day 14. The responses we observed are specific to HPV-driven tumors and one would expect different cancers to exhibit varying sensitivity to such therapy. This is illustrated by the work of Poeck *et al*, who showed that bifunctional siRNAs, 5'-triphosphate-siRNAs (3p-siRNA) targeting *Bcl*2, led to better melanoma tumor reduction than OH-siRNA or 5' triphosphate siRNAs containing target mismatches[4]. While Poeck *et al* demonstrated that the structure of 3p-siRNA was critical for activation of immune responses[4], the D-siRNAs in our study rely exclusively on sequence differences to elicit differential TLR activation. Many reports have suggested that modifications to the siRNA structure impacts its immunostimulatory ability. Modifications to the siRNA 5' end with triphosphate [4] or addition of miRNA-like non-pairing uridine bulges to the siRNA backbone [28, 37] increases siRNA-induced immunostimulation while addition of methyl groups[3, 27] removes immunostimulation. For the D-siRNAs used here, we found that sequence alone play a role in immunostimulation. However, we observed no consistent sequence motifs in our D-

siRNAs that resembled known immunostimulatory motifs. Neither the amount of "GU" motifs nor the overall numbers of U were significantly different[7, 26]. However, IDT-4 does have the "UGUGU" motif, which Heil *et al* have reported as immunostimulatory [26]. However, this motif was absent in other highly active siRNAs (e.g. IDT-5, -6, -8 and -14). We did note that high immunostimulation in these D-siRNAs appeared to be associated with a UXUCU motif starting at position 9 or 10 downstream from the 5' end of the antisense strand, with IDT-4 having both UXUCU and UGUGU motifs (Supplementary Figure S3).

Our study also demonstrates that the immunostimulatory profiles of our D-siRNAs are similar *in vivo* to their initial *in vitro* profiles with IDT-4 consistently demonstrating the highest level of immunostimulation in both settings. We also observed TLR7 specificity pertaining to siRNA-induced immunostimulation. IDT-4 induced immunostimulation was concurrent with the activation of DCs and NK cells. Furthermore, we showed that *in vivo* induction of IFNα by immunostimulatory siRNAs is transient, lasting 4 to 8 hours. Although we have established that immunostimulation by our D-siRNAs is sequence-dependent, we also observed that addition of methyl groups to the antisense strand of these siRNAs abolishes their *in vivo* immunostimulatory ability, thus supporting results previously published [3, 27, 37]. However, the RNAi ability by these modified siRNAs was retained, allowing us to directly compare the effects of TLR7 activation on tumor reduction.

SiRNAs delivered systemically will target not only to tumor cells, but also to cells of the liver, lung, and spleen - the primary sites of liposomal deposition [29,34], hence activating immune cells such as macrophages, dendritic cells and monocytes,

benefiting anti-tumor activity. To address the activation of these immune cells by siRNAs, we treated established tumors in mice with systemically-delivered siRNAs on days 5, 8, and 12, and observed tumor sizes on days 10 and 14. We observed that siRNA with both RNAi and immunostimulatory abilities gave persistent anti-tumor effects. We suggest that TLR7 activation played a significant role in this tumor reduction, as loss of TLR7 activity via siRNA sequence modification or ablation of myD88 pathway impaired this siRNA-mediated tumor reduction. Further, immunostimulation alone had potent anti-tumor activity as demonstrated by the activity of BP1 Mod2 on day 10; an effect not seen in MyD88$^{-/-}$ mice treated with this immunostimulatory siRNA. We also found these non-specific immunostimulatory effects by BP1 Mod2 to be transient as tumor sizes returned to control levels by day 14. This data point suggests that gene silencing-independent anti-tumor effects through potential TLR7 activation to be transient.

The BP1 Mod2 data are consistent with findings from Kleinman *et al* who showed that non-targeting siRNAs can have biological activity via activation of TLR3 causing down-regulation of vascular endothelial growth factor (VEGF) gene in macular degeneration[38]. However, the contribution of TLR3 or cytosolic RIG-I to siRNA driven immunostimulation is minor using a DOTAP-based delivery system [8, 28], as is the case with our cationic liposomes [29]. This suggests that the anti-tumor effects observed here were due to TLR7 activation. Further work relying on TLR and RIG-I deficient animal models should help address the direct contribution of TLR3/7 and RIG-I on assisting tumor reduction and tumor clearance.

Overall our data show for the first time that siRNA-mediated innate immune

activation has significant but transient anti-tumor effects in HPV-driven cancers. Our data suggests that siRNA-induced immunostimulation improved RNAi anti-tumor ability in HPV-driven tumors. Conversely, abolishing immunostimulation of our bifunctional siRNA reduced its anti-tumor ability. Further improvements on bifunctionality may be gained by incorporation of novel sequence modifications that confer stronger immunostimulatory effects such as addition of uridine bulges recently reported for human TLR8 activation [28] or by conjugation to a CpG-linker to incorporate adaptive immune responses for enhanced anti-tumor ability[5, 39] in combination with RNAi in such cancer models. With these improvements, RNAi can be developed as a single-molecule approach with multiple activities, and could therefore provide a promising therapeutic strategy against HPV-driven cancers.

METHODS

Cell lines

Caski cells line was obtained from the American Type Culture Collection (ATCC). TC-1 cells (murine C57BL/6 lung epithelial cells transformed with HPV16 E6, E7 and *ras* oncogenes) were obtained from TC Wu [24]. TC-1-luci cells were developed in-house by transducing the luciferase gene into TC-1 cells via lentiviral transfection[40]. TC-1 and TC-1-luci cells were maintained in Dulbecco's modified Eagle's medium, (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS) (Bovogen, VIC, AU), 100units/ml penicillin G, 100µg/ml streptomycin sulfate, and 0.29mg/ml L-glutamine (P/S/G) (Gibco Invitrogen, Mount Waverley, Australia); referred to as complete DMEM. Caski cells were maintained in Roswell Park Memorial Institute (RPMI-1640; Invitrogen, Carlsbad, CA) media supplemented as above; referred to as complete RPMI-1640. Stable TLR7$^{-/-}$ BMMs and TLR2/4$^{-/-}$

BMMs were generated using the J2 retrovirus encoding v-*raf* and v-*myc* as previously reported [28, 41, 42], and grown in DMEM with 10% FCS and 20% L-929 conditioned cell medium. Cells were kept at 37°C in a 5% CO_2 atmosphere.

SiRNA

D-siRNAs (Supplementary Table 1) and the modified D-siRNAs (Supplementary Table 2) used for *in vivo* experiments were designed[22] and supplied by Integrated DNA Technologies (IDT) (Iowa, USA) as single-stranded, HPLC-purified, lyophilized sodium salt. They were re-constituted into solution using DEPC water to a concentration of 100μM. An equal volume of sense and its respective antisense strand was added to a tube which was heated to 99°C for 10 minutes, and then left at room temperature for 30 minutes. Duplexed D-siRNAs were checked on non-denaturing polyacrylamide gels (4.95ml 19:1 Acrylamide/Bis (BioRad, Hercules, CA), 7.05ml dH_2O, 3ml 5×TBE (54g Tris, 27.5g Boric Acid, 20ml 0.5M EDTA pH 8), 50μL 10% Ammonium Persulfate (APS), and 15μL 1,2-bis(dimethylamino)-ethane (TEMED)) and stored as 20μM aliquots for *in vitro* work and 40μg/5μL for *in vivo* work. SiRNAs s10 [1], βgal 924 [7], and BP1 Mod2 [7] were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) as double-stranded, lyophilized, desalted form and reconstituted to 100μM using DEPC water.

Transfection

Caski cells were transfected using Oligofectamine™ reagent (Invitrogen, Mount Waverley, Australia) according to manufacturer's instructions. On the day prior to transfection, cells were plated out at a density of 300,000 cells/well in a 6-well plate. D-siRNA (1μL of 20μM stock) was diluted in 4μL dH_2O, to which 80μL of Opti-MEM I

reduced-serum media (Invitrogen) was added. For each transfection, 2μL Oligofectamine™ reagent was added to 13μL Opti-MEM I and incubated at RT for 5 minutes. The mixture was then added to the prepared D-siRNA and the mixture incubated at room temperature for 20 minutes before being added to 400μL Opti-MEM I and added to cells. After 4 hours incubation at 37°C in 5% CO_2 the transfection mix was removed, cells washed with 1× PBS, and complete RPMI-1640 added and incubation continued overnight at 37°C in a 5% CO_2 atmosphere. TC-1/TC-1-luci cells were transfected using cationic liposomes [29] (see below) as follows: On the day prior to transfection, cells were plated at a density of 100,000 cells/well in a 6-well plate. D-siRNA (2μL of 20μM stock) was diluted in 3μL dH_2O and brought up to a volume of 100μL with Opti-MEM I reduced-serum media. Cationic liposomes were diluted in 5% dextrose/dH_2O to achieve a liposome to D-siRNA N:P ratio of 4:1 in a final volume of 100μL. The liposome was then added to the D-siRNA and the mixture incubated at RT for 20 minutes. Liposome/D-siRNA was mixed with 800μL OptiMEM I before being added to the cells and incubated for 4 hours at 37°C in 5% CO_2. The transfection mix was then removed; cells washed with 1× PBS and complete DMEM added and incubation continued overnight at 37°C in a 5% CO_2 atmosphere. Transfection of control siRNAs were also done in a similar manner to that described above.

For transfection of human PBMCs to measure IFNα induction, fresh blood from healthy male donors was collected in heparin-treated tubes, and submitted to ficoll-paque plus (GE Healthcare) gradient purification following the manufacturer's guidelines (Add reference to our review Gantier and Williams, Methods in Mol. Biology, 2010). Isolated cells were plated in a 96-well plate at 200,000 cells per well

in RPMI 1640+L-glutamine medium (Gibco) complemented with 1X Antibiotic/Antimycotic (Invitrogen) and 10% FBS (referred to as complete RPMI), and incubated for 4 hours at 37°C in a 5% CO_2 atmosphere prior to stimulation with TLR agonists. Cells were then transfected with D-siRNAs using DOTAP (Roche, Castle Hill, Australia) at a DOTAP to siRNA ratio of 5.3 µg/µl of 80µM siRNA. DOTAP was first diluted in 75µl RPMI for 5 minutes before being mixed with an equal volume of RPMI containing the D-siRNAs. The resulting mix was incubated for at least 10 minutes before 50µl was added per well of a 96-well plate, resulting in a final volume of 150 or 200µl.

For stimulation of mouse bone marrow macrophages with siRNAs, bone marrow extraction and differentiation were carried out following standard procedures [43]. Briefly, femurs from wild type C57BL/6 mice were flushed with complete RPMI, and cells were plated in complete RPMI supplemented with 20% L-cells condition medium[44] on 10 cm bacteriological plastic plates for 6 days at 37°C in a 5% CO_2 atmosphere. 80,000 cells per 96-well were plated on day 6 in 150uL of complete RPMI supplemented with 20% L-929 cell conditioned medium. Cells were then transfected with D-siRNAs/siRNAs using DOTAP (Roche, Castle Hill, Australia) at a DOTAP to siRNA ratio of 3.74 µg/µl of 80µM siRNA, with D-siRNA concentration of 750nM. 3M-002 (human TLR8 agonist and mouse TLR7 agonist, cat. no. tlrl-C75), ODN2216 (TLR9 agonist, cat. no. tlrl-hodna), Pam3CSK4 (TLR2/1 agonist, cat. no. tlrl-pms), and CL-75 (mouse TLR7 human TLR8 ligand) were used as controls and purchased from Invivogen (Willoughby, Australia). SiLam-N-MIS was also included as TLR7-dependent immunostimulatory siRNA in the experiments involving immortalized BMMs, as previously reported [28]. Controls were added directly to

medium and used at a final concentration of 1µg/ml (3M-002), 3µmol/l (ODN2216) and 100ng/ml (PamSCSK4). Transfections were carried out in triplicate in all experiments.

Immunoblotting

Protein extraction was performed on cells 24 hours after transfection. Protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 50mM Tris, pH 8.0) with the addition of 2mM phenylmethlsulfonyl fluoride (PMSF) and 1µL/mL Protease Inhibitor Cocktail (PIC) (Sigma, Sydney, Australia). Lysis buffer was freshly prepared and added to transfected cells in 6-well plates (200µL/well), and incubated for 10 minutes on ice. Protein concentration was determined using protein assay kit (Bio-Rad, Hercules, CA) with the appropriate standards. Cell extracts were boiled for 10 minutes at 99°C in the presence of Loading Buffer (200nM Tris-HCL, 8% SDS, 0.4% Bromophenol Blue, 40% Glycerol) and β-mercaptoethanol. 60-75µg protein samples were separated on a 10-15% SDS-PAGE gel, transferred unto Immobilin-P™ polyvinylidine fluoride (PVDF) membrane (Millipore, Australia) and immunoblotted using HPV16E7 (Santa Cruz, USA) antibody on the SNAP I.D. system (Millipore, Australia) according to manufacturer's instructions. Bound antibodies were detected using their appropriate secondary antibodies followed by enhanced chemiluminescence using in-house reagents.

Quantitative real-time PCR

RNA was extracted from cells using Tri-Reagent (Invitrogen, Auckland, New Zealand) according to protocol. cDNA synthesis was performed using Omniscript

Reverse Transcriptase kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Real-time PCR was carried out using SYBR® Green PCR Master Mix (ABI, Warrington, UK) according to manufacturer's protocol with primers as described in Supplementary Table 3.

The PCR was carried out on Rotorgene 6000 (Corbett) as follows: initial denaturing at 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds (denature), 60°C for 15 seconds (anneal), and 72°C for 20 seconds (extension), and melt conditions at 75°C to 95°C rising at 1°C each step. Data was analysed using the Rotor-Gene 6000 series software.

Detection of cytokines

Human IFNα in culture supernatants was quantified by sandwich ELISA using 0.5 µg/ml mouse monoclonal (PBL Biomedical) and rabbit polyclonal antibodies (PBL Biomedical) respectively. A goat anti-rabbit HRP-conjugated antibody (Pierce) at 0.8µg/ml was used for detection. Mouse TNFα was measured using the BD OptEIA ELISA set (BD Biosciences, North Ryde, Australia). In both IFNα and TNFα ELISAs, TMB substrate (Sigma, Castle Hill, NSW, Australia) was used for quantification of the cytokines on the Fluostar OPTIMA plate-reader.

ELISA for murine IFNα was performed using Verikine™ Mouse Interferon Alpha (Mu-IFNα) ELISA kit (42100-1; PBL Biomedical) according to manufacturer's protocol and quantification of cytokine was carried out on the Labsystems Multiskan plate reader.

***In Vitro* activation of mouse splenocytes**

C57BL/6 mice were euthanased by CO_2 narcosis and spleens removed under aseptic conditions. Spleens were then homogenized followed by a 30 minute incubation with 1 mg/ml Collaginase D, 100 µg/ml DNAse (Roche) at 37°C before cells were passed through a 70 µM cell strainer (Beckton Dickinson, San Jose, USA) and red cells removed by a 2 minute incubation with ACK lysing solution. Splenocytes were then resuspended to $2x10^6$ cells/ml in RPMI1640 media supplemented with 5% FBS, P/S/G, 1 x 10^{-3} M Sodium Pyruvate (Gibco) and 5 x 10^{-5} M 2-mecaptoethanol (Sigma). SiRNA encapsulated in PEGylated Liposomes (as described below) were added to culture at a final concentration of 90nM. Isotonic sucrose was used as a negative control and 25 ng/ml of the TLR7 agonist Imiquimod (InVivogen, San Diego, CA) was used as a positive control. Cells were incubated for 24 hrs at 37°C before removal from culture and staining with antibodies against CD3ε-PE, CD11c-APC, CD69-FITC, CD86-PE, NK1.1-APC and MHC class II I-A^b-FITC (Biolegend, San Diego, CA) for 30 minutes on ice. Samples were then washed and run on a Gallios Benchtop flow cytometer (Beckmann Coulter, Villepinte, France). Flow cytometry data was analysed using Flowjo7.6 (Flowjo, Ashland, OR).

Cationic liposomes

For transfection of TC-1/TC-1-luci cells, cationic liposomes were prepared using dioleoyl trimethylammonium propane (DOTAP) (Sigma, St Louis, MO) and dioleoyl phosphatidylethanolamine (DOPE) (Northern Lipids, Vancouver, Canada) at a 1:1 molar ratio as previously described[45], resulting in a dried, thin lipid film. The lipid film was hydrated with sterile 5% dextrose solution to give a final concentration of $2.5x10^{-6}$ mole DOTAP/mL. The resulting liposomes was left to stabilize at room temperature for two hours followed by size reduction of the multilamellar liposomes

performed according to the procedure described in Wu *et al* [29]. The resulting small unilamellar liposomes were subsequently complexed with siRNA at Nitrogen to Phosphate (N:P) ratios of 4:1 for transfection as described above.

***In vivo* experiments**

C57BL/6 mice were purchased from Animal Research Centre (ARC) (Perth, WA, Australia). For delivery of siRNA into C57BL/6 mice and subsequent tumor reduction experiments, 40µg of each D-siRNA/mouse was complexed with Polyethylene Glycol (PEG)ylated liposomes according to the protocol described in Wu *et al* [29]. Briefly, to prepare the PEGylated liposomes, 0.352mg DOTAP, 0.136mg cholesterol (Sigma, St Louis, MO), 0.037mg DOPE and 0.263mg PEG_{2000}-C16-ceramide (Avanti Polar Lipids, Alabaster, AL) were dissolved in *tert*-butanol at 37°C before the required volumes of lipids for each D-siRNA to be tested were aliquoted into round-bottomed flasks. Sterile sucrose solution was prepared and 40µg/mouse D-siRNA was added to this solution. The D-siRNA/sucrose solution was then added to the lipids solution and mixed well by gentle shaking. The solution was then snap-frozen in dry ice/ethanol or acetone bath and lypophilised at least overnight on the freeze-dryer (ALPHA 1–2 LDplus, Martin Christ, Germany) at a condensing temperature of −80°C and pressure of less than 0.1 mbar. Once the samples were dry, each sample was rehydrated with 300µL sterile water per 40µg D-siRNA and allowed to stand at room temperature for one hour. Control siRNAs were also prepared as described above. TC-1 cells were subcutaneously injected into female C57BL/6 mice at 1×10^6 cells/mouse (Day 0). D-siRNAs/siRNAs complexed in liposomes were then injected intravenously into mice 5, 8, and 12 days after subcutaneous TC-1 tumors were established. Tumor size was measured using caliper on days 10 and 14. For IFNα

induction experiments, D-siRNAs/siRNAs complexed in liposomes as above were injected intravenously into mice and blood collected 4, 8, 24, and 48 hours after I.V. injection. Serum was then collected according to Sakurai *et al* [46] and used for mIFNα ELISA. All animal work was approved by The University of Queensland Animal Ethics Committee, Queensland, Australia.

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Conflict of interest statement: The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: siRNAs knockdown of HPV16 E6/E7 in cervical cancer cells and immunostimulatory ability. (a) HPV16 E6/E7 mRNA levels in CaSki cells analysed by qRT-PCR and normalised against β-actin as a housekeeping gene. Following transfection with 40nM D-siRNAs for 4 hours, cells were collected 20 hours post-transfection. RNA was extracted from cells and cDNA synthesized and used for quantitative real-time PCR. Error bar represents ±SEM. Significant differences between scrambled and siRNAs are indicated (***P < 0.001; two-sided t-test). Data from two independent experiments each with triplicate samples. **(b)** HPV16 E6/E7 mRNA levels in TC-1 cells analysed by qRT-PCR and normalised against β-actin as a housekeeping gene. Following transfection with 40nM of selected D-siRNAs for 4 hours, cells were collected 20 hours post-transfection. RNA was extracted from cells and cDNA synthesized and used for quantitative real-time PCR. Error bar represents ±SEM. Significant differences between scrambled and siRNAs are indicated (* P < 0.05, **P < 0.01; two-sided t-test). The difference between scrambled and IDT-3 was not significant according to student's t-test and noted as "ns". Data from two independent experiments with triplicate samples. **(c)** Fresh human PBMCs from healthy male donors were treated with 90nM D-siRNAs before supernatants were collected after 16 hours and IFNα detected by ELISA. CpG DNA (3 μM) and 3M-002 were used as positive control (see Materials and Methods). There were no detectable levels of IFNα in scrambled, mock, and medium samples (noted as "nd"). Error bar represents ±SEM. Data from two independent experiments with triplicate samples.

Figure 2: siRNAs exhibit varying levels of immunostimulation. (a) Mouse bone

marrow macrophages were stimulated with 90nM siRNA, inclusive of modified siRNAs, overnight and supernatants were collected after 16 hours before mouse TNFα levels were detected by ELISA. Significant difference between IDT-4 and IDT-4m7 is indicated (*P < 0.05). Error bar represents ±SEM. Data from two independent experiments with triplicate samples. **(b)** TLR7$^{-/-}$ immortalized BMMs were stimulated with 750nM of DOTAP-complexed siRNAs and incubated for 16 hours at 37ºC. PAM3C (Pam3CSK4), siLam-N-Mis and CL75 were used as controls (see Materials and Methods). Following overnight stimulation, mouse TNFα levels were measured by ELISA. The difference between IDT-4 and IDT-4m7 is not significant according to student's *t*-test (noted as "ns"). **(c)** TLR2/4 double knockout cells (TLR7$^{+/+}$) were stimulated as described in (b), and mouse TNFα levels measured by ELISA. The significant difference between IDT-4 and IDT-4m7 is indicated (***P <0.001). **(b and c)** Error bars represent ±SEM. Data is representative of two independent experiments in biological triplicate. **(d)** Following selected siRNA treatment inclusive of modified IDT-4 (IDT-4m7) and scrambled (scrambled-m7) at 40nM for 4 hours, TC-1 cells were harvested at 20 hours post transfection and HPV16 E6/E7 mRNA levels were analysed by qRT-PCR. SiRNA s10 was included as positive control for gene knockdown. Error bar represents ±SEM. Significant differences between scrambled-m7 and IDT-4, scrambled-m7 and IDT-4m7 and scrambled-m7 and s10 are indicated (**P < 0.01, ***P < 0.001; two-sided *t*-test). Data from two independent experiments with triplicate samples. **(e)** C57BL/6 mice are treated with a single I.V. injection containing 40μg siRNA encapsulated in liposomes. Blood was collected 4, 8, 24, and 48 hours post injection. Serum from the blood was collected and murine IFNα (mIFNα) levels determined by ELISA. Error bar represents ±SEM with n=3. No detectable levels of mIFNα (marked as "nd") was observed at 24 and 48 hours for

samples scrambled, IDT-4 and empty liposome, and no detectable levels of mIFNα was observed at 48 hours for sample BP1 Mod2, a highly-immunostimulatory siRNA that do not target the HPV16 E6/E7 oncogene.

Figure 3: Bifunctional siRNA activates DCs and NK cells. Splenocytes were stimulated with liposome-encapsulated siRNAs at a final concentration of 90nM for 24 hours at 37ºC. Isotonic sucrose and Imiquimod (TLR7 agonist) were used as controls. Cells were then stained with CD3ε-PE, CD11c-APC, CD69-FITC, CD86-PE, NK1.1-APC and MHC class II I-A^b-FITC and read by flow cytometry. **(a)** Presentation of DC on the cells' surface following siRNA stimulation, represented as mean fluorescence intensity (MFI). Significant difference between IDT-4 and IDT-4m7 is indicated (*P < 0.05). **(b)** NK cells' activation following siRNA stimulation. Significant difference between IDT-4 and IDT-4m7 is indicated (**P < 0.01). **(c)** T cells' activation following siRNA stimulation. There is no significant difference between IDT-4 and IDT-4m7 according to student's *t*-test (noted as "ns"). Error bar on all these experiments represents ±SEM. Data from two independent experiments, each with triplicate samples.

Figure 4: Pre-treatment of TC-1 cells with siRNAs impacts tumor growth *in vivo*. TC-1 cells were transfected *in vitro* with 40nM of IDT-4, -12 or scrambled as control for 4 hours before 1×10^6 cells were injected subcutaneously into C57BL/6 mice and tumor growth was observed for up to 14 days. **(a)** Tumors were visualized on day 14 by injecting mice with D-luciferin and imaging using a Kodak *In Vivo* Imager. **(b)** Tumor weight was measured on day 14. Error bar represents ±SEM with n=5. Significant differences between control and IDT-4, and control and IDT-12

are indicated (*P < 0.05; two-sided t-test). **(c)** Tumors were measured by caliper on subsequent days (days 8, 10, and 13) post initial S.C. injection of tumor cells. Error bar represents ±SEM with n=5.

Figure 5: Treatments of established tumors with siRNAs show a reduction in tumor growth. Treatments of established tumors with intravenously delivered siRNAs. TC-1 cells (1×10^6 cells) were subcutaneously injected into C57BL/6 mice on day 0 before mice were treated intravenously with 40μg siRNAs encapsulated in liposomes on days 5, 8, and 12. **(a-b)** Tumor growth reduction by RNAi is independent of TLR7 activation. Treatments carried out with IDT-4 as an immunostimulatory D-siRNA and IDT-4m7 as a non-immunostimulatory D-siRNA, both with RNAi ability for HPV16 E6/E7. Caliper measurements of tumors were carried out on (a) day 10 and (b) day 14 post initial subcutaneous injections. Error bar represents ±SEM with n=6. Significant differences between control and IDT-4, and control and IDT-4m7 are indicated (*P < 0.05, **P < 0.01; two-sided t-test). **(c-d)** Activation of TLR7 independent of RNAi results in a transient anti-tumor effect. Treatments carried out with 40μg siRNA/mouse per I.V. injection as previously described with BP1 Mod2 (highly immunostimulatory for IFNα) and βgal 924 (non-immunostimulatory for IFNα), both not targeting HPV16 E6/E7. Caliper measurements of tumors were carried out on (c) day 10 and (d) day 14 post initial subcutaneous injections. Significant difference between control and BP1 Mod2 is indicated (***P < 0.001; two-sided t-test). Error bars represents ±SEM with n=5. **(e)** Quantification of immunoblots of TC-1 cell lysates in Supplementary Figure S1b using Image J software. siRNA treated samples in HPV16 E7 blots were corrected against β-tubulin blots, and compared against untreated samples. Error bar

represents ±SEM. Significant difference between control and s10 is indicated (*P < 0.05; two-sided t-test). Data from two independent experiments with triplicate samples. **(f)** Tumor growth reduction by immunostimulatory siRNA is dependent on immunostimulation. Treatments carried out with 40µg siRNA/mouse per I.V. injection as previously described, with IDT-4 as immunostimulatory siRNA targeting E6/E7 and BP1 Mod2 as immunostimulatory siRNA but not targeting E6/E7, in MyD88-knockout mice. Caliper measurements of tumors were carried out on day 10 post initial subcutaneous injections. Error bars represents ±SEM with n=4. Non-significant differences were noted as "ns".

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: **siRNAs are able to knockdown HPV16 E6/E7 in cervical cancer cells.** Selected D-siRNAs were used to transfect murine lung fibroblast cells harbouring HPV16 E6/E7, TC-1. **(a)** Detection of E7 protein by immunoblotting of TC-1 cell lysates following transfection with 40nM D-siRNAs IDTs -3, -4, -12, -13, and -15 for 4 hours. Lysates were collected 20 hours post transfection for western blotting. Controls include untreated TC-1 cells and treatment with a scrambled siRNA, and a conventional siRNA, s10 as a positive control for gene knockdown. **(b)** Detection of E7 protein by immunoblotting of TC-1 cell lysates following transfection with 40nM non-HPV targeting siRNAs βgal 924 and BP1Mod2 siRNAs for 4 hours. SiRNA s10 was used as a positive HPV16 E7 knockdown control and scrambled siRNA used as a negative knockdown control. Lysates were collected 20 hours post transfection for western blotting. **(a-b)** Data is the best representation of at least three independent

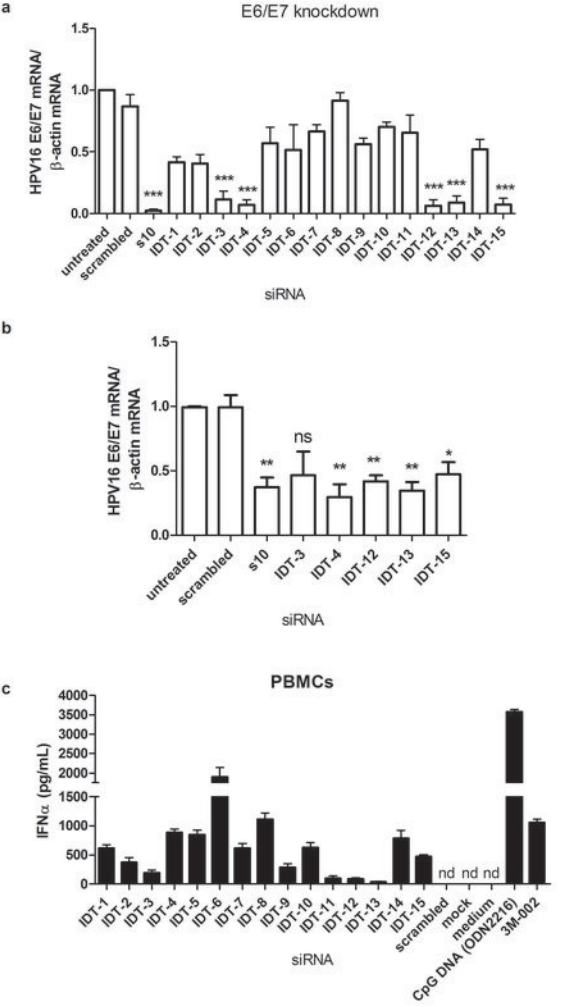
experiments.

Figure S2: Treatments of established tumors with intravenously delivered siRNAs. TC-1 cells (1×10^6 cells) were subcutaneously injected into C57BL/6 mice on day 0 before mice were treated intravenously with 20μg siRNAs encapsulated in liposomes on days 5, 8, and 12. **(a-b)** Tumor growth reduction by RNAi is independent of TLR7 activation. Treatments carried out with IDT-4 as an immunostimulatory D-siRNA and IDT-4m7 as a non-immunostimulatory D-siRNA, both with RNAi ability for HPV16 E6/E7. Caliper measurements of tumors were carried out on (a) day 10 and (b) day 14 post initial subcutaneous injections. Error bar represents ±SEM with n=6. Significant differences between control and IDT-4 is indicated (**$P < 0.01$; two-sided *t*-test). Non-significant differences are marked as "ns".

Figure S3: **Immunostimulatory motifs for D-siRNAs.** A schematic diagram showing the sequences of highly immunostimulatory D-siRNAs. The sequence motif UGUGU reported by Heil *et al* to be immunostimulatory are in bold[26], while the motif consistent with high immunostimulation in D-siRNAs (UXUCU) are in bold and underlined. IDT-5 has an extra "U" within the motif shown as italicised. The motif UXUCU was observed starting at position 9 or 10 from the 5'-end of the antisense strand for D-siRNAs IDTs-5, -6, -8, and -14; while this motif was observed starting at position 6 from the 5'-end of the antisense strand for IDT-4. IDT-4 was noted to have both UXUCU and UGUGU motifs, suggesting that these motifs are responsible for its high immunostimulation profile. We also noted that BP1 Mod2 siRNA used as control for high immunostimulation has the UGUGU motif[7].

Supplementary Tables: SiRNAs and qRT-PCR primers used in this study. Table 1: Sequences of siRNAs used in this study. Table 2: Addition of the 2'OMe to the oligo backbone of IDT-4 and scrambled. Table 3: Primer sequences for qRT-PCR.

FIGURE 1


a
E6/E7 knockdown
HPV16 E6/E7 mRNA/ β-actin mRNA
1.5
1.0
0.5
0.0
***
***
***
***
***
untreated
scrambled
s10
IDT-1
IDT-2
IDT-3
IDT-4
IDT-5
IDT-6
IDT-7
IDT-8
IDT-9
IDT-10
IDT-11
IDT-12
IDT-13
IDT-14
IDT-15
siRNA
b
HPV16 E6/E7 mRNA/ β-actin mRNA
1.5
1.0
0.5
0.0
ns
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*
untreated
scrambled
s10
IDT-3
IDT-4
IDT-12
IDT-13
IDT-15
siRNA
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PBMCs
IFNα (pg/mL)
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3500
3000
2500
2000
1500
1000
500
0
nd nd nd
IDT-1
IDT-2
IDT-3
IDT-4
IDT-5
IDT-6
IDT-7
IDT-8
IDT-9
IDT-10
IDT-11
IDT-12
IDT-13
IDT-14
IDT-15
scrambled
mock
medium
CpG DNA (ODN2216)
3M-002
siRNA

FIGURE 2

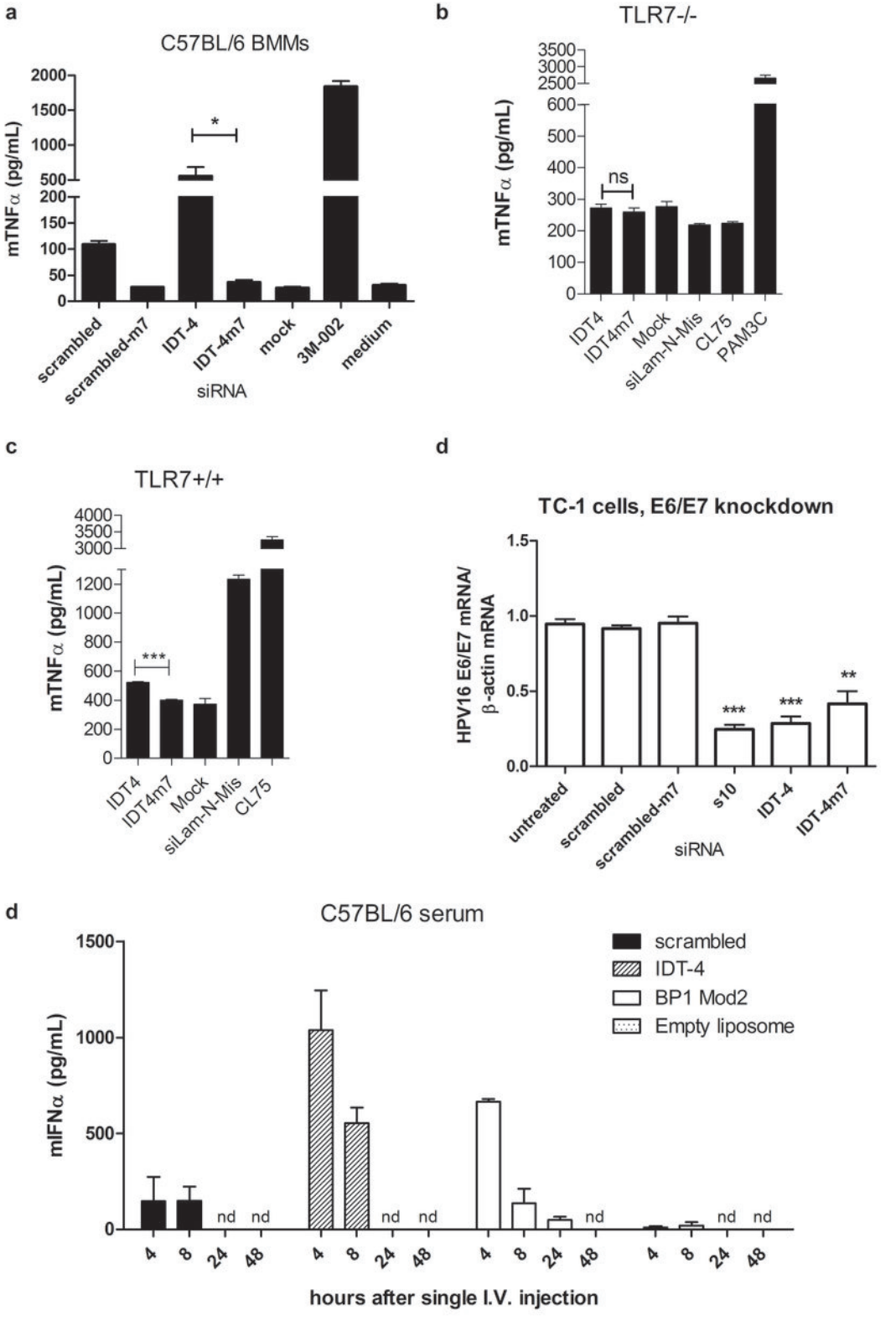

a
C57BL/6 BMMs
mTNFα (pg/mL)
2000
1500
1000
500
200
150
100
50
0
*
scrambled
scrambled-m7
IDT-4
IDT-4m7
mock
3M-002
medium
siRNA
b
TLR7-/-
mTNFα (pg/mL)
3500
3000
2500
600
500
400
300
200
100
0
ns
IDT4
IDT4m7
Mock
siLam-N-Mis
CL75
PAM3C
c
TLR7+/+
mTNFα (pg/mL)
4000
3500
3000
1200
1000
800
600
400
200
0
***
IDT4
IDT4m7
Mock
siLam-N-Mis
CL75
d
TC-1 cells, E6/E7 knockdown
HPV16 E6/E7 mRNA/
β-actin mRNA
1.5
1.0
0.5
0.0
***
***
**
untreated
scrambled
scrambled-m7
s10
IDT-4
IDT-4m7
siRNA
d
C57BL/6 serum
mIFNα (pg/mL)
1500
1000
500
0
scrambled
IDT-4
BP1 Mod2
Empty liposome
nd
4
8
24
48
hours after single I.V. injection

FIGURE 3

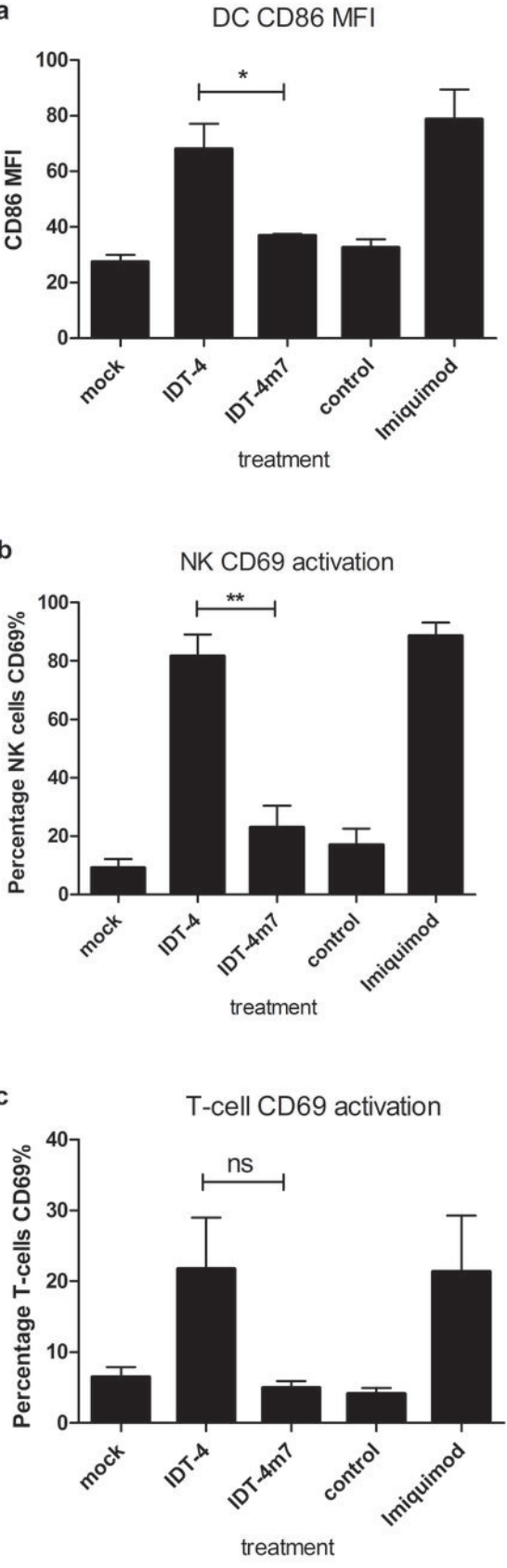

a
DC CD86 MFI
CD86 MFI
100
80
60
40
20
0
*
mock
IDT-4
IDT-4m7
control
Imiquimod
treatment
b
NK CD69 activation
Percentage NK cells CD69%
100
80
60
40
20
0
**
mock
IDT-4
IDT-4m7
control
Imiquimod
treatment
c
T-cell CD69 activation
Percentage T-cells CD69%
40
30
20
10
0
ns
mock
IDT-4
IDT-4m7
control
Imiquimod
treatment

FIGURE 4a

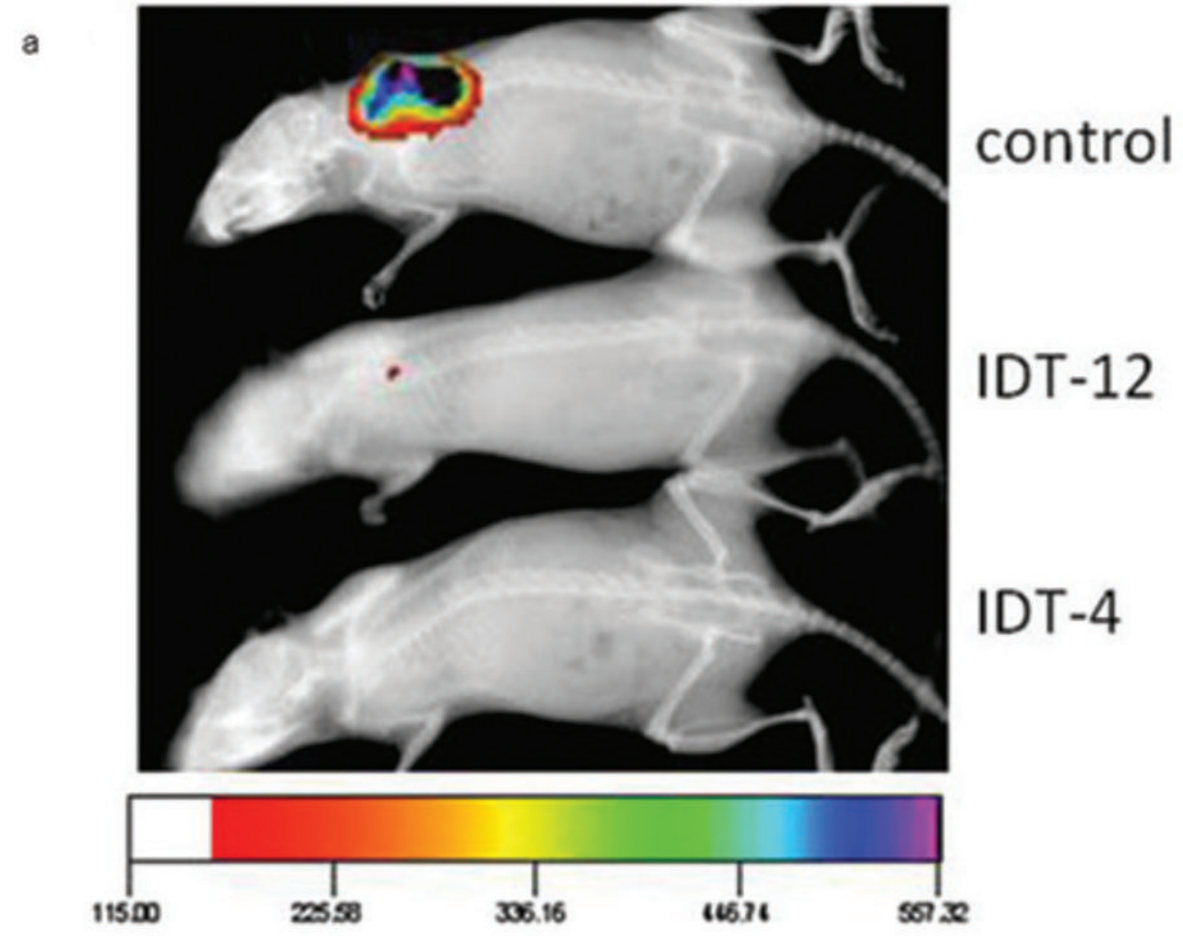


FIGURE 4

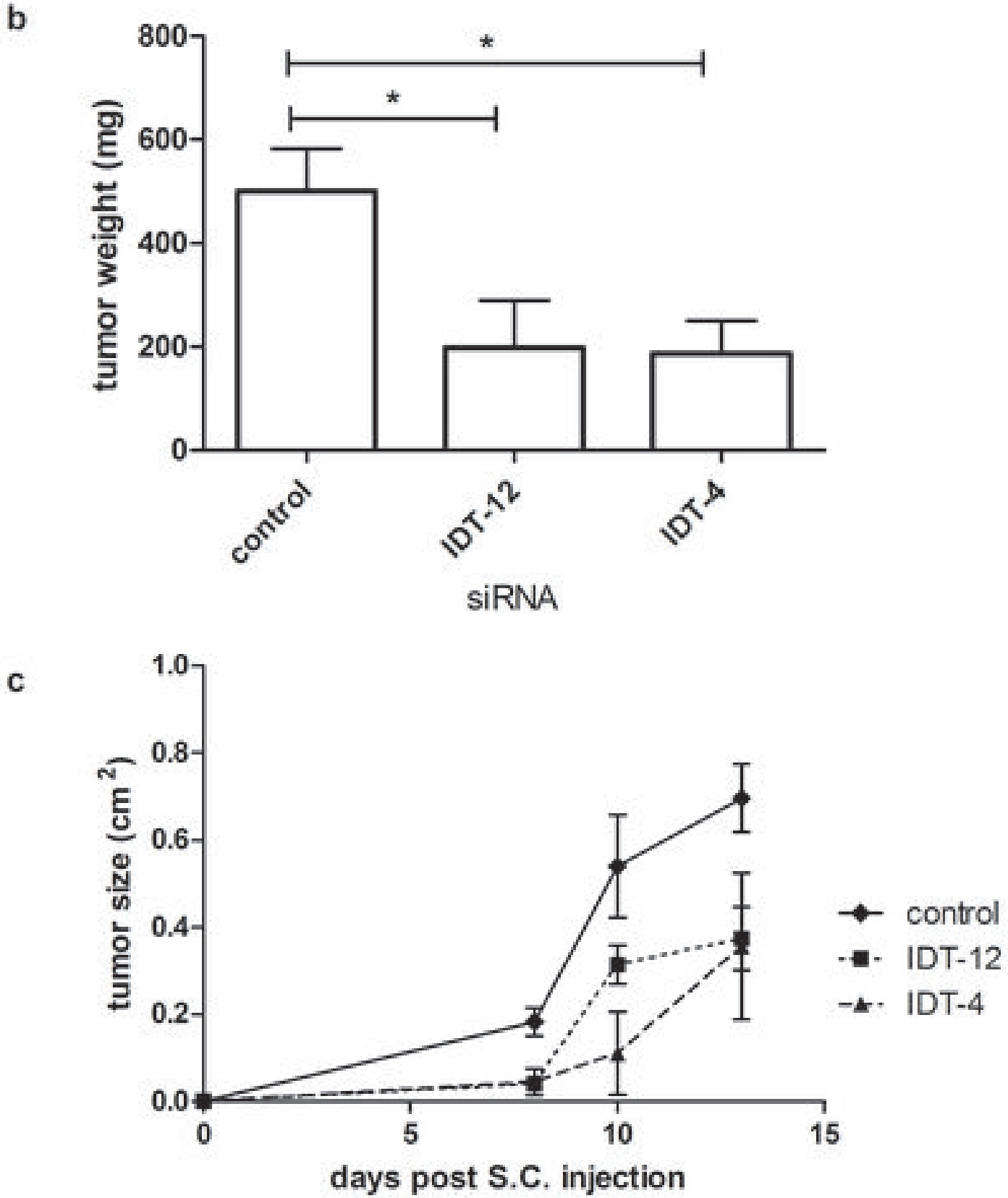
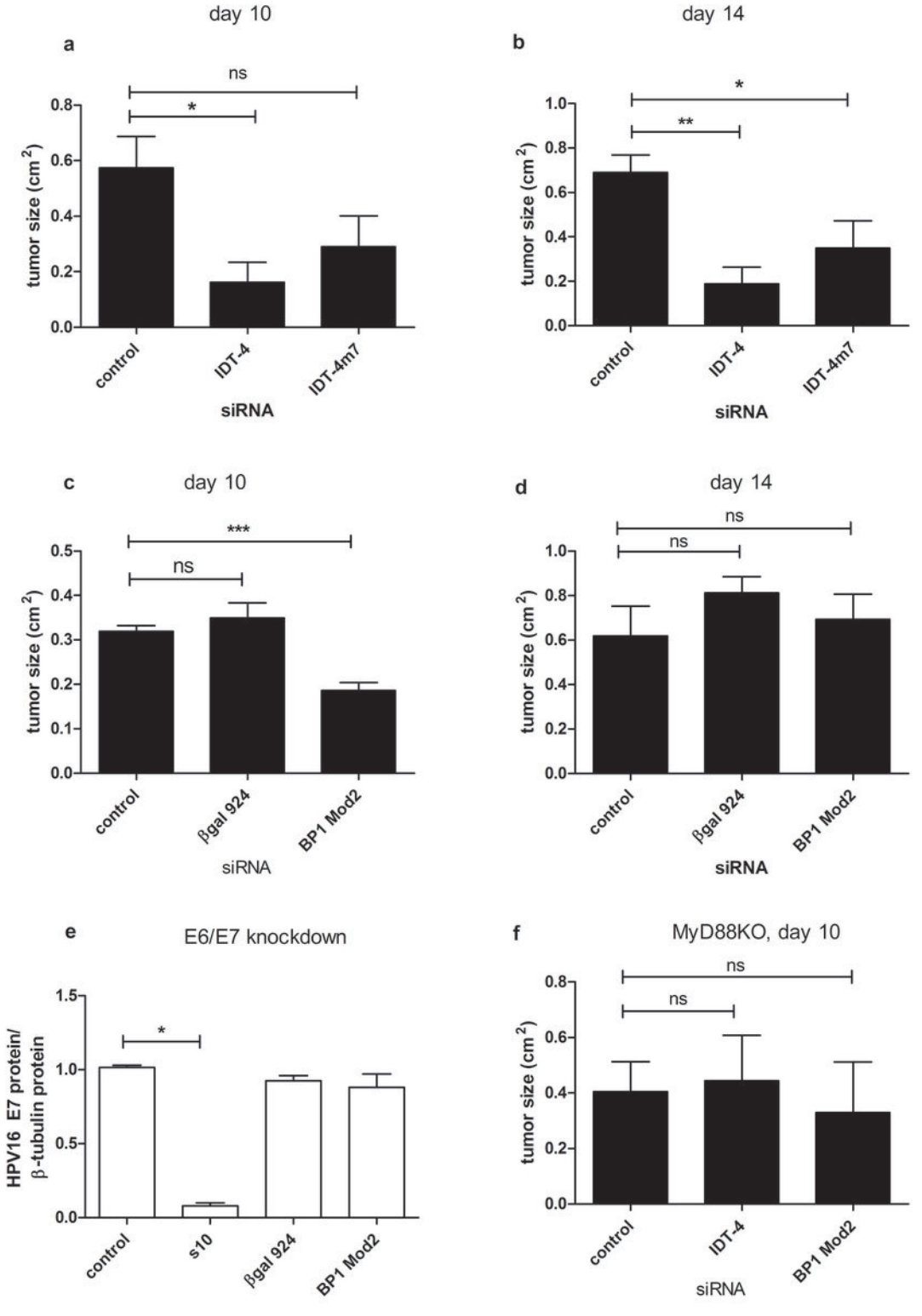

b
800
600
400
200
0
tumor weight (mg)
*
*
control
IDT-12
IDT-4
siRNA
c
1.0
0.8
0.6
0.4
0.2
0.0
tumor size (cm 2)
0
5
10
15
days post S.C. injection
control
IDT-12
IDT-4

FIGURE 5


day 10
a
ns
*
tumor size (cm 2)
0.0
0.2
0.4
0.6
0.8
control
IDT-4
IDT-4m7
siRNA
day 14
b
*
**
tumor size (cm 2)
0.0
0.2
0.4
0.6
0.8
1.0
control
IDT-4
IDT-4m7
siRNA
c
day 10
***
ns
tumor size (cm 2)
0.0
0.1
0.2
0.3
0.4
0.5
control
βgal 924
BP1 Mod2
siRNA
d
day 14
ns
ns
tumor size (cm 2)
0.0
0.2
0.4
0.6
0.8
1.0
control
βgal 924
BP1 Mod2
siRNA
e
E6/E7 knockdown
*
HPV16 E7 protein/
β-tubulin protein
0.0
0.5
1.0
1.5
control
s10
βgal 924
BP1 Mod2
f
MyD88KO, day 10
ns
ns
tumor size (cm 2)
0.0
0.2
0.4
0.6
0.8
control
IDT-4
BP1 Mod2
siRNA