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ORIGINAL ARTICLE

Expression of a soluble IL-10 receptor enhances the therapeutic effects of a papillomavirus-associated antitumor vaccine in a murine model

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Abstract

The presence of IL-10, produced either by tumor cells or immunosuppressive cells, is frequently associated with a poor prognosis for cancer progression. It may also negatively impact anticancer treatments, such as immunotherapies, that otherwise would promote the activation of cytotoxic T cells capable of detecting and destroying malignant cells. In the present study, we evaluated a new adjuvant approach for anticancer immunotherapy using a plasmid vector encoding a soluble form of the IL-10 receptor (pIL-10R). pIL-10R was coadministered to mice with a DNA vaccine encoding the type 16 human papillomavirus (HPV-16) E7 oncoprotein genetically fused with glycoprotein D of herpes simplex virus (HSV) (pgDE7h). Immunization regimens based on the coadministration of pIL-10R and pgDE7h enhanced the antitumor immunity elicited in mice injected with TC-1 cells, which express HPV-16 oncoproteins. The administration of the DNA vaccines by in vivo electroporation further enhanced the anticancer effects of the vaccines, leading to the activation of tumor-infiltrating polyfunctional E7-specific cytotoxic $CD8^+$ T cells and control of the expansion of immunosuppressive cells. In addition, the combination of immunotherapy and pIL-10R allowed the control of tumors in more advanced growth stages that otherwise would not be treatable by the pgDE7h vaccine. In conclusion, the proposed treatment involving the expression of IL-10R enhanced the antitumor protective immunity induced by pgDE7h administration and may contribute to the development of more efficient clinical interventions against HPV-induced tumors.

Keywords Cancer · DNA vaccine · IL-10 · gDE7 · TC-1 cells · Cancer immunotherapy

Abbreviations

ATCC	American Type Culture Collection
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA	Ethics Committee on the Use of Animals in Experimentation
c-Ha-*Ras*	v-Ha-*ras* Harvey rat sarcoma viral oncogene homolog
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CONCEA	National Council for Control of Animal Experimentation
E6	Early protein 6
E7	Early protein 7
EP	Electroporation
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FSC	Forward scatter
gD	Glycoprotein D
ICGEB	International Centre for Genetic Engineering and Biotechnology
mAb	Monoclonal antibodies
PBMC	Peripheral blood mononuclear cell

Parts of the present study have been presented as a poster presentation at the 3rd International Centre for Genetic Engineering and Biotechnology (ICGEB) workshop: Human papillomavirus and associated malignancies: biology, prevention and therapy. The poster was entitled "*In vivo* neutralization of IL-10 enhances the antitumor effects of a therapeutic vaccine to HPV-associated tumor", and the meeting was held in São Paulo, Brazil, on September 2nd, 2017.

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pgDE7h	Vaccine plasmid composed of gD fused to HPV-16 E7
pIL-10R	Interleukin-10 receptor plasmid
SSC	Side scatter

Introduction

Cancer immunotherapies aim to boost the ability of the immune system to recognize and eliminate tumor cells. However, the therapeutic efficacy of such procedures may be compromised by tumor-induced immune escape mechanisms that inhibit the cytotoxic responses capable of eliminating cancerous cells [1]. Interleukin IL-10 (IL-10) has emerged as an important mediator of tumor-associated immune evasion mechanisms. IL-10 levels are elevated in the context of human papillomavirus (HPV) infection in both vaginal secretions from non-altered cervix and premalignant lesions of the cervix, and the IL-10 levels show a positive correlation with the grade of the lesion [2, 3]. IL-10 plays an important role in inhibiting antiviral responses, which favors the progression of premalignant lesions resulting in a predisposition for the development of uterine carcinoma [4, 5]. In addition, similar to other cancer types, HPV-positive cervical cancer, oral squamous cell carcinoma and head and neck cancer frequently show enhanced IL-10 expression, which is considered a prognostic indicator of poor clinical outcome [3, 6–8].

IL-10 contributes to the induction of immune tolerance, causing reduced expression of costimulatory and major histocompatibility complex class II (MHC-II) molecules in dendritic cells (DCs) and, therefore, inhibits DC differentiation and maturation; IL-10 also compromises DC migration to secondary lymphoid organs [9–11]. Increased levels of IL-10 in cancer patients correlate with the downregulation of MHC-II expression in cancer cells and the inhibition of T lymphocyte-mediated cytotoxicity [12]. Additionally, IL-10 promotes the expansion of systemic and tumor-infiltrating regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs), which creates a favorable environment for tumor progression and reduces local antigen-specific $CD8^+$ T cell responses even in patients with detectable cellular immune responses [13, 14]. Therefore, the control of IL-10 levels represents a key step in tumor control, especially in advanced disease stages.

Previous evidence has demonstrated that silencing the gene encoding the IL-10 receptor (IL-10R) in DCs increases their antigen presentation and expression of costimulatory molecules, leading to enhanced tumor growth control in HPV-16 E6 and E7-expressing tumor cells (TC-1 cells) [15]. In addition, the combined silencing of IL-10 and TGF-β receptors in DCs significantly increases the number of activated E7-specific $CD8^+$ T lymphocytes and enhances protective antitumor immunity in mice [16]. Another experimental approach developed to reduce the in vivo levels of IL-10 was based on the administration of a plasmid encoding the soluble extracellular region of the IL-10 receptor (pIL-10R) [17]. Such an approach led to increased survival and activation of proinflammatory responses in a murine melanoma model, but the strategy has not been tested as an adjuvant with other anticancer therapeutic interventions.

Our group has developed an immunotherapy against HPV-associated tumors based on immunization, either with a DNA vaccine or purified recombinant protein, with a chimeric protein derived from the fusion of the HPV-16 E7 oncoprotein with the HSV-1 glycoprotein D (gD) (pgDE7h) [18, 19]. The vaccines proved to be capable of activating E7-specific $CD8^+$ T cell responses in mice and conferred therapeutic protection in mice grafted with tumor cells expressing HPV-16 oncoproteins [18]. However, this immunotherapeutic approach loses efficacy when it is applied to animals with tumors at more advanced stages. In light of this evidence, this study sought to evaluate the adjuvant effects of IL-10R expression on the immune responses mediated by pgDE7h. Our results demonstrate that the coadministration of pIL-10R and pgDE7h increased the activation and tumor infiltration of the E7-specific $CD8^+$ T lymphocytes induced by immunotherapy and therefore generated more efficient control of tumors at more advanced stages.

Materials and methods

Plasmids

The pgDE7h vector contains a codon-optimized sequence encoding the gDE7 hybrid protein cloned into pUMVC3 (Aldevron, USA) [20]. pIL-10R encodes the soluble extracellular portion of the murine IL-10 receptor corresponding to amino acids 1–189 cloned into the *NotI* site of pcDNA3 (Invitrogen, USA) [17]. Plasmid purification was performed using a Giga Prep plasmid purification kit (QIAGEN, USA) according to the manufacturer's instructions.

TC-1 cells

The TC-1 cell line is derived from primary lung epithelium cells of C57BL/6 mice that were transformed with c-Ha-*ras*- and HPV-16 E6- and E7-encoding genes [21]. TC-1 cells were cultured in RPMI (Gibco, USA) medium supplemented with 50 U/ml penicillin/streptomycin, 0.4 mg/ml G418, 10% fetal bovine serum (FBS) (Vitrocell, Campinas, São Paulo, Brazil) at 37 °C and 5% CO_2. On the day of tumor grafting, the cells were treated with trypsin–EDTA (Vitrocell, São Paulo, Brazil) and suspended in serum-free RPMI medium at the proper concentrations for inoculation.

Tumor challenge

Six–eight-week-old C57BL/6 mice were transplanted with 7.5×10^4 TC-1 cells in 100 µl of RPMI medium by subcutaneous injection in the right dorsolateral region. Tumor growth was monitored 2–3 times a week with calipers, and survival was recorded for at least 60 days. The smaller (d) and larger (D) diameters of the tumors were measured, and the tumor volume was calculated according to the formula $D \times d^2/2$. Mice with tumors of approximately 1 mm^3 were considered tumor-bearing mice, and mice with tumors greater than 700 mm^3 were euthanized.

Immunization protocols

Mice were immunized by injection of one dose of pgDE7h (5 or 50 µg) by the intramuscular route (i.m.) in the tibialis anterior muscle or by electroporation (EP) 5 or 14 days after tumor grafting. For EP, a total of six 130 V 450 ms electrical pulses were applied using a NEPA21 Super Electroporator (NepaGeneCo., Ltd., Chiba, Japan). In the combined immunization regimen, mice received one or two doses of pIL-10R (50 µg) administered 5 days apart either by injection or EP. The first dose was coadministered with pgDE7h, and the second dose was delivered alone.

Intracellular cytokine staining (ICS) and MDSC detection

Peripheral blood mononuclear cell (PBMC) or spleen samples were collected 14 days after pgDE7h administration and incubated at a concentration of 10^6 cells per well for 12 h at 37 °C in a 5% CO_2 atmosphere, in the presence of Brefeldin A (5 µg/ml) (GolgiPlug; BD Biosciences, USA) and IL-2 (5 ng/ml) and with or without MHC class I-specific E7-derived peptide ($_{49}$RAHYNIVTF$_{57}$) at a final concentration of 300 ng/well. In some experiments, 5 µg/ml anti-CD107a-FITC antibody (BD Biosciences, USA), 10 µg/ml monensin (GolgiStop; BD Biosciences, USA) and 2 µg/ml anti-CD28 antibody (BD Biosciences, USA) were added during the overnight stimulation. The cells were labeled with anti-CD8α-BV605 or -BB515-conjugated antibodies (BD Bioscience, USA). After fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences, USA), the samples were intracellularly stained with anti-IFN-γ-PE or anti-IFN-γ-Alexa Fluor 700 and anti-TNF-α-PE-Cy7 antibodies (BD Bioscience, USA). MDSCs were tracked using anti-CD11b-FITC and anti-Gr1-PE antibodies (BD Bioscience). The samples were analyzed on an LSRFortessa® flow cytometer (BD Bioscience, San Jose, CA, USA), and the data were analyzed with FlowJo software. The analyses were performed after the initial exclusion of doublets using the FSC and SSC parameters. Subsequently, the percentages of $CD8^+$ IFN-γ^+, $CD8^+$ TNF-α^+ and $CD8^+$ $CD107a^+$ cells in the total $CD8^+$ T cell population were determined. MDSCs were characterized as $CD11b^{high}$ GR-1^{int}, $CD11b^{high}$ $Gr1^{high}$ or total $CD11b^+$ Gr-1^+.

Detection of tumor-infiltrating lymphocytes

Tumor samples were collected 28 days after tumor transplantation and digested with collagenase D (1 mg/mL) for 1 h at 37 °C and macerated to obtain a cell suspension. The samples were labeled with an APC-conjugated MHC class I dextramer carrying the E7-specific peptide (Immudex, USA) and surface stained with anti-CD8α-BB515 (BD Bioscience) and anti-CD45-PerCP Cy5.5 antibodies (BD Bioscience, USA). For some experiments, cells were fixed, permeabilized (Cytofix/Cytoperm; BD Biosciences, USA), and stained with anti-IFN-γ-PE monoclonal antibodies (mAb) (#554,412, BD Bioscience, USA) after stimulation overnight with IL-2 (5 ng/ml) in the presence of $_{49}$RAHYNIVTF$_{57}$ peptide (300 ng/well). Samples were analyzed by flow cytometry using an LSRFortessa™ (BD Bioscience San Jose, CA, USA). Doublets were excluded using the FSC and SSC parameters, and $CD45^+$ cells were subsequently analyzed to obtain the percentages of $CD8^+$ IFN-γ^+ or $CD8^+$ dextramer E7-specific$^+$ cells with the total $CD8^+$ T cell population.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). One- or two-way ANOVA was used with the Bonferroni multiple comparison test or Student's t test was used for comparisons of unpaired samples. The log-rank test followed by the Mantel–Cox posttest was used for the survival analysis. $p < 0.05$ was considered statistically significant with a confidence interval of 95%.

Results

pIL-10R administration enhances the antitumor effects of pgDE7h

First, we determined whether the administration of pIL-10R would impact the therapeutic antitumor effects of pgDE7h. To address this question, mice were immunized with one dose of pgDE7h (50 µg) or underwent a combined immunization regimen consisting of two doses of pIL-10R (50 µg for each injection) injected 5 days apart by the intramuscular route (i.m.) starting five days after the TC-1 cell graft, and one dose of pgDE7h delivered at the same time as the first dose of pIL-10R (Fig. 1a). Mice immunized with

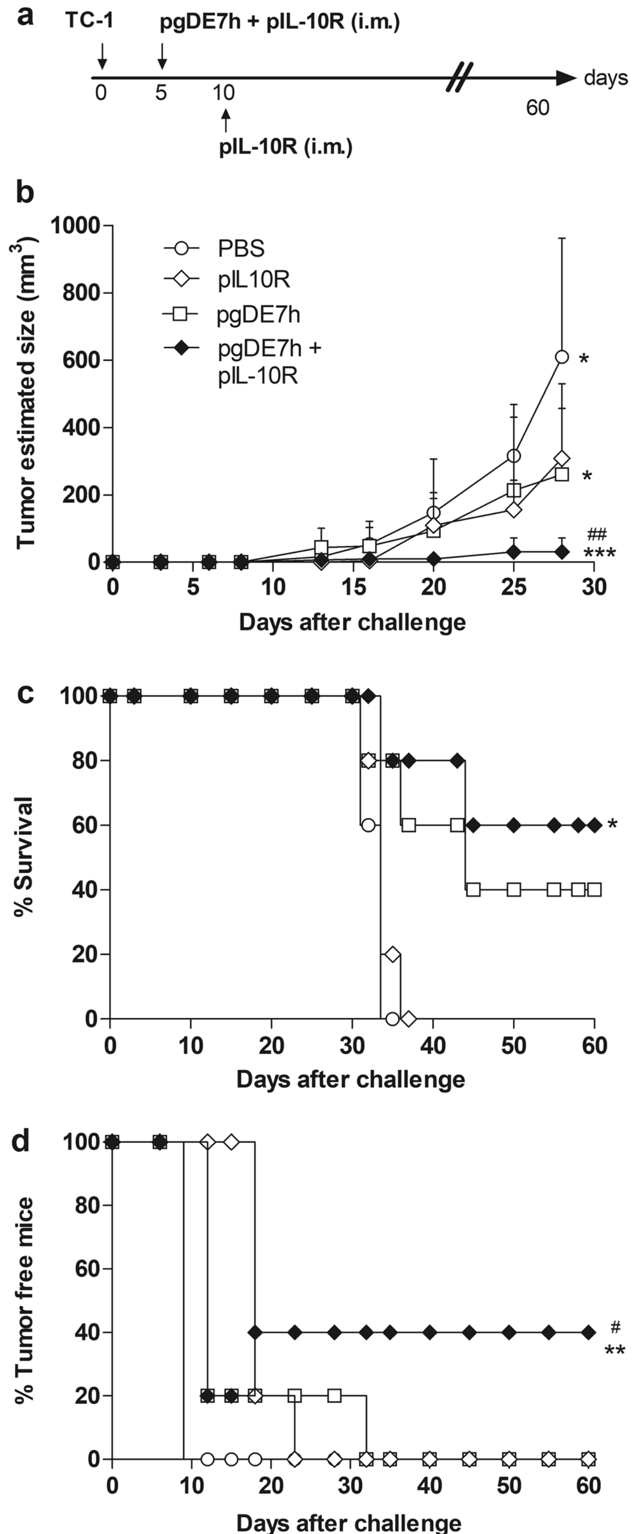


Fig. 1 Administration of pIL-10R exhibits adjuvant effects on the tumor protection elicited by the pgDE7h vaccine. Mice were grafted with TC-1 tumor cells and i.m. immunized with one dose of pgDE7h 5 days later with or without two 50 µg doses of pIL-10R administered 5 days apart. Tumor growth was monitored 2–3 times per week for a period of 60 days. **a** Schematic immunization schedule. **b** Estimated tumor size over time (two-way ANOVA). **c** Percentage of surviving or **d** tumor-free mice over time (log-rank–Mantel–Cox). Data from 3 independent experiments ($n=10$–15) (*) vs PBS, (#) vs pgDE7h. *$p<0.05$, **$p<0.01$, ***$p<0.001$

pgDE7h showed delayed tumor growth compared with the sham-treated mice (PBS), and 40% of the vaccinated mice survived up to 2 months after the challenge, but all mice developed tumors (Fig. 1b–d). Mice immunized with two doses of pIL-10R showed reduced tumor size in comparison with the sham-treated mouse group (PBS), but by day 40 after challenge, all mice had to be euthanized due to the progression of the tumors (Fig. 1b, c). In contrast, mice immunized with pIL-10R combined with pgDE7h showed a significant decrease in tumor growth compared with the other mouse groups, and 60% of them survived until the end of the follow-up period (Fig. 1b, c). Tumor-free mice were only observed in the group that received the combined immunization regimen (pgDE7h plus pIL-10R) (Fig. 1d). These results indicate that the administration of pIL-10R enhanced the therapeutic antitumor effects of pgDE7h.

EP administration enhances the antitumor effects of pgDE7h and pIL-10R and halts the expansion of immunosuppressive cells

In an attempt to increase the potency of the combined immunotherapeutic treatment, we delivered pgDE7h and pIL-10R to mice by in vivo EP. For this purpose, mice were injected with TC-1 cells and immunized with 5 µg of pgDE7h admixed with or without 50 µg of pIL-10R in a single dose regimen (Fig. 2a). As shown in Fig. 2b, mice immunized with pgDE7h showed partial tumor growth control and 80% survival up to 60 days after tumor challenge. However, mice immunized with pgDE7h and pIL-10R showed more pronounced tumor growth control and 100% survival (Fig. 2b, c). More importantly, complete tumor protection was observed in 90% of the mice vaccinated with pgDE7h/pIL-10R but only in 50% of the mice vaccinated with pgDE7h (Fig. 2c).

In a second step, we aimed to evaluate the impact of the treatment in mice with more advanced tumors. The vaccine regimen was started 14 days after the tumor grafting, a point at which the tumors were palpable (1–2 mm^2). Mice were immunized with a single dose of pgDE7h (5 µg) admixed with pIL-10R (50 µg) and followed by a second dose of pIL-10R administered 5 days later; both doses were delivered by EP (Fig. 3a). The combined therapy led to efficient tumor control, and 100% of the animals survived the challenge at the end of the observation period (60% tumor-free mice, Supplementary Fig. 1d) (Fig. 3b, c). Mice immunized only with pgDE7h showed delayed tumor development, and 80% of them were still alive at the end of the observation period

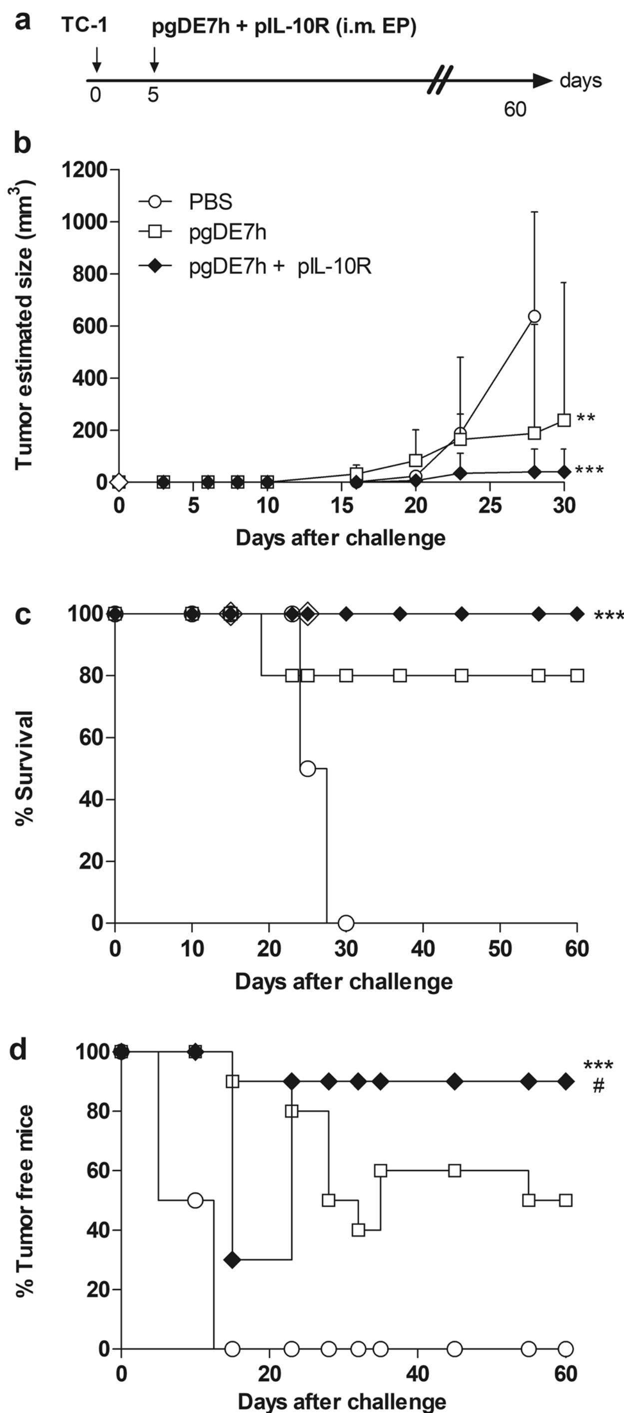


Fig. 2 EP potentiates the effect of the combined immunotherapy with the pgDE7h vaccine and pIL-10R. Mice were injected with TC-1 tumor cells and immunized 5 days later using in vivo EP with one 5 µg dose of pgDE7h with or without one 50 µg dose of pIL-10R. Tumor growth was monitored 2–3 times per week for a period of 60 days. **a** Schematic immunization schedule. **b** Estimated tumor size over time (two-way ANOVA). **c** Percentage of surviving or **d** tumor-free mice over time (log-rank–Mantel–Cox). Data from 3 independent experiments ($n=10$) (*) vs PBS, (#) vs pgDE7h. *$p<0.05$, **$p<0.01$, ***$p<0.001$

(30% tumor-free, data not shown) (Fig. 3b, c). Immunization with pIL-10R alone was less efficient in tumor growth control, and mice treated with this approach showed slightly augmented survival compared to PBS-immunized mice (Fig. 3b, c). We also investigated whether a third dose of pIL-10R, administered 5 days after the second dose, would further improve the antitumor therapeutic protection. Mice that received 2 or 3 doses of pIL-10R showed no significant differences in their control of tumor growth or survival (Supplementary Fig. 1). By the end of the follow-up period, the tumors and spleens of mice given the combined immunization regimen were smaller than those from mice immunized with pgDE7h (Fig. 3d). The enlarged spleens in mice vaccinated with pgDE7h reflected the accumulation of MDSCs, as previously shown [22]. In accordance with the tumor protection results, mice immunized with pgDE7h and pIL-10R controlled the expansion of immunosuppressive cells. Administration of the combined immunotherapy led to a significant decrease in the frequency of the total $CD11b^+$ $GR\text{-}1^+$ and $CD11b^+$ $GR\text{-}1^{high}$ cell populations in the spleens from vaccinated mice compared with those from mice immunized with pgDE7h (Fig. 3e). Taken together, these data indicate that immunization with pIL-10R delivered by EP enhances the therapeutic antitumor effects of pgDE7h, even in mice with pre-established tumors, and halts the expansion of MDSCs triggered by tumor progression.

Administration of pIL-10R promotes activation of polyfunctional tumor-infiltrating $CD8^+$ T lymphocytes

Following the conditions described above, we next evaluated the induction of antigen-specific $CD8^+$ T cell responses in mice given the pgDE7h/pIL-10R treatment by EP. As shown in Fig. 4a, b, a higher frequency of E7-specific IFN-γ-producing $CD8^+$ T cells was detected in the blood of the mice given the combined therapy compared with that of mice given the sham treatment (PBS). Comparable results were observed in the spleens collected from the mice given the other immunization therapies. Notably, administration of pgDE7h/pIL-10R enhanced CD107a expression concomitantly with enhanced IFN-γ and TNF-α expression compared to the administration of pgDE7h (Fig. 4c). Furthermore, the combination of pgDE7h and pIL-10R enhanced the numbers of splenic IFN-γ/TNF-α- or IFN-γ/CD107a-producing $CD8^+$ T cells compared with pgDE7h alone (Fig. 4c). When single cytokine production by $CD8^+$ T lymphocytes was analyzed, we observed that the numbers of IFN-γ producing cells were increased in mice immunized with pgDE7h/pIL-10R compared with sham-treated mice (PBS), but the numbers of TNF-α producing cells were not increased and were instead similar in the two groups (Fig. 4c–e).

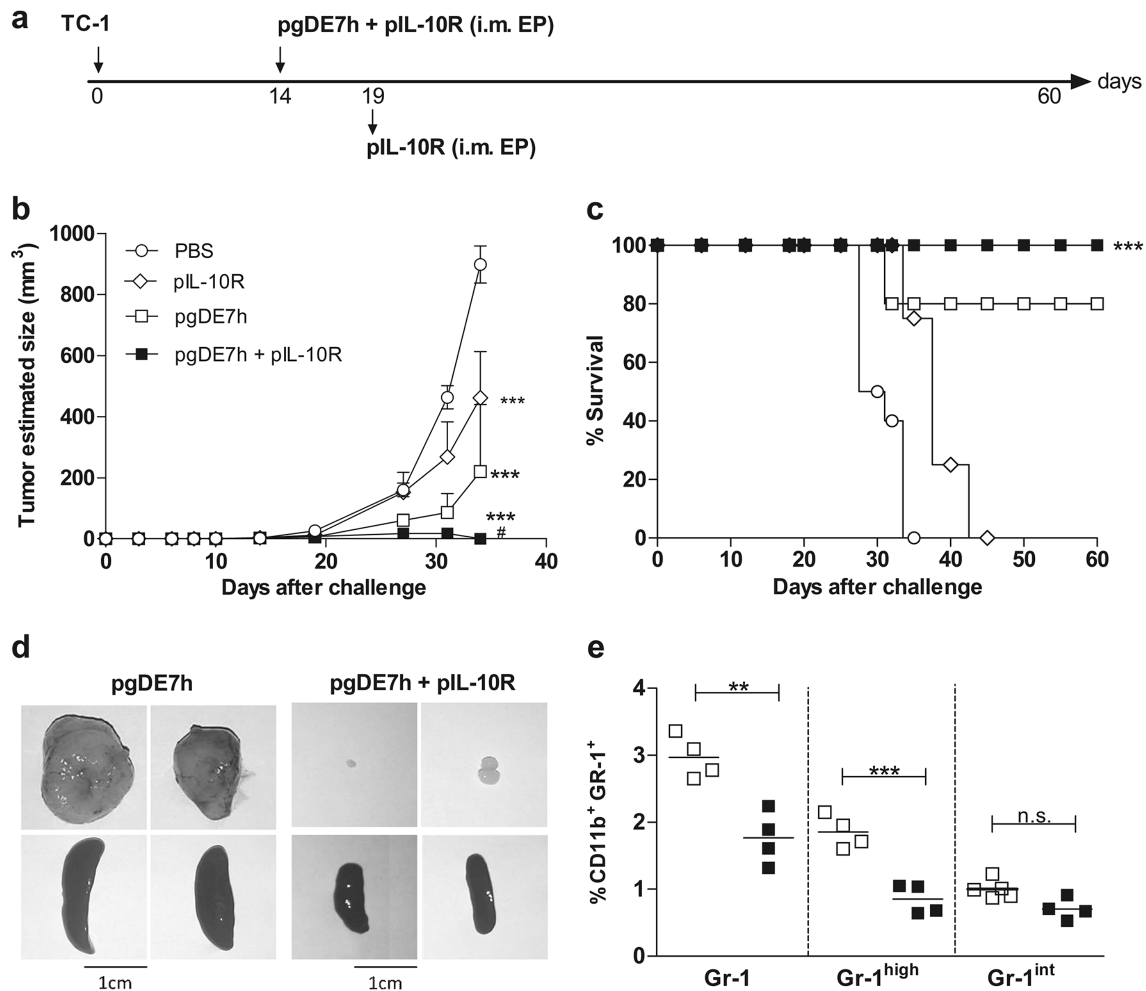


Fig. 3 Combination of pgDE7h and pIL-10R delivered by EP induces the regression of pre-established tumors and halts the accumulation of immunosuppressive cells. Mice were injected with TC-1 tumor cells and immunized 14 days later by in vivo EP with one 5 µg dose of pgDE7h with or without two 50 µg doses of pIL-10R administered 5 days apart. Tumor growth was monitored 2–3 times per week for a period of 60 days. **a** Schematic immunization schedule. **b** Estimated tumor size over time (two-way ANOVA). **c** Percentage of surviving mice over time (log-rank–Mantel–Cox). Data from 3 independent experiments ($n=10$–15). **d** Representative pictures of tumors (top row) and spleens (lower row) harvested 28 days after the challenge from mice treated with the pgDE7h vaccine alone or in combination with pIL-10R. **e** Frequencies of total CD11b$^+$ Gr-1$^+$ or CD11b$^+$ Gr-1^{high} and CD11b$^+$ Gr-1^{int} subsets of MDSCs in the spleen were determined by flow cytometry (Student's *t* test) ($n=6$). (*) vs PBS, (#) vs pgDE7h. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *ns* not significant

Finally, we determined the presence of tumor-infiltrating E7-specific CD8$^+$ T cells in mice given the combined immunization regimen. As indicated in Fig. 5a, compared with PBS-treated mice, mice immunized with pgDE7h and pIL-10R showed a significant increase in the influx of total and E7-specific CD8$^+$ T cells to the tumor (Fig. 5a, b), and these cells were not detected in PBS- or pIL-10R-immunized mice (data not shown). In addition, approximately 15% of the total tumor-infiltrating CD8$^+$ T cells were capable of producing IFN-γ upon ex vivo peptide stimulation (Fig. 5c, d). Collectively, these results indicate that the administration of pgDE7h/pIL-10R enhances the frequency and activation of systemic and tumor-infiltrating E7-specific CD8$^+$ T cells, leading to more efficient control of tumor cell growth under experimental conditions.

Discussion

In clinical trials, immunotherapies have been shown to have therapeutic efficacy that supports the regression of precancerous HPV-associated lesions, but in advanced stages, the clinical efficacy still needs improvement. Targeting cancer-induced immunosuppressive mechanisms in parallel with the induction of immune responses to tumor antigens can provide a more appropriate environment for T cell priming

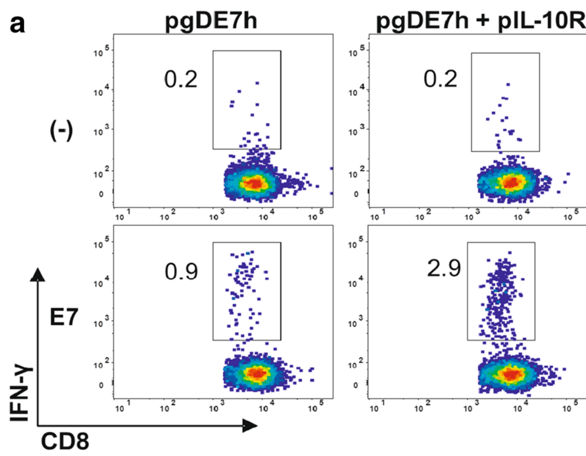
and functioning. In light of this, in the present report, we described a novel experimental approach based on the coadministration of a DNA antitumor vaccine (pgDE7h) and a plasmid vector encoding the IL-10 receptor (pIL-10R) as a strategy to halt tumor-induced immunosuppressive mechanisms and, therefore, act as an adjuvant for antigen-specific immunotherapy. Our results demonstrated that the combination of pIL-10R with pgDE7h increased the activation of systemic and tumor-infiltrating E7-specific T lymphocytes and most importantly, the antitumoral therapeutic effects elicited by the DNA vaccine pgDE7h and thus may contribute to the development of more efficient anticancer immunotherapies.

Cytokines, such as IL-10, can be neutralized in vivo using monoclonal antibodies, interfering RNAs or plasmids expressing the corresponding cytokine receptor. In the present study, the decrease in circulating IL-10 levels caused by the injection of the plasmid encoding the soluble fraction of the IL-10 receptor in conjunction with pgDE7h immunization generated robust therapeutic antitumor effects in mice grafted with TC-1 tumor cells. In a melanoma mouse model, the administration of pIL-10R to tumor-bearing mice neutralized the IL-10 expression induced by the tumor and promoted more efficient activation of dendritic cells able to capture tumor antigens and trigger an antitumor inflammatory response [17]. Plasmid-based therapy offers some advantages, as DNA vaccines are considered safe, stable, relatively inexpensive and feasible for use in humans, particularly when administered by in vivo electroporation [23]. Furthermore, repeated administrations of anti-IL-10 neutralizing antibodies may have side effects, such as chronic gastrointestinal inflammation [24]. Other therapeutic strategies have been shown that blocking IL-10R can increase the potency of antitumoral effects. IL-10R gene silencing in dendritic cells has improved the cell functions related to the presentation of HPV-16 antigens, increased the expression of costimulatory molecules and controlled tumor growth in mice challenged with TC-1 cells [15]. Another study combining IL-10R and TGF-β-receptor silencing in dendritic cells demonstrated the presence of increased numbers of E7-specific $CD8^+$ T lymphocytes and more pronounced antitumor effects [16]. Our results add another piece of information about the beneficial aspects of the reduction of IL-10 levels in the therapeutic control of tumors and describe a new feasible experimental approach that can induce similar IL-10-reducing effects and improve the potency of anticancer vaccines.

EP further improved the therapeutic protection elicited in mice immunized with pgDE7h/pIL-10R, as seen by the induction of a robust cellular antigen-specific response. These data suggest that EP can increase the expression of pIL-10R and the duration of the induced antitumor immunity. The use of EP for DNA vaccine delivery is known to increase the number of transfected cells and extend the antigen expression time, therefore enabling a stronger activation of protective immune responses [25]. Previous studies by our group have shown that under experimental conditions, EP promotes activation of antigen-specific $CD8^+$ T cells and protection against HPV-associated tumors in mice [26]. Notably, the phase 2/3 clinical trial with the VGX-3100 DNA vaccine delivered by EP demonstrated that the immunization procedure is well tolerated by patients and permits the control of high-grade lesions in patients with cervical intraepithelial neoplasia [27]. This evidence supports the use of EP for the delivery of DNA vaccines and may contribute to the development of active immunotherapeutic strategies capable of controlling the growth of tumors in more advanced stages.

In the current study, coadministration of pIL-10R and pgDE7h followed by EP increased the magnitude of the activated $CD8^+$ T cell responses and the number of cells simultaneously expressing IFN-γ, TNF-α and CD107a. The induction of polyfunctional $CD8^+$ T cells is related to the control of HPV-associated cancer progression in murine models and in patients [28–30]. Accordingly, it has been suggested that the development of this type of cell correlates positively with the clinical efficacy of therapeutic vaccines against high-grade cervical lesions [31]. Other groups have shown that blocking IL-10/IL-10R signaling during antigen presentation favors the induction of immune responses with stronger $CD8^+$ T cell responses and increased efficacy against tumors [15]. However, there is clinical evidence that IL-10 has a dual role in cancer therapy, as IL-10 can promote the enhancement of antigen-specific $CD8^+$ cytotoxic T lymphocyte responses and expression of Th1 cytokines and can activate tumor-resident $CD8^+$ T cells [32–36]. Our results provide experimental evidence that the neutralization of IL-10 by the expression of IL-10R improves the activation of polyfunctional $CD8^+$ T lymphocytes in the presence of a vaccine vector capable of inducing a protective immune response. Thus, the conflicting results regarding the therapeutic effects of IL-10 may be, at least in part, explained by the specific features of the experimental model used in the present study. The coadministration of pIL-10R and pgDE7h clearly promoted an adjuvant effect, particularly in situations in which the tumors had reached larger sizes that downregulated the activation of the effector immune responses capable of recognizing and destroying the tumor cells. The main question to be answered in the future will be the fate of this new adjuvant treatment under clinical conditions.

In our experimental conditions, the expression of pIL-10R led to the enhanced migration of activated E7-specific $CD8^+$ T cells into the tumor mass. Importantly, our findings suggest that the cells induced in this specific condition and present in the tumor microenvironment are not anergic and may contribute to tumor control. Administration of an anti-IL-10R mAb associated with immunization with E7-derived long peptides makes the tumor microenvironment more accessible for $CD8^+$ T cell infiltration [37]. Nonetheless, other pathways that do not involve modulation by IL-10 can interfere with the activation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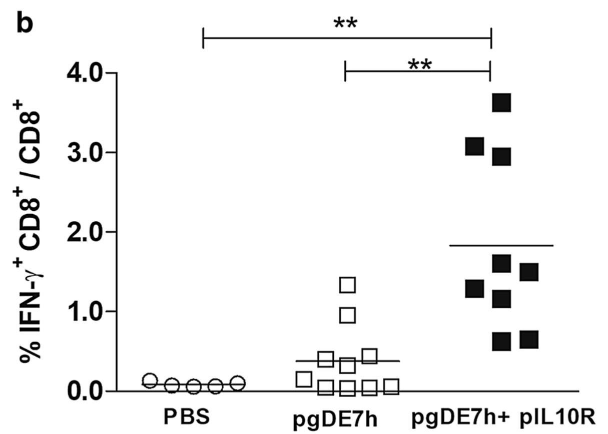
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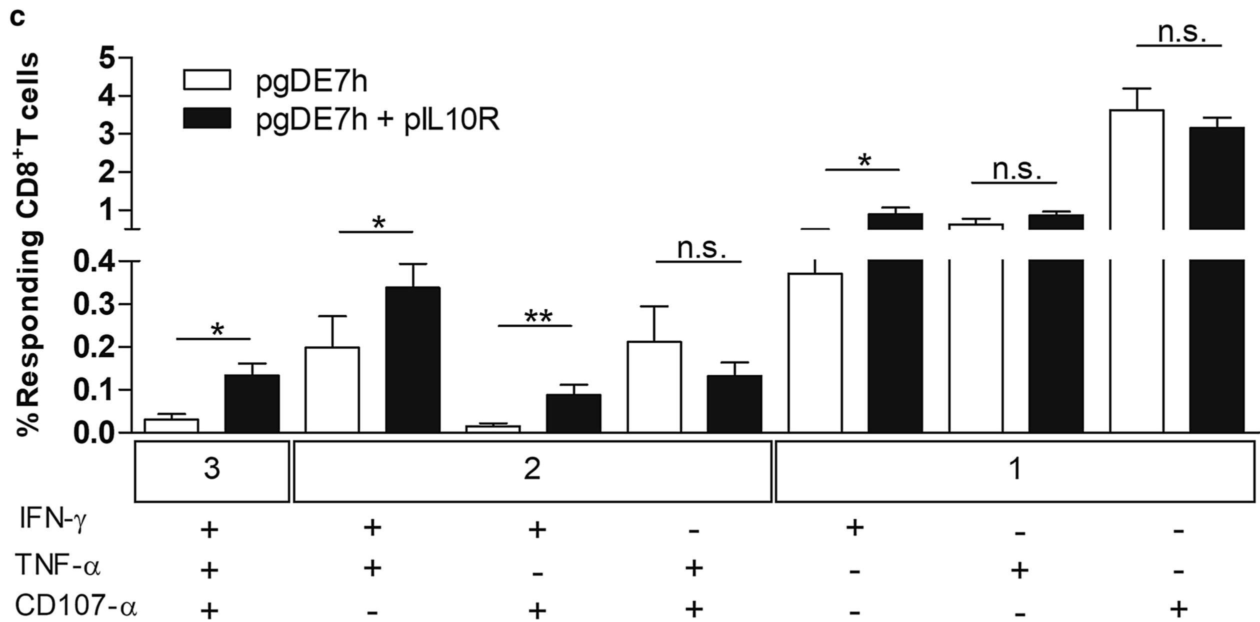
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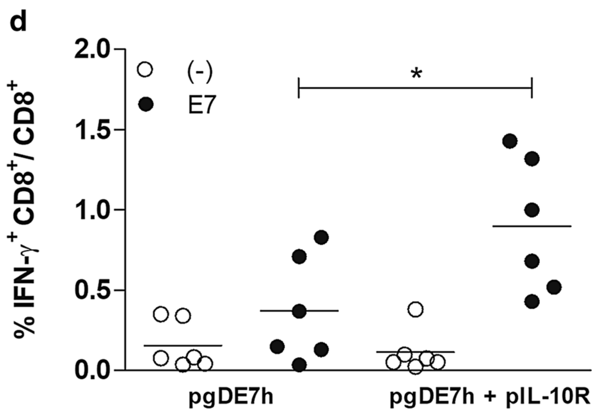
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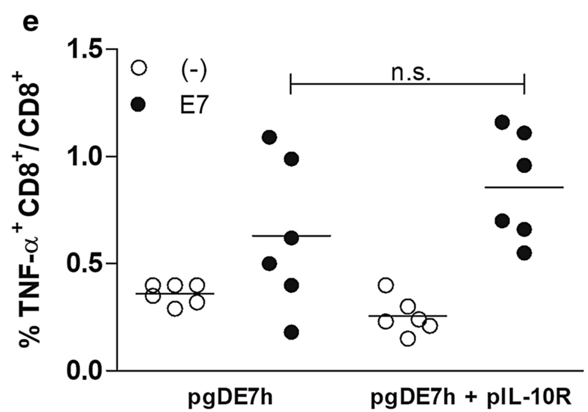
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pgDE7h
pgDE7h + pIL-10R

◂**Fig. 4** Coadministration of pIL-10R with pgDE7h enhances the activation of E7-specific CD8⁺ T cells. Mice were injected with TC-1 tumor cells and immunized 14 days later by in vivo EP with one 5 µg dose of pgDE7h with or without two 50 µg doses of pIL-10R administered 5 days apart by in vivo EP. **a** and **b** Blood samples and splenocytes **c–e** were harvested 28 days after immunization and stimulated overnight with the HPV-16 E7 K^b MHC class I-restricted immunodominant epitope peptide. The frequencies of IFN-γ-producing $CD8^+$ T cells were determined by flow cytometry and are shown as representative dot plots **a** or percentages **b**. Unstimulated (-) IFN-γ-producing $CD8^+$ T cell frequencies were below 0.2%. ($n=5$–10) (ANOVA, posttest: Bonferroni). **c** The percentages of $CD8^+$ T cells expressing IFN- γ, TNF-α and CD107a were determined by flow cytometry. **d** Frequencies of $CD8^+$ T cells producing IFN-γ or **e** TNF-α (Student's *t* test) ($n=6$). $*p<0.05$, $**p<0.01$, *ns* not significant

and migratory behavior of T cells, leading to the blockade of cytotoxic activity at the tumor site. Checkpoint-blocking mAbs, such as those targeting PD-1/PD-L1 and CTLA4, are also alternatives to increase the potency of therapeutic vaccines [38, 39]. Our findings add a new and alternative approach that may impact both the activation of cytotoxic T cells and the immunosuppressive mechanisms triggered by tumor cells.

Together, the data presented in this study demonstrate that pIL-10R has an adjuvant effect when coadministered with the pgDE7h vaccine, improving both the protective antitumor immunity and therapeutic efficacy against HPV-associated tumors. The results represent a clinically translatable therapeutic approach capable of controlling immunological escape mechanisms induced by tumors and improving the mounting of specific cytotoxic responses to HPV-associated malignancies.

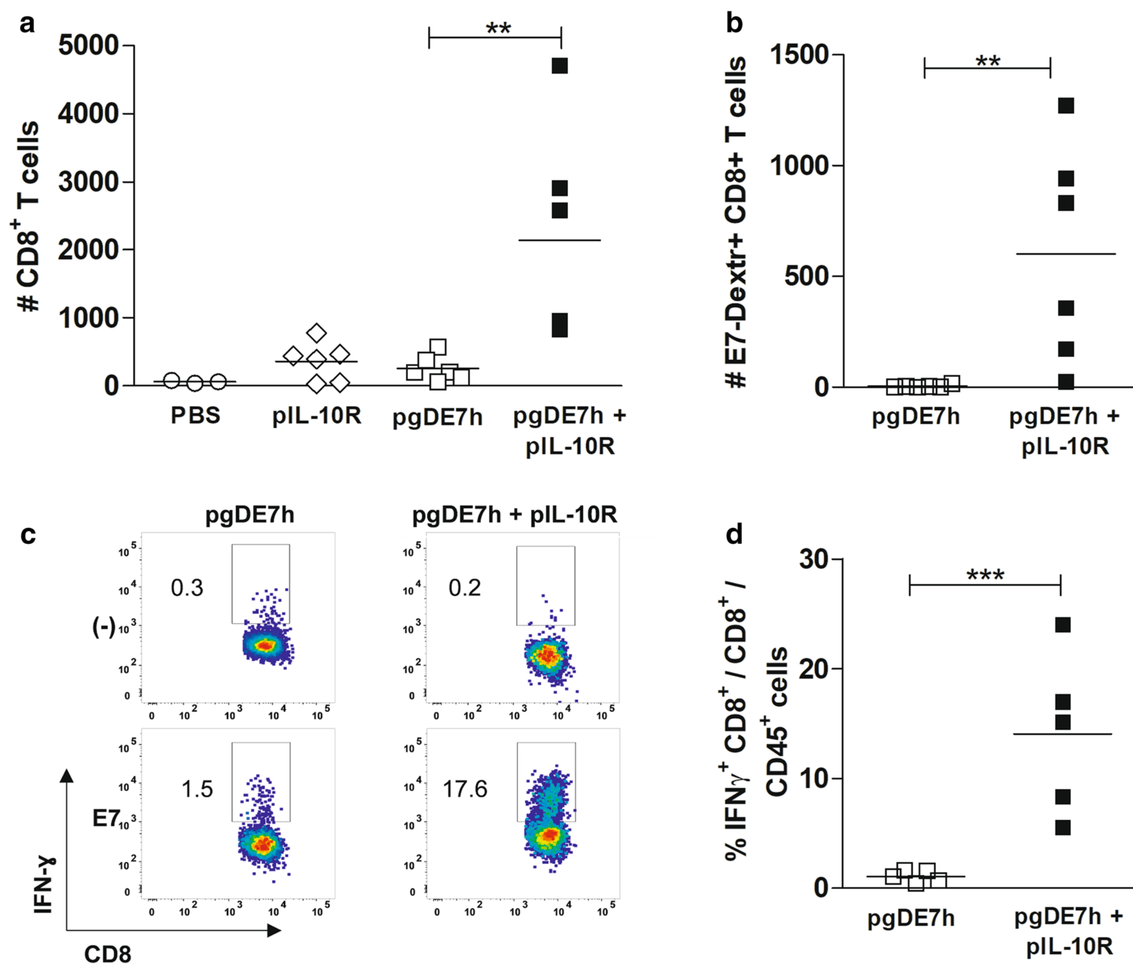

Fig. 5 Combination of pgDE7h and pIL-10R induces the infiltration of antigen-specific $CD8^+$ T lymphocytes into the tumor site. Mice were injected with TC-1 tumor cells and immunized 14 days later by in vivo EP with one 5 µg dose of pgDE7h with or without two 50 µg doses of pIL-10R administered 5 days apart. The tumors were harvested 28 days after the challenge and analyzed by flow cytometry. **a** Number of total or **b** E7-specific $CD8^+$ T cells that infiltrated the tumor site (ANOVA, Bonferroni). **c** Representative dot plots and **d** percentages of IFN-γ-producing $CD8^+$ T cells after overnight stimulation with HPV-16 E7-derived peptide (Student's *t* test). Unstimulated (-) IFN-γ-producing $CD8^+$ T cell frequencies were below 0.3%. $**p<0.01$, $***p<0.001$

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Author contributions JRS, MD and LCSF conceived the study and the experimental design. MOD supervised the experimental work. JRS, NSS, MOS, LRMMA, ACRM, and MOD carried out the experiments, processed the experimental data, and participated in the interpretation of the results. EGR discussed the results and contributed to the writing of the manuscript. JRS wrote the manuscript with support from MOD and LCSF.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All handling procedures were performed according to the protocol approved by the Ethics Committee on the Use of Animals (CEUA) of the Institute of Biomedical Sciences of the University of São Paulo (Brazil) on August 18, 2014 (project number 92/2014) and according to standard rules approved by the National Council for Control of Animal Experimentation (CONCEA), Brazil.

Animal source Six–eight-week-old C57BL/6 mice were purchased from the Animal Breeding Center of the Institute of Biomedical Sciences of the University of São Paulo (Brazil) and housed in the Animal Facility at the same Institute.

Cell line authentication The TC-1 cell line was originally created and kindly provided by Dr. T.C. Wu, Johns Hopkins University, Baltimore, MD, USA. The cells tested negative for mycoplasma by PCR and were cultured for no more than 2 weeks after thawing. The cells showed consistent morphologies and growth rates prior to use in in vivo experiments. The cells tested positive for the expression of the HPV-16 E6 and E7 proteins by Western blotting. The TC-1 cell line is not currently available from the ATCC bank.

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