



# Intratumoral delivery of an HPV vaccine elicits a broad anti-tumor immune response that translates into a potent anti-tumor effect in a preclinical murine HPV model

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## Abstract

Therapeutic cancer vaccines have met limited clinical success. In the setting of cancer, the immune system is either tolerized and/or has a limited tumor-specific T cell repertoire. In this study, we explore whether intratumoral (IT) vaccination with an HPV vaccine can elicit quantitative and qualitative differences in immune response as compared to intramuscular (IM) vaccination to overcome immune resistance in established tumors. We report that IT administration of an HPV-16 E7 peptide vaccine formulated with polyinosinic–polycytidylic acid [poly(I:C)] generated an enhanced antitumor effect relative to IM delivery. The elicited anti-tumor effect with IT vaccination was consistent among the vaccinated groups and across various C57BL/6 substrains. IT vaccination resulted in an increased frequency of PD-1<sup>hi</sup> TILs, which represented both vaccine-targeted and non-vaccine-targeted tumor-specific CD8<sup>+</sup> T cells. Overall, the CD8<sup>+</sup>/Treg ratio was increased within the tumor microenvironment using IT vaccination. We also assessed transcriptional changes in several immune-related genes in the tumor microenvironment of the various treated groups, and our data suggest that IT vaccination leads to upregulation of a broad complement of immunomodulatory genes, including upregulation of interferon gamma (IFN $\gamma$ ) and antigen presentation and processing machine (APM) components. IT vaccine delivery is superior to traditional IM vaccination routes with the potential to improve tumor immunogenicity, which has potential clinical application in the setting of accessible lesions such as head and neck squamous cell carcinomas (HNSCCs).

**Keywords** HPV vaccine · PD-1 · Tim-3 · Lag-3 · Intratumoral vaccination · Immune checkpoints

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## Abbreviations

APM	Antigen presentation and processing machinery
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
CTLs	Cytotoxic T lymphocytes
GLA-SE	Glucopyranosyl lipid adjuvant formulated in a stable emulsion
HNSCC	Head and neck squamous cell carcinoma
IS	Immune system
IFN $\gamma$	Interferon gamma
IM	Intramuscular
IT	Intratumoral
JAX	Jackson Laboratories
MGH	Massachusetts General Hospital
MDSC	Myeloid-derived suppressor cells
OCT	Optimal cutting temperature (compound)
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
Poly(I:C)	Polyinosinic–polycytidylic acid
Tim-3	T cell immunoglobulin and mucin-domain containing-3
TAC	Taconic Farms
TLRs	Toll like receptors
TAA	Tumor-associated antigen(s)
TILs	Tumor-infiltrating lymphocytes
TSA	Tumor-specific antigen(s)
SNP	Single nucleotide polymorphism
Untx	Untreated

## Introduction

Chronic infection with human papillomavirus (HPV) is associated with an increased risk of developing cervical, anal, and oropharyngeal cancers. Prophylactic HPV vaccines have ushered in a new era of cancer prevention for HPV-associated cancers, in particular for pre-cancerous lesions [1]. However, incidence rates of HPV-associated cancers, particularly anal and oropharyngeal squamous cell carcinomas continue to rise [2–5], which highlights the unmet need to develop novel therapies for people infected with HPV and/or suffering from HPV-associated diseases. In 2016, immune checkpoint inhibitors for programmed cell death 1 (PD-1, CD279), pembrolizumab and nivolumab, were approved for the treatment of recurrent and/or metastatic head and neck squamous cell carcinoma (HNSCC) [6]. However, new therapeutic approaches are needed since approximately 80% of patients with HNSCC do not respond to immune checkpoint inhibitors alone [7] and current therapies offer little durable benefit [8–10].

Therapeutic cancer vaccines have been investigated over the past 4 decades [11, 12], but have met limited clinical

success [13]. In the setting of therapeutic vaccine administration, the immune system (IS) is ‘experienced’ having been exposed to foreign pathogens (i.e., HPV) and/or tumor-associated antigens (TAA), and the IS fails to effectively clear the tumor; whereas in the prophylactic vaccine setting, the IS is naïve to the antigen and has time to be educated prior to subsequent antigen (i.e., pathogen) exposure. Much of our understanding of effective vaccines has been derived from studies from the naïve IS rather than ‘retraining’ a chronically antigen-exposed, exhausted or tolerized IS that has been permissive to cancer growth. To overcome the multiple mechanisms of immune evasion in a chronic viral setting [14, 15], effective therapeutic cancer vaccines need to generate new populations of viral or tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs) or reverse exhausted TAA-specific CTLs.

The use of adjuvants in cancer vaccine formulation is an effective strategy to activate both the innate and humoral components of the IS [10, 16, 17]. Adjuvants sculpt the immune response to a foreign peptide or protein by extending and modulating an antigen’s immunogenicity. Identification of receptors and signaling pathways for pathogen-associated molecular patterns (PAMPs) has allowed the development of sophisticated molecular adjuvants. Pattern recognition receptors (PRR) encompass a variety of receptors, including Toll like receptors (TLR) that are differentially expressed on professional and non-professional antigen presenting cells [18]. Activation of TLR pathways has been shown to be an effective adjuvant strategy for both HPV polypeptides and DNA-based vaccines. Specifically, TLR3, TLR4, and TLR7 agonists, polyinosinic–polycytidylic acid [poly(I:C)], GLA-SE (glucopyranosyl lipid adjuvant formulated in a stable emulsion), and resiquimod [19], respectively, have been utilized as adjuvants in therapeutic vaccines. Here, we employed poly(I:C) as an adjuvant because TLR3 activation promotes a Th1-type response, which leads to increases in cytokines involved in T cell differentiation (i.e., IL12, TNF and IFN $\gamma$ ) and cross priming of CD8<sup>+</sup> T cells [20].

In this study, we explored whether IT vaccination with a E7 peptide formulated with poly I:C elicited an enhanced immune response as compared to IM vaccination in an HPV-related TC-1 syngeneic mouse model. We hypothesized that the IT route would result in an enhanced anti-tumor effect due to (1) increased T cell infiltration into the tumor and (2) elicitation of a broader immune response against both the vaccine-targeted antigen (HPV16 E7) as well as non-vaccine-targeted TAAs. We also compared IT to IM vaccination routes with or without adjuvant. In the context of activating the innate immune system via TLR3 using a TLR3 agonist, poly(I:C) with the highly immunogenic HPV E7 epitope, non-vaccine targeted TAA-specific CTLs were elicited which translated into enhanced anti-tumor responses

and improved survival with IT vaccination. These results suggest that IT vaccine delivery is superior to traditional IM vaccine routes, which has clinical application in the setting of accessible lesions such as HNSCCs.

## Materials and methods

### Animals

Six- to -eight-week-old female C57BL/6 mice were maintained under specific pathogen-free conditions in the Massachusetts General Hospital (MGH) (Boston, MA, USA) vivarium for the duration of these studies.

### Reagents

Collagenase IV, hyaluronidase IV, and DNase I were purchased from Sigma. Fluorochrome-conjugated antibodies for multi-color flow cytometry were used (see Table 1).

### Cell line

TC-1 tumor cells [21] were maintained in RPMI medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum.

### Vaccine and formulation

For the HPV peptide vaccines, the H-2Db-restricted HPV16 E7aa49-57 peptide, RAHYNIVTF, was generated at the Peptide Synthesis Core at the Massachusetts General Hospital (Boston, MA) and dissolved in 10% acetic acid and formulated in nucleic acid-free, sterile water with poly(I:C). Adjuvant poly(I:C) was purchased by InvivoGen (San Diego, CA, USA) and added to the peptide at an equal concentration of 20 µg. Animals were dosed with a 1:1 (w:w) suspension in

phosphate buffered saline (PBS) resulting in a 0.8 µg dose per kg (20 µg/25 g mouse).

### Tumor implantation and vaccination

For the therapeutic tumor experiments, C57BL/6 mice were inoculated subcutaneously in the right flank with  $1 \times 10^5$  TC-1 cells re-suspended in PBS per mouse. When the tumor volume reached 50 mm<sup>3</sup>, mice were vaccinated either via IM or IT injection of 20 µg of H-2Db-restricted HPV16 E7aa49-57 peptide, RAHYNIVTF, with or without 20 µg of poly(I:C). Subsequently, the mice were boosted at 5-day intervals for a total of three vaccinations. IM vaccinations were administered in the ipsilateral side of tumor inoculation. Mice were monitored for tumor growth by measuring tumor diameter with digital calipers twice a week. Tumor volume was calculated using the formula [largest diameter × (perpendicular diameter)<sup>2</sup>] × 0.5. To determine the overall survival (OS) of the treated tumor-bearing mice, mice with a tumor diameter greater than 2 cm were euthanized and counted toward death. Tumor-bearing mice that died from causes unrelated to their tumors were not included in these analyses.

### Preparation of single-cell suspensions from TC-1 tumor

Based on the specific experiment being performed, mice were sacrificed 5 days after the last vaccination, and TC-1 tumors were surgically excised, placed in RPMI-1640 medium containing 100 U/ml penicillin and 100 µg/ml streptomycin, and washed with PBS. Tumors were then minced into 1- to 2-mm<sup>3</sup> pieces and immersed in serum-free RPMI-1640 medium containing 0.05 mg/ml collagenase I, 0.05 mg/ml collagenase IV, 0.025 mg/ml hyaluronidase IV, 0.25 mg/ml DNase I, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37 °C with periodic agitation. The resulting tissue digest was then filtered through a

**Table 1** Fluorochrome-conjugated antibodies used for multi-color flow cytometry

Primary antibody	Clone	Fluorescence	Vendor
CD3	17A2	Alexa Fluor (AF)-700	Biologend
CD4	GK1.5	Brilliant ultraviolet (UVB)-395	BD Bioscience
CD8	53-6.7	UVB 805	BD Bioscience
FoxP3	FJK-16s	AF-488	eBioscience
CD279 (PD-1)	29F.1A12	Brilliant Violet (BV)-605	Biologend
Tim-3	RMT3-23	Allophycocyanin-(APC)	Biologend
CD223 (Lag-3)	C9B7W	PerCP/eF710	eBioscience
HPV16 E7 49-57 peptide (RAHYNIVTF) loaded H-2Db tetramer		Phycoerythrin (PE)	MBL International Corporation
Live/dead dye		Near infrared (NIR)	Biologend

40- $\mu$ m nylon filter mesh to remove undigested tissue fragments. Single-cell suspensions were washed twice in PBS (400 g for 10 min), re-suspended in staining buffer (0.5% BSA in PBS), and viable cells were enumerated using trypan blue dye exclusion.

### Flow cytometric analysis

Single-cell preparations from the tumor were incubated with live/dead dye (Zombie NIR) to exclude the dead cells. After the wash, the cells were stained with a cocktail containing HPV16 E7 tetramer, CD3, CD4, CD8alpha, Foxp3, CD223 (Lag-3), CD279 (PD-1), and Hepatitis A virus cellular receptor 2 (also known as T-cell immunoglobulin and mucin-domain containing-3, Tim-3) antibodies for 30 min at 4 °C. For detection of intracellular Foxp3, single-cell suspensions were washed with FACS staining buffer, fixed, and permeabilized with a FoxP3 fixation/permeabilization buffer kit as per manufacturer's instructions (eBioscience) before adding AF488-conjugated anti-mouse Foxp3 for 30 min at 4 °C. Cells were acquired with an LSRII flow cytometer (BD biosciences) and analyzed with FlowJo software (Tree star, Ashland, OR, USA). In some experiments when the tumor volume measured less than 0.5 cm, single-cell suspensions from individual mice of the same treatment group were combined to obtain a sufficient number of cells for flow analyses.

### Intratumoral differential gene expression

To determine changes in gene expression, tumors were excised 10 days after vaccination and embedded in optimal cutting temperature (OCT) compound. Embedded tissues were stored at  $-80$  °C until use. The tissue was cryo-sectioned at 5  $\mu$ m thickness and total RNA was extracted using RNeasy Plus Micro Kit (Qiagen). 100 ng of total RNA was hybridized with the GX Mouse Immunology Panel from Nanostring and detection of mRNA hybridized to specific fluorescent probes was done using nCounter SPRINT™. The nSolver™ analysis software package (V2.5) was used to normalize the dataset to the geometric mean of 15 housekeeping genes and the changes in counts for 561 immunology-related mouse genes (Mouse Immunology CodeSet) were analyzed. Changes in gene expression in the IM and IT vaccination cohorts were plotted as fold change relative to untreated tumor samples.

### Statistical analysis

GraphPad software (La Jolla, CA) was used to present the data. Data are representative of two or more independent experiments, and data are represented as mean and error bars represent standard deviation of the mean. Statistical significance was determined using the Kruskal–Wallis

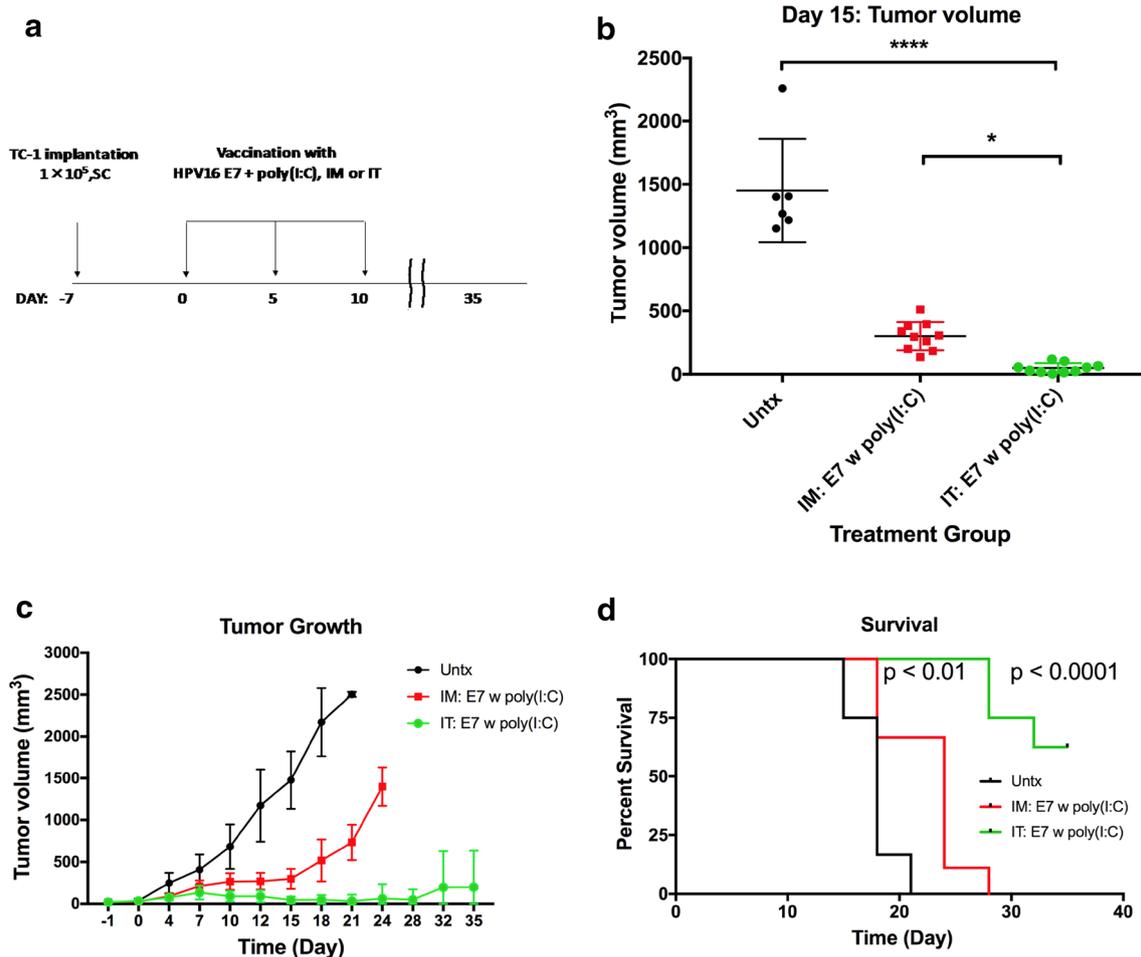
test followed by Dunn's test for multiple comparisons or Mann–Whitney for comparison of two groups. Survival distributions for mouse cohorts represented using Kaplan–Meier curves and significance between cohorts was determined using log-rank (Mantel–Cox) tests with  $p$  values as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , unless otherwise specified in the figure legends.

## Results

### IT administration of an HPV-16 E7 peptide vaccine generated an enhanced antitumor effect relative to IM delivery

We compared the anti-tumor effects of administering an HPV16 E7 peptide vaccine via different routes in an HPV-related preclinical tumor model. TC-1 tumor-bearing mice were treated via IT or IM routes with an HPV16 E7 peptide formulated with poly(I:C), and compared to mice whose tumors were untreated (i.e., control group). This vaccination regimen was repeated every 5 days for a total of three treatments over 10 days as shown in Fig. 1a. We found that IT administration slowed tumor growth when compared to administration of the same vaccine via IM or when tumor-bearing mice were left untreated. Statistically significant differences were observed between the untreated, IM-treated, and IT-treated groups (Fig. 1b). Although IM vaccination initially delayed tumor growth, the tumors began to grow at a rate similar to the untreated controls by day 15 (Fig. 1c). The anti-tumor effect translated into improved survival in the mice treated with a vaccine administered directly into the tumor ( $p < 0.0001$  and  $p < 0.01$ ) as compared to the untreated and IM-treated groups, respectively (Fig. 1d). Furthermore, at day 35 after IT vaccination with the HPV16 E7 peptide and poly(I:C), four of the nine mice were tumor-free, whereas all of the mice in the IM-vaccinated and untreated cohorts had to be euthanized due to tumor burden. These data demonstrate that the IT vaccination route with HPV16 E7 peptide and poly(I:C) elicits a stronger anti-tumor effect with durable responses as compared to the IM route.

In order to determine whether the enhanced anti-tumor effects observed with IT vaccination could be attributed simply to the intralesional administration of poly(I:C), we evaluated the IT administration of the HPV16 E7 peptide alone, poly(I:C) alone, and the combination of E7 peptide with poly(I:C), administered via IM or IT (Supplemental Figure 1a). We observed that IT administration of poly(I:C) alone had a better anti-tumor effect than E7 peptide alone or E7 peptide formulated with poly(I:C) administered IM (Supplemental Figure 1b and 1c). However, the anti-tumor effect with IT administration of poly(I:C) alone was not durable



**Fig. 1** Intratumoral administration of an HPV vaccine elicits potent anti-tumor effects with prolonged survival compared to intramuscular administration. **a** In vivo vaccination formulated with an HPV-16 E7 peptide and with poly(I:C) was administered every 5 days for a total of three treatments in mice bearing TC-1 tumors. **b** At day 15, tumors from IT-treated mice were significantly smaller than tumors that were vaccinated IM ( $p < 0.05$ ) or left untreated ( $p < 0.0001$ ). There was no statistical significance in tumor growth between the untreated or

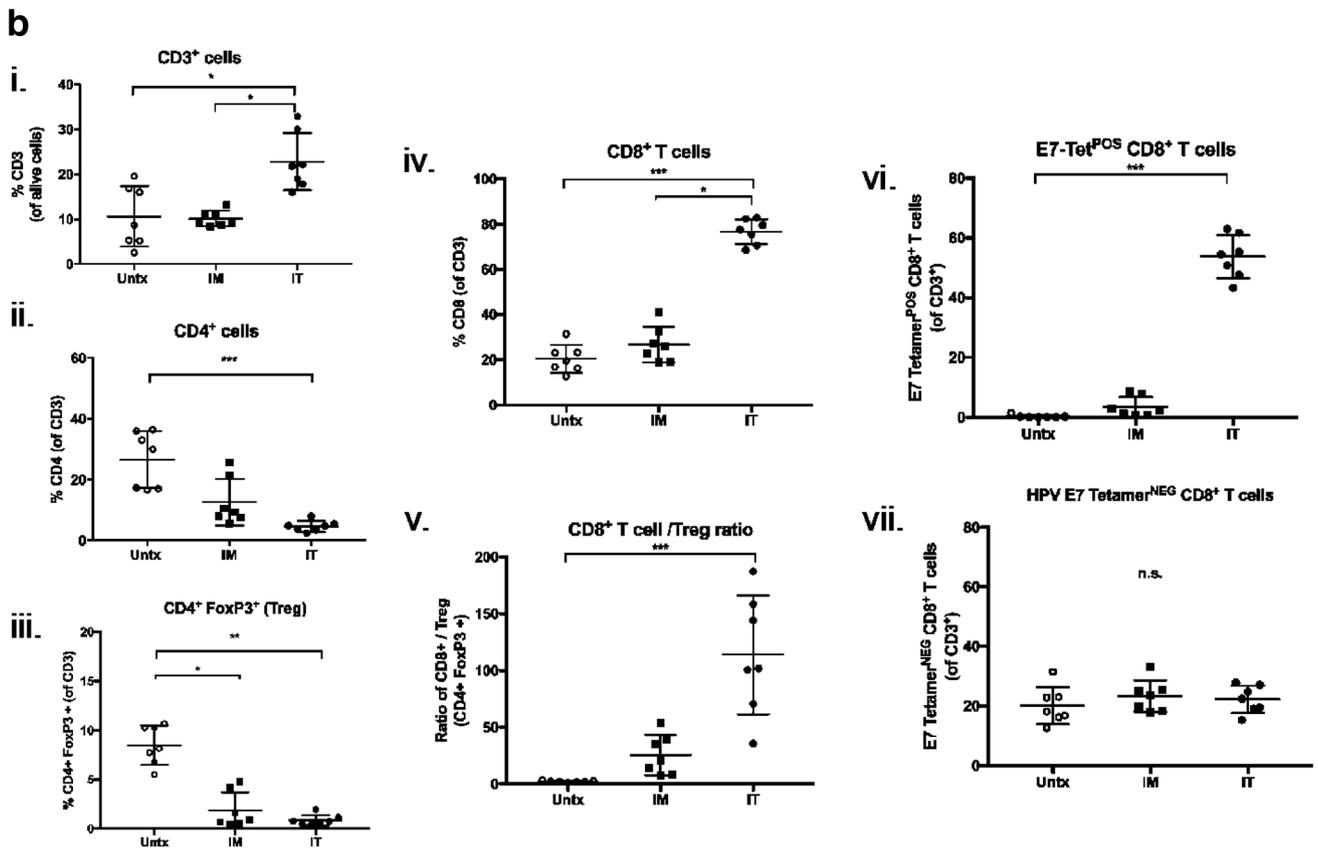
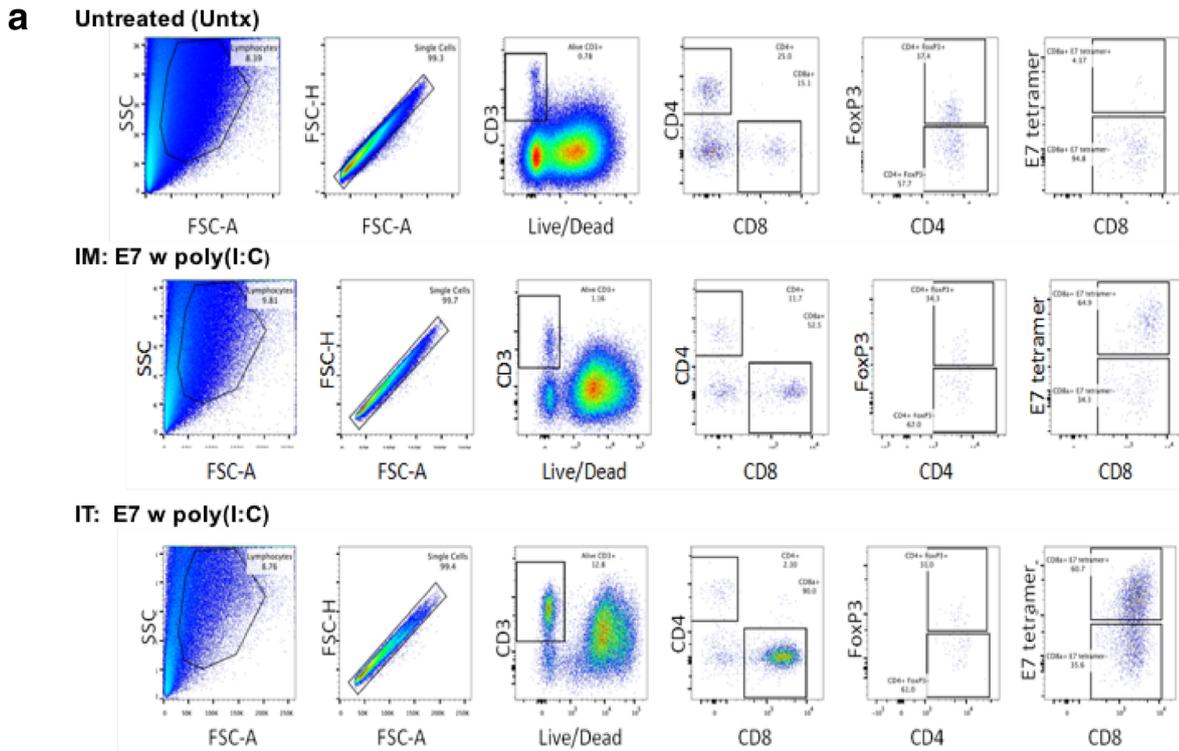
with the eventual growth of tumor over time. Interestingly, IT administration of poly(I:C) did not elicit a high frequency of CD8<sup>+</sup> tumor-infiltrating lymphocytes (TIL) (data not shown), suggesting that the anti-tumor effect observed with poly(I:C) alone—a potent TLR3 agonist—failed to adequately drive an adaptive, memory T cell immune response.

### Anti-tumor effects with IT vaccination achieved across C57BL/6 substrains

To ensure scientific rigor and reproducibility, we repeated the HPV16 E7 vaccination experiments in another C57BL/6 sub-strain that was purchased from Taconic (TAC) Farms. C57BL/6 mice bred at TAC and Jackson Laboratories (Jax) have genetic differences, including the deletion of the

IM-vaccinated mice. **c** Tumor growth was significantly delayed when vaccine was administered intratumoral (IT,  $N = 10$ ) as compared to intramuscular (IM,  $N = 10$ ) and untreated tumors (Untx,  $N = 10$ ). **d** The survival of IT-vaccinated mice was significantly improved compared to IM vaccination ( $p < 0.001$ ) or no treatment ( $p < 0.0001$ ). Error bars represent standard deviation of the mean with  $p$  values as follows: \* $p < 0.05$ , and \*\*\*\* $p < 0.0001$

nicotinamide nucleotide transhydrogenase (*Nnt*) gene as well as 11 single nucleotide polymorphism (SNP) loci [22]. Six- to eight-week old female C57BL/6 mice from TAC and Jax were housed in the same facility in separate cages for at least 1 week prior to tumor inoculation and during the treatment phase of these experiments. When the tumors reached 50 mm<sup>3</sup>, mice were treated either via IT or IM routes with the HPV16 E7 peptide with or without poly(I:C) formulation. Tumor growth was measured as described and, 5 days after the second vaccination, tumors were harvested. IT vaccination stimulated a strong anti-tumor response in both C57BL/6 substrains (Supplemental Figure 2). At day 10, there was no significant difference in tumor growth in the various treatment groups between the TAC mice compared to the Jax mice (Supplemental Figure 2b) and IT vaccine



**Fig. 2** IT vaccination generates more TILs, which have the highest CD8<sup>+</sup>/Treg ratio. Immunophenotyping of tumor infiltrating lymphocytes (TILs) in TC-1 tumor-bearing mice after IM or IT vaccination with HPV-16 E7 peptide and poly(I:C) was performed as described in Fig. 1. Tumors were harvested 5 days after the second vaccination, processed and stained for CD3, CD4, CD8, FoxP3 and HPV16 E7 49–57 peptide loaded H-2Db tetramer, and then analyzed by flow cytometry as described in “Materials and methods”. **a** Gating strategy to assess immunologic responses is shown for TILs from a mouse from each cohort (Untx, untreated=top row, IM=middle row, IT=bottom row). **b** The percentage of total CD3<sup>+</sup> T cells of all live cells, CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>FoxP3<sup>+</sup>(Treg), CD8<sup>+</sup>/Treg ratio, E7<sup>POS</sup>- and E7<sup>NEG</sup>-CD8<sup>+</sup> T cells are shown (**bi–bvii**, respectively). Each treatment group comprised C57BL/6 mice ( $N=7$ ) with TAC ( $N=4$ ) and Jax ( $N=3$ ); see Supplemental Figure 1. Error bars represent standard deviation of the mean with  $p$  values as follows: \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , and *n.s.* not significant

administration in either substrain was superior to the IM delivery route (Supplemental Figure 2c and 2d). The lack of differences in tumor growth observed between tumor-bearing mice from TAC and Jax in the various treatment groups indicates that the genetic differences between these C57BL/6 substrains and/or gut microbiome do not alter the anti-tumor responses generated by IT delivery of an HPV peptide vaccine.

### IT vaccination resulted in an increased CD8<sup>+</sup>/Treg ratio within the tumor microenvironment

We next characterized the nature of the TIL responses in the tumor to better understand the anti-tumor effects observed with IT vaccination. Five days after the second vaccination, tumors were harvested, and single-cell suspensions were prepared and analyzed using flow cytometry. We found that IT vaccination significantly increased the frequency of CD3<sup>+</sup> and CD8<sup>+</sup> T cells as compared to those mice treated via IM administration with the same vaccine formulation (2.5-fold CD3; 2.5-fold CD8), or the untreated mice (2-fold CD3; 4-fold CD8) (Fig. 2bi, iv). As the C57BL/6 mice purchased from TAC or Jax mice did not demonstrate any significant differences in their immunologic responses (Supplemental Figure 2c and d), the results from these two substrains were combined for these analyses.

Interestingly, IT vaccination generated the lowest frequency of CD4<sup>+</sup> T cells and CD4<sup>+</sup> FoxP3<sup>+</sup> T regulatory (Treg) cells (4.7% and 0.9%, respectively) as compared to the IM (12.6% and 1.9%, respectively) or the untreated groups (26.7% and 8.5%, respectively). Overall, IT vaccination resulted in the highest CD8<sup>+</sup>/Treg ratio as compared to the IM vaccination or no treatment. Within the CD8<sup>+</sup> T cell population, the greatest frequency of E7-positive CD8<sup>+</sup> T cells was elicited with IT vaccination (53.8%) as compared to IM (3.4%) or no treatment (Fig. 2bvi). A E7-negative CD8<sup>+</sup> T cell population was also observed after IT and IM vaccination as well as in the tumors of untreated mice. No

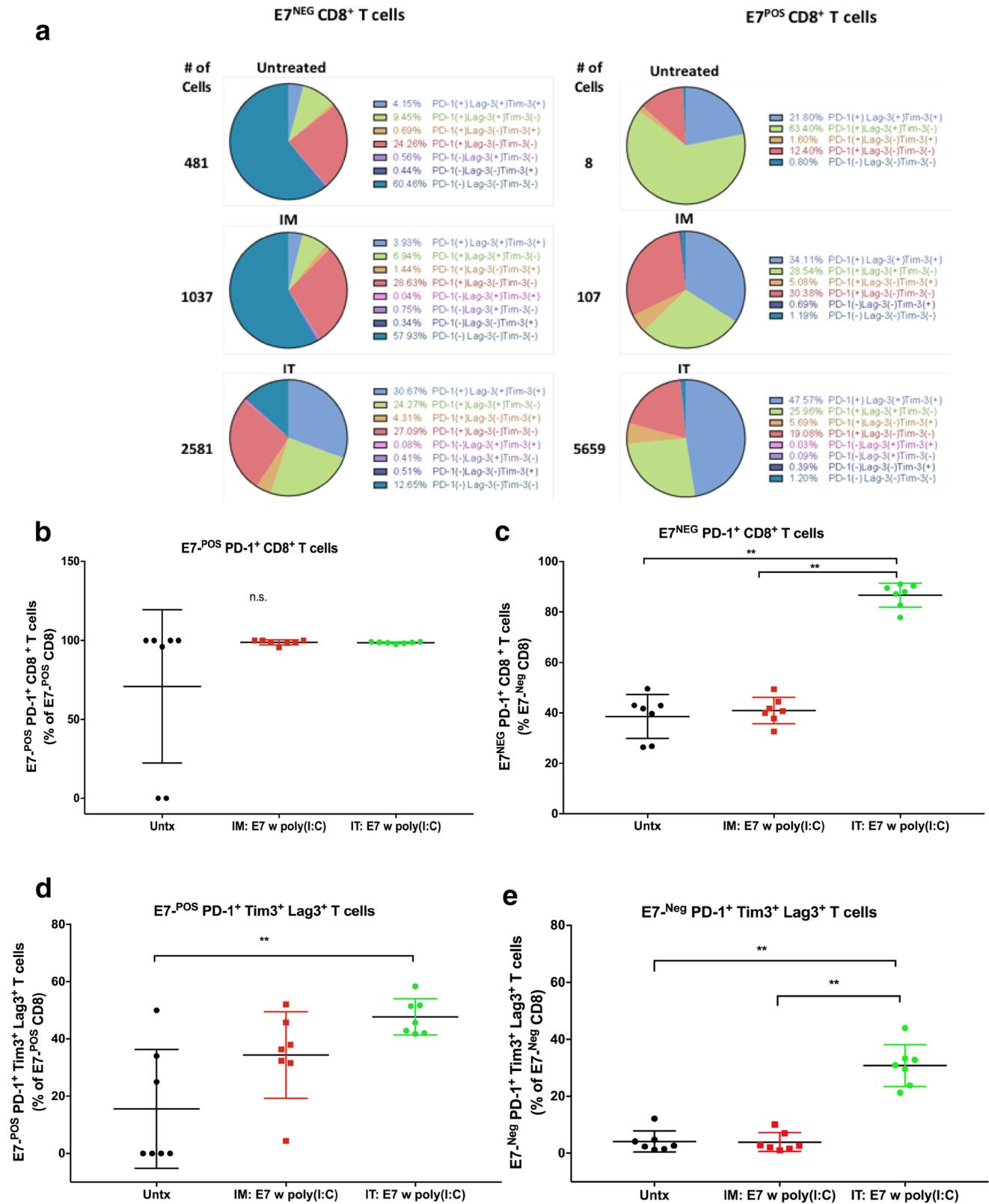
significant difference in the frequency of this E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> population was observed across treatment groups (Untx 22.3%; IM 23.3%; IT 20.1%) (Fig. 2bvii). However, we found that the non-E7-specific CD8<sup>+</sup> T cell populations among the various treatment groups were very different in their activation status, as discussed below.

### IT vaccination increased the frequency of PD-1<sup>hi</sup> TILs and TAA-specific PD-1<sup>+</sup>Tim-3<sup>+</sup>Lag-3<sup>+</sup> CD8<sup>+</sup> TILs

Although the expression of immune checkpoint receptors such as PD-1, Tim-3 and Lag-3 can represent activation or exhaustion, depending on the immunologic context, the presence of high levels of PD-1 and/or the co-expression of multiple immune checkpoint receptors on CD8<sup>+</sup> TILs has been shown to identify TAA-specific T cells [23, 24]. Using multiparametric flow cytometry, we evaluated both the vaccine-targeted (E7-tetramer<sup>POS</sup>) and non-vaccine-targeted (E7-tetramer<sup>NEG</sup>) CD8<sup>+</sup> TILs in terms of: (1) PD-1 expression levels and (2) fraction of PD-1<sup>+</sup>Tim-3<sup>+</sup>Lag-3<sup>+</sup> “triple-positive” cells (Fig. 3). While the number of CD8<sup>+</sup> TILs (described above) differed across the treatment groups, the E7-tetramer<sup>POS</sup> CD8<sup>+</sup> TILs population was predominantly unimodal and expressed a high frequency of PD-1 expression levels, which was comparable across the IT, IM, and untreated groups (Fig. 3b).

In contrast, the PD-1 expression profile of the E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> populations differed significantly across treatment groups. We observed the untreated and IM-treated TILs were multimodal, containing both low and high levels of PD-1 expression, whereas for the IT vaccine group, the E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> TILs consisted predominantly of PD-1<sup>hi</sup> CD8<sup>+</sup> T cells with almost no PD-1<sup>lo</sup> CD8<sup>+</sup> TILs present (Figs. 3c, 4). In fact, the PD-1 expression profile of the E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> TIL population appeared to be very similar to the vaccine-specific (E7-tetramer<sup>POS</sup>) CD8<sup>+</sup> populations (Fig. 3b, c). Overall, within the E7-tetramer<sup>NEG</sup> population, the fraction of CD8<sup>+</sup> TILs with low PD-1 expression was 0.91% (Untx); 1.07% (IM); 2.56% (IT), whereas the fraction of CD8<sup>+</sup> PD-1<sup>hi</sup> was 1.17% (Untx); 3.24% (IM); 10.08% (IT) (Fig. 4c, e). IT vaccination generated a statistically significant higher frequency of PD-1<sup>+</sup> non-E7-specific TILs as compared to the IM-vaccinated or untreated groups ( $p<0.0001$ ) (Fig. 3c).

Expression of multiple checkpoint receptors on TILs may more stringently identify a population of TAA-specific CD8<sup>+</sup> T cells than PD-1 expression alone. Therefore, we performed multiparametric flow cytometric analysis of PD-1, Tim-3 and Lag-3 on CD8<sup>+</sup> TILs isolated from tumors of mice, which received IM or IT vaccination or no vaccine (Fig. 3d, e). IT vaccination also elicited the highest frequency of PD-1<sup>+</sup>Tim-3<sup>+</sup>Lag-3<sup>+</sup> CD8<sup>+</sup> TILs—both in the E7-tetramer<sup>POS</sup> and E7-tetramer<sup>NEG</sup> populations of CD8<sup>+</sup>



TILs. Within the vaccine-targeted (E7-tetramer<sup>POS</sup>) CD8<sup>+</sup> TIL population the mean percent “triple-positive” CD8<sup>+</sup> T cells was 47.70% (IT) versus 34.35% (IM) versus 15.57%

(UnTx) ( $p < 0.01$ ). Importantly, IT vaccination also generated the highest frequency of “triple-positive” CD8<sup>+</sup> T cells within the non-E7-vaccine-targeted (E7-tetramer<sup>NEG</sup>) CD8<sup>+</sup>

**Fig. 3** IT vaccination elicits the greatest frequency of PD-1, Tim-3, and Lag-3 expression on both E7-specific and non-E7-specific CD8<sup>+</sup> TILs. TC-1 tumor-bearing mice were vaccinated as described in Fig. 1, and single tumor cell suspensions were generated as described in “Materials and methods”. **a** Pie chart depicting the percentage of PD-1, Tim-3, and Lag-3 single, double, and triple expression on E7<sup>POS</sup>- and E7<sup>NEG</sup>-CD8<sup>+</sup> T cells of total CD8<sup>+</sup> TILs in the various treatment groups is shown. The frequency of PD-1 expressing CD8<sup>+</sup> T cells in the various treatment groups is summarized. **b, c** IT vaccination generated the greatest frequency of PD-1 expressing E7<sup>POS</sup>- and E7<sup>NEG</sup>-CD8<sup>+</sup> T cells as compared to IM vaccination or no treatment. **d, e** IT vaccination resulted in the greatest frequency of PD-1+Tim-3+Lag-3 E7<sup>POS</sup>- and E7<sup>NEG</sup>-CD8<sup>+</sup> T cells. Error bars represent standard deviation of the mean with *p* values as follows: \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001, *n.s.* not significant

TIL population: IT (30.78%); IM (3.93%); Untx (4.15%) (*p*<0.01), suggesting that IT vaccination induced a higher TAA-specific immune response, possibly extending beyond the vaccine-specific antigens. Our results indicate that IT vaccination with E7 peptide formulated with poly(I:C) increased the frequency and altered the activation status of both vaccine-targeted and non-vaccine-targeted CD8<sup>+</sup> TILs and increased the CD8<sup>+</sup>/Treg ratio, both of which contributed to an improved anti-tumor response.

### IT vaccination elicited an inflammatory gene signature

In order to better understand how IT vaccination can significantly enhance the generation of CD8<sup>+</sup> TILs and the anti-tumor response, we assessed transcriptional changes of immune genes in the tumor microenvironment of the HPV-related TC-1 tumor-bearing C57BL/6 mice 5 days after IM or IT vaccination. Changes in tumor gene expression were analyzed using the Nanostring™ nCounter platform. IT vaccination resulted in coordinated increases and decreases in gene expression as shown by hierarchical agglomerative clustering of 561 immune-related genes for six out of seven mice whose tumors received IT vaccination (Fig. 5a). IT-vaccinated mice led to increased gene expression of lymphocyte-specific genes (i.e., e.g., *Cd3e*, *Cd4*, *Cd8a*, *Cd8b*) and myeloid-lineage cells (e.g., *Cd11b*, *Emr1*, *Cd14*, *Cd11c*) within the tumors compared to mice receiving IM vaccination or non-vaccinated mice (Fig. 5b) suggesting that IT vaccination activated both innate immune cells and stimulated lymphocyte migration into the tumor.

Interferon- $\gamma$  (IFN $\gamma$ ) is strongly expressed by T cells, activated through TCR signaling upon binding its cognate antigen. This ability to secrete IFN $\gamma$  is retained in partially exhausted, tumor-specific PD-1+ CD8<sup>+</sup> TILs, which likely explains why the presence of an intratumoral IFN $\gamma$  gene signature correlates strongly with response to anti-PD-1 blockade. We found that tumors from IT-vaccinated mice demonstrated a robust gene signature reflective of IFN $\gamma$

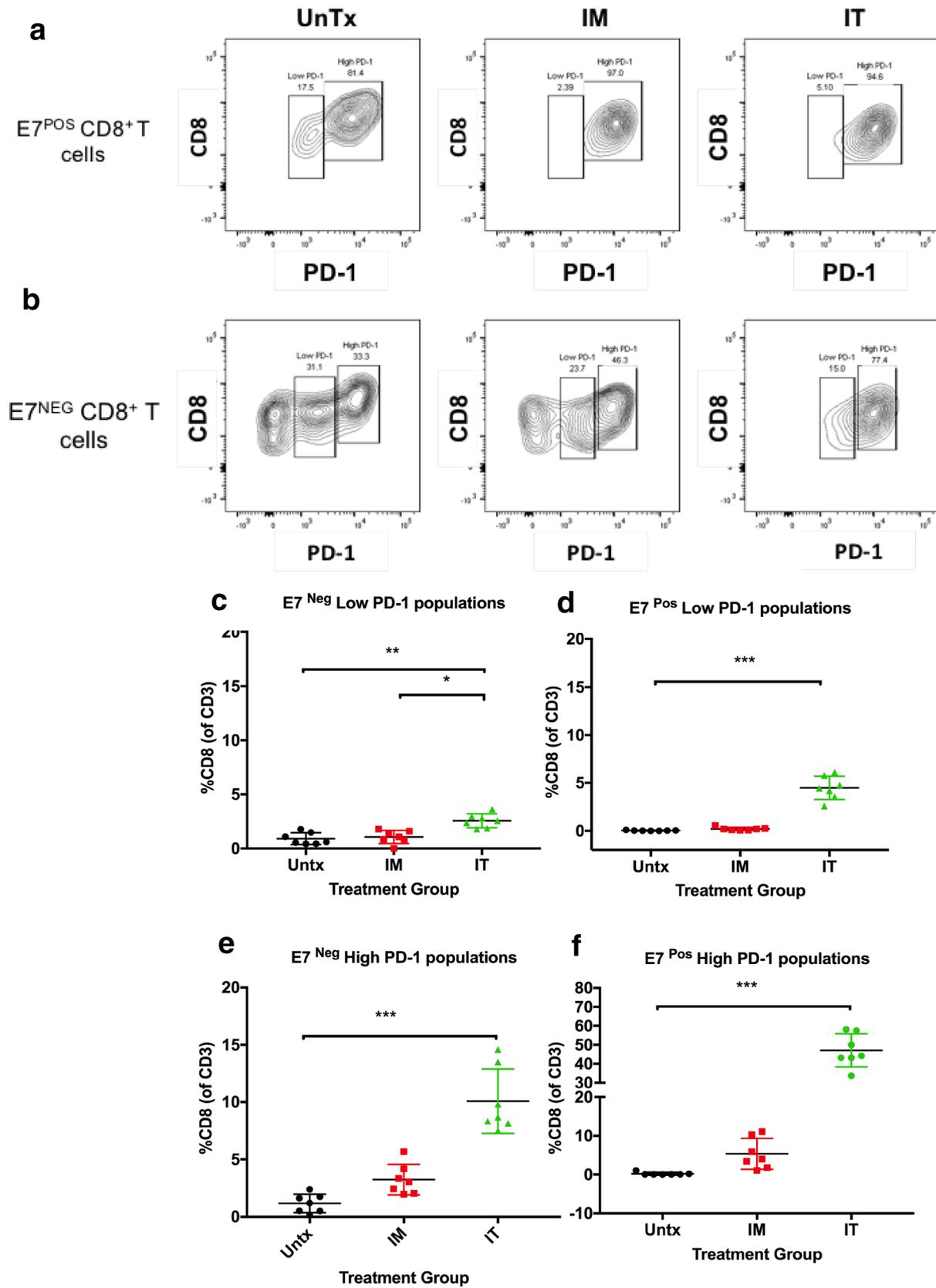
signaling and T cell activation (Fig. 5c). Modest increases in this IFN $\gamma$  signature were also seen in tumors from mice receiving IM vaccination, but they were less pronounced than those seen with IT vaccination. As IFN $\gamma$  also plays a critical role in modulating genes involved in the antigen processing machinery (APM), we examined the changes of these IFN $\gamma$ -inducible genes. Transcripts involved in processing and presentation of antigen on both MHC class I (*H2-K1*, *Tap1*, *Tapbp*, *Psmb9* and *10*, *B2m*) and MHC class II (*H2-Aa*, *Ciita*, *Cd74*) were significantly up-regulated after IT vaccination compared to IM vaccination, particularly for *Ciita*, the master regulator of MHC class II (Fig. 5d). Taken together, these data suggest that IT vaccination leads to upregulation of a broad complement of immunomodulatory genes, indicating activation of both innate and adaptive immunity, including upregulation of IFN $\gamma$  and APM components.

### Discussion

In this study, we explored whether IT administration of an HPV E7 peptide vaccine in combination with a TLR3 agonist, poly(I:C), was superior to IM administration of the same vaccine formulation. We observed significant tumor regression and mortality when the vaccine was delivered directly into the tumor as compared to either (1) IM vaccination with E7 peptide + poly(I:C) or (2) IT administration of poly(I:C) or E7 peptide alone.

In order to better understand the mechanism underlying this observed difference in anti-tumor effect, we first analyzed the vaccine-targeted CD8<sup>+</sup> T cell response, using an E7-specific tetramer. The fraction of E7-tetramer<sup>POS</sup> CD8<sup>+</sup> TILs was greatly expanded (15-fold) in the TILs from mice, which received IT as compared to IM vaccination. Although the flow cytometric analysis as performed in these studies only allow the quantitation of relative percentages of cells within a population, not the absolute number, Nanostring-based gene expression of cell-type associated genes (e.g., *CD274*(*CD3e*)) provided an independent means of quantifying TIL composition. IT vaccination increased *CD3e* transcript levels threefold over IM and 25-fold over untreated controls, reflecting not just an alteration in the phenotypic frequencies, but a bona fide increase in the absolute number of T cells (i.e., CD3+), including E7-specific CD8<sup>+</sup> TILs.

The E7-tetramer<sup>POS</sup> CD8<sup>+</sup> TILs in the IT-treated mice appear similar to the E7-tetramer<sup>POS</sup> CD8<sup>+</sup> TILs in the IM-treated mice, in terms of consisting of a large proportion of PD-1<sup>hi</sup> and/or PD-1+Tim-3+Lag-3+ phenotypes. Controversy remains as to whether these phenotypes accurately describe whether the CD8<sup>+</sup> TILs are activated or exhausted. However, in our study, the strong correlation between the increased numbers of these CD8<sup>+</sup> TILs and the generation



**Fig. 4** IT vaccination elicits the greatest frequency of both E7-specific and non-E7-specific CD8<sup>+</sup> TILs expressing high levels of PD-1. **a, b** CD8<sup>+</sup> TILs in the various treatment groups were separated into PD-1 expression low or high based on relative MFI. **c–f** The frequency of PD-1- low or -high expressing E7<sup>POS</sup>- and E7<sup>NEG</sup>-CD8<sup>+</sup> TILs from the various treatment groups is shown ( $N=7$ ). IT vaccination generates the highest frequency of high PD-1-expressing E7-specific and non-E7-specific CD8<sup>+</sup> T cells as compared to IM vaccination or no treatment. Error bars represent standard deviation of the mean with  $p$  values as follows: \*\* $p < 0.01$ , \*\*\* $p < 0.001$

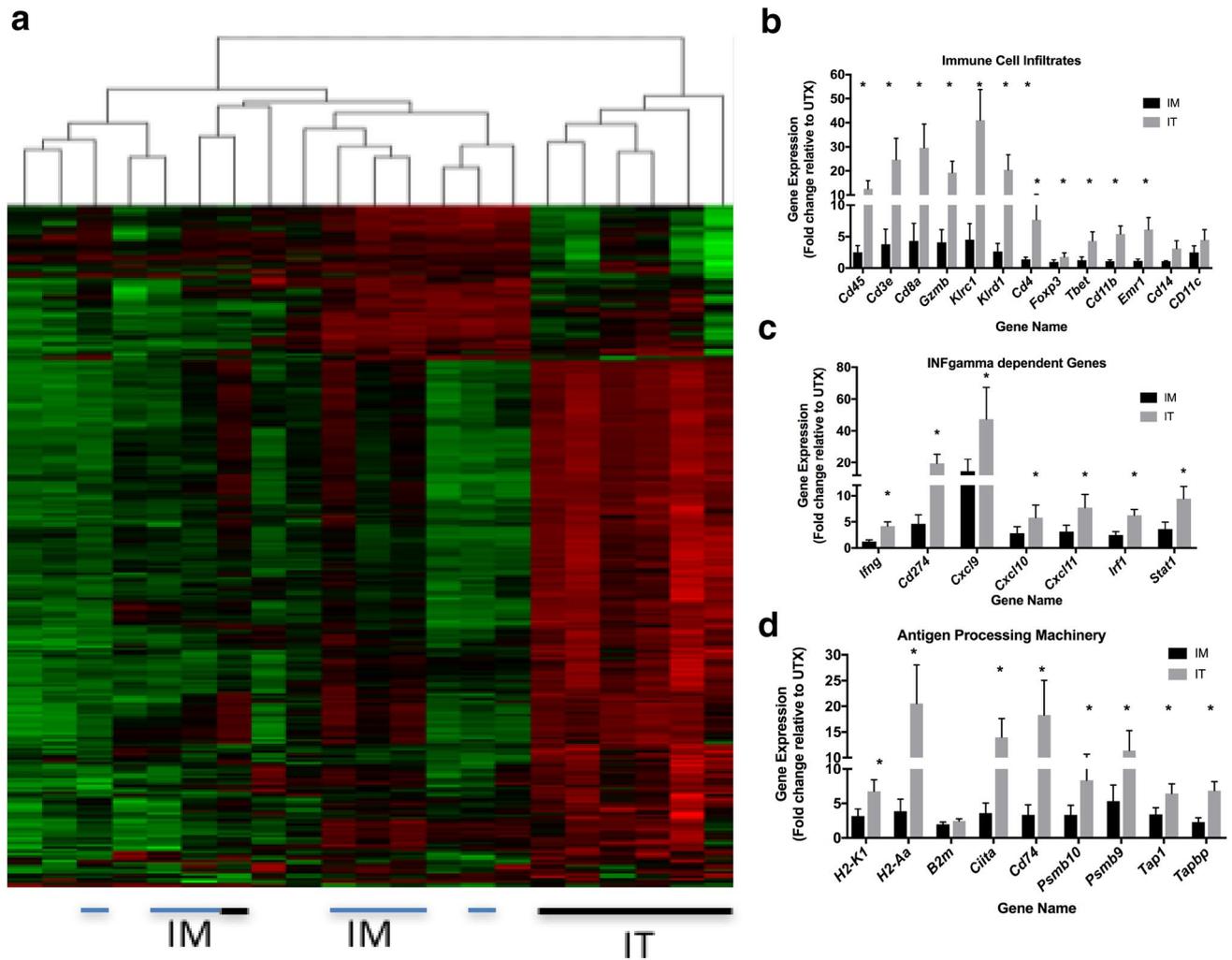
of an effective anti-tumor response—without additional PD-1 blockade—suggests that these cells remain adequately cytotoxic. Less controversial, however, is that the PD-1<sup>hi</sup> and PD-1<sup>+</sup>Tim-3<sup>+</sup>Lag-3<sup>+</sup> TIL phenotypes represent TAA-specific CD8<sup>+</sup> TILs. That the majority of E7-specific CD8<sup>+</sup> T cells in the tumor are PD-1+TIL (PD-1<sup>med</sup> or PD-1<sup>hi</sup>) or “double-positive” CD8<sup>+</sup> TILs (PD-1+Tim-3+Lag-3+; PD-1+Tim-3+; PD-1+Lag-3+; Figs. 3, 4) supports this conclusion.

As described above, IT administration of an HPV E7 vaccine led to a significant increase in the frequency and number of E7-specific CD8<sup>+</sup> TILs as compared to IM vaccination (15-fold). Importantly, however, the IT delivery of the vaccine [E7 peptide + poly(I:C)] had an even more pronounced effect on the phenotype of E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> TILs. Although the total fraction of E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> cells (% of CD3) was relatively unchanged, the proportion of E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> TILs that demonstrated a PD-1+ (PD-1<sup>med</sup> or PD-1<sup>hi</sup>) or “triple-positive/double-positive” phenotype (PD-1<sup>+</sup>Tim-3<sup>+</sup>Lag-3<sup>+</sup>; PD-1<sup>+</sup>Tim-3<sup>+</sup>; PD-1<sup>+</sup>Lag-3<sup>+</sup>; Fig. 3) increased significantly, indicating that they are tumor antigen-specific. Based on the dramatic increase in CD274 (*CD3e*) transcript counts (25-fold), we conclude through gene expression analyses that not only the frequency, but absolute number of E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> TILs expressing the PD-1<sup>hi</sup> or “double-positive” phenotype increased. In the IT-treated groups, we propose this phenotype represents a strong, multi-specific TAA-specific T cell response. The E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> TILs may represent antigen-activated T cells, which have downregulated the E7-specific T cell receptor (TCR), which has been described in the literature [25]. However, the downregulation of the TCR in activated T cells is often associated with CD8 loss. We report here of a CD8 expressing TIL population with high markers of activation (PD-1, Lag-3, and/or Tim-3) which are E7-tetramer<sup>NEG</sup>. Thus, an alternative explanation for our findings is that the E7-tetramer<sup>NEG</sup> CD8<sup>+</sup> TILs may represent TCRs, which react to either non-E7 HPV antigens (i.e., E6) and/or mutation-derived neopeptides, to possibly represent epitope spreading beyond the E7-vaccine target. The clinical translation and relevance of our preclinical findings with IT vaccination is highlighted in human studies in HPV-associated cancers which demonstrate the importance

of immunodominant T cell reactivities against mutated neoantigen(s) or cancer germline antigen(s), beyond HPV tumor-specific antigen(s), in eliciting anti-tumor effects in vivo [26].

In order to broadly understand the unique effects of IT vaccination and how it leads to an enhanced anti-tumor immune response, we performed Nanostring-based transcriptional analysis on tumors from untreated, IM and IT-vaccinated mice. Hierarchical agglomerative clustering tightly grouped 6 out of 7 IT-vaccinated mice; the single non-clustered IT-treated mouse had the worst tumor regression and was one of the two IT-treated mice with residual tumor at day 35. We observed that the transcriptional signatures of tumors from IM and untreated mice often co-clustered together, suggesting that the generation of E7-specific T cells through IM vaccination failed to exert a strong, consistent reprogramming of the tumor microenvironment (TME). We did identify, however, a small but significant increase in transcripts reflecting increased immune cell infiltrates in the IM-vaccinated group, correlating with the flow cytometric results. IT vaccination led to a markedly enhanced, broad immune cell infiltrate characteristic of a so-called inflamed or “hot” tumors compared to either untreated or IM-vaccinated mice. In particular, both IFN $\gamma$  itself and many IFN $\gamma$ -inducible genes (“IFN $\gamma$  signature”) were significantly enhanced in the IT-vaccinated mice as compared to IM vaccinated and untreated controls. IM vaccinated mice demonstrated a smaller but significant upregulation of these genes over the untreated baseline, likely reflecting some degree of antigen-dependent activation of anti-E7 CD8<sup>+</sup> T cells within the TME. Although NK cells may well be playing a role in the local production of IFN $\gamma$  in this model, the dramatic increase in these IFN $\gamma$  signature genes in the IT-vaccinated mice likely reflects a much higher level of T cell/antigen engagement within these tumors—as demonstrated by the flow cytometric assessment of PD-1<sup>hi</sup> and/or “triple/double-positive” CD8<sup>+</sup> TILs.

One important consequence of IT expression of IFN $\gamma$  is that it can enhance expression of the APM. As expected, given the levels of expression of IFN $\gamma$  and IFN $\gamma$ -signature genes, tumors from IM-vaccinated mice demonstrated a significant upregulation of APM genes compared to untreated mouse controls, whereas IT-vaccinated mice showed a marked increase in these genes. Particularly noteworthy is that *CIIa*, the master transcriptional regulator of class II antigen processing was upregulated as compared to untreated control mice and IM-vaccinated mice, suggesting that myeloid cells in the immunosuppressive TC-1 TME can be converted through IT vaccination into potential immunogenic APC. A similar IFN $\gamma$ -dependent conversion of myeloid-derived suppressor cells (MDSC) into immunogenic APC was shown by Kerkar et al. though the introduction of IL-12 into tumors using genetically engineered anti-pmel



**Fig. 5** Transcriptional up-regulation inflammatory genes in IT vaccination TC-1 tumors. **a** Gene expression profiles cluster in six out of seven mice after IT vaccination (black bar), whereas gene expression profiles for IM-vaccinated mice (blue bar) and untreated mice cluster together. **b** IT vaccination leads to increased expression in genes associated with inflammatory cells that infiltrate tumors, including lymphocytes (CD3, CD4, CD8) and myeloid-lineage cells. **c** IM and IT vaccination results in a significant increase in genes known to be part of an interferon-gamma signature, which are downstream transcrip-

tional targets of IFN $\gamma$  signaling and associated with T cell activation in the TME. **d** APM gene expression was increased after IT and IM vaccination. Tumor inoculation and vaccination are described in “Materials and methods” and Fig. 1. RNA was prepared from tumors harvested on day 10 after initiation of treatment. Changes in gene expression were determined using the Nanostring™ platform with the mouse Immunology probe set and analyzed using nSolver software™. Data are presented as fold change relative to Untx mice. \* $p < 0.05$

transgenic T cells [27]. Although in this study, we do not formally demonstrate that IT myeloid cells become active APC, the generation of such a strong non-vaccine-targeted (E7-tetramer<sup>NEG</sup>) CD8<sup>+</sup> TIL response suggests that IT vaccination is leading to immunogenic cell death and that non-E7 tumor antigens are being processed and presented effectively, whether the locus of antigen presentation and T cell priming is in the tumor itself and/or draining lymph node.

The question remains as to why IT vaccination with a tumor-specific antigen (TSA) is superior to an antigen agnostic IT vaccine approach like poly(I:C) alone and whether this is a generalizable phenomenon or unique to the TC-1

syngeneic tumor model. We report that IT administration of a poly(I:C) adjuvant alone results in a limited anti-tumor effect (Supplemental Figure 1) and only with the combination of a TSA can an enhanced and durable tumor-specific anti-tumor effect be achieved. Our working hypothesis is that delivery of an antigen like HPV E7 into the tumor in the presence of an appropriate adjuvant leads to antigen loading and activation of tumor APC, which then drives the generation of an adaptive anti-tumor T cell response. Upon a second and third vaccination, primed anti-tumor T cells are recruited to the tumor because of poly(I:C)-induced activation of innate immune cells, leading to coordinated

upregulation of chemokines and integrins, which facilitate T cell trafficking. Antigen delivered into the tumor site may generate T cells, which more effectively “home” back to the site of antigen exposure. Subsequent to infiltration of the tumor, these TAA-specific T cells now encounter antigen, kill tumor cells and release antigens and produce IFN $\gamma$ , which upregulates further antigen processing presentation. Thus, by delivering antigen and adjuvant directly into the tumor, IT vaccination mimics a naturally effective anti-tumor response through a coordinated (spatially and temporally) activation of *both* innate and adaptive immunity, leading to epitope spreading and a broadening of the immune response. Our findings suggest that IT administration of an antigen-specific vaccine may provide a significant benefit over traditional routes of therapeutic cancer vaccine administration.

Recent studies with PD1/PD-L1 checkpoint inhibitors suggest that antigen-specific CD8<sup>+</sup> TILs that express PD-1 respond initially to blocking antibodies, but these T cells released from their checkpoint have a limited ability to undergo multiple rounds of proliferation. In this study, we provide evidence that IT vaccine delivery may help to convert a tolerogenic TME as well as elicit broad anti-tumor-specific immune responses, all of which translates into effective and potent anti-tumor responses.

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## Compliance with ethical standards

**Conflict of interest** Anandaroop Mukhopadhyay is employed by Oncosec Med Inc. The authors declare that they have no other conflicts of interest.

**Ethical approval and ethical standards** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in this study involving animals were in accordance with the ethical standards of Massachusetts General Hospital (MGH), where experiments were conducted in accordance with recommendations for the proper use and care of laboratory animals as MGH is an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-certified institution, and after IACUC approval (#2014N000059).

**Animal source** C57BL/6 mice were purchased from Taconic Farms (Hudson, NY, USA, C57BL/6NTac) and the Jackson Laboratory (Farmington, CT, USA, C57BL/6J, Stock Number 000664).

**Cell line authentication** The TC-1 tumor cell lines were kindly provided by Dr. T-C Wu (Johns Hopkins Hospital, Baltimore, MD, USA). The HPV-16 E6, E7, and *ras* oncogenes were used to transform pri-

mary C57BL/6 mice lung epithelial cells to generate the TC-1 tumor cell lines as previously described [21]. Cell line authentication was performed by the laboratory of TC Wu, who generated these cell lines, and tested negative for mycoplasma as determined by testing performed by Taconic Laboratories.

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