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ORIGINAL RESEARCH

Adaptive resistance to anti-PD1 therapy by Tim-3 upregulation is mediated by the PI3K-Akt pathway in head and neck cancer

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ABSTRACT

Programmed Death 1 (PD-1) and T cell Ig and mucin domain-3 protein (Tim-3) are immune checkpoint receptors that are expressed on tumor-infiltrating lymphocytes (TIL) in tumor-bearing mice and humans. As anti-PD-1 single agent response rates are only <20% in head and neck squamous cell carcinoma (HNSCC) patients, it is important to understand how multiple inhibitory checkpoint receptors maintain suppressed cellular immunity. One such receptor, Tim-3, activates downstream proliferative pathways through Akt/S6, and is highly expressed in dysfunctional TIL. We observed that PD-1 and Tim-3 co-expression was associated with a more exhausted phenotype, with the highest PD-1 levels on TIL co-expressing Tim-3. Dampened Akt/S6 phosphorylation in these PD-1$^+$Tim-3$^+$ TIL, when the PD-1 pathway was ligated, suggested that signaling cross-talk could lead to escape through Tim-3 expression. Indeed, PD-1 blockade of human HNSCC TIL led to further Tim-3 upregulation, supporting a circuit of compensatory signaling and potentially permitting escape from anti-PD-1 blockade in the tumor microenvironment. Also, in a murine HNC tumor model that is partially responsive to anti-PD-1 therapy, Tim-3 was upregulated in TIL from persistently growing tumors. Significant antitumor activity was observed after sequential addition of anti-Tim-3 mAb to overcome adaptive resistance to anti-PD-1 mAb. This increased Tim-3-mediated escape of exhausted TIL from PD-1 inhibition that was mediated by phospho-inositol-3 kinase (PI3K)/Akt complex downstream of TCR signaling but not cytokine-mediated pathways. Taken together, we conclude that during PD-1 blockade, TIL upregulate Tim-3 in a PI3K/Akt-dependent manner, providing further support for dual targeting of these molecules for more effective cancer immunotherapy.

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Introduction

Tumor-infiltrating lymphocytes (TIL) can recognize and eliminate tumor cells in the microenvironment. Upon activation, these T cells express immune checkpoint receptors such as programmed death (PD)-1 to maintain tolerance to self-antigens. During chronic activation, T cells in the tumor microenvironment acquire a dysfunctional status, leading to lack of responsiveness to antigen stimulation.[1-3] These "exhausted" T cells share several important features: loss of effector function, an altered transcriptional state and sustained expression of multiple inhibitory receptors.[1] Among the inhibitory receptors expressed by exhausted T cells, PD-1-blocking mAbs are effective in treatment of multiple cancer types,[4,5] including head and neck squamous cell carcinoma (HNSCC). In HNSCC cells, membrane and/or intracytoplasmic PD-L1 expression is frequent (40–70%),[6,7] coinciding with PD-1 upregulation on the majority of CD8$^+$ TIL.[6] This heightened PD-1/PD-L1 axis implies that anti-PD-1 therapy should be more effective at boosting antitumor immunity in HNSCC. However, recent clinical trial data revealed only a modest response rate to anti-PD-1 therapy of 15–20%.[8,9] Thus, it is important to understand dynamic changes in the tumor microenvironment after anti-PD-1 therapy and elucidate potential mechanism(s) of escape to design rational combination therapies.

T cell immunoglobulin mucin (Tim)-3 is a type 1 membrane receptor that is highly expressed in Th1/Tc1 T cells.[10,11] Several lines of evidence suggest that Tim-3 acts as an inhibitory receptor in the setting of chronic activation,[12-14] whereas other data suggest that it may enhance T cell receptor (TCR) signaling.[14] In cancer patients, Tim-3 expression on PD-1$^+$ TIL marks the most dysfunctional CD8$^+$ T cells,[15-18] and concurrent Tim-3 blockade appears to enhance the effects of anti-PD-1 mAbs in restoring T cell functionality.[19] However, dynamic changes in Tim-3 expression and signaling during the response to anti-PD-1 treatment, including potential signaling cross-talk in exhausted TIL, have not been investigated in detail.

In both freshly isolated tumor specimens and a murine HNSCC model, we found that 30–50% of CD8$^+$ TIL are Tim-3$^+$, and this expression correlates with the highest level of PD-1 expression and greater exhaustion. In this study, we show that Tim-3 expression is upregulated in response to PD-1 blockade, both *in vitro* and *in vivo*. We also show that the

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PI3K/Akt/mTOR pathway mediates cross-talk between PD-1 and Tim-3, providing a potential mechanism underlying Tim-3 upregulation in the setting of PD-1 blockade. Our findings support the concept of rationally combining blockade of Tim-3 with anti-PD- mAb therapy. Our data also suggest that targeted signaling pathway interference may help to prevent adaptive Tim-3-mediated escape from anti-PD-1 therapy.

Material and methods

Patients and specimens

Peripheral blood samples and fresh tumor specimens were obtained from previously untreated, stage III/IV HNSCC patients (n = 36). All patients were treated in the Department of Otolaryngology at the University of Pittsburgh Medical Center, and all subjects signed a written informed consent approved by the Institutional Review Board of the University of Pittsburgh (UPCI# 99–069). The clinical-pathological features of the HNSCC patients in this study are shown in Table 1. The patient cohort included 11 females and 25 males with a mean age of 59 y (range: 24–80 y).

Collection of peripheral blood mononuclear cells (PBMC) and tumor-infiltrating lymphocytes (TIL)

Venous blood from HNSCC patients was drawn into heparinized tubes and centrifuged on Ficoll-Hypaque gradients (GE Healthcare Life Sciences, Piscataway, NJ). PBMC were recovered, washed in RPMI-1640 medium (Sigma, St. Louis, MO), and either used immediately for experiments or re-suspended in freezing media containing 10% DMSO, transferred to Mr. Frosty containers (Thermo Scientific, Waltham, MA), and stored at −80°C until flow cytometry analysis. For TIL isolation, fresh tumors from HNSCC patients were minced into small pieces manually or using a gentle MACS Dissociator (Miltenyi Biotec, Auburn, CA), then transferred to 70-μm cell strainers (BD) and mechanically separated using the plunger of a 5-mL syringe. The cells passing through the cell strainer were collected and subjected to Ficoll-Hypaque gradient centrifugation. After centrifugation, mononuclear cells were recovered and stored at −80°C until flow cytometry analysis or immediately used for experiments.

Table 1. Clinicopathological features of the HNSCC patients in this study.

HN#	Gender	Age (at diagnosis)	Tumor site	T-stage	N-stage
HN15–8172	MALE	52	Oropharynx	T2	N2
HN15–8088	MALE	51	Oropharynx	T1	N2
HN14–7943	MALE	43	Larynx	T4	N2
HN14–7928	MALE	54	Larynx	T3	N0
HN14–7800	FEMALE	77	Oral cavity	T4	N0
HN15–8068	FEMALE	57	Oral cavity	T2	N2
HN14–7900	MALE	49	Oropharynx	TX	N2
HN14–7893	MALE	75	Oral cavity	T4	N0
HN14–7927	MALE	66	Larynx	TX	NX
HN15–8010	FEMALE	80	Oral cavity	T4	N0
HN15–8021	MALE	24	Oral cavity	T3	N0
HN15–8110	MALE	74	Oropharynx	T4	N2
HN15–8144	FEMALE	67	Oral cavity	T3	N0
HN15–8173	MALE	63	Larynx	T4	N0
HN15–8202	FEMALE	69	Oral cavity	T4	N2
HN15–8228	MALE	60	Oropharynx	T3	N2
HN15–8253	MALE	60	Oropharynx	T3	N2
HN15–8290	MALE	27	Oral cavity	T2	N2
HN15–8296	MALE	67	Larynx	T3	N2
HN15–8294	MALE	64	Oropharynx	T4	N2
HN15–8180	MALE	50	Oral cavity	T4	N1
HN14–7881	MALE	54	Oropharynx	T1	N2
HN14–7909	FEMALE	58	Oral cavity	T2	N1
HN14–7949	MALE	72	Larynx	T3	N2
HN14–7960	MALE	61	Larynx	T4	N2
HN14–7961	MALE	57	Oral cavity	T2	N0

Antibodies and flow cytometry

The following anti-human antibodies were used for staining: CD3-Alexa Fluor 700, $CD8^+$-PE, $CD4^+$-PercP/Cy5.5, CD25-APC-Cy7, FOXP3-PerCP/Cy5.5, purchased from BD Biosciences (San Jose, CA), Tim-3-BV421, CD25-PE-Cy7, IFN-γ-APC-Cy7 and TNF-α-FITC purchased from Biolegend (San Diego, CA), CD4-PE-TR purchased from Life Technologies (Carlsbad, CA), phospho-AKT(Ser473)-APC, TIGIT-APC purchased from eBioscience (San Diego, CA), phospho-S6 (Ser235/236)-Alexa Fluor 488 (Cell Signaling Technology, Danvers, MA), CTLA4-FITC purchased from Ancell (Stillwater, MN). The following anti-mouse antibodies were used for staining: PD-1-BV421, CD25-PE, $CD4^+$-APC-Cy7, $CD8^+$-PE-Cy7 purchased from Biolegend (San Diego, CA), Tim-3-APC purchased from eBioscience (San Diego, CA), MHC pentamer (H-2Db RAHYNIVTF)-PE purchased from Proimmune (Oxford OX4 4GA, United Kingdom).

Intracellular staining of p-Akt, p-S6, IFNγ and TNF-α was performed as follows: PBMC or TIL were stained with surface marker antibodies, fixed with fixation/permeabilization buffer (eBioscience), washed, and stained for intracellular antigens in 1X permeabilization buffer. Cells were analyzed on an LSR Fortessa (BD Biosciences) flow cytometer, and data analyzed using Flow Jo (Treestar, Ashland, OR). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward and side scatter. Dead cells were excluded based on viability dye staining (Zombie Aqua Fixable Viability Dye, Biolegend, San Diego, CA).

Sorting of TIL subsets

TIL were isolated from tumor specimens as described above and T cells were purified using EasySep™ Human T Cell Enrichment Kit (Stemcell technologies, Vancouver, Canada). Then, the T cells were stained with $CD4^+$-PercP-Cy5.5, $CD8^+$-PE, CD25-PE-Cy7, PD-1-APC and Tim-3-BV421 antibodies. $PD\text{-}1^+Tim\text{-}3^-$, $PD\text{-}1^-Tim\text{-}3^+$, $PD\text{-}1^+Tim\text{-}3^+$ and $PD\text{-}1^-Tim\text{-}3^-$ $CD8^+$ and $CD4^+CD25^{lo/-}$ cells were sorted using Beckman Coulter MoFlo Astrios.

Re-stimulation of TIL using anti-CD3/CD28 beads

T lymphocyte subsets were sorted based on PD-1 and Tim-3 expression from freshly isolated TIL from HNSCC patients, rested in 30% Human AB serum overnight and then subjected to re-stimulation experiments. Sorted TIL were cultured with Dynabeads® Human T-Activator CD3/CD28 beads at a fixed cell: bead ratio of 1:5. The cultures were incubated at 37°C with

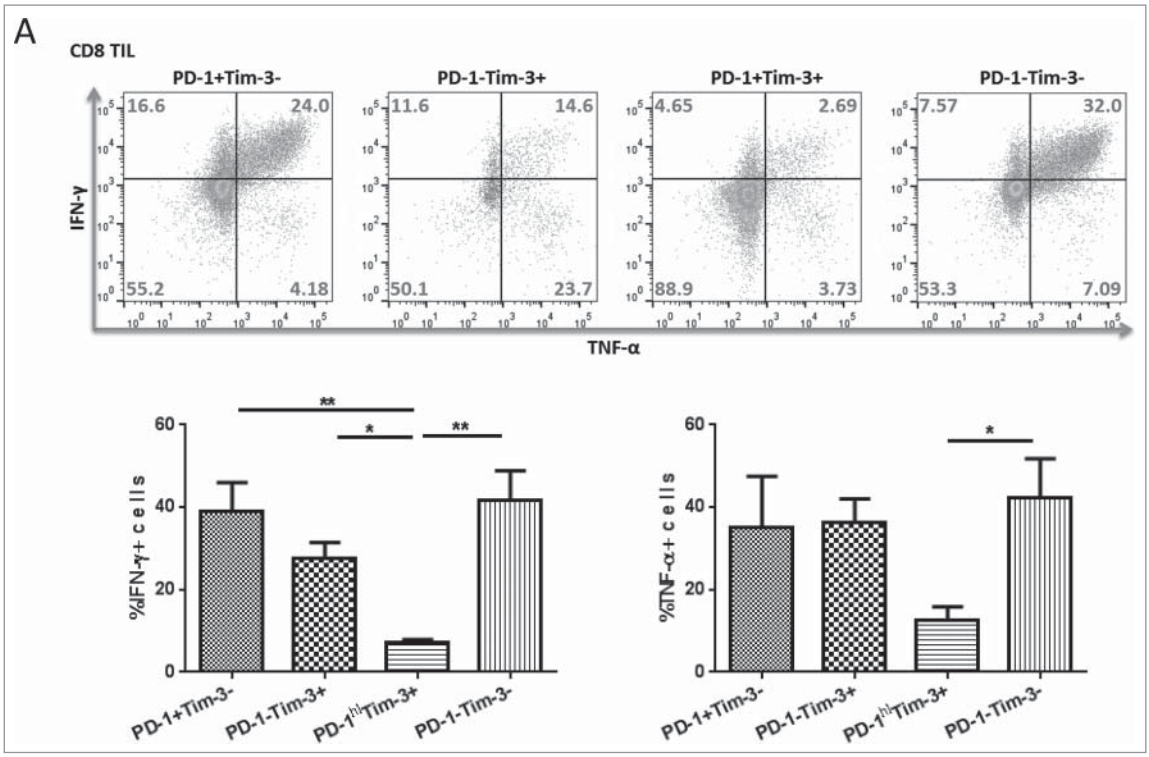

Figure 1. PD-1$^+$Tim-3$^+$ TIL subsets are dampened in IFNγ and TNF-α secretion upon TCR stimulation. Freshly isolated TIL from HNSCC patients were sorted based on PD-1, Tim-3 expression. Sorted cells were stimulated *in vitro* with anti-CD3/CD28 beads and protein transport inhibitor (BD GolgiPlug™) for 6 h. Cells were then stained intracellularly for cytokines. (A) Intracellular staining results of TNFα and IFNγ in sorted CD8$^+$ T cells are shown. (n = 3) Significance were calculated by RM-one-way ANOVA, $^*p < 0.05$.

5% CO_2 for indicated time periods. Magnetic beads were removed before analysis.

Freshly isolated HNSCC TIL were cultured with Dynabeads® Human T-Activator CD3/CD28 beads at a fixed cell: bead ratio of 1:1 in the presence of protein transport inhibitor (BD GolgiPlug™) from BD Biosciences (San Jose, CA) at 37°C with 5% CO_2 for indicated time period, flow cytometry analysis is used for cytokine detections.

Murine in vivo tumor studies

Female C57BL/6 mice were purchased at 6 to 8 weeks from Jackson laboratories (Bar Harbor, Maine). On arrival, animals were acclimated for 48 h prior to MEER tumor cells (1×10^6 cell per mice at the back of neck) injections, after ketamine (90 mg/kg body weight), xylazine (4.5 mg/kg body weight) mediated anesthesia. All animals were maintained in a pathogen-free facility and cared for and used in accordance with protocol approved by the Institutional Animal Care and Use Committee (IACUC) University of Pittsburgh, which follows the US Public health Service's guide for the care and use of animals. Anti-PD-1 mAb (clone: 4H2) were given at 3 mg/kg starting from day 12 for five doses, anti-Tim-3 mAb (clone: F38–2E2) (eBioscience; San Diego, CA) were given at 5 mg/kg for four doses upon anti-PD-1 treatment completion. Tumor measurement was performed with Vernier Caliper (tumor volume = (Longest diameter^2 × smallest diameter)/2), and animals were killed when tumor size was larger than 20 mm in its greatest dimension, the animal was substantially emaciated, or pain or functional impairment was apparent.

Pharmacologic inhibition studies

TIL were cultured for 48 h in medium with Dynabeads® Human T-Activator CD3/CD28 beads at a fixed cell: bead ratio of 1:1 in the presence of either DMSO control or one of the five inhibitory drugs, which were dissolved in DMSO: LY294002 (Cell Signaling) a pan-PI3K inhibitor, Akt 1/2 (EMD Bioscience) an isozyme selective Akt inhibitor VIII, CAY10626 (Abcam) a PI3K α/mTOR inhibitor, GS-1101 (Abcam) PI3K p110δ inhibitor, AS-041164 PI3K p110γ inhibitor (Abcam) together with anti-PD-1 mAb (nivolumab, Bristol Myers-Squibb).

Results

PD-1$^+$Tim-3$^+$ marks exhausted TIL subsets indicated by dampened IFNγ and TNF-α production upon TCR stimulation

In order to define the functional exhaustion status of TIL based on PD-1 and Tim-3 expression,[1] we sorted freshly isolated TIL from HNSCC patients (n = 3) into four subsets: PD-1$^+$ Tim-3$^-$ (28% ± 6%), PD-1$^-$Tim-3$^+$ (23% ± 10%), PD-1$^+$Tim-3$^+$ (33% ± 14%), and PD-1$^-$Tim-3$^-$ (21% ± 14%) in CD8$^+$ T cells. Upon stimulation with anti-CD3/CD28 coated beads for 6 h, PD-1$^+$Tim-3$^+$ and PD-1$^-$Tim-3$^+$ TILs showed the least IFNγ and TNF-α production in both CD8$^+$ T cells (Fig. 1). Thus, PD-1$^+$Tim-3$^+$ T cells appeared to be the most dysfunctional HNSCC TIL subset,[18,20] and the expression of Tim-3 correlates with more prominent defects in functionality to single positive PD-1$^+$ cells. These results raised the question of whether, and how, co-expression of Tim-3 might be

modulated in response to blockade of the PD-1 pathway, which suffers from a relatively low single-agent response rate.[6,9,21]

Tim-3 is upregulated after PD-1 blockade in vitro in human TIL

Although anti-PD-1 therapy shows promising therapeutic results in patients with HNSCC, only a minority of patients experience therapeutic benefit.[8] Thus, we hypothesized that during PD-1 blockade, PD-1 and Tim-3 co-expression might maintain the exhaustion status of TIL, rendering the cells less responsive to PD-1 blockade. We first investigated expression of Tim-3 before and after *in vitro* PD-1 blockade. Total TIL extracted from freshly excised HNSCC tumors were incubated with anti-PD-1 mAb at 10 μg/mL for 24 h. As shown in Fig. 2A, cell surface expression of Tim-3 was increased by approximately 50% in $CD8^+$ T cells ($p = 0.0009$) and by 40% in $CD4^+CD25^{lo/-}$ effector T cells ($p = 0.003$). %Tim-3^+ T cells

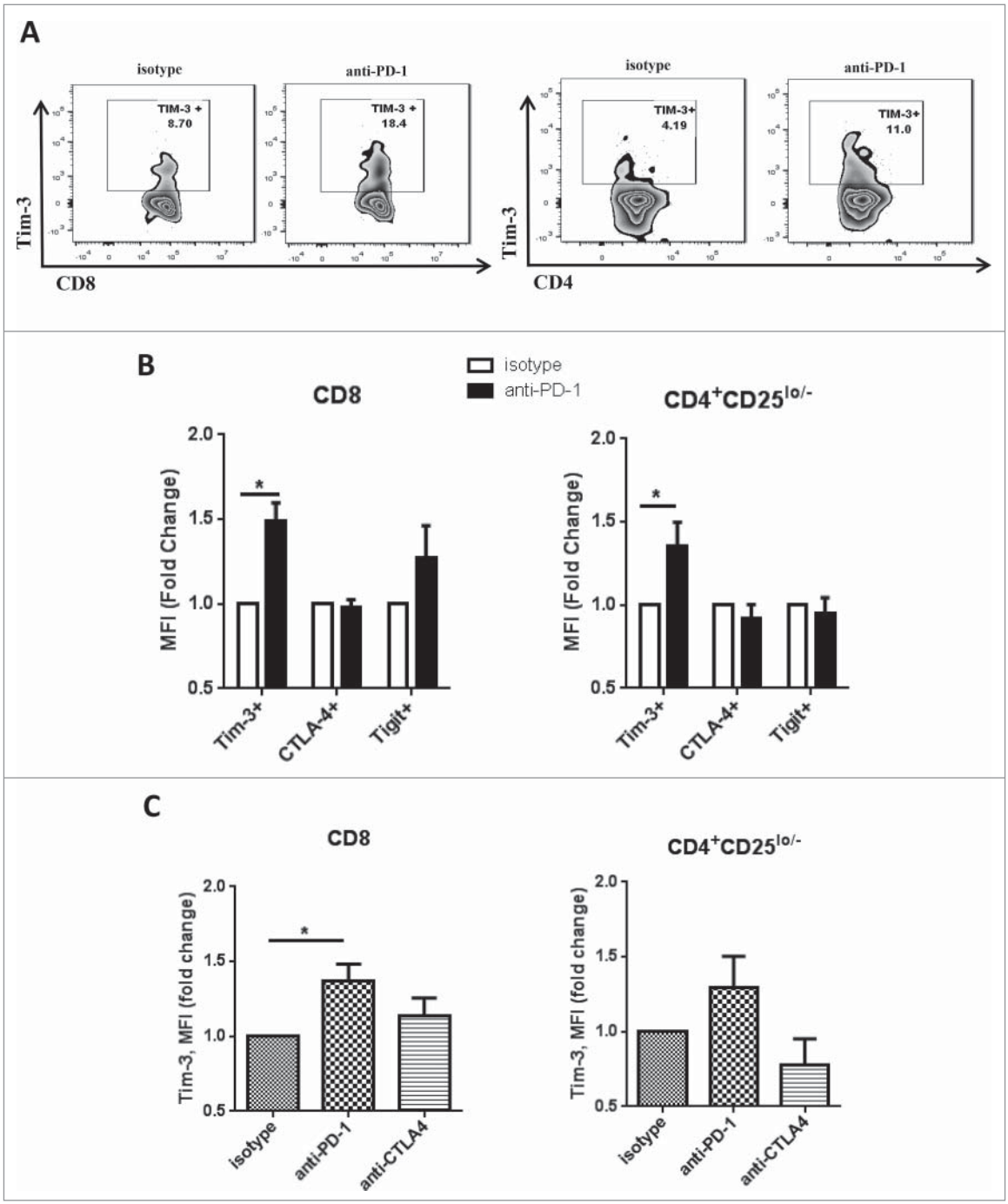

Figure 2. Tim-3 is upregulated after PD-1 blockade *in vitro* in human TIL. Freshly isolated tumor-infiltrating lymphocytes from HNSCC patient were treated with anti-PD-1 mAb—Nivolumab, or anti-CTLA-4 mAb—Ipilumumab, and IgG4/IgG1 as isotype control. All the treatments were given at the concentration of 10 μg/mL *in vitro* for 24 h. Tim-3, CTLA-4, TIGIT expressions were assessed by flow cytometry. (A) Representative flow plots showing Tim-3 expression in $CD8^+$ and $CD4^+CD25^{lo/-}$ cells. (B) Summary data showing MFI fold change of Tim-3, CTLA-4 and TIGIT. MFI of isotype control group is normalized to 1. (n = 6) Significance was calculated with multiple t test, $^*p < 0.05$. (C) Summary data showing Tim-3 MFI fold change in isotype, anti-PD-1(nivolumab) or anti-CTLA-4 (ipilumumab) treatment. (n = 5) Significance was calculated with RM-one-way ANOVA, $^*p < 0.05$. All data represent average ± SEM.

were also increased in both $CD8^+$ and $CD4^+CD25^{lo/-}$ effector TIL T cells ($p = 0.0006$) (Fig. S2B). Meanwhile, this effect was relatively selective for Tim-3, since upregulation of other checkpoint receptors such as CTLA-4 and TIGIT was not observed under these conditions (Fig. 2B and Fig. S2A). Furthermore, this upregulation of Tim-3 after anti-PD-1 mAb treatment was not observed in response to CTLA-4 blockade using ipilimumab at 10 ug/mL (Fig. 2C), despite baseline expression of CTLA-4 on the majority of these TIL.[22] This finding suggests that compensatory upregulation of Tim-3 might represent a specific adaptive response that functions to sustain the exhaustion status of TIL in response to PD-1 blockade.

PD-1 blockade upregulates IFNγ and TNF-α expression primarily in Tim-3- cells

In line with previous reports that blockade of the PD-1:PD-L1 pathway can significantly enhance antitumor immune responses,[7] we observed significant overall upregulation of IFNγ and TNF-α expression after *in vitro* PD-1 blockade, by both $CD8^+$ and $CD4^+CD25^{lo/-}$ effector T cells.[20] Based on our observation that PD-1 blockade induces Tim-3 upregulation, we investigated whether this upregulation of Tim-3 attenuates the Th1/Tc1 skewing promoted by anti-PD-1 treatment. As shown in Fig. 3A, $Tim\text{-}3^+$ cells displayed significantly higher baseline PD-1 expression compare with Tim-3- cells in both $CD8^+$ (80% vs. 57%, $p = 0.01$) and $CD4^+CD25^{lo/-}$ (70% vs. 48%, $p = 0.03$) T cells. Despite the higher level of PD-1 expression in $Tim\text{-}3^+$ cells, greater IFNγ ($p = 0.0003$) and TNF-α ($p = 0.0015$) production was observed during PD-1 blockade in Tim-3- cells. This finding suggests that augmented induction of Tim-3 expression after PD-1 blockade provides an escape mechanism to enforce dysfunction and exhaustion, since the T cells that manifested higher Tim-3 expression after PD-1 inhibition were more defective in Th1/Tc1 cytokine expression.

Anti-PD-1 mAb therapy upregulates Tim-3 expression in murine HNC model

HNSCC is highly associated with HPV16 infection,[23,24] and recent data indicate that HPV^+ HNC are more immunogenic and anti-PD-1 responsive.[7,9] In order to identify mechanisms of anti-PD-1 resistance and strategies to enhance this partial anti-PD-1 response, we used an immunocompetent, orthotopic murine HPV^+ HNC model. MEER (E6-E7-H-ras) transformed murine tonsillar carcinoma[25] syngeneic tumors were injected into C57/BL6 mice. Since we had observed that Tim-3 is upregulated in human TIL after *in vitro* PD-1 blockade, we investigated whether this upregulation also occurred *in vivo*. After tumor progression, mice were randomized to receive anti-PD-1 mAb or isotype mAb therapy (3 mg/kg), and tumor growth was measured every 3 d. Within 36 d, anti-PD-1 mAb treatment modestly decreased tumor growth ($p = 0.1$, isotype mAb vs. anti-PD-1 mAb). Analysis of the TIL from these mice showed that Tim-3 was significantly upregulated in both $CD8^+$ (average: 5% vs. 30%, $p = 0.00024$) and $CD4^+CD25^{lo/-}$ (average: 22% vs. 30%, $p = 0.04$) T cells from the anti-PD-1 mAb treated mice (Figs. 4A–C). Additionally, *ex vivo* anti-PD-1 treatment of splenocytes from these mice led to further induction of Tim-3 expression in both $CD8^+$ and $CD4^+CD25^{lo/-}$ T cells from both untreated and anti-PD-1 mAb treated mice (Fig. 4D). Since anti-PD-1 treatment failed to show significant therapeutic benefit in this murine HNSCC model, we hypothesized that tumor-infiltrating T cells may develop adaptive resistance to PD-1 blockade in part through compensatory upregulation of Tim-3 expression.

Sequential anti-Tim-3 treatment enhances therapeutic benefit of anti-PD-1 immunotherapy in a murine HNC model

Anti-PD-1 treatment alone showed only modest therapeutic effects in the murine HNC model (Fig. 5A). Based on our observation that Tim-3 was upregulated as a potential compensatory mechanism in response to PD-1 blockade, we administered anti-Tim-3 mAb treatment in mice whose tumors progressed during initial anti-PD-1 therapy. After tumor establishment on day 10, anti-PD-1 mAb was given every 2 d at 3 mg/kg for four doses, and a second group of mice received subsequent anti-Tim-3 treatment after completion of anti-PD-1 treatment. As shown in Fig. 5A, anti-PD-1 treatment alone showed a modest benefit ($p = 0.13$), whereas sequential anti-Tim-3 treatment significantly suppressed tumor growth, beginning at day 29 ($p = 0.02$) compare with untreated mice. We then wished to determine whether tumor-specific immunity was stronger in the setting of combination PD-1/Tim-3 blockade. Analyzing TIL from combination vs. single Ab-treated mice, we found that the HPV $E7_{49-57}$-tetramer-specific $CD8^+$ T cells were significantly upregulated in the sequential anti-Tim-3 treated group, suggesting that the delayed blockade of Tim-3 increased T cell activation against tumor antigens expressed in the tumor microenvironment. By analyzing immune phenotypes in $CD8^+$ TIL from all the treatment groups, we found that the proportion of $PD\text{-}1^{hi}$ (exhausted) T cells was significantly reduced in both anti-PD-1 treated and anti-PD-1/anti-Tim-3-treated TIL[17](Fig. S3A). Thus, tumor-infiltrating T cells in combination Ab-treated mice possessed stronger antitumor activity, and were more likely to restore functionality in response to anti-PD-1 therapy, compare with single anti-PD-1 mAb treated or untreated mice. We conclude that additional inhibitory immune checkpoint receptors (such as Tim-3) may be upregulated upon blockade of PD-1, supporting dual targeting of immune checkpoint receptors in order to maximize tumor-specific immunity.

Tim-3 upregulation upon PD-1 blockade requires activation of PI3K/Akt

Phosphoinositide3-kinases (PI3Ks) plays an important role in inflammatory response in both innate and adaptive immune cells by catalyzing the phosphorylation of inositol phospholipids in the 3-position of the inositol ring. In mammalian cells, there are four class I isoforms of PI3Ks (PI3Kα, β, γ, δ), they differ in catalytic subunit that synthesize the phospholipid "PIP3" which regulates cell movement, growth, survival and differentiation as a "second messenger." These four isoforms are further divided into two groups: class IA and class IB based

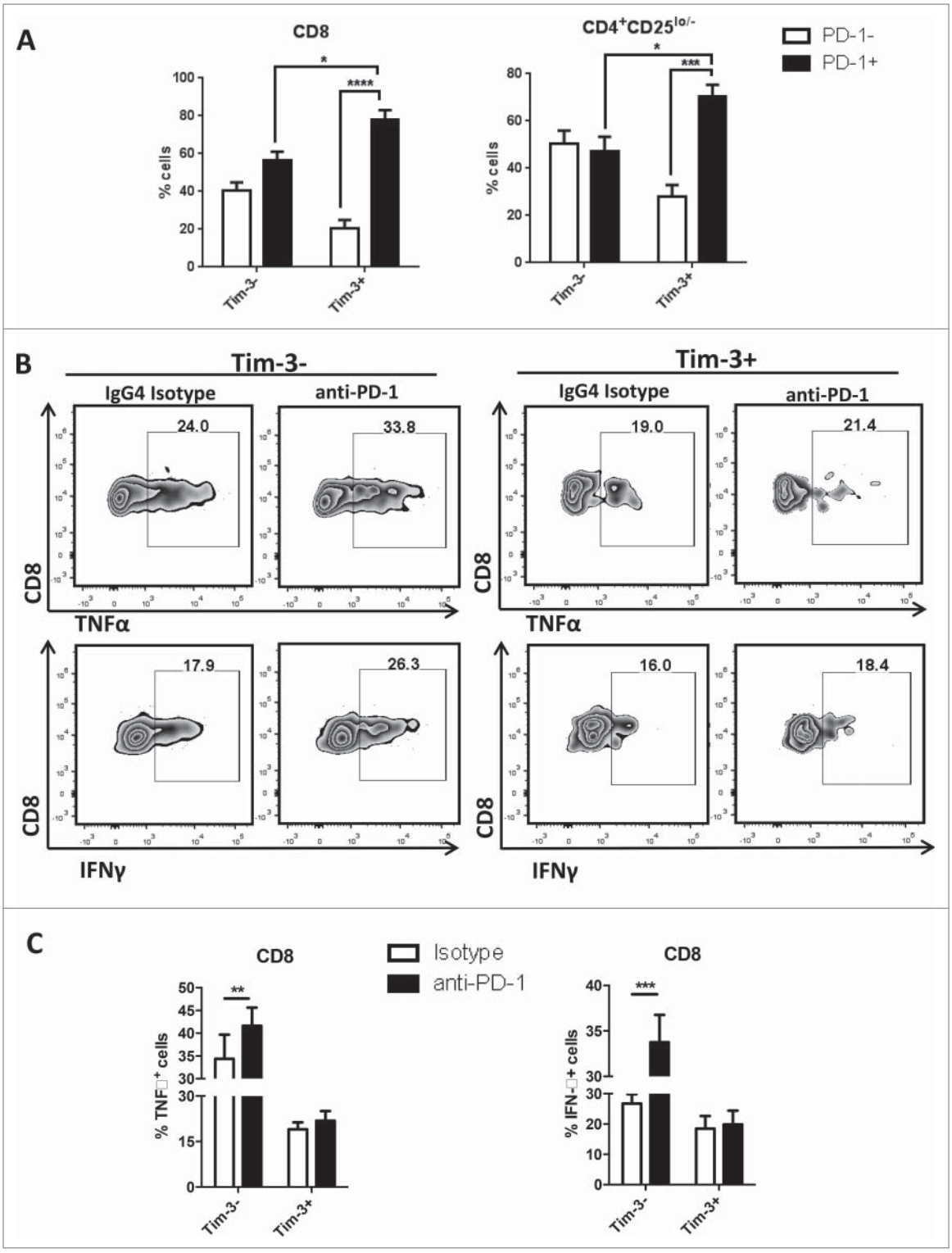

Figure 3. PD-1 blockade induced upregulation of IFNγ and TNF-α expression are mostly seen in Tim-3- cells. Freshly isolated tumor-infiltrating lymphocytes from HNSCC patient were treated with anti-PD-1 mAb—Nivolumab and IgG4 as an isotype control. All treatments were given at the concentration of 10 μg/mL *in vitro* for 48 h. Anti-CD3/CD28 beads and protein transport inhibitor (BD GolgiPlug™) were added according to manufacture's instruction at least 6 h of cell culture for cytokine detection. Cytokine expressions were assessed by flow cytometry. (A) Summary data of baseline PD-1 expression in Tim-3+ and Tim-3- subsets.(n = 5) (B) Flow plot of IFNγ, TNFα expression in Tim-3$^+$ and Tim-3$^-$ subsets. (C) Summary data of IFNγ, TNFα expression in Tim-3$^-$ and Tim-3$^+$ subsets. (n = 5) All data represent average ± SEM. Significance were calculated by two-way ANOVA, $^*p < 0.05$, $^{**}p < 0.001$.

on their structure and general mode of regulation. Class I PI3Ks catalyze same reaction with different kinetic parameters, the main distinction between the four isoforms is their adaptation to upstream regulation by receptor transduction pathways. Importantly, class IA PI3K isoforms (PI3Kα, β, δ) are adapted to regulation by receptors signal through protein tyrosin kinases, whereas class IB PI3K (PI3Kγ) is adapted to regulation by GPCRs via direct binding to G$\beta\gamma$ subunits.[26-29] Tim-3 expression, in response to certain cytokines, has been reported to be induced by activation of the PI3K pathway.[26] We previously

showed that pS6 is decreased in TIL after PD-1 stimulation,[30] potentially linking PD-1 to the PI3K-Akt-mTOR pathway. Since TCR/CD28 co-stimulation leads to PI3K/Akt activation, and PD-1 ligation causes inhibition of TCR proximal signaling through SHP-2[27], leading to decreased PI3K activity, we hypothesized that Tim-3 upregulation after PD-1 blockade might be caused by increased PI3K activity due to de-repression by PD-1 blockade.

To test this hypothesis, we used PI3K p110 subunit-selective inhibitors to determine if the upregulation of Tim-3 upon PD-1 is due to enhanced TCR proximal signaling. Among the four isoforms of PI3Ks, p110α, p110γ and p110δ are reported

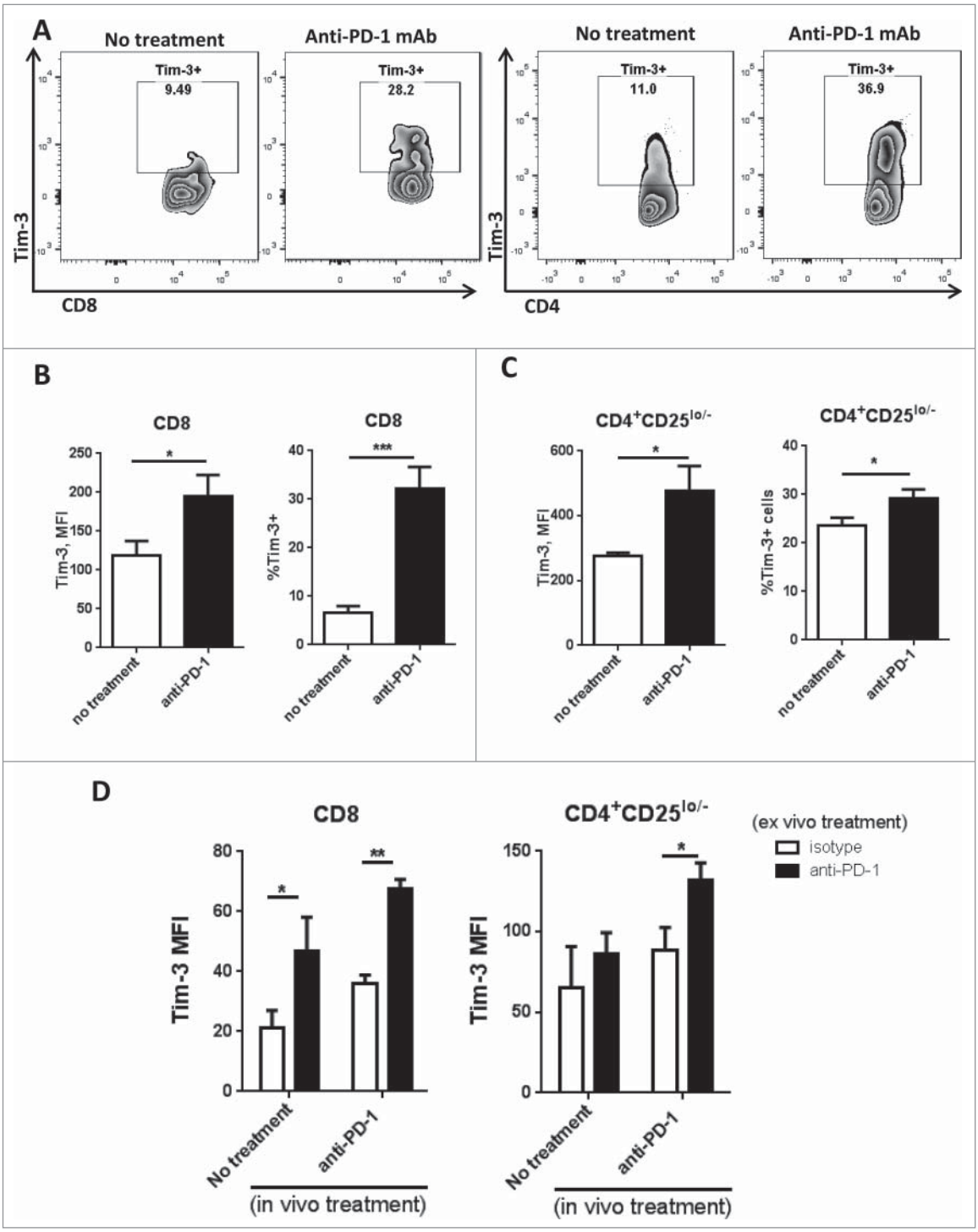

Figure 4. Tim-3 is upregulated in TIL after *in vivo* anti-PD-1 treatment, and is further upregulated upon *in vitro* PD-1 blockade in murine HNC model. C57BL/6 mouse were injected 1×10^6 MEER cells in the neck, anti-mouse PD-1 mAb was given as treatment; all the mouse were scarified around day 36 after injection of tumor cells. Freshly isolated tumor-infiltrating lymphocytes from murine HNC model were analyzed for Tim-3 expression by flow cytometry. (A) Representative flow plots of Tim-3 expression pattern in no treatment group and anti-PD-1 treated group were shown in $CD8^+$ and $CD4^+CD25^{lo/-}$ T cells. (B, C) Summary data of Tim-3 MFI and %Tim-3^+ cells in tumor-infiltrating $CD8^+$ and $CD4^+CD25^{lo/-}$ T cells. (n = 6) Significance were calculated by unpaired *t* test, $^*p < 0.05$, $^{**}p < 0.001$. (D) Freshly isolated splenocytes were incubated with anti-PD-1 mAb or IgG4 for 48 h and Tim-3 expression was assessed by flow cytometry. Summary data of Tim-3 MFI in $CD8^+$ and $CD4^+$ T cells from splenocytes.(n = 6) Significance were calculated by two-way ANOVA, $^*p < 0.05$, $^{**}p < 0.001$.

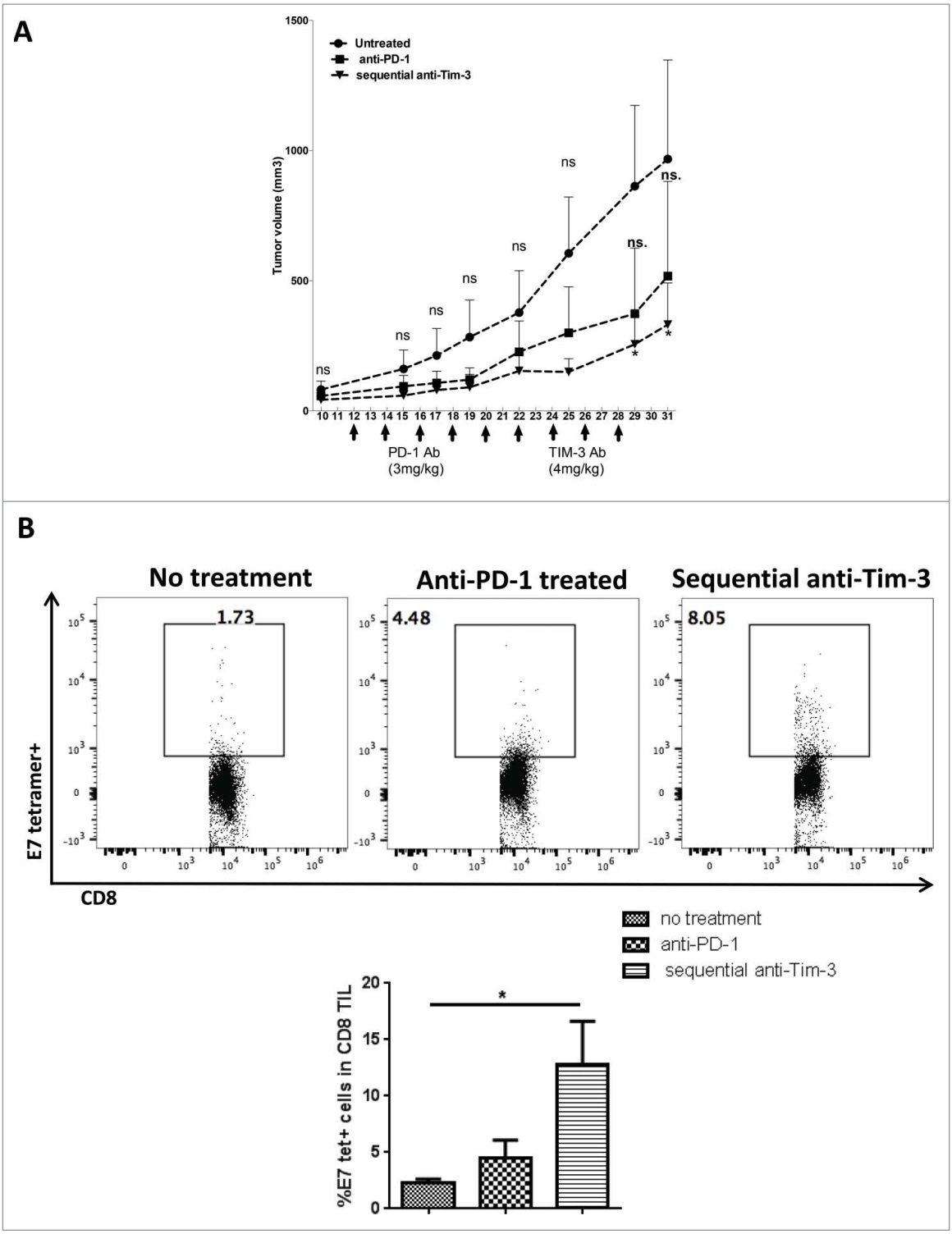

Figure 5. Sequential anti-Tim-3 treatment suppresses tumor growth and show better antitumor immune response. C57BL/6 mouse were injected 1×10^6 MEER cells in the neck, after tumor progression (day 10) anti-mouse PD-1 mAb was given at five doses every 2 d, sequential anti-Tim-3 mAb treatment were given to one group of mice starting from day 22 for four doses every 2 d. Tumor volume were measured every 2 d, Mice TIL were harvested at day 31, and HPV E7-tetramer and PD-1 expression were assessed by flow cytometry. (A) Tumor growth curve up to day 31 was shown, (n = 5) significance was calculated by two-way ANOVA, $^*p < 0.05$. (B) Representative flow plots and summary data showing %E7-tetramer$^+$ cells in CD8$^+$ T cells. (n = 5) Significance was calculated by ordinary one-way ANOVA, $^*p < 0.05$.

to have important roles in mediating immune cell activation.[28] Specifically, p110γ and p110δ are critical for T cell activation, where p110δ mediates T cell activation through TCR signaling, and p110γ is more likely to be mediating signaling downstream of chemokine receptors.[29] We used a broad-spectrum PI3K inhibitor (LY294002), class IA PI3K inhibitors: PI3K p110δ subunit inhibitor (GS-1101), PI3K p110α/mTOR inhibitor (CAY10626), and a class IB PI3K inhibitor: selective p110γ inhibitor (AS-041164). TIL were incubated with each inhibitor in the presence of anti-CD3/CD28 bead stimulation for 48 h, and Tim-3 expression was assessed using flow cytometry. As shown in Fig. 6A, TCR stimulation increased baseline Tim-3

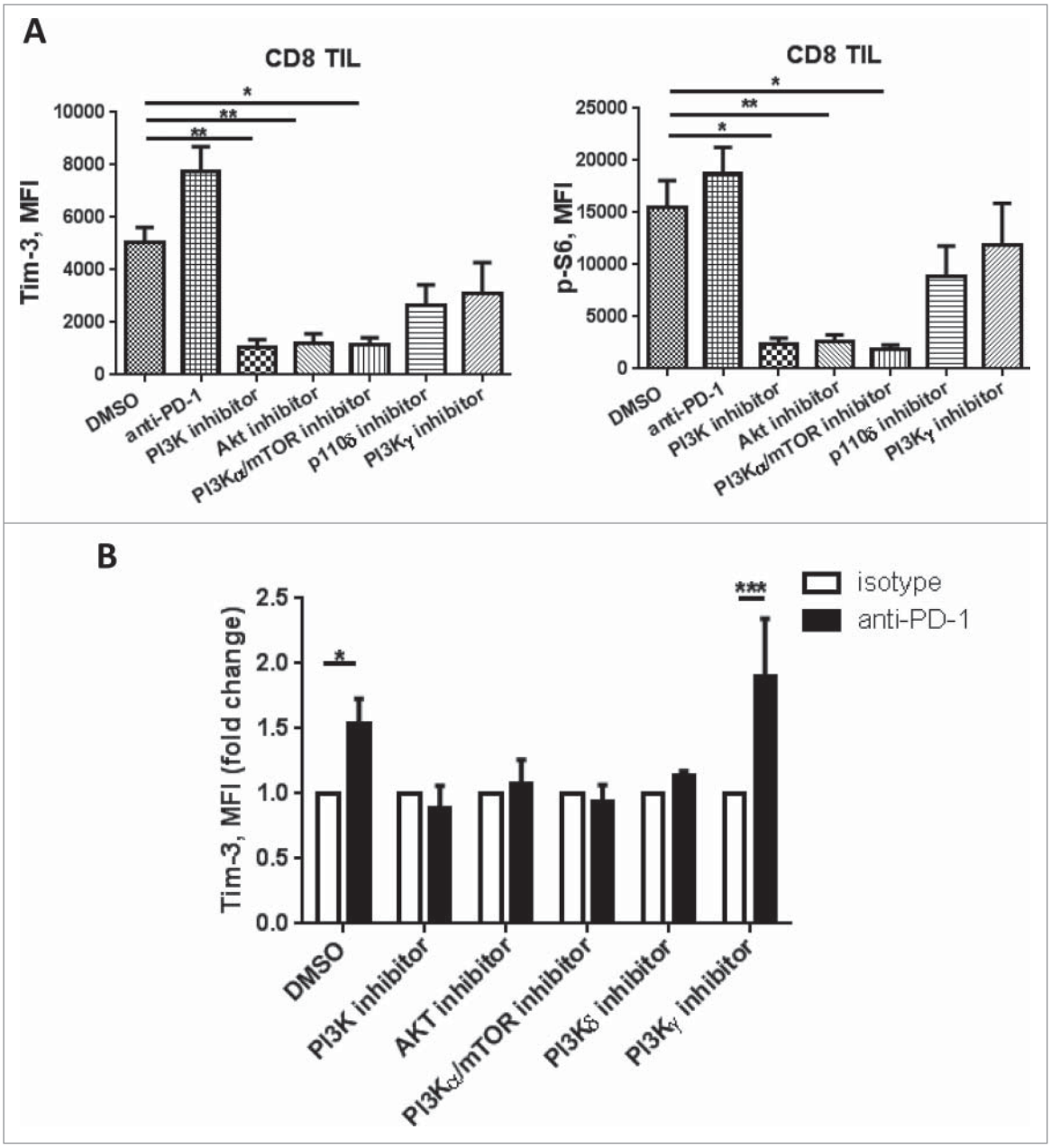

Figure 6. Tim-3 upregulation after PD-1 blockade is PI3K/Akt pathway dependent. TIL from HNSCC patient were treated with anti-CD3/CD28 beads in combination with DMSO, broad PI3K inhibitor (LY294002), PI3K p110α/mTOR inhibitor (CAY10626), Akt inhibitor, PI3K p110δ inhibitor (GS-1101) and PI3K p110γ inhibitor (AS041164) with or without anti-PD-1 mAbs (Nivolumab) for 48 h. Cells are collected and Tim-3, p-S6 expressions were assessed by flow cytometry. (A) Summary data showing Tim-3 and p-S6 MFI, (n = 4), significance were calculated with ordinary one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.001$. (B) Summary data showing Tim-3 MFI fold change in the presence of selective PI3K inhibitors with anti-PD-1 or IgG4. (n = 4) Significance was calculated with unpaired *t* test, $^{*}p < 0.05$.

expression, and addition of anti-PD-1 mAb further induced Tim-3 upregulation. All the class IA PI3K subunit inhibitors and Akt inhibitor had similar effects on abrogating upregulation of Tim-3 expression as the broad-spectrum PI3K inhibitor upon TCR stimulation. This result is in line with previous findings[26] that activation of the PI3K/Akt pathway mediates upregulation of Tim-3 in response to cytokines.

Next, we tested whether the upregulation of Tim-3 upon PD-1 blockade is PI3K/Akt dependent. As shown in Fig. 6B, addition of anti-PD-1 mAb further upregulated baseline Tim-3 expression in the presence of TCR activation ($p = 0.003$). This effect was abrogated in the presence of the broad PI3K inhibitor (LY294002), class IA PI3K inhibitors: PI3K p110α/mTOR inhibitor (CAY10626), PI3K p110δ inhibitor (GS-1101); and Akt inhibitor, while the upregulation effect is still seen in the presence of the class IB PI3K p110γ (AS-041164) ($p = 0.04$) inhibitor. We conclude that upon PD-1 blockade, upregulation of Tim-3 is mediated by enhanced TCR-proximal signaling to the PI3K/IAkt/mTOR pathway. Based on a previous report that selective PI3K or Akt inhibition results in enhanced antitumor immune response in a HPV+ TC-1 mouse model in a Treg-dependent manner without attenuating conventional T cell activity,[31] our work raises a potential therapeutic strategy of combining anti-PD-1 mAb with selective PI3K/Akt inhibition to promote antitumor immunity by overcoming compensatory Tim-3 upregulation.

Discussion

The PD-1:PD-L1 pathway is upregulated in HNSCC and anti-PD-1 therapy has shown some therapeutic benefit in approximately 20% of patients.[8] However, the majority of patients still fail to benefit from anti-PD-1 treatment, and the underlying mechanism of resistance to PD-1 blockade is not fully understood. In this study, we show that Tim-3 expression is upregulated in response to PD-1 blockade on both murine and human T cells, an effect that appears to be mediated through enhanced PI3K/Akt/mTOR signaling. We observed that PD-1^{hi}Tim-3^{+} T cells are the most dysfunctional subset in HNSCC TIL, which is in line with previous reports in melanoma[15] and leukemia.[17] In addition, blockade of PD-1 led to Tim-3 upregulation on HNSCC TIL both *in vitro* and *in vivo*. In order to clarify if the upregulation of Tim-3 expression is a sign of T cells dysfunction, we investigated Th1 cytokine production after PD-1 blockade in Tim-3^{+}/$^{-}$ subsets. Anti-PD-1 treatment induced upregulation of IFNγ and TNF-α in Tim-3- cells. Despite the expansion of Tim-3^{+} T cells, these inflammatory cytokines were not further induced upon PD-1 blockade. At this point, we cannot specifically define the cells which initially are responsible for IFNγ production. Indeed, Tim-3^{+} cells after anti-PD-1 blockade may originate from Tim-3 negative cells at the start of the activation conditions (Fig. 3).

HPV^{+} HNSCC has a high incidence rate and HPV^{+} cancers are generally immunogenic.[23,25] And it has been reported that PD-1 and Tim-3 expressions are enriched in HPV^{+} HNSCC.[32,33] We used a murine orthotopic HNSCC model expressing HPV16 E6 and E7 oncogenes[25] to investigate the effect of anti-PD-1and anti-Tim-3 therapy on antitumor immunity. Tim-3 upregulation was observed in TIL of the *in vivo* anti-PD-1-treated mice, and *ex vivo* PD-1 blockade on splenocytes further induced Tim-3 expression. Sequential anti-Tim-3 treatment following PD-1 blockade significantly suppressed tumor growth. The observation of enriched antigen-specific CD8^{+} cytotoxic T cells with expansion of PD-1^{int} CD8^{+} TIL, together with a reduction of PD-1^{hi} cells, indicates that sequential anti-PD-1/anti-Tim-3 treatment induced a more robust antitumor immune response in the orthotopic murine HNC model.

Our findings corroborate a recent report that Tim-3 is significantly upregulated in response to PD-1 blockade in lung cancer.[34] Also, other groups have shown that genetic PD-1 depletion led to a more exhausted T cell phenotype, marked by upregulation of multiple inhibitory receptors, including Tim-3.[35] Our work extends these findings, revealing a possible mechanism underlying Tim-3 upregulation in the setting of PD-1 blockade. Multiple studies have shown that upon ligation, PD-1 downstream signaling pathway suppresses TCR proximal signaling through SHP-2 phosphorylation[36] and increased PTEN phosphatase activity.[37] Interestingly, Tim-3 expression has been shown to be induced through Akt and T-bet,[38] downstream of cytokine receptor signaling. Thus, we hypothesized that the Tim-3 upregulation induced upon PD-1 blockade may be caused by reduced suppression of the PI3K/Akt/mTOR pathway. By using selective PI3K subunit inhibitors, we find that Tim-3 upregulation can still be observed in the presence of class Ib PI3K inhibitor: p110γ inhibitor, where the inhibition of class Ia PI3K-mediated Akt/mTOR pathway abrogated the upregulation effect of Tim-3 in the setting of PD-1 blockade. Our study is in line with a previous report linking Tim-3 expression to activation of PI3K pathway. However, we studied this mechanism in short-term *in vitro* settings. Further investigation is needed to elucidate the *in vivo* mechanism(s) underlying the Tim-3 upregulation observed in response to PD-1 blockade, and the clinical significance of this upregulation in anti-PD-1 treated patients.

We conclude that upon PD-1 blockade in HNSCC, Tim-3 expression is upregulated more significantly, compare with other inhibitory ICRs such as CTLA-4 and TIGIT, and that this upregulation is dependent, at least in part, on activation of PI3K/Akt pathway. However, further observations and experiments will be necessary to document that PD-1 blockade induced Tim-3 upregulation is responsible for adaptive resistance, as our *in vivo* data suggest, preferably using treated cancer patients. Overall, our study provides a rational combination therapeutic strategy in the treatment of HNSCC, supporting dual or multiple inhibitory receptors targeting, possibly including transient small molecular inhibition of the PI3K pathway.

Disclosure of potential conflicts of interest

Robert L. Ferris: consulting or advisory role: AstraZeneca, Bristol- Myers Squibb, Merck, ONO Pharmaceutical and Celgene. Research funding: Amgen (Inst), Bristol-Myers Squibb (Inst.) AstraZeneca (Inst.) and VentiRx (Inst.)

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