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益田, 昌吾

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Shogo Masuda[1,2], Hideya Onishi[1], Naoya Iwamoto[1], Akira Imaizumi[1], Satoko Koga[1], Shinjiro Nagao[1], Keita Sakanashi[1], Shinsaku Itoyama[1,2], Akiko Fujimura[1,2], Noritaka Komune[2], Ryunosuke Kogo[2], Masayo Umebayashi[3], Takashi Morisaki[3], and Takashi Nakagawa[2]

[1]Department of Cancer Therapy and Research
[2]Department of Otorhinolaryngology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
[3]Fukuoka General Cancer Clinic, Fukuoka, Japan

Correspondence to: Hideya Onishi, Department of Cancer Therapy and Research
Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku
Fukuoka 812-8582, Japan
Tel: +81-92-642-6220
Fax: +81-92-642-6221
E-mail: ohnishi@surg1.med.kyushu-u.ac.jp

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Abstract

In the tumor microenvironment, wherein cytotoxic lymphocytes interact with cancer cells, lymphocyte exhaustion, an immune checkpoint inhibitor target, is promoted. However, the efficacy of these inhibitors is limited, and improving response rates remains challenging. We previously reported that protein tyrosine phosphatase nonreceptor type (PTPN) 3 is a potential immune checkpoint molecule for activated lymphocytes and that PTPN3 inhibition should be a focus area for cancer immunotherapy development. Therefore, in this study, we focused on PTPN3 suppressive therapy in terms of lymphocyte exhaustion under hypoxic conditions, which are a cancer microenvironment, and investigated measures for improving the response to anti-programmed death receptor (PD)-1 antibody drugs. We found that PTPN3 expression was upregulated in activated lymphocytes under hypoxic conditions, similar to the findings for other immune checkpoint molecules, such as PD-1, T cell immunoglobulin mucin-3, and lymphocyte-activation gene-3; furthermore, it functioned as a lymphocyte exhaustion marker. In addition, PTPN3-suppressed activated lymphocytes promoted mammalian target of rapamycin (mTOR)-Akt signaling pathway activation and enhanced proliferation, migration, and cytotoxic activities under hypoxic conditions. Furthermore, PTPN3 suppression in activated lymphocytes increased PD-1 expression and enhanced the antitumor effects of anti-PD-1 antibody drugs against head and neck cancer in vitro and in vivo. These results suggest that the suppression of PTPN3 expression in activated lymphocytes enhances the therapeutic effect of anti-PD-1 antibody drugs in head and neck cancer, especially under hypoxic conditions that cause lymphocyte exhaustion.

Keywords: PTPN3, Cancer immunotherapy, Hypoxia, Head and neck cancer, Nivolumab

INTRODUCTION

Immune checkpoint inhibitors, such as nivolumab, an anti-programmed death receptor (PD)-1 antibody drug that targets lymphocyte exhaustion, have considerably advanced cancer therapy [1,2]. While these immune checkpoint inhibitors have elicited long-term clinical responses, including cures, in several histologic types of tumors, some histologic types of tumors do not respond to these immune checkpoint inhibitors [3]. Therefore, to improve response rates, various kinds of approaches have been explored. These include the use of biomarkers for selecting appropriate immune checkpoint inhibitors for each patient, the development of new immune checkpoint inhibitors, and the conduct of clinical trials of new combination therapies that involve existing anticancer agents and immune checkpoint inhibitors [3]. In the case of head and neck cancer, the sixth most common cancer worldwide, [4], the

efficacy of nivolumab and pembrolizumab in patients with recurrent or metastatic squamous cell carcinoma was shown in the CheckMate 141 and KEYNOTE-048 trials; these drugs are thus widely used [5, 6]. However, the response rate of these drugs as single agents is only 10%–20%, which requires improvement [5, 6].

Cancer microenvironments, such as hypoxic environments, chronic cancer antigen stimulation, low nutritional status, and metabolic abnormalities have been implicated in lymphocyte exhaustion [7]. Among these, we previously focused on the hypoxic environment and reported the malignant transformation of cancer cells under these conditions [8-10]. In vivo oxygen concentrations differ from those under normoxic conditions: blood, 10%–12.5%; normal tissues, 3%–6%; and tumor tissues, less than 2% [11,12]. Because of this, it should be carefully noted that results from in vitro studies under normoxic conditions may differ from actual in vivo results. In head and neck cancers, increased hypoxic regions within tumors have been reported to be associated with chemoradiotherapy resistance, decreased immune cell infiltration, and decreased overall survival [13]. Under hypoxic conditions, T cells show suppressed functions, primarily in relation to proliferative capacity, and increased expression of multiple inhibitory receptors, such as PD-1, T cell immunoglobulin mucin (TIM)-3, and lymphocyte-activation gene (LAG)-3 [14-17]. Although immune checkpoint inhibitors may be effective in restoring lymphocyte exhaustion, increased hypoxic areas have been reported to be associated with resistance to anti-PD-1/PD-L1 antibody drugs in preclinical models and clinical specimens of head and neck cancer [18,19]. Therefore, the hypoxic environment is an important factor to be considered in studies on immune checkpoint inhibitor therapy.

Protein phosphatases have attracted increasing attention as targets in cancer immunotherapy [20]. In eukaryotes, protein phosphatases reverse the phosphorylation of proteins using protein kinases and are crucial regulators of intracellular processes related to the maintenance of cellular homeostasis [21]. We focused on protein tyrosine phosphatase nonreceptor type (PTPN) 3, a member of the protein tyrosine phosphatase family that has been reported to negatively regulate T cell receptor (TCR) signaling in T cells by dephosphorylating tyrosine residues [22]. In our previous study, we reported that suppression of PTPN3 in activated lymphocytes increased their proliferative capacity, migration ability, and cytotoxic activity and that PTPN3 could act as an immune checkpoint [23].

In the current study, we clarified the function of PTPN3 as a lymphocyte exhaustion marker and its interrelationship with other immune checkpoint molecules in terms of lymphocyte exhaustion under hypoxic conditions, a cancer microenvironment. We also evaluated the function of PTPN3 suppression-activated lymphocytes under the same conditions. Based on these results, we investigated the possibility of combining immunotherapy involving anti-PD-1 antibody drugs with PTPN3-suppressive therapy for head and neck cancer to further improve the antitumor effect of existing immune checkpoint inhibitors.

MATERIALS AND METHODS

Induction of Lymphocyte Activation

Peripheral blood mononuclear cells (PBMCs) were obtained via density gradient centrifugation performed using HISTOPAQUE-1077 (Merck KGaA, Darmstadt, Germany); blood samples were obtained from healthy volunteers in heparin-containing blood collection tubes. PBMCs were incubated in Roswell Park Memorial Institute (RPMI)-1640 (Nacalai Tesque, Kyoto, Japan) with 0.5% human albumin, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Nacalai Tesque), and 200 U/mL interleukin (IL)-2 (Primmune, San Diego, CA, USA); suspended in culture medium; and cultured in 6-well plates coated with anti-CD3 (OKT3; BioLegend, San Diego, CA, USA) and anti-CD28 (BioLegend) antibodies. Cultures under normoxic conditions were performed at 37 °C in 5% CO_2 and 95% air, and cultures under hypoxic conditions were performed in a multigas incubator (Sanyo, Osaka, Japan) at 37 °C in 1% O_2, 5% CO_2, and 94% N_2 for 3–7 days.

All experiments involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards and were approved by the Kyushu University Ethics Committee (permit number: 2021-465). Informed consent for the use of PBMCs for research and publication was obtained from all participants in the study prior to blood collection.

RNA interference (RNAi)

The nucleotide sequence of the short hairpin RNA (shRNA) targeting PTPN3 was as follows: GACAGCTACTTAGTCTTGATCCGTA. According to the manufacturer's protocol, the oligonucleotides were ligated into a plasmid vector (pcDNATM6.2-GW/Em-miR, #K4934-00; Thermo Fisher Scientific, Waltham, MA, USA), which was then transfected into the 293FT cell line (Thermo Fisher Scientific) using Lipofectamine 2000 (Thermo Fisher Scientific) together with the ViraPower™ Packaging Mix (included in BLOCK-iT™ Lentiviral Poll l miR RNAi Expression Systems; Thermo Fisher Scientific). The culture supernatant was collected, filtered through a 0.22 μm filter (Millex-GV, Merck), and added to the target cells together with LentiBlast Premium (OZ Biosciences, Marseille, France) as a lentiviral vector.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using a High Pure RNA Isolation Kit (Roche, Basel, Switzerland) and quantified using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific). RNA (1.0 μg) was reverse-transcribed to cDNA by using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. According to the manufacturer's protocol, real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) with the PowerUp SYBR Green Master Mix (Applied Biosystems). PCR primers for human PTPN3

(PrimePCR™ Probe Assay: PTPN3, Human) were purchased from Bio-Rad (Hercules, CA, USA). β-actin PCR primer sequences were as follows: forward, 5′-TTGTTACAGGAAGTCCCTTGCC-3′; reverse, 5′ATGCTATCACCTCCTGTG-3′. The target gene expression level for each sample was normalized to that of β-actin for the same sample by using the ΔΔCt value method.

Flow Cytometry Assay

Cell surface antigens were stained using fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and anti-CD69 antibodies (BD Pharmingen, San Diego, CA, USA) or phycoerythrin (PE)-conjugated anti-PD-1, anti-TIM-3, anti-LAG-3, anti-NKG2D, and Fas-L antibodies (BD Pharmingen), as previously described [23]. In the proliferation experiment, activated lymphocytes were labeled with CFSE (CellTrace CFSE Cell Proliferation Kit for flow cytometry, Thermo Fisher Scientific) according to the manufacturer's protocol. The labeled cells were incubated for 2 or 3 days before flow cytometry analysis.

The intracellular staining method for phosphorylated antigens in lymphocytes (phosphoflow method) is briefly described below. Cultured cells were collected and resuspended in 1×10^6 cells/50 μL RPMI-1640, conjugated with anti-CD3 and anti-CD28 antibodies on ice, and stimulated in a 37 °C water bath for 2 or 5 min. The reaction was stopped by fixing with 4% paraformaldehyde for 10 min (room temperature), followed by permeabilization with 99% cold methanol for 30 min (on ice). Primary antibodies against phosphorylated antigens were prepared for pAkt (1:100, Ser473; No. 9271; Cell Signaling Technology, Danvers, MA, USA), pAkt (1:100, Thr308; No. 4056; Cell Signaling Technology), pS6 (1:100, No. 2211; Cell Signaling Technology).

For intracellular PTPN3 staining, the cells were collected, fixed, and permeabilized without antibody restimulation and were conjugated with anti-PTPN3 antibody (1:100, sc-9789; Santa Cruz Biotechnology, Dallas, TX). Alexa Fluor 488-conjugated antibody (1:100, A11001; Thermo Fisher Scientific) was used as the secondary antibody. The average fluorescence intensity or cell positivity was measured using a FACSCalibur flow cytometer (BD Pharmingen) and analyzed using CELLQuest (BD Pharmingen). Activated lymphocyte gating was based on forward- and side-scattered light as previously described [23].

Cancer Cell Culture

FaDu, a human hypopharyngeal squamous cell carcinoma cell line, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque) supplemented with 10% fetal calf serum (Thermo Fisher Scientific), 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque).

Proliferation Assay

Proliferation assays were performed by culturing lymphocytes in 24-well plates coated with anti-CD3 and anti-CD28 antibodies at 1×10^6 cells/mL per well in three wells under normoxia or hypoxia, and counting the number of cells on each day for 4 days.

Migration Assay

Lymphocyte migration capacity was analyzed using time-lapse images obtained with the JuLI FL system (NanoEnTek, Seoul, Korea); the random migration distance of any lymphocyte per hour was measured using DIPP-Motion V ver 1.2.5 (DITECT, Tokyo, Japan).

Cytotoxicity Assay

Target cells (FaDu) were seeded in 96-well flat-bottomed plates (Thermo Fisher Scientific) and allowed to adhere overnight; effector cells (activated lymphocytes) were added at an effector:target (E:T) ratio of 10:1, and target cells and effector cells were cocultured for 24 h, as reported in a previous study [23]. Viable cancer cells were quantified by adding the viable cell assay reagent SF (WST-8; Nacalai Tesque) and measuring the absorbance after 1 h, as previously reported [23]. Briefly, the absorbance of viable cancer cells was calculated by subtracting the absorbance of the lymphocyte monoculture sample from that of the coculture sample.

Xenograft Tumor Mouse Model

Four-week-old BALB/c-nu female mice were purchased from Charles River Laboratories Japan (Japan) (15 mice in total, three mice per group). After the mice were acclimated for 10 days, tumor cells—1×10^6 FaDu cells suspended in 100 μL of a 1:1 mixture of DMEM (Nacalai Tesque) and Matrigel (Corning, Corning, NY, USA)— were subcutaneously injected into both flanks of mice, and the tumors were transplanted. From day 4 after tumor implantation, the treatment group received twice weekly intraperitoneal injections of each type of treated lymphocyte (shcontrol or shPTPN3) suspended in RPMI-1640 (100 μL), nivolumab (200 μg/100 μL; anti-PD-1 antibody; Ono Pharmaceutical Co., Ltd., Osaka, Japan), or saline (100 μL) once a week, and the tumor diameter was measured. The tumor volume was calculated using the following formula: $A \times B^2 \times 0.5$, where A is the largest tumor diameter and B is the vertical tumor diameter. Mice were euthanized 20 days after subcutaneous tumor cell injection, and the tumors that formed were resected for histological analysis. All animal care methods and experimental procedures were performed in accordance with the Kyushu University Animal Experiment Regulations, and animal experiments were approved by the Kyushu University Animal Experiment Committee (permit number: A21-332-1). Mice were bred and maintained at the specific pathogen-free animal facility of Kyushu University, and care was taken to minimize the number of animals used and their suffering. All experiments were conducted in strict accordance with the Guidelines for the Proper Conduct of Animal Experiments (Science Council of

Japan).

Immunofluorescence Staining

Tumor tissues were excised from the mice, fixed in formalin (Mildform 10NM, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and embedded in paraffin. Immunofluorescence staining was performed as previously described [24]. Briefly, staining was performed as follows: the slides were deparaffinized with xylene and rehydrated with alcohol. Antigen activation was performed by boiling the slides under high pressure in Target Retrieval Solution (pH 6.0; DAKO, Tokyo, Japan) for 10 min. Next, blocking was performed with 1% bovine serum albumin at room temperature for 10 min. The slides were reacted with PE-conjugated anti-CD8 antibody (BD Pharmingen) and Alexa Fluor 488-conjugated CA-IX antibody (bs-4029R-A488; Bioss Antibodies, Woburn, MA) at 4 °C and mounted with nuclear staining inclusion agent (DAPI-Fluoromount-G; Southern Biotechnology, Birmingham, AL, USA). The slides were observed under a fluorescence microscope (Axio Imager A1, Carl Zeiss, Oberkochen, Germany). The number of stained $CD8^+$ cells in 10 randomly selected regions from the samples was measured. Cell counts were measured using ImageJ 1.53 (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data have been presented as the mean ± SD. Student's *t*-test was used to compare means between two groups, and p-values less than 0.05 were considered significant. For in vivo experiments, the incidence of tumor volume of 150 mm^3 or greater was plotted using the Kaplan–Meier method and analyzed using the log-rank test. Statistical analysis was performed using JMP 13 (SAS Institute Japan, Tokyo, Japan) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

RESULTS

PTPN3 Expression in Activated Lymphocytes was Greater under Hypoxic than under Normoxic Conditions and was Associated with Other Immune Checkpoint Molecules

Lymphocyte exhaustion marker levels are elevated under hypoxic conditions [14,15]. Therefore, to analyze PTPN3 expression in activated lymphocytes under hypoxic conditions, PBMCs obtained from healthy volunteers were activated with IL-2 and anti-CD3 and anti-CD28 antibodies for 2 days, as previously reported [23]. The activated lymphocytes were then cultured under normoxic or hypoxic conditions. The expression of PTPN3 and other lymphocyte exhaustion markers (PD-1, TIM-3, and LAG-3) was analyzed over time from day 3 to day 5. PTPN3 expression was found to be significantly greater under hypoxic conditions than under normoxic conditions (*p <.05, **p <.01), and the expression of other lymphocyte exhaustion markers was also elevated under the same conditions as previously reported [14,15] (Figure 1A, B, C; see Figure, Supplemental Digital Content 1A, B).

We examined changes in each lymphocyte exhaustion marker (PD-1, TIM-3, and LAG-3) in activated lymphocytes upon PTPN3 suppression. Lymphocytes were activated under the same conditions as in Figure 1A, B, and control shRNA or PTPN3 shRNA was transfected into activated lymphocytes using lentiviral vectors, followed by culture under normoxic or hypoxic conditions (Figure 1D). Suppression of PTPN3 expression was confirmed by real-time PCR and flow cytometry (Figure 1E, F). In activated lymphocytes, CD69, a lymphocyte activation marker, was upregulated by PTPN3 suppression under both normoxic and hypoxic conditions (Figure 1G). PD-1 expression in $CD8^+$ cells was upregulated by PTPN3 suppression and was the highest under hypoxic conditions, whereas the expression of TIM-3 and LAG-3 on these cells was downregulated by PTPN3 suppression (Figure 1H).

These results suggested that PTPN3, in addition to known immune checkpoint molecules, was upregulated in activated lymphocytes under hypoxic conditions, which promote lymphocyte exhaustion, and functioned as a lymphocyte exhaustion marker. In addition, PTPN3 suppression in activated lymphocytes increased PD-1 expression, decreased TIM-3 and LAG-3 expression, and increased CD69 expression under hypoxic conditions, suggesting that PTPN3 suppression would aid lymphocyte recovery from the exhaustion state.

PTPN3 Suppression in Activated Lymphocytes Enhanced their Function, Even under Hypoxic Conditions via mTOR-Akt Signaling Pathway Activation

Activated lymphocyte function has been reported to be suppressed under hypoxic conditions, primarily in terms of proliferative capacity [16, 17]. Therefore, we analyzed the function of PTPN3 suppression-activated lymphocytes under hypoxic conditions. Proliferative capacity was analyzed by determining the cell count. Under normoxic conditions, PTPN3 suppression has been reported to enhance the proliferative potential of activated lymphocytes and increase the cell count [23]. In the current study, the proliferative potential of activated lymphocytes was significantly enhanced by PTPN3 suppression under hypoxic conditions; however, it was lower than that under normoxic conditions (Figure 2A). The CFSE dilution assay was performed to confirm these results, which were consistent with those depicted in Figure 2A (Figure 2B, C). Time-lapse photography was used to calculate the migration distance per hour. Under hypoxic conditions, migration was not lesser than that under normoxic conditions; however, it was enhanced under both conditions by PTPN3 inhibition (Figure 2D, E). Cytotoxic activity was assessed by coculturing the target cancer cells (FaDu, hypopharyngeal carcinoma cell line) with activated lymphocytes at an E:T ratio of 10:1 and measuring the absorbance of the remaining cancer cells. Enhanced cytotoxic activity was observed in the PTPN3 suppression group under both normoxic and hypoxic conditions (Figure 2F, G).

PTPN3-suppressed activated lymphocytes did not show considerable survival rate changes under normoxia or hypoxia compared to the controls (see Figure, Supplemental Digital Content 2A).

Furthermore, NK group 2D (NKG2D) and Fas ligand (Fas-L), molecules that affect cytotoxic activity, were analyzed. Under hypoxic conditions, NKG2D expression was decreased in PTPN3-suppressed activated lymphocytes, whereas Fas-L expression was increased (see Figure, Supplemental Digital Content 2B).

Activated lymphocytes that become dysfunctional under hypoxic conditions have been reported to show reduced signaling pathway activation [17]. Therefore, we analyzed the mTOR-Akt, which are associated with lymphocyte function, by restimulating activated lymphocytes cultured under normoxic and hypoxic conditions via TCR stimulation (Figure 2H). In the mTOR-Akt signaling pathway, Akt and S6 phosphorylation was lower under hypoxic than under normoxic conditions in the control group. However, PTPN3 inhibition enhanced signaling pathway phosphorylation under both normoxic and hypoxic conditions, which was more evident after 5 min of stimulation (Figure 2I).

These results suggested that PTPN3 suppression in activated lymphocytes enhanced proliferation, migration, and cytotoxic activities under both hypoxic and normoxic conditions, thereby enhancing antitumor effects. In addition, PTPN3-suppressed activated lymphocytes did not enter a dysfunctional state under hypoxic conditions, and mTOR-Akt signaling pathway activation was observed upon TCR stimulation.

PTPN3 Suppression in Activated Lymphocytes Enhanced the Antitumor Effects of Anti-PD-1 Antibody (Nivolumab) Therapy under Hypoxic Conditions

Among the groups analyzed, PTPN3-suppressed activated lymphocytes showed the greatest increase in PD-1 expression under hypoxic conditions (Figure 1H); therefore, we hypothesized that synergistic effects with the anti-PD-1 antibody drug nivolumab might be observed under the same conditions. To determine the optimal nivolumab concentration for PD-1 inhibition, we added nivolumab to activated lymphocytes in vitro and analyzed PD-1 on $CD8^+$ cells using flow cytometry, as per the procedure reported by Wang et al [25]. The detection of PD-1 on $CD8^+$ cells by flow cytometry plateaued when nivolumab was added at a concentration of 10 μg/mL to 1×10^6 activated lymphocytes. Therefore, nivolumab was used at this concentration in the subsequent in vitro study (Figure 3A, B). Real-time PCR analysis showed that PTPN3 expression was upregulated in activated lymphocytes after nivolumab addition (10 μg/mL) (Figure 3C).

To further analyze the functional changes induced by nivolumab addition, we examined proliferative and cytotoxic activities. Nivolumab was added to a control and a PTPN3-suppressed group of activated lymphocytes under normoxic and hypoxic conditions, respectively, and proliferative activity was measured; nivolumab did not affect proliferative activity in either group (Figure 3D). For cytotoxic activity, activated lymphocytes and FaDu cells were cocultured with or without nivolumab at an E:T ratio of 10:1 under normoxic or hypoxic conditions, and the absorbance of the surviving cancer cells was measured. Under normoxic conditions, the cytotoxic activity was

enhanced in the PTPN3-suppressed activated lymphocyte group, similar to the results presented in Figure 2F; however, nivolumab addition did not lead to significant changes in either group (Figure 3E). However, under hypoxic conditions, nivolumab addition enhanced cytotoxic activity in both control and PTPN3-suppressed activated lymphocytes groups, with the greatest enhancement observed in the group in which nivolumab was added to PTPN3-suppressed activated lymphocytes (Figure 3F).

These results suggested that in vitro, PTPN3-suppressed activated lymphocytes showed enhanced antitumor effects when combined with nivolumab, only under hypoxic conditions.

PTPN3 Inhibition Improved Tumor Response to Anti-PD-1 Antibody Drug (Nivolumab), Especially in Hypoxic Environments

The in vitro results showed that the combination of PTPN3-suppressed activated lymphocytes and nivolumab increased cytotoxic activity; therefore, we performed in vivo experiments with a xenograft model in which FaDu cells were subcutaneously grown in immunodeficient (BALB/c-nu) mice. Tumor cells were subcutaneously implanted in both flanks of mice, following which control or PTPN3-suppressed activated lymphocytes, nivolumab, or saline was intraperitoneally administered on days 4, 7, 11, and 14, and changes in tumor size were observed (Figure 4A). The PTPN3-suppressed activated lymphocytes alone and the control activated lymphocytes plus nivolumab groups did not show significant changes in tumor size compared to those in the control activated lymphocyte alone group. However, the PTPN3-suppressed activated lymphocytes plus nivolumab group showed significantly lesser tumor size and weight than the control group (Figure 4B–D).

The number of infiltrating $CD8^+$ cells in the excised tumor tissues was compared using fluorescence immunostaining. This number increased with nivolumab treatment and was the highest in the PTPN3-suppressed activated lymphocytes plus nivolumab group (Figure 4E, F). CA-IX, which is upregulated in hypoxic regions and is reported to be a poor prognostic factor in head and neck cancer [18,26], was also stained. All groups showed CA-IX-stained areas inside the tumor (see Figure, Supplemental Digital Content 3), and the number of $CD8^+$ cells infiltrating the area significantly increased in the PTPN3-suppressed activated lymphocytes plus nivolumab group (Figure 4G, H).

The results suggested that the combination of nivolumab plus PTPN3-suppressed activated lymphocytes exerted a synergistic effect on tumors for which treatment with either nivolumab or PTPN3-suppression therapy alone was ineffective in vivo and that increased $CD8^+$ T cell infiltration into hypoxic areas may have contributed to the enhanced antitumor effect.

DISCUSSION

Hypoxic environments, such as the cancer microenvironment, have been reported to contribute to resistance to immune checkpoint inhibitors [16-19]. In this study, we showed that the function of

PTPN3-suppressed activated lymphocytes is enhanced under hypoxic conditions and that combination therapy using existing anti-PD-1 antibody therapy for head and neck cancer and PTPN3 suppression therapy in activated lymphocytes is particularly useful under hypoxic conditions. The schema of the present study's findings is shown in Figure 5. Similar to that of other immune checkpoint molecules, PTPN3 expression was upregulated under hypoxic conditions in activated lymphocytes, and the proliferative capacity and cytotoxic activity of which decreased via the mTOR-Akt signaling pathway; however, these functions were restored upon PTPN3 suppression. In activated lymphocytes, among PD-1, TIM-3, and LAG-3, only PD-1 expression was upregulated upon PTPN3 suppression; further enhancement of cytotoxic activity was observed upon PD-1 inhibition by nivolumab.

Lymphocyte exhaustion is primarily defined by the progenitor exhaustion and terminal exhaustion stages [16,17,27]. Some studies suggest that terminally exhausted T cells do not respond to immune checkpoint inhibitors but rather act in an immunosuppressive manner, causing therapy resistance [16]. Hypoxia is one of the factors identified to induce terminal T cell exhaustion; sustained antigen stimulation under hypoxic conditions causes this rapid induction of terminal exhaustion in T cells [17]. Although we did not clearly classify this stage in the current study, the PD-1, TIM-3, LAG-3, and PTPN3 expression in activated lymphocytes was upregulated and their functions were downregulated under hypoxic conditions and upon TCR stimulation in vitro, suggesting that the terminal exhaustion state had been induced. Under hypoxic conditions, PTPN3-suppressed activated lymphocytes showed decreased TIM-3 and LAG-3 expression, whereas PD-1 expression was further upregulated. Functional analysis suggested that T cells were further activated by PTPN3 suppression under hypoxic conditions. However, only PD-1 expression was upregulated, suggesting that PTPN3, similar to PTPN11 (SHP2; member of the PTPN family), may directly affect PD-1 signaling [28,29] and that there may be a crosstalk in the signaling pathway. The in vitro and in vivo results showed that the dead cell percentage did not increase in PTPN3-suppressed activated lymphocytes (see Figure, Supplemental Digital Content 2A) and that the antitumor effect was enhanced on combination with nivolumab. Thus, the findings indicate that PTPN3-suppressed activated lymphocytes do not undergo activation-induced cell death or terminal exhaustion under hypoxic conditions but instead show enhanced function and maintain their activated state.

Lymphocyte exhaustion causes its dysfunction, primarily in terms of proliferative capacity, via mTOR-Akt signaling pathway inactivation [16]. In the current study, under hypoxic conditions that promote lymphocyte exhaustion, control activated lymphocytes showed a decrease in Akt phosphorylation after 2–5 min of transient TCR stimulation, whereas PTPN3-suppressed activated lymphocytes responded to TCR stimulation, leading to enhancement of the mTOR-Akt signaling pathway. These results suggest that PTPN3 suppression may contribute to the enhanced function of the mTOR-Akt signaling pathway in response to TCR stimulation.

In basic studies involving PD-1 antibody drugs in combination with other immune checkpoint

inhibitors, multiple immune checkpoint molecules were found to be upregulated in tumor-infiltrating T cells after anti-PD-1 antibody treatment in preclinical models of head and neck cancer and lung adenocarcinoma, and the antitumor effect was enhanced by additional administration of anti-TIM-3 antibody drugs [30,31]. In the current study, PD-1 expression was upregulated in PTPN3-suppressed activated lymphocytes, especially under hypoxic conditions, and additional nivolumab administration enhanced the antitumor effect, which is consistent with previous findings [30,31] and may be useful for the future development of combined immunotherapies.

This study has two main limitations. First, the human leukocyte antigen (HLA) type of the activated lymphocytes and tumor cells was not matched. The cytotoxic function of cytotoxic T lymphocytes is originally performed by TCR-recognizing autologous MHC class I and antigens presented by tumor cells; therefore, it is necessary to match the HLA types of both cells. However, because it was difficult to perform this study in an autologous model, we examined its antitumor effects in an allogeneic experimental model. In our previous studies, we reported that PTPN3-suppressed activated lymphocytes enhanced the antitumor effect in a similar experimental system, regardless of HLA match or mismatch [23,32]. In the current study, we also investigated the possible involvement of NKG2D/MICA and MICB and Fas/Fas-L in this antitumor effect. Under hypoxic conditions, NKG2D expression decreased in PTPN3-suppressed activated lymphocytes, whereas Fas-L expression increased, which may have contributed to the enhanced antitumor effect observed under hypoxic conditions (see Figure, Supplemental Digital Content 2A, B). In addition, because all fractions were collected without separating only T lymphocytes when PBMCs were activated, it is possible that natural killer cells were involved in the antitumor effect. Second, this is a preclinical model of adoptive immunotherapy involving PTPN3-suppressed activated lymphocytes in combination with nivolumab, rather than systemic PTPN3-suppressive therapy. To date, PTPN3-specific inhibitors have not been developed; therefore, we intend to develop a PTPN3 inhibitor for systemic administration. In the current xenograft model experiment, we used lentiviral vectors to suppress PTPN3 in activated lymphocytes, thus limiting the protocol for PTPN3 suppression in activated lymphocytes followed by nivolumab administration. In vitro results of PTPN3 upregulation in activated lymphocytes after nivolumab administration suggested that synergistic effects between nivolumab and PTPN3 suppression may be achieved, even if nivolumab is administered before PTPN3 suppression. When PTPN3 inhibitors are developed, it would be important to conduct a trial in which PTPN3 inhibitors are administered after nivolumab treatment and to analyze systemic adverse events.

In conclusion, we report that PTPN3 is a lymphocyte exhaustion marker and that suppression of PTPN3 expression in activated lymphocytes may help enhance the therapeutic effect of anti-PD-1 antibody drugs for head and neck cancer, especially in a hypoxic environment that contributes to lymphocyte exhaustion. Our findings will contribute to the development of novel combined immunotherapies involving nivolumab.

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Oncol. 2021;14:101152.

FIGURE LEGENDS

Figure 1. PTPN3 expression in activated lymphocytes is greater under hypoxic than under normoxic conditions, similar to findings for other lymphocyte exhaustion markers.

Lymphocytes activated with anti-CD3 and CD28 antibodies were cultured separately under normoxic and hypoxic conditions; PTPN3 expression analyzed using real-time PCR (N, normoxia; H, hypoxia) (A) and flow cytometry in triplicate (filled histogram, shcontrol; dotted line, shPTPN3; solid line, isotype control) (B). Values in the graph represent MFIs. PD-1, TIM-3, and LAG-3 expression (C) was analyzed via flow cytometry each day after culture. (D) Activated lymphocytes were transfected with shRNA using lentiviral vectors and cultured under normoxic and hypoxic conditions. Suppression of PTPN3 expression in activated lymphocytes was confirmed using real-time PCR (E) and flow cytometry (filled histogram, shcontrol; dotted line, shPTPN3; solid line, isotype control) (F). The values shown in the graph represent MFIs. Data have been presented as the mean ± SD. *p <.05, **p <.01. Cell surface antigens were analyzed using flow cytometry. (G) Percentage of CD69 expression in the control or PTPN3-suppressed activated lymphocytes under normoxic and hypoxic conditions. Blue line, shcontrol; red line, shPTPN3; black line, isotype control. (H) PD-1, TIM-3, and LAG-3 percentages in CD8+ cells were analyzed by flow cytometry. MFI, mean fluorescence intensity; SSC, side scatter; PD-1, programmed death receptor-1; TIM-3, T cell immunoglobulin mucin-3; LAG-3, lymphocyte-activation gene 3.

Figure 2. PTPN3-suppressed activated lymphocytes show enhanced function under hypoxic and normoxic conditions, mediated by mTOR-Akt signaling.

Lymphocyte proliferative capacity was analyzed by measuring cell counts and CFSE dilution assay. (A) Cultures were grown on anti-CD3 antibody- and anti-CD28 antibody-coated plates, and cell counts were measured in triplicate on each day. (B) After lymphocytes activation, cells were labeled with CFSE. The fluorescence intensity of CFSE in activated lymphocytes was analyzed 3 or 4 days after incubation, using flow cytometry in triplicate. Values in the graph represent MFIs. (C) Data in the graph are presented as the mean of MFIs ± SD. *p <.05, **p <.01. (D) Lymphocyte migration ability was evaluated by time-lapse photography. The lines on the image show the lymphocyte migration trajectory. (E) Lymphocyte migration per hour was analyzed from the time-lapse movies obtained. Cytotoxic activity under normoxic (F) and hypoxic (G) conditions was analyzed by coculturing activated lymphocytes and a target cancer cell line (FaDu) at an E:T ratio of 10:1 and calculating the absorbance of the remaining cancer cells. Data have been presented as the mean ± SD. *p <.05, n.s., not significant. (H) Activated lymphocytes transfected with shRNA using lentiviral vectors were cultured under normoxic or hypoxic conditions and restimulated with anti-CD3 and anti-CD28 antibodies, followed by analysis of phosphoproteins in the mTOR-Akt signaling pathways by flow cytometry. (I) Phosphoprotein analysis findings for the mTOR-Akt signaling pathway under

conditions of 2 (left row) and 5 (right row) min restimulation under normoxic and hypoxic conditions are shown. The values shown are MFIs. MFI, mean fluorescence intensity; CFSE, Carboxyfluorescein Diacetate Succinimidyl Ester.

Figure 3. PTPN3-suppressed activated lymphocytes enhance the antitumor effects of anti-PD-1 antibody drug therapy under hypoxic conditions.
(A) The effect of PD-1 blockade in $CD8^+$ cells after nivolumab addition was evaluated using flow cytometry. The numbers shown are the percentages of $CD8^+$ PD-1-positive cells. (B) The percentages in (A) are shown in the graph. (C) PTPN3 expression in activated lymphocytes after nivolumab addition (10 μg/mL) was analyzed by real-time PCR. Nivo, nivolumab. (D) Changes in proliferative potential of control or PTPN3-suppressed activated lymphocytes upon nivolumab addition were evaluated by determining the cell count. Cultures were performed on anti-CD3 antibody- and anti-CD28 antibody-coated plates, and cell counts were measured in triplicate on each day. Activated lymphocytes and the target cancer cells (FaDu cell line) were cocultured and nivolumab was added; the absorbance of the remaining cancer cells was calculated to evaluate cytotoxic activity under normoxic (E) and hypoxic (F) conditions. Data have been presented as the mean ± SD. *p <.05, **p <.01, n.s., not significant.

Figure 4. PTPN3-inhibitory treatment is effective in preclinical models, especially in hypoxic environments, for enhancing the antitumor effects of anti-PD-1 antibody drug therapy.
(A) Immunocompromised mice (BALB/c-nu) were subcutaneously injected with FaDu cells on day 0 for tumor growth, and activated lymphocytes and nivolumab or saline were intraperitoneally administered. Activated lymphocytes were administered on days 4, 7, 11, and 14 and nivolumab or saline on days 4 and 11. All mice were euthanized on day 20. (B) Mean tumor size of each group is shown. Photographs (C) and mean weights (D) for each group of tumors resected on day 20 after tumor injection are shown. Images of immunofluorescence-stained tumor sections (E) and the average number of tumor-infiltrating $CD8^+$ cells in multiple regions for each group (F) are shown. Red, $CD8^+$; Blue, DAPI; Nivo, nivolumab. Images of immunofluorescence-stained tumor sections (G) and the mean number of tumor-infiltrating $CD8^+$ cells in hypoxic regions (H) are shown for each group. Red, $CD8^+$; Blue, DAPI; Green, CA-IX; Nivo, nivolumab. Magnification, 200×. Data have been presented as the mean ± SD. *p <.05, **p <.01, n.s., not significant.

Figure 5. Schema of the study findings.
Under hypoxic conditions, activated lymphocytes showed elevated PTPN3 expression, similar to the findings for known markers of lymphocyte exhaustion and impaired function. However, PTPN3 suppression enhanced these functions via mTOR-Akt signaling pathway activation. PTPN3

suppression increased PD-1 expression in activated lymphocytes, especially under hypoxic conditions, and additional administration of the anti-PD-1 antibody drug nivolumab further enhanced the antitumor effect.

List of Supplemental Digital Content

Supplemental Digital Content 1. TIFF

PD-1, TIM-3, and LAG-3 expression in activated lymphocytes is elevated under hypoxia.

(A) Flow cytometry gating strategy is shown. (B) Flow cytometry analysis of PD-1, TIM-3, and LAG-3 expression on $CD8^+$ cells cultured under normoxic and hypoxic conditions on days 3 and 4.

Supplemental Digital Content 2. TIFF

PTPN3 suppression in activated lymphocytes under hypoxic conditions does not alter the percentage of cells that undergo apoptosis.

(A) The percentage of activated lymphocytes that undergo apoptosis because of PTPN3 suppression under normoxic and hypoxic conditions was analyzed via flow cytometry. PI, propidium iodide. (B) The NKG2D and Fas-L percentage for lymphocytes activated because of PTPN3 suppression under normoxic and hypoxic conditions was analyzed via flow cytometry. SSC, side scatter; Fas-L, Fas ligand.

Supplemental Digital Content 3. TIFF

All the tumors in each group showed hypoxic regions under treatment conditions in the xenograft model of head and neck cancer.

Stained images of CA-IX (left), DAPI (medium), and merge (right) for each group are shown. Blue, DAPI; Green, CA-IX. Magnification, 100×.

Figure 1

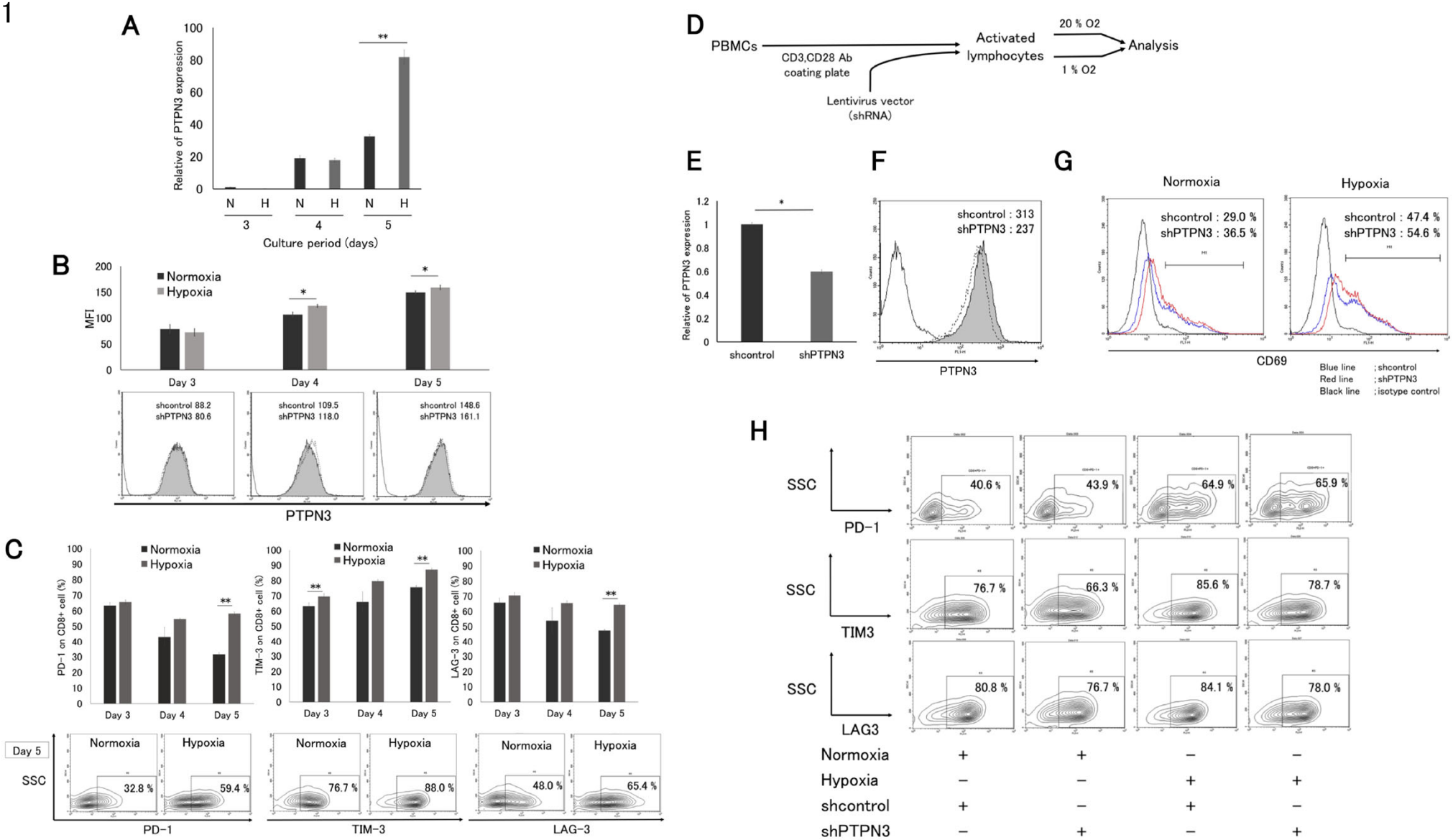

A
Relative of PTPN3 expression
Culture period (days)
N H N H N H
3 4 5
B
Normoxia
Hypoxia
MFI
Day 3
Day 4
Day 5
shcontrol 88.2
shPTPN3 80.6
shcontrol 109.5
shPTPN3 118.0
shcontrol 148.6
shPTPN3 161.1
PTPN3
C
PD-1 on CD8+ cell (%)
TIM-3 on CD8+ cell (%)
LAG-3 on CD8+ cell (%)
Day 5
SSC
32.8 %
59.4 %
76.7 %
88.0 %
48.0 %
65.4 %
PD-1
TIM-3
LAG-3
D
PBMCs
CD3,CD28 Ab
coating plate
Activated
lymphocytes
20 % O2
1 % O2
Analysis
Lentivirus vector
(shRNA)
E
Relative of PTPN3 expression
shcontrol
shPTPN3
F
shcontrol : 313
shPTPN3 : 237
PTPN3
G
Normoxia
Hypoxia
shcontrol : 29.0 %
shPTPN3 : 36.5 %
shcontrol : 47.4 %
shPTPN3 : 54.6 %
CD69
Blue line ; shcontrol
Red line ; shPTPN3
Black line ; isotype control
H
SSC
PD-1
40.6 %
43.9 %
64.9 %
65.9 %
TIM3
76.7 %
66.3 %
85.6 %
78.7 %
LAG3
80.8 %
76.7 %
84.1 %
78.0 %
Normoxia + + − −
Hypoxia − − + +
shcontrol + − + −
shPTPN3 − + − +

Figure 2

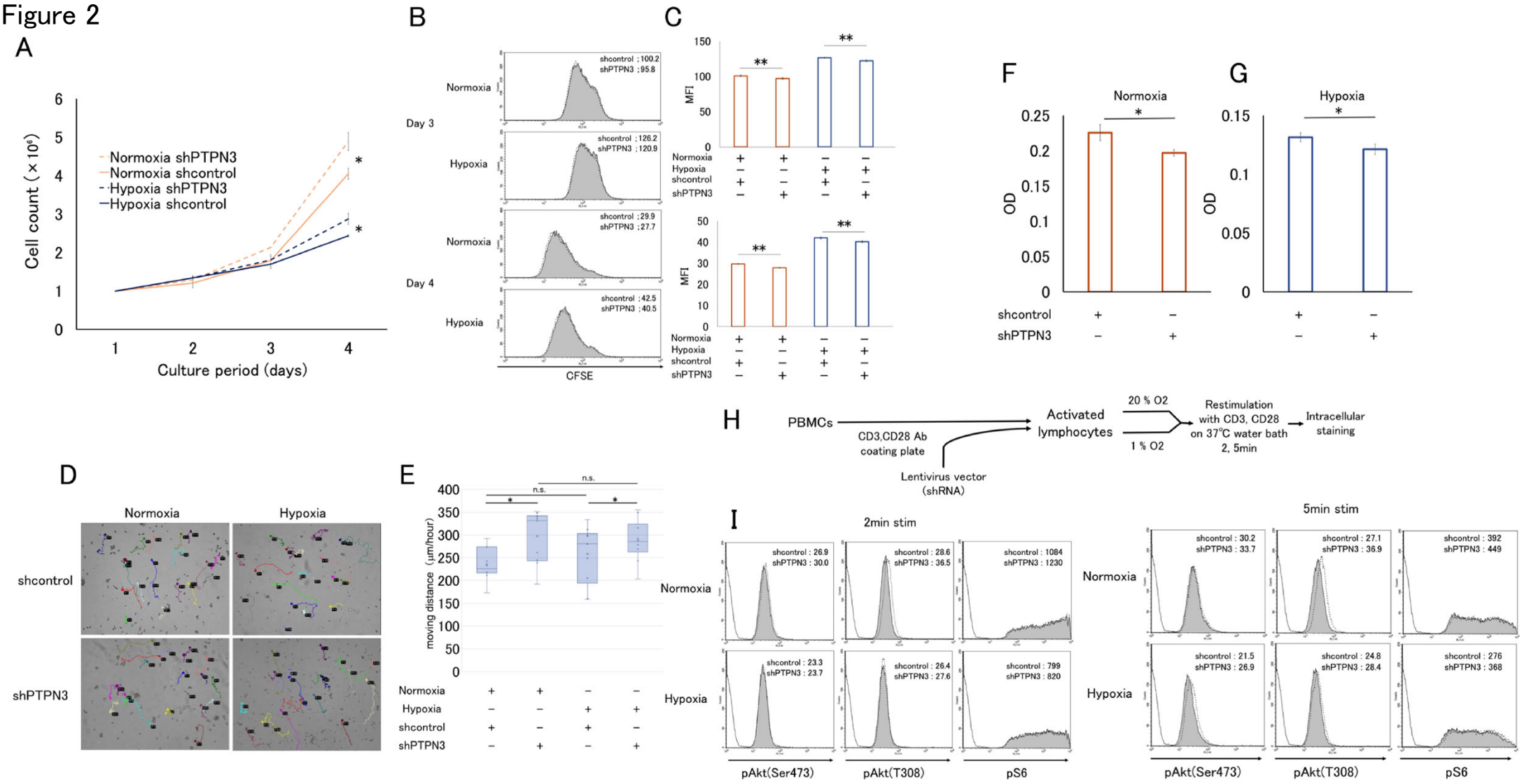

A
Cell count (×10⁵)
Normoxia shPTPN3
Normoxia shcontrol
Hypoxia shPTPN3
Hypoxia shcontrol
Culture period (days)
B
Day 3
Day 4
Normoxia
Hypoxia
shcontrol ; 100.2
shPTPN3 ; 95.8
shcontrol ; 126.2
shPTPN3 ; 120.9
shcontrol ; 29.9
shPTPN3 ; 27.7
shcontrol ; 42.5
shPTPN3 ; 40.5
CFSE
C
MFI
Normoxia
Hypoxia
shcontrol
shPTPN3
D
Normoxia
Hypoxia
shcontrol
shPTPN3
E
moving distance (μm/hour)
n.s.
F
Normoxia
OD
shcontrol
shPTPN3
G
Hypoxia
H
PBMCs
CD3,CD28 Ab coating plate
Lentivirus vector (shRNA)
Activated lymphocytes
20 % O2
1 % O2
Restimulation with CD3, CD28 on 37℃ water bath 2, 5min
Intracellular staining
I
2min stim
5min stim
shcontrol : 26.9
shPTPN3 : 30.0
shcontrol : 28.6
shPTPN3 : 36.5
shcontrol : 1084
shPTPN3 : 1230
shcontrol : 23.3
shPTPN3 : 23.7
shcontrol : 26.4
shPTPN3 : 27.6
shcontrol : 799
shPTPN3 : 820
shcontrol : 30.2
shPTPN3 : 33.7
shcontrol : 27.1
shPTPN3 : 36.9
shcontrol : 392
shPTPN3 : 449
shcontrol : 21.5
shPTPN3 : 26.9
shcontrol : 24.8
shPTPN3 : 28.4
shcontrol : 276
shPTPN3 : 368
pAkt(Ser473)
pAkt(T308)
pS6

Figure 3

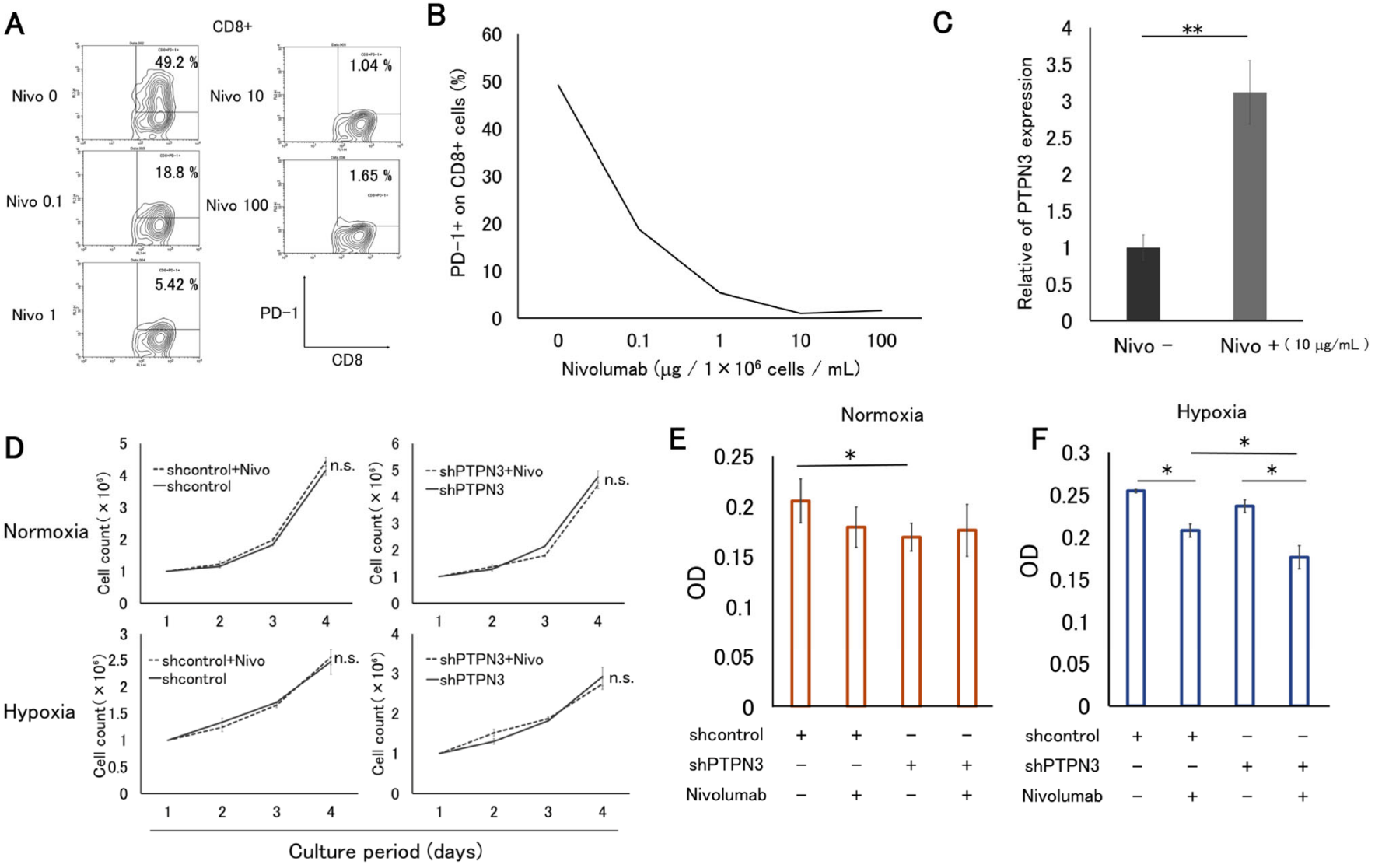
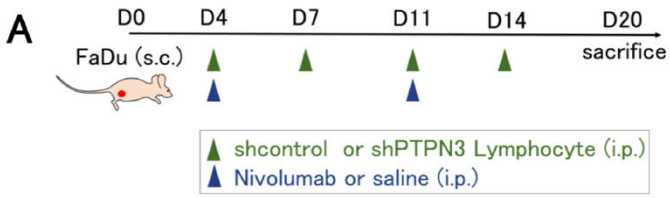
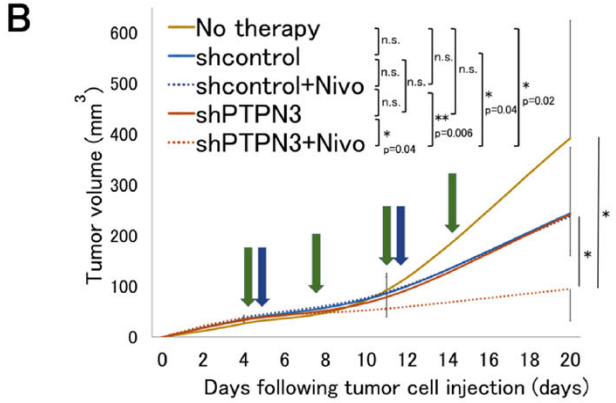
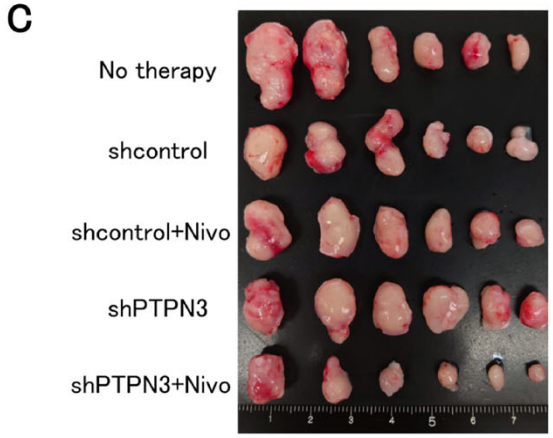
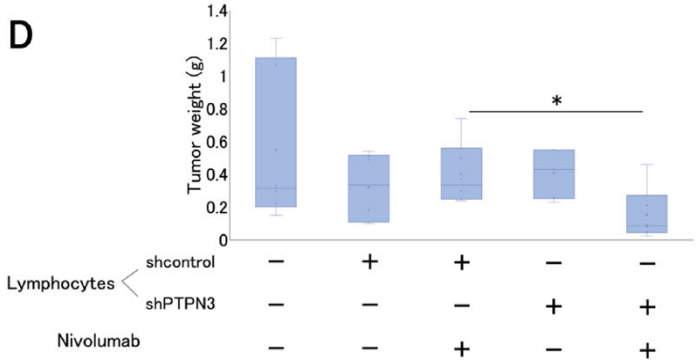

A
CD8+
Nivo 0
49.2 %
Nivo 10
1.04 %
Nivo 0.1
18.8 %
Nivo 100
1.65 %
Nivo 1
5.42 %
PD-1
CD8
B
PD-1+ on CD8+ cells (%)
Nivolumab (μg / 1 × 10⁶ cells / mL)
C
Relative of PTPN3 expression
Nivo −
Nivo + (10 μg/mL)
D
Normoxia
Hypoxia
shcontrol+Nivo
shcontrol
shPTPN3+Nivo
shPTPN3
n.s.
Cell count (× 10⁶)
Culture period (days)
E
Normoxia
OD
shcontrol
shPTPN3
Nivolumab
F
Hypoxia
OD
shcontrol
shPTPN3
Nivolumab

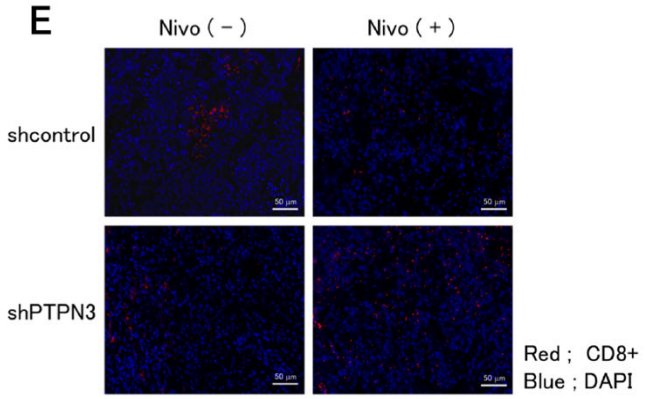
Figure 4

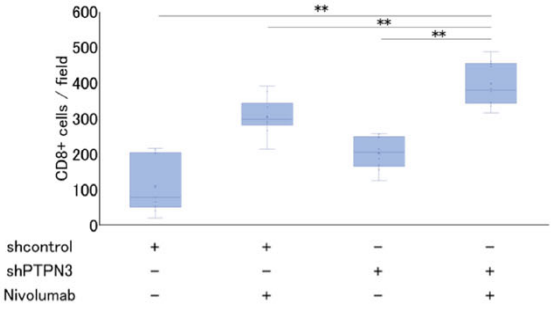

A
D0
D4
D7
D11
D14
D20
sacrifice
FaDu (s.c.)
shcontrol or shPTPN3 Lymphocyte (i.p.)
Nivolumab or saline (i.p.)

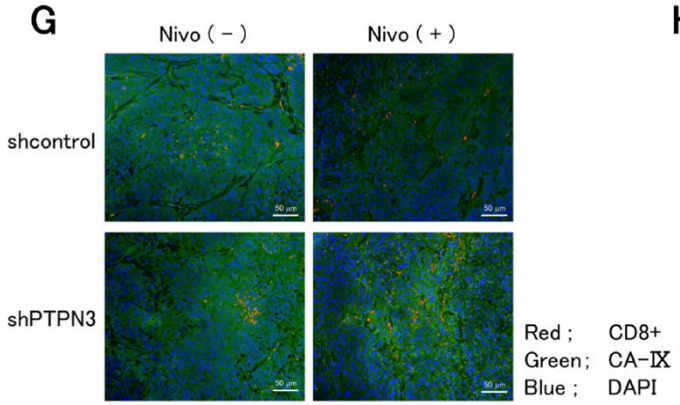

B
No therapy
shcontrol
shcontrol+Nivo
shPTPN3
shPTPN3+Nivo
Tumor volume (mm3)
Days following tumor cell injection (days)
n.s.
p=0.04
p=0.006
p=0.02


C
No therapy
shcontrol
shcontrol+Nivo
shPTPN3
shPTPN3+Nivo


D
Tumor weight (g)
Lymphocytes
shcontrol
shPTPN3
Nivolumab


E
Nivo (−)
Nivo (+)
shcontrol
shPTPN3
Red ; CD8+
Blue ; DAPI


F
CD8+ cells / field
shcontrol
shPTPN3
Nivolumab


G
Nivo (−)
Nivo (+)
shcontrol
shPTPN3
Red ; CD8+
Green ; CA−IX
Blue ; DAPI

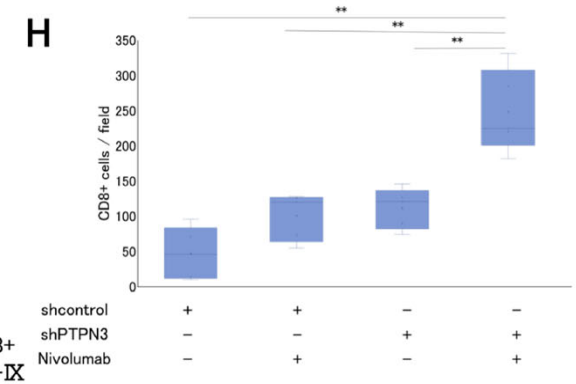
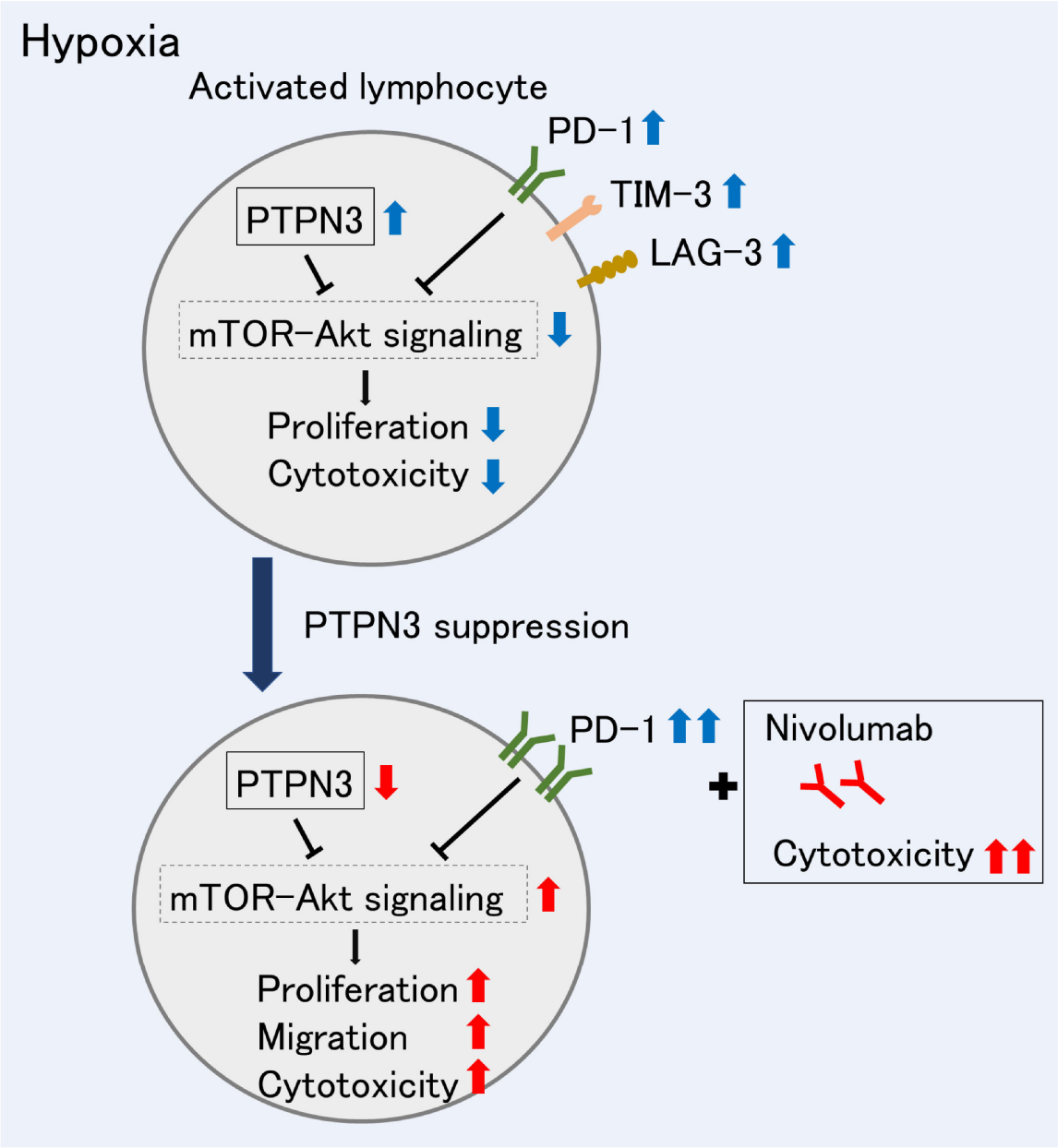
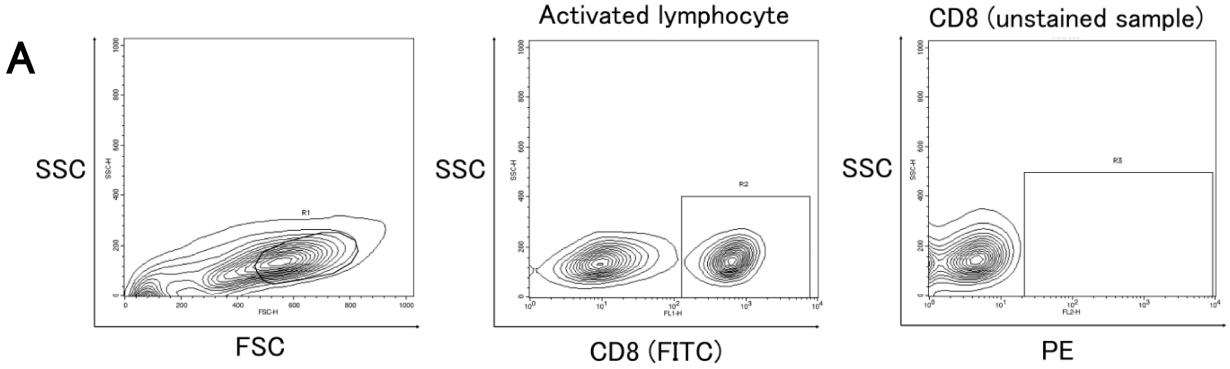
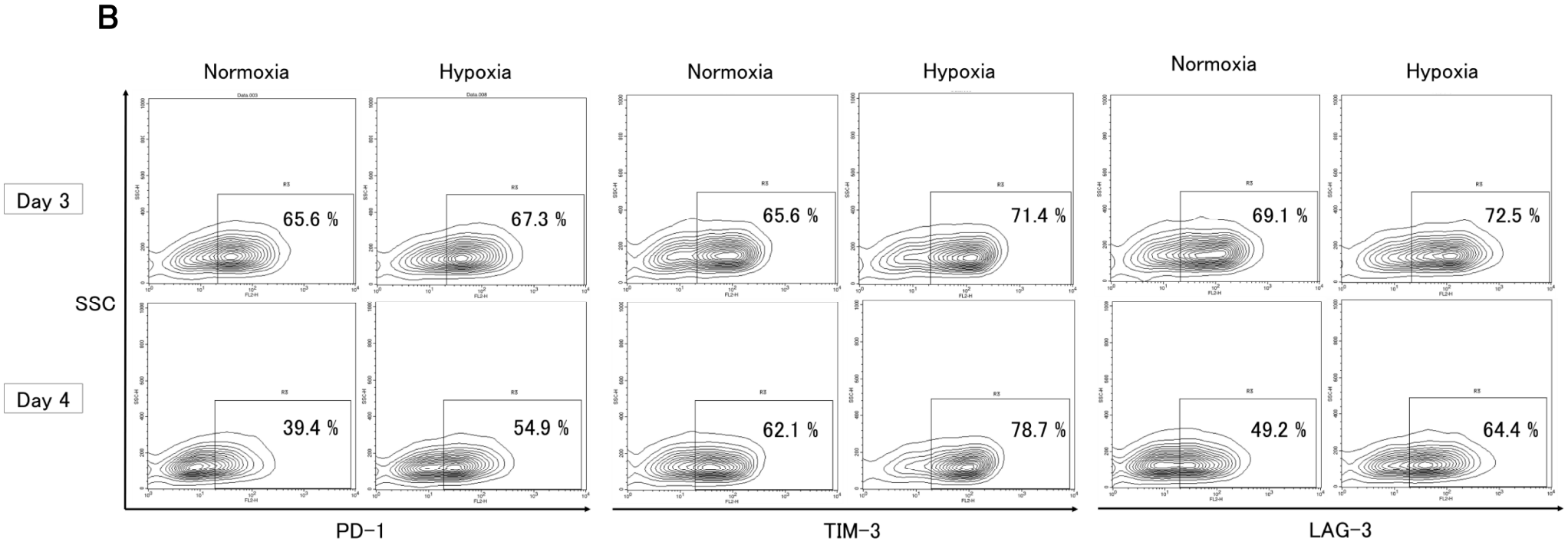

H
CD8+ cells / field
shcontrol
shPTPN3
Nivolumab

Figure 5

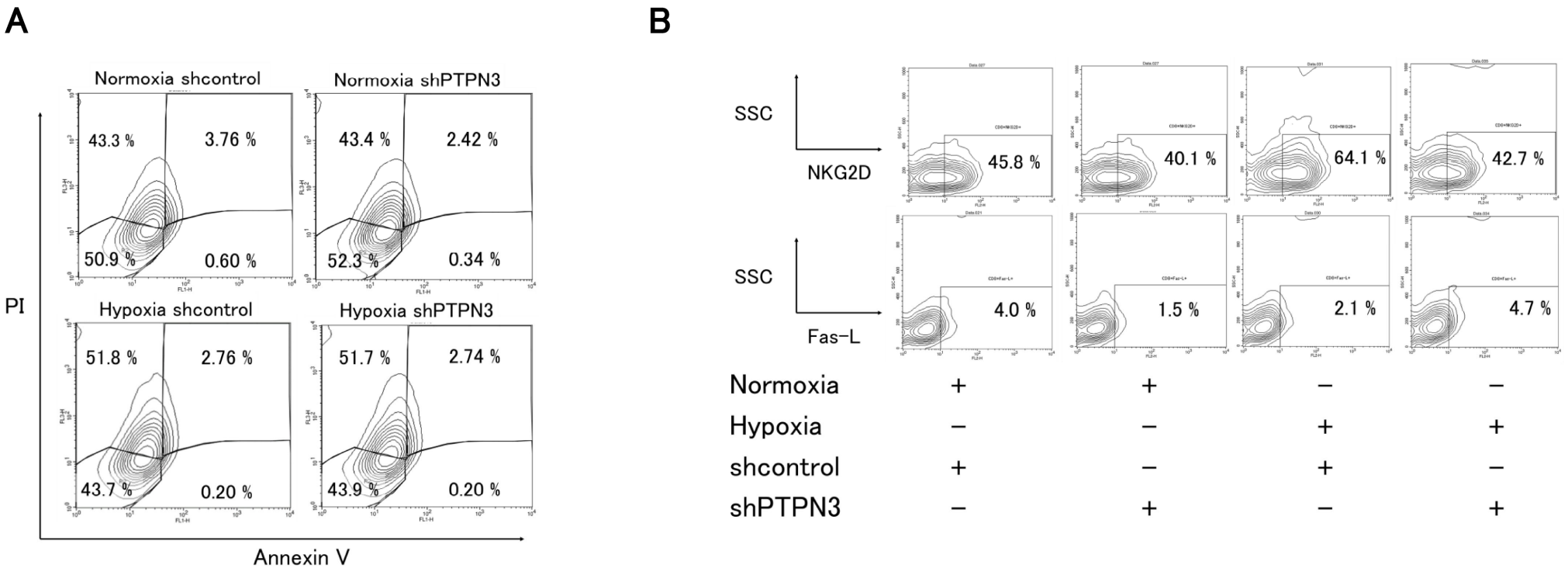

Hypoxia
Activated lymphocyte
PD-1
TIM-3
LAG-3
PTPN3
mTOR-Akt signaling
Proliferation
Cytotoxicity
PTPN3 suppression
PD-1
Nivolumab
PTPN3
mTOR-Akt signaling
Cytotoxicity
Proliferation
Migration
Cytotoxicity

Supplemental Digital Content 1





Supplemental Digital Content 2



Supplemental Digital Content 3