

SOX10 Inhibits T Cell Recognition by Inducing Expression of the Immune Checkpoint Molecule PD-L1 in A375 Melanoma Cells

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Abstract. *Background/Aim:* Malignant melanoma is a fatal skin cancer and is among the most immunogenic malignancies expressing melanoma-differentiation antigens and neoantigens. SRY-related HMG-box 10 (SOX10) is a transcription factor and a neural-crest differentiation marker that is used as a diagnostic marker for melanoma whilst playing a role in melanoma initiation through activation of the SOX10-MITF axis. SOX10 was shown to play a role in melanoma initiation by inducing expression of immune checkpoint molecules (e.g., HVEM and CEACAM1). In this study, we aimed to investigate the relationship between SOX10 and the expression an immune checkpoint molecule, programmed death-1 ligand 1 (PD-L1). *Materials and Methods:* SOX10 overexpression and knockdown was performed using SOX10 gene transfection and SOX10 siRNA transfection into A375 melanoma cells. PD-L1 expression was assessed by flow cytometry and western blotting. T cell response was evaluated using NY-ESO-1 specific TCR-transduced T (TCR-T) cells by IFN γ ELISPOT assay. *Results:* SOX10 overexpression increased the expression of PD-L1, whereas SOX10 knockdown, using siRNA, decreased its expression. IFN γ ELISPOT assay revealed that overexpression of SOX10 decreased the susceptibility of cells

to NY-ESO-1-specific TCR-T cells. *Conclusion:* SOX10 has a role in the intrinsic immune suppressive mechanisms of melanoma through expression of PD-L1.

Malignant melanoma is a major skin malignancy, with a projected 99,780 new cases and 7,650 deaths in the United States 2022 (1). Immunotherapy using immune checkpoint inhibitors (ICIs) such as PD-1/PD-L1 blockade, CTLA-4 blockade, and LAG-3 blockade has improved the prognosis of metastatic melanoma (2). Accordingly, the efficacy of immunotherapy using PD-1/PD-L1 blockade is critical in the prognosis of melanoma. PD-L1 expression is induced via several mechanisms, including oncogenic and inflammatory signaling, genomic alteration, and epigenetic regulation (3).

SOX10 belongs to the SOX transcription factor family and is expressed in neural crest cells (4). SOX8, SOX9, and SOX10 have a redundant DNA-binding motif, which suggests redundant functions; however, SOX10 is expressed during differentiation of peripheral nerves and melanoblasts, indicating that SOX10 might be essential for these processes (4). SOX10 is expressed at high rates in melanoma cells, which are a malignant counterpart of melanocytes, and thus SOX10 is a useful specific diagnostic marker for melanoma (5). SOX10 induces the expression of microphthalmia-associated transcription factor, an important transcription factor for melanoma, and plays a role in cancer stem cells, cell growth, and the invasion of melanoma cells (6). A recent study revealed that SOX10 also plays a role in melanomagenesis, partly evading immune response by inducing immune checkpoint molecules such as CEACAM1

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and HVEM (7). However, another study reported that SOX10 suppressed the expression of PD-L1, thereby inhibiting the IRF4-IRF1 axis (8). Considering these conflicting results, in the present study, we aimed to further analyze the relationship between SOX10 and the immune checkpoint molecule PD-L1, and whether PD-L1 expression induced by SOX10 might have a role in immune escape.

Materials and Methods

Cell lines and cell culture. A375, 888-mel, and Sk-mel-28 melanoma cell lines and T2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell lines were maintained in Dulbecco's modified Eagle's medium or RPMI (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (5 mg/ml penicillin, 5 mg/ml streptomycin; Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in an incubator at 37°C with humidified air and 5% CO₂.

SOX10 overexpression and down-regulation by siRNA. SOX10 cDNA was cloned from the 888-mel cell line. Total RNA was isolated from 888-mel cells by RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized by RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific), using 1 µg of total RNA. The SOX10 gene was amplified using PrimeSTAR GXL (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed using primers 5'-GGATCCATGGCGGAGGAGCAGGACCT-3' and 5'-CTCGAGTACTTGTCTGTCATCGTCTTTGTAGTCGCCCTTGTCGTCATCGTCTTTGTAGTCGCCGGCCGGGACAGTGTCTGAT-3'; the PCR product was then digested by *Bam*HI and *Xho*I and subcloned into a pMXs-Puro expression vector. The sequence was confirmed by Sanger sequencing.

SOX10 cDNA transfection was performed using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. For SOX10 knockdown, siRNA (Thermo Fisher Scientific) was transfected using Lipofectamine RNAi MAX (Thermo Fisher Scientific) according to manufacturer's protocol.

Quantitative reverse transcription polymerase chain reaction. The cells were transfected with SOX10 gene or siRNA, 36 h later the cells were treated by IFN-γ, and then 12 h later mRNA was extracted from cells using RNeasy Mini Kit (Qiagen). Total mRNA from each sample was reverse-transcribed using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR (RT-PCR) was performed in triplicate for each sample, using following primer sequences: human *EF1a* forward, 5'-CTGAACCATCCAGGCCAAAT-3' and reverse, 5'-GCCGTGTGGCAATCAAT-3'; SOX10 forward, 5'-GCTGCTGAACGAAAGTGACA-3' and reverse, 5'-GCCTGGGCTGGTACTTGTAG-3'; PD-L1 forward, 5'-TGAGGATATTTGCTGTCTTTATATTC-3' and reverse, 5'-GTCCTTGGGAACCGTGACAGT-3'. Amplification of specific PCR products was detected using PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Forty cycles of PCR were performed using QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). Fold change of mRNA expression was calculated as 2^{ΔCt before - ΔCt after}, in which ΔCt=Ct specific probe - Ct internal control.

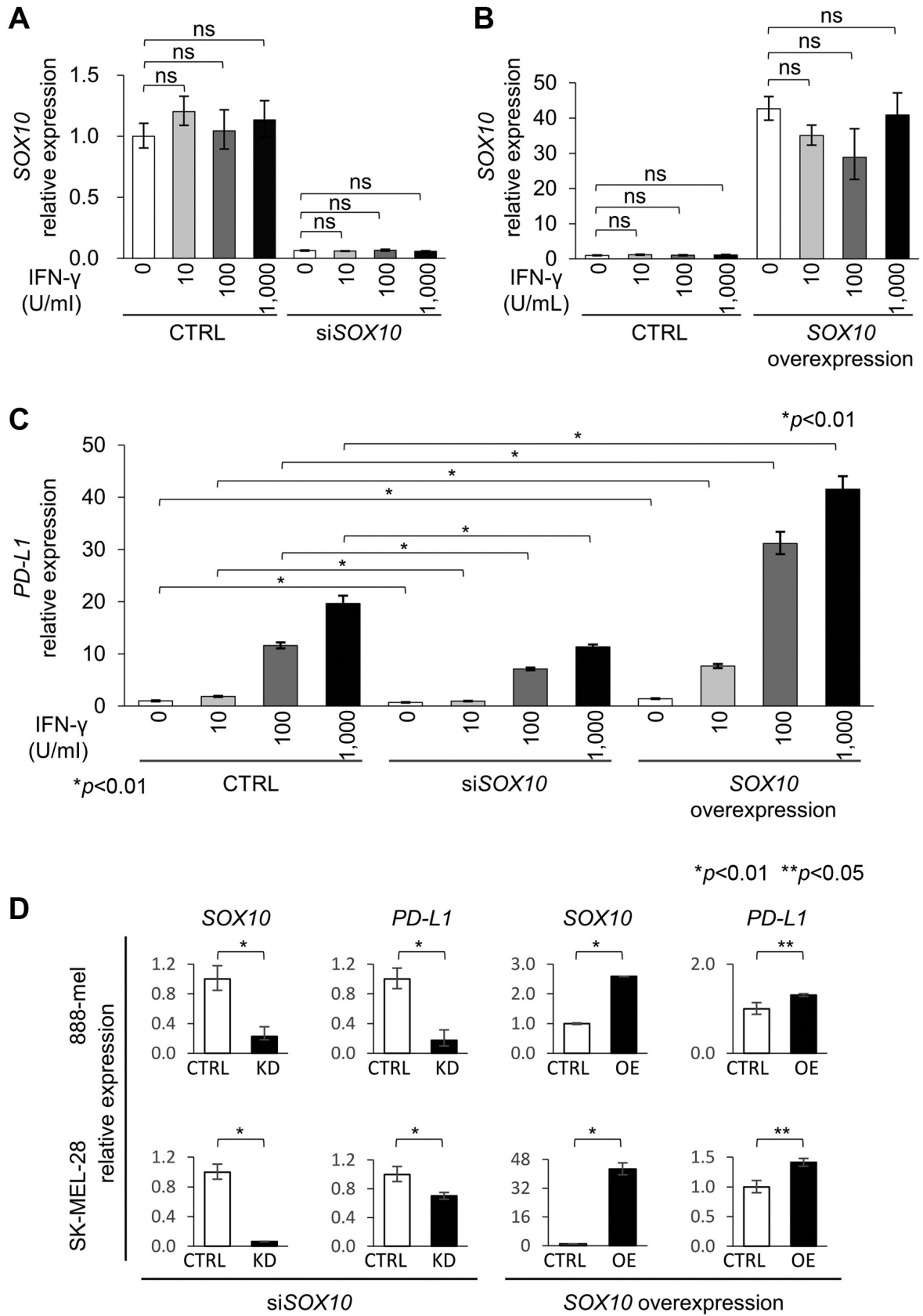
Flow cytometry and western blot. Flow cytometry was performed using FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA) as previously described (9). Anti-human PD-L1 (clone MIH3; BioLegend, San Diego, CA, USA) and anti-human PD-1 (clone EH12.2H7, BioLegend) were used to assess the surface expression of PD-L1 and PD-1. Isotype control was used as a negative control.

Western blot was performed as previously described (10). Total cellular proteins were extracted from A375 cells at 4°C using the RIPA lysis buffer and then resolved on a 10% SDS-PAGE and transferred to Immobilon-P membranes (Merck Millipore, Burlington, MA, USA). Western blots were probed with the following antibodies: anti-PD-L1 (clone E1L3N, Cell Signaling Technology, Danvers, MA, USA), anti-IRF-1 (clone D5E4, Cell Signaling Technology), and anti-β-actin (clone AC-15, Sigma-Aldrich). Anti-mouse IgG and anti-rabbit IgG second antibodies (KPL, Gaithersburg, MD, USA) were used at a dilution of 1:2,000. The membrane was visualized with Chemiluminescent HRP Substrate (Merck Millipore). Imaging was performed using the Odyssey XF imaging system (LI-COR Biosciences Inc., Lincoln, NE, USA), and the bands were quantified using LI-COR Image Studio software (LI-COR Biosciences).

Establishment of TCR-T cells and IFNγ ELISPOT assay. A*02:01/NY-ESO-1₁₅₇₋₁₆₅ TCR (clone 1G4LY) was used to generate T cell receptor gene-transduced T cells (TCR-T) cells as previously described (11). TCRα and TCRβ sequences encoding 1G4 TCR specific for HLA-A2 NY-ESO-1 peptide (12) were synthesized using GeneArt (Thermo Fisher Scientific). PLAT-A amphotropic packaging cells (Cosmo Bio) were transfected with the pMXs retroviral vector encoding 1G4, using Lipofectamine 2000 reagent (Thermo Fisher

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Figure 1. SOX10 increased the expression of PD-L1 in A375 cells. (A) Expression of SOX10 in SOX10-knockdown cells. A375 cells were transfected with SOX10 siRNA, and 48 h later, the cells were treated with IFNγ at several concentrations for 12 h, after which total RNA was obtained and the expression of SOX10 was assessed by real-time quantitative RT-PCR. Data are shown as means±SEM. (B) Expression of SOX10 in SOX10-overexpressed cells. A375 cells were transfected with SOX10 plasmid, and 48 h later, the cells were treated with IFNγ at several concentrations for 12 h, after which total RNA was obtained and the expression of SOX10 was assessed by real-time quantitative RT-PCR. Data are shown as means±SEM. The expression of levels of SOX10 showed no significant difference by IFNγ treatment. (C) Expression of PD-L1 in SOX10-knockdown and -overexpressing cells. A375 cells were transfected with SOX10 siRNA or SOX10 plasmid, and 48 h later, the cells were treated with IFNγ at several concentrations for 12 h, after which total RNA was obtained and the expression of PD-L1 was assessed by real-time quantitative RT-PCR. Data are shown as means±SEM. SOX10 knockdown (KD) significantly decreased the expression of PD-L1, on the other hand SOX10 overexpression (OE) significantly increased the expression of PD-L1. (D) PD-L1 expression under SOX10 knockdown and overexpression in 888-mel cells and SK-MEL-28 cells. 888-mel cells and SK-MEL-28 cells were transfected with SOX10 siRNA or SOX10 plasmid, 2 days later the expression of PD-L1 was addressed by qRT-PCR. Data are shown as mean±SEM. SOX10 knockdown (KD) significantly decreased the expression of PD-L1, on the other hand SOX10 overexpression (OE) significantly increased the expression of PD-L1. CTRL: Control.



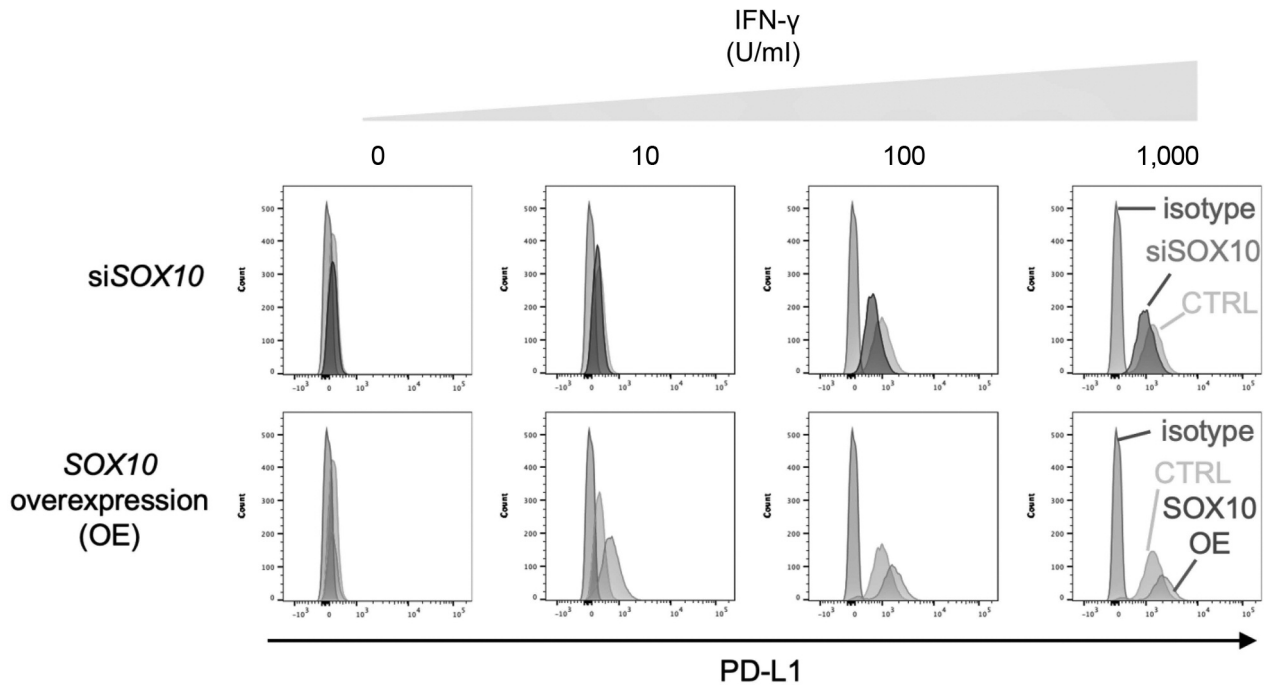


Figure 2. *PD-L1* expression in *SOX10* overexpression and knockdown. A375 cells were transfected with *SOX10* siRNA or *SOX10* plasmid, and 48 h later, the cells were treated with $IFN\gamma$ at several concentrations for 12 h, after which the expression of *PD-L1* was assessed by FACS. Data are shown as means \pm SEM. *SOX10* knockdown (KD) decreased the expression of *PD-L1*, on the other hand *SOX10* overexpression (OE) significantly increased the expression of *PD-L1* at protein levels. CTRL: Control.

Scientific), and the culture supernatant was transduced onto PG13 cells (ATCC). Then, the peripheral blood mononuclear cells were transduced with the PG13-derived retrovirus supernatants after stimulation with 50 ng/mL anti-CD3 mAb (OKT3; BioLegend) for 3 days. The transduced cells were cultured in a complete AIM-V medium (Thermo Fisher Scientific) containing 10% human AB serum, rhIL-2 (100 IU/ml; PeproTech, Cranbury, NJ, USA), and rhIL-15 (10 ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany) and used for flow cytometry or the interferon- γ enzyme-linked immunospot assay ($IFN\gamma$ ELISPOT assay) as previously described (13). Antigen-presenting cells (T2) were pre-incubated with 20 μ M synthetic peptides for 2 h at room temperature. Fifty-thousand TCR-T cells were cultured with an equal number of target cells.

Statistical analysis. Student's *t*-test was used to compare control group and experimental group in qRT-PCR analysis and $IFN\gamma$ ELISPOT assay. $p < 0.05$ was considered to indicate a significant difference.

Results

Expression of *PD-L1* was induced by *SOX10* in melanoma cells. *SOX10* is a neural crest differentiation marker that is expressed in tumors such as melanoma derived from the neural crest, making *SOX10* a useful diagnostic marker for melanoma (5, 14). A previous study reported that *SOX10* suppressed the expression of *PD-L1* by inhibiting *IRF1-IRF4*

(8). However, there are several molecular mechanisms by which expression of *PD-L1* can be induced (3), and the data presented by the authors in that study varied according to the cell line used. In the present study, we further analyzed the relationship between *SOX10* and *PD-L1* expression in melanomas. We transfected *SOX10* cDNA into melanoma cells, including A375, 888-mel, and SK-MEL-28 (Figure 1A and D). Then, *PD-L1* gene expression was assessed by quantitative RT-PCR (qRT-PCR). The expression of *PD-L1* was increased by *SOX10* overexpression, a finding that conflicts the results of the previously mentioned study (8). We thus further analyzed the expression of *PD-L1* in protein by using FACS analysis and western blot. FACS analysis revealed that *PD-L1* expression was obviously increased in A375 melanoma cells (Figure 2). Based on these results, we used A375 cells in the subsequent experiments.

To confirm that *SOX10* induces *PD-L1* expression, we performed *SOX10* knockdown using siRNA. *SOX10* siRNA transfection decreased expression at the mRNA and protein levels (Figure 1B, C and Figure 2).

***SOX10* overexpression increased *IRF1* protein expression.** *PD-L1* expression is induced by $IFN\gamma$ signaling (3), and we thus investigated the effects of $IFN\gamma$ under *SOX10* expression.

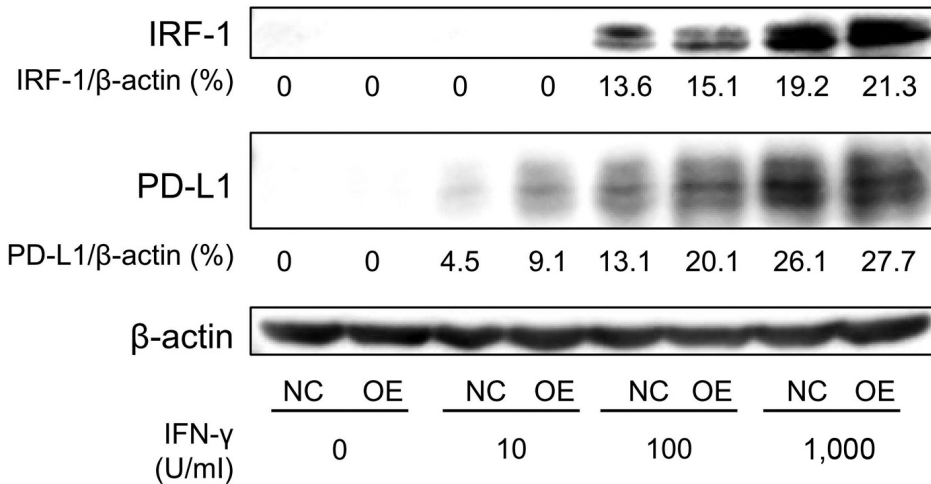


Figure 3. Western blots of PD-L1 and IRF-1 under SOX10 overexpression. A375 cells were transfected with SOX10 plasmid, and 48 h later, the cells were treated with IFN γ at several concentrations for 12 h, after which the expression of IRF-1 and PD-L1 were assessed by western blotting. β -actin was used as an internal positive control. The numerical data indicate the relative quantified data of the bands. SOX10 overexpression (OE) increased the expression of PD-L1 and upstream protein IRF-1 at protein levels.

IFN γ stimulation increased PD-L1 at the mRNA and protein levels (Figure 1C and Figure 2). A previous study revealed that the expression of SOX10 decreased the transcription factor IRF1 downstream of IFN γ signaling, and thus we analyzed the expression of IRF1 under IFN γ stimulation. IFN γ stimulation increased the protein expression of IRF1 in a dose-dependent manner (Figure 3). In addition, overexpression of SOX10 increased IRF1 protein expression and downstream expression of PD-L1 (Figure 3) in A375 cells.

SOX10 overexpression attenuates reactivity of TCR-T cells. Given that overexpression of SOX10 increased the immune checkpoint molecule PD-L1, we hypothesized that T cell recognition might be inhibited by overexpression of SOX10. In this study, we used NY-ESO-1-specific TCR-T cells because A375 cells express NY-ESO-1 endogenously and can be recognized by NY-ESO-1 (12). We established an HLA-A2-restricted 157-165 peptide of NY-ESO-1-specific 1G4 TCR-transduced TCR-T cells and the specificity was confirmed by tetramer assay (data not shown). The TCR-T cells specifically recognized NY-ESO-1 peptide-pulsed T2 cells (Figure 4A).

We then investigated the TCR-T reactivity to SOX10-overexpressed A375 cells. The overexpression of SOX10 did not decrease TCR-T recognition by IFN γ ELISPOT assay (Figure 4C). Next, we performed FACS analysis to investigate the expression of PD-1, the receptor for PD-L1. FACS analysis revealed that TCR-T cells did not express PD-1. We thus exhausted the TCR-T cells by treatment with CD3/CD28 beads, which increased the expression of PD-1

(Figure 4B). Overexpression of SOX10 decreased recognition by PD-1-positive TCR-T cells treated with CD3/CD28 beads (Figure 4C).

Discussion

Historically, melanoma has been considered a representative immunogenic malignancy (15). Numerous cases of spontaneous regression have been reported (16), and immunotherapy using tumor-infiltrating lymphocytes (TILs) resulted in favorable results, with 38%-56% objective responsive rates (17). Furthermore, human tumor-associated antigens (TAAs) were identified in melanoma for the first time in human malignancies (18, 19). Genetic analysis revealed that melanoma carries the highest mutational signature among human malignancies, suggesting that melanoma expresses high rates of neoantigens encoded by gene mutations (20, 21). However, most clinical cases continue growing without therapeutic intervention, suggesting that clinical melanoma might be in the escape phase (22). The great success of immunotherapy using immune checkpoint blockades indicates that immune checkpoint molecules play a major role in immunological escape in melanoma (23). Currently, small-molecule targeting immune checkpoints have been developed (24). Therefore, mechanical analysis of immune checkpoint molecules is essential to overcome immunological escape in melanoma. In the present study, we focused on SOX10 because it is a neural crest lineage marker that is expressed at high rates in melanoma and plays a transcriptional role. The immune checkpoint molecules HVEM and CEACAM1 are reported to be downstream of SOX10,

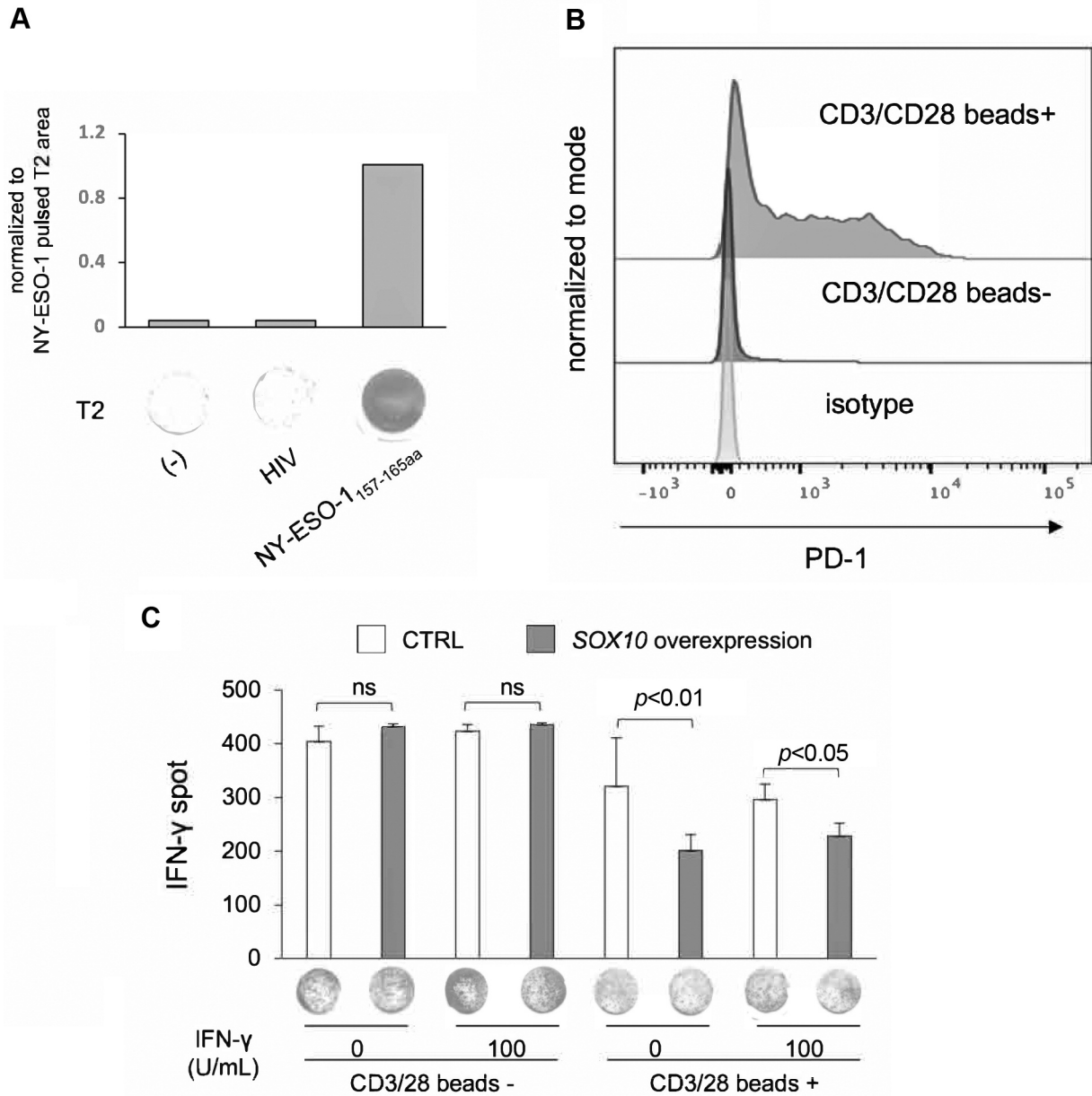


Figure 4. Overexpression of SOX10 enabled melanoma cell escape from TCR-T cells. (A) IFN γ ELISPOT assay of TCR-T cells. NY-ESO-1₁₅₇₋₁₆₅-specific TCR-transduced TCR-T cells were used. T2 cells were pulsed with NY-ESO-1₁₅₇₋₁₆₅ peptide and used for IFN γ ELISPOT assay. HIV peptide pulsed (+) and un-pulsed (-) samples were used as negative controls. (B) Expression of PD-1 in TCR-T cells treated with CD3/CD28 beads. NY-ESO-1₁₅₇₋₁₆₅-specific TCR-T cells were treated with CD3/CD28 beads for 3 days, and the expression of PD-1 was evaluated by FACS. An untreated sample (CD3/CD28 beads-) was used as a negative control. Isotype antibody was used as a negative control for FACS. (C) IFN γ ELISPOT assay of PD-1⁺ TCR-T cells for SOX10-overexpressed A375 cells. SOX10-overexpressed A375 cells were used for IFN γ ELISPOT assay. CD3/CD28 beads+ TCR-T cells were used as effector cells. CD3/CD28 beads- TCR-T cells were used as a negative control. Data are shown as means \pm SEM. Statistical significance was evaluated by the Student's *t*-test. SOX10 overexpression decreased the TCR-T reaction of CD3/CD28 beads treated PD-1-positive cells, whereas did not decrease the CD3/CD28 bead un-treated PD-1-negative cells.

which plays a role in the tumorigenesis of melanoma via immune escape (7). Similarly, microRNAs might also have a role in immune escape (25). The expression of HVEM is reported to be related to poorer prognosis in melanoma cases

(26). HVEM has a broader expression compared with PD-L1, indicating that HVEM might be a novel target for immunotherapy. However, another study reported that SOX10 inhibits IFN γ signaling by suppressing the expression of IRF1

and downstream PD-L1 (8). Their results are not in accordance with ours; however, we used different melanoma cell lines, and both studies found that reactivity to SOX10 varies according to the cell line used. Thus, the SOX10 downstream reaction might vary among cells given that SOX10 functions not only as transcription factor but also as transcriptional repressor in many target genes, reflecting the diversity of its functions (27, 28).

Conclusion

At present, it seems difficult to conclude whether SOX10 increases or decreases the expression of PD-L1. However, PD-L1 can be induced by SOX10 in melanoma cells and PD-L1 induced by SOX10 has a role in immunological escape from CTLs. Therefore, SOX10 may be related to the intrinsic immunological escape of melanoma cells.

Conflicts of Interest

The Authors have no financial conflicts of interest to disclose.

Authors' Contributions

All Authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Kenta Sasaki, Yoshihiko Hirohashi, Kenji Murata, Tomoyuki Minowa, Munehide Nakatsugawa, Aiko Murai, Yuka Mizue, Terufumi Kubo, Takayuki Kanaseki, Tomohide Tsukahara, Hisashi Uhara, and Akemi Ishida-Yamamoto. Data analysis was performed by Sadahiro Iwabuchi and Shinichi Hashimoto. The first draft of the manuscript was written by Kenta Sasaki, Yoshihiko Hirohashi, Akemi Ishida-Yamamoto, and Toshihiko Torigoe, and all authors commented on the revised versions of the manuscript. All Authors read and approved the final manuscript.

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