약학석사학위논문

Simultaneous immunotherapy with PD-L1 checkpoint blockade and interferon-beta augments anti-tumor therapeutic efficacy

PD-L1 체크포인트 억제와 인터페론 베타의 동시적 면역치료를 통한 항암치료효능 연구

2019년 2월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과 병태생리학 전공

홍 은 혜

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이 논문을 병태생리학 석사학위논문으로 제출함

2018년 11월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과 병태생리학 전공

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홍은혜의 약학석사 학위논문을 인준함

2019년 01월

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Abstract

Simultaneous immunotherapy with PD-L1 checkpoint blockade and interferon-beta augments anti-tumor therapeutic efficacy

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Recently, PD-L1 checkpoint inhibitors, kinds of immunotherapy, have revolutionized the treatment of cancer. This therapy has shown dramatic response in patient by increasing overall survival as well as showing a better safety profile compared to traditional cancer therapy. However, only a few patients correspond to this benefit. There are many reasons for this, but lack of tumor infiltrating lymphocyte (TIL) in tumor microenvironment (TME) is considered the most significant cause. Therefore, combination therapies that is designed to bring T cells into tumor site would be considered for the future promising cancer therapy.

We hypothesized that combination therapy with interferon-beta (IFN-β) and PD-L1 checkpoint inhibitor could enhance the efficacy of anti-tumor immune responses and complement each other. IFN-β has various immune modulatory function. Especially, it recruits lymphocytes into tumor site and regulates sub-population of the surrounding immune cell.

We confirmed that administered IFN-β promoted chemokines such as CCL5 and CXCR3 ligand, which is contributed to recruiting lymphocyte in TME. In addition, we found that tumor infiltrating $CD4^+$ and $CD8^+$ T cell are accumulated in respond to IFN-β. Furthermore, IFN-β treatment significantly decreased mRNA expression of Th2 related chemokine, which suppressed the recruitment of Treg in TME. However, increased expression level of PD-L1 has been also observed in tumor. The PD-L1 molecule is associated with the inhibitory signaling of T cell. We thought that PD-L1 checkpoint inhibitors could offset this effect. Thus, we blocked the PD-1/PD-L1 interaction by using the anti-PD-L1-mAb to reactivate the T cell. This combination treatment result in not only increasing the overall survival but also inhibiting tumor growth in syngenic mouse model as well as a humanized mouse model.

Our data suggests that simultaneous immunotherapy with interferon-beta and PD-L1 blockade is potential immune combination therapy.

Keywords: Immunotherapy, PD-L1 checkpoin inhibitor, Interferon-beta(IFN-β), Immune modulatory function, Tumor infiltrating lymphocyte (TIL), Tumor microenvironment (TME), PD-L1 (programed death-ligand 1), Melanoma

Student ID: 2017-29374

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TME	Tumor microenvironment
TIL	Tumor infiltrating lymphocyte
IFN-β	Interferon beta
PD-L1	Programed death-ligand 1
rhIFN-β1a	Recombinant human interferon-beta 1 a
CHO	Chinese hamster ovary
mAb	Monoclonal antibody
MHC-I	Major histocompatibility Complex
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
PD-1	Programed death-1
ANOVA	Analysis of variance

INTRODUCTION

1. Immune checkpoint inhibitors

Over the recent decade, cancer immunotherapy has been emerging in the field of cancer therapy. This therapy uses patient's immune system to help them fight against cancer. There are several types of immunotherapy such as immune checkpoint inhibitors, cancer vaccine, adoptive cell transfers (ACT) and so on [1]. Among these immunotherapies, immune checkpoint inhibitors are FDA approved for numerous cancer indications since 2011 and has been successfully used in patients to benefit them (Tab1) [2]. These inhibitors are treatment that can block inhibitory checkpoints, which is related to suppress anti-tumor T-cell activity, by restoring immune system function to fight against cancer. Currently, approved checkpoint inhibitors drugs were Ipmilmumab(target molecule of CTLA-4), Pembrolizumab, Nivolumab(target molecule of PD-1) and Atezolizumab, Duvalumab, Avalumab (target molecule of PD-L1) (Fig1) [2].

These immune checkpoint inhibitors have shown remarkable results for various types of cancer compared to traditional therapy including 1st generation chemotherapy and 2ndgeneration target therapy. The traditional therapies only increase short term survival. However, the 3rd generation immunotherapy increases long term survival and also has a durable response.

(Fig2). Therefore, it's one of the newer strategies to fight cancer compared to conventional therapy.[3]

However, the efficacy of immune checkpoint inhibitors is limited to only certain patients. This is due to patients having heterogeneous tumor microenvironment between individuals. [4]

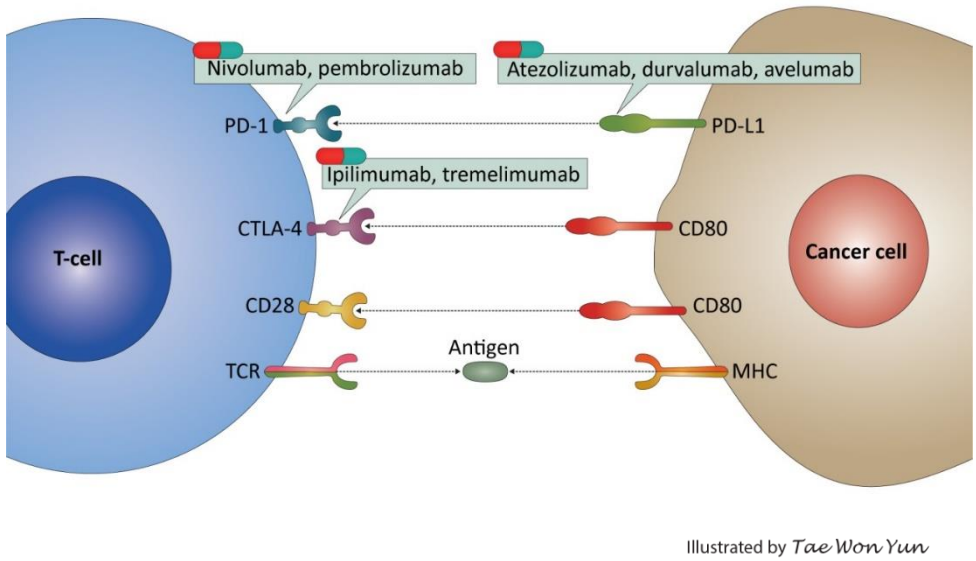


Figure 1. Therapeutic targets of immune checkpoint inhibitors

Drug	target	Approved	Indication
Iplimumab	CTLA-4	2011	Adjuvant therapy for melanoma Metastatic melanoma
Pembrolizumab	PD-1	2014	Locally advanced/metastatic Urothelial ca Metastatic colorectal ca Metastatic gastric ca Metastatic melanoma Metastatic NSCLC Metastatic squamous cell ca of head and neck Microsatellite instability high tumor Relapsed Hodgkin lymphoma Primary mediastinal large B-cell lymphoma Metastatic cervical cancer
Nivolumab	PD-1	2015	Urothelial advanced/metastatic urothelial ca Metastatic colorectal ca Metastatic liver ca Metastatic melanoma Metastatic NSCLC Metastatic renal cell ca Metastatic head and neck Microsatellite instability high colorectal cancer Relapsed Hodgkin lymphoma
Atezolizumab	PD-L1	2016	Advanced/metastatic urothelial ca Metastatic NSCLC
Duvalumab	PD-L1	2017	Locally advanced/metastatic urothelial ca Adjuvant therapy for stage II NSCLC before/after chemoradiation
Avalumab	PD-L1	2017	Locally advanced/metastatic urothelial ca

Ca: cancer, FDA: Food and Drug Administration, NSCLC: non-small cell lung cancer
CTLA4: cytotoxic T-lymphocyte antigen 4, PD-1: programmed cell death 1
PD-L1: programmed cell death ligand 1

Table 1. Immune checkpoint inhibitors approved by the Food and Drug Administration. (As of July 2018)

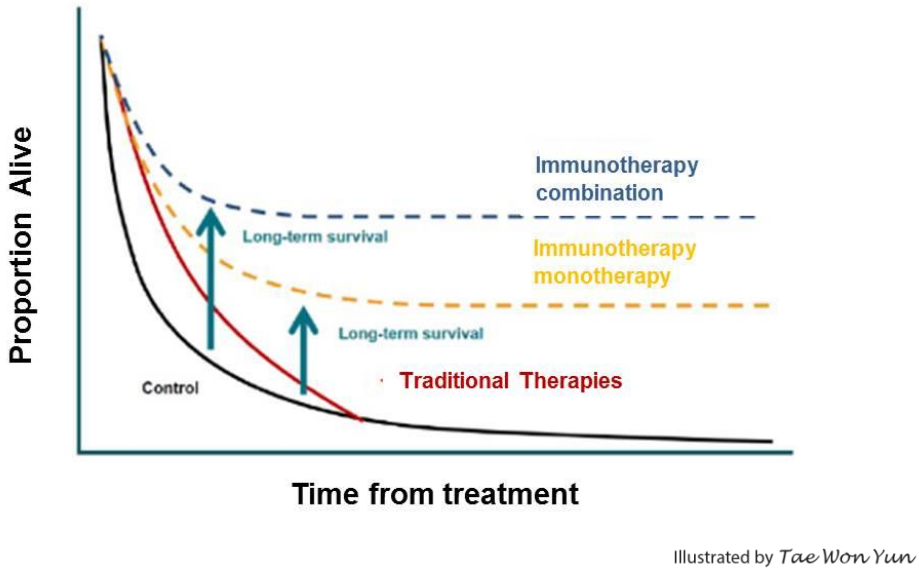


Figure 2. Immunotherapy increases long-term survival in cancer treatment

2. Types of patients based on TIL and PD-L1 status of expression.

The first indication of anti-PD-1/L1 antibodies, one of the immune checkpoint inhibitors, is melanoma. Melanoma patients are classified into 4 groups based on their PD-L1 expression level and absence or presence of TIL in the TME. These include Type I ($PD\text{-}L1^{+}$, TIL^{+}), Type II ($PD\text{-}L1^{-}$, TIL^{-}), Type III ($PD\text{-}L1^{+}$, TIL^{-}) and Type IV ($PD\text{-}L1^{-}$, TIL^{+}). This classification can be applied to not only melanoma but also various human tumors, as defined by PD-L1 expression/TILs. [6] In human melanoma, this classification is the most mature one. [5,7]

- **Type I cancer ($PD\text{-}L1^{+}TILs^{+}$)**

  In Type I patients ($PD\text{-}L1^{+}$, TIL^{+}), 38% of patients with melanoma are under this condition. [7,8] Patients under this category already have enough T cells in the TME to turn off the inhibition signal associated with PD-L1. Therefore, only single agent anti PD-1/L1 blockade is benefits the patients

- **Type II cancer (PD-L1^{-}TIL^{-})**

  A large number (about 41%) of melanoma patients are under type II tumor microenvironment. In these patients, TIL as well as PD-L1 expression is not sufficient, thus, the single agent checkpoint blockade is not a successful therapy and have very poor prognosis. Therefore, combination therapy with other reagents such as type I interferon that bring T cell to the TME is required. Type I interferon not only attracts T cell to the tumor site but also promotes PD-L1 expression level.[9] Thus, immuno combination treatment with type I interferon may be a useful therapeutic strategy for PD-1/PD-L1 axis. Another strategy to attract T cell to the tumor site would be chemotherapies, poly:IC, cancer vaccination and chimeric antibody receptor-specific T cells.[10]

- **Type III cancer (PD-L1^{+}TIL^{-})**

  Only 1% of patients with melanoma have type III tumor microenvironment. However, this type is the highest percentage in NSCLC. Because these patients do not have sufficient lymphocytes in the tumor site, immune system does not respond to fight against cancer. So in this type, there is a need to perform combination reagent that can bring TIL that is similar to type II. For instance, there are radiotherapy, chemotherapy, Type I interferon and CAR-T [11]

- **Type IV cancer (PD-L1^{-}TIL^{+})**

  Approximately 20% of patients with melanoma have type IV tumor microenvironment These patients have low expression level of PD-L1 but enough T cell. In this type, another suppressive pathway such as M2 polarized macrophages, adenosine (IDO) or non-T cell effects are dominant. The therapeutic approaches of this type are still in its infancy.

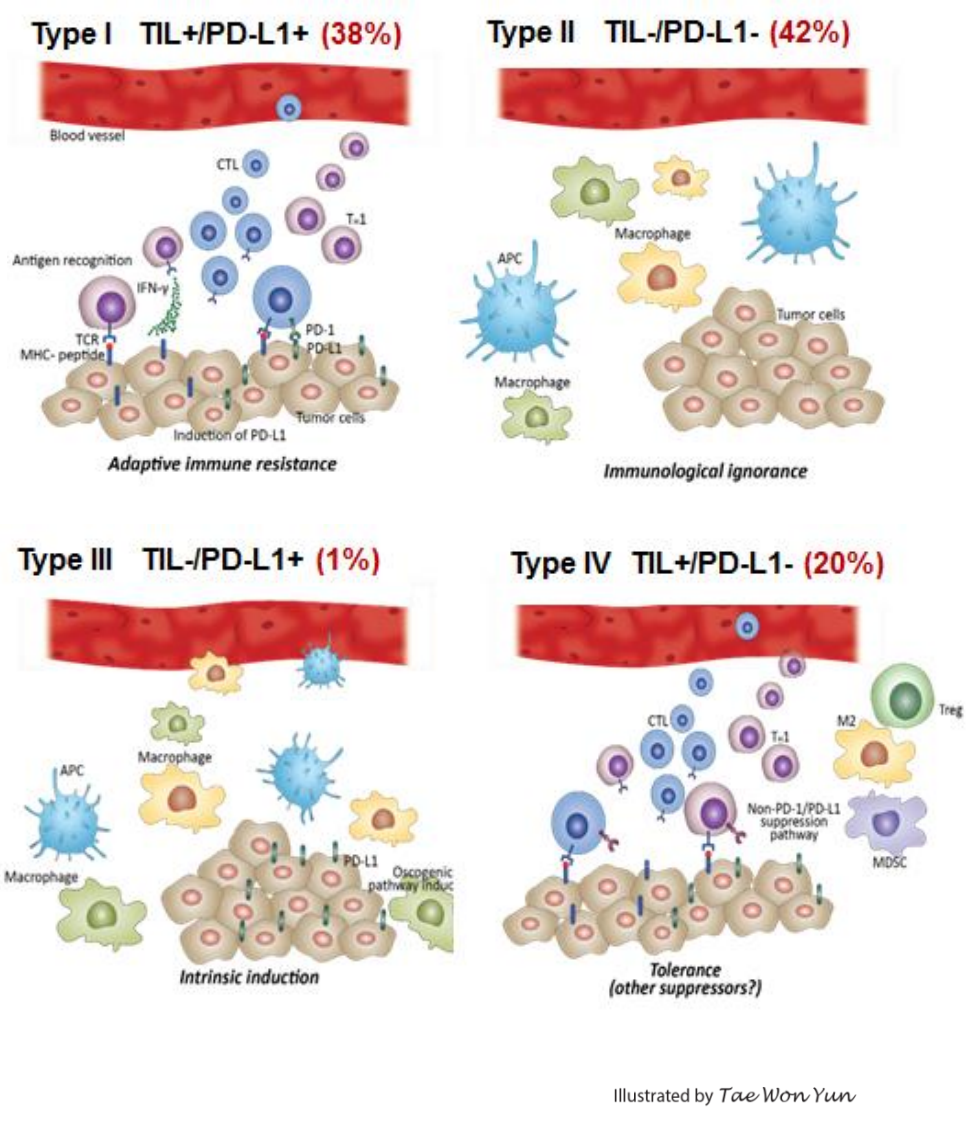


Figure 3. Melanoma patients have been categorized into 4 different tumor microenvironments based on PD-L1 expression and presence or absence of TIL.

3. Interferon-beta

Type I Interferon include IFN-α,IFN-β,IFN-ε,IFN-κ and IFNω subtype. This type has a variety of biological functions such as direct effect (antiproliferation, antiangiogenesis) and indirect effect to modulate surrounding immune cells. [9] Of the various subtypes, IFNα/β have specifically been known and approved in several diseases. IFN-α was approved for treatment of melanoma and renal cancer cell. Low dose of IFN-β have also been used in Japan. However, IFN-β has a high binding affinity to IFNAR1/2, which forms more stable ternary complexes than IFN-α [9]. Because of this, IFN-β regulates cellular function at very lower concentration than IFN-α subtype.[9] Therefore, IFN-β is potential and powerful cytokines to modulate the immune system. IFN-β induce chemokine (CXCL9, CXCL10) to attract activated lymphocyte, which recruit immune cells to the tumor site. Moreover, IFN-β regulates MHC class I expression[14], enhancing antigen recognition[12,15] and certain immune cells, such as DC for the activation of naive T cells and NK cells for enhancing to kill the target. [13,15] Thus, these IFN- β driven immune stimulatory pathways offer opportunities to devise immune combination therapeutic strategies. [15]

4. Expected anti-tumor therapeutic efficacy of simultaneous immunotherapy with PD-L1 checkpoint blockade and interferon–beta

Our purpose of this research is to convert patients' melanoma type from type II and III into type I by combination treatment using type I IFN. IFN-β has immune modulate function that promotes the expression of MHC-I in dendritic cells and cancer cells and induce inflammation to the TME and secretes various chemokines to strongly induce TIL recruitment. Although IFN-β has anti-tumor immunomodulatory function, it also has pro-tumor immune regulatory function such as increasing PD-L1 expression level. PD-L1 is one of the inhibitory signals to make T cell to be exhausted.

Therefore, the purpose of this study is to confirm the anti-tumor therapeutic efficacy of Simultaneous immunotherapy with PD-L1 checkpoint blockade and interferon. As a result, we were able to observe that if the interferon beta, one of the type I interferon, is first treated in the cancers, the MHC-I of the cancer is increased and chemokines are induced to recruit lymphocyte. Recruited Lymphocyte might kill the cancer. However, one of the negative effects of the interferon is its potential to increase the level of PD-L1. For this increased expression, T cells are exhausted. Therefore, PD-L1 blockade is required to reactivate the function of T cell for this effect. (Fig4)

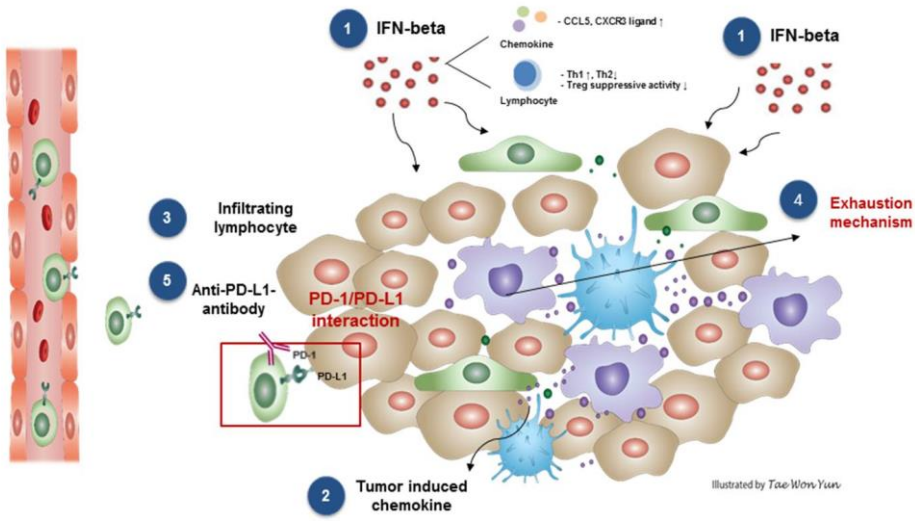


Figure4. Expected anti-tumor therapeutic efficacy of simultaneous immunotherapy with PD-L1 checkpoint blockade and interferon –beta

MATERIALS AND METHODS

1. Gene construction, Expression and Purification of anti-PD-L1-mAb.

The plasmids freedom pCHO1.0 (Life Technologies) was used as expression vector. The codon-optimized synthetic gene of the heavy chain and light chain of Anti-PD-L1-antibody (sequence derived by Atezolizumab, which is approved by FDA) for CHO-S cells were ordered from Cosmogenetech (Korea). This plasmid vector was transfected into CHO-S cells and stable clone were established according to the manufacturer's manual (Thermos Fisher Scientific). Antibody was produced in Established stable CHO clone and followed by purification using Protein A affinity chromatography (Mabselectsure, GE Healthcare). This protein was eluted with 250mM sodium citrate, pH 3.6. Eluted fractions were neutralized immediately with 1M Tris-HCl, pH 9.0 and dialyzed in PBS, pH 7.0. To verify protein purifies, SDS-PAGE was run on and stained with Coomassie blue. The Concentration of this portion was measured by the cedex bio system (Roche).

2. Anit-PD-L1-mAb binding assay by Flow cytometric analysis

To confirm Anti-PD-L1-mAb, binding to PD-L1, flow cytometry was performed using B16F10 mouse melanoma cell and MDA-MB23-1 human TNBC cell lines that expresses high levels of PD-L1. These cells were harvested using cell dissociation buffer (Gibco) and incubated at 4℃ for 1h in FACS buffer (PBS containing 2% FBS and 0.05% Sodium azide) with 1ug of Anti-PD-L1-mAb or commercial mouse/human anti-PD-L1mAb (BioXcell, Clone 10F.9G2 and 29E.2A3) or Human IgG(Jackson immunoresearch). Cells were rinsed twice with FACS buffer and incubated with PE-labeled goat anti-human IgG or anti-Rat-IgG secondary antibody(biolegend) in FACS buffer for 1hr at 4℃. The fluorescence signal was obtained by using a FACS Calibur (BD Biosciences) operated by Cell Quest pro software and data was reanalysis by flowjo program.

3. PD-1/PD-L1 Competitive inhibition assay by ELISA

Recombinant human and mouse PD-L1 Fc fusion protein (BPS Bioscience) were coated into 96-well half plate (corning) at 5ug/ml by overnight incubation at 4℃. After day, plates were washed with PBS-T (Tween0.5%) three times and blocked with blocking buffer (3% skim milk), followed by incubation for 1h at room temperature. The solution was then discarded, and

plates were washed three times with washing buffer. Serial dilution of Anti-PD-L1-mAb (clone HYE3) or commercial mouse/human anti-PD-L1-mAb or appropriate dilution of Biotinylated PD-1 were sequentially added, and the plates were incubated for 4h at 37℃ in shaking incubator. After another washing procedure, Blocking buffer(3% skim milk) was added, followed by incubation for 10min. The plates were washed, and Horseradish peroxide conjugated streptavidin (1:500) in 3% skim milk was added. After incubating at room temperature for 1hr, Plates were washed. And TMB colorimetric substrate was added. Reaction was stopped using 2M H2SO4. The absorbance at 450nm was detected on multi-reader (molecular devise, spectra MAX M5)

4. T cell Activation assay

For-T-cell activation assay, 96-well flat-bottomed plates (SPL) were coated overnight with anti-CD3 (clone OKT3; e-bioscience) at 2.5ug/mL mixed together with 3ug/ml recombinant PD-L1-Fc fusion protein (R&D system) in PBS at 4℃. After day, Wells were washed twice with PBS before adding cells. Splenocytes were seeded at $4X10^5$ cells per well in complete RPMI media. (RPMI 1640, 10% heat-inactivated fetal bovine serum, 1% penicillin/ Streptomycin, 1% L-Glutamine, 0.1% 2-mercaptoethanol). Appropriated serial diluted Anti-PD-L1-mAb (HYE3 clone) or commercial anti-PD-

L1mAb (Clone 10F.9G2, BioXcell) or Human IgG (Jackson immune research) were added at the plate. Plate was incubated in a humidified 38℃, 5% Co2 incubator. Supernatant on day 4 were analyzed by IFN-γ ELISA kits (R&D systems) according to the manufacturer's instruction.

5. Cell culture

Mouse cell line (4T1, CT26, B16F10, RENCA) and human cell line (MDA-MB-231, A375P, HCC827, SK-OV3, SK-BR3) were purchased from Korean cell line bank (Seoul, Korea). These cells were maintained at 37℃ in a humidified 5% CO2 incubator and cultured in complete Dulbecco's modified Eagle's Medium or RPMI-1640 from Hyclone (GE healthcare) supplemented with 10% heat-inactivated fetal bovine serum(FBS), 1% of penicillin/streptomycin solution.

6. Cell cytotoxicity assay

Cell cytotoxicity of IFN-β on the various mouse cell and humans were determined using a WST assay. Mouse cell line (4T1, CT26, RENCA, MC38, B16F10) and human cell lines (A375P, MDA-MB-231, SK-OV3, SK-BR3, HCC827) were seeded to 96-well plates at an appropriate concentration. After day, mIFN-β (Biolegend) or R27T (glycoengineered hIFN-β1a) were treated with or without various concentration for 48hr. WST reagent (Ez-cytox,

Dogen) was added to each well and cells were incubated for 1hr. Absorbance at 450nm was detected on Multi-reader (molecular Devices, Spectra MAX M5). The percentage of cell viability was calculated using the following formula: % Viability = [(Absorbance of experimental – absorbance of blank)/(Absorbance of untreated control-absorbance of blank)]X100

7. Stimulated PD-L1 and MHC-I expression level in respond to IFN-β in-vivo

Mouse and human melanoma cell (B16F10, A375P) were cultured with various concentration of mIFN-β or hIFN-β1a for 48hr. After 48hr, cells were harvested for FACS analysis or determination of mRNA expression. For FACS analysis, cells were stained with APC-label anti-$H2K^b$ (clone AF6-88.5, biolegend) or $H\text{-}2D^b$ (clone KH95, biolegned) or HLA-ABC(clone W6/32, biolegend) or PE-labeled with anti-mouse or human PD-L1(Clone MIH5 , 29E.2A3, respectively). For determination of mRNA expression, Total RNA was extracted using Hybrid-RTM kit (Geneall, Seoul, Korea), and First-strand cDNA was generated using a Transcriptor First strand cDNA synthesis kit (Roche, Indianapolis, IN, USA). SYBR premix Ex Taq (Takara) was performed according to the manufacturer's instruction. All primer sets used in this study are shown in Table 2. Beta-actin was used for normalization and Relative expression of each mRNAs was calculated using ΔΔCt method.

8. Stimulated PD-L1 and MHC-I expression level in respond to IFN-β in-ex-vivo

To confirm increased expression level of PD-L1 and MHC-I in ex-vivo, B16F10 $5X10^5$ cells were subcutaneously inoculated and mIFN-β was treated every other day. Tumor tissues were resected from B16F10-bearing mice on day after three times injection of mIFN-β. The tissues were incubated with collagenase type IV (Worthington Biochemical corporation) for 30min at 37℃ by using Gentle MAX(MACS) to homogenize the tumor tissue. Single tumor cells were obtained by lymphocyte separation medium (MP) according to manufacturer's protocol. Isolated single tumor cells were washed in FACS buffer (PBS containing 2% FBS and 0.05% Sodium azide) and stained with PE-labeled anti-mouse PD-L1(clone MIH5, biolegend) and APC-label anti-$H2K^b$ (clone AF6-88.5, biolegend) or $H\text{-}2D^b$ (clone KH95, biolegned) Stained cells were analyzed using a FACS Calibur (BD Biosciences) operated by Cell Quest pro software and data was re-analyzed by Flowjo program.

9. Chemokine analysis in vitro.

To perform chemokine analysis in vitro, B16F10 mouse melanoma cells were cultured with 1000ng/ml of mIFN-β or PBS for 48hr. After 48hr, supernatant were collected for chemokine ELISA or total RNA were extracted by using

Hybrid-RTM kit (Geneall, Seoul, Korea). Chemokine in the supernatant were measured using ELISA kit (mouse CCL5/RANTES Duoset, mouse CXCL10/IP-10/CRG-2 Duoset, R&D system) according to each manufacturer's instructions. Extracted RNA reversibly transcribed to cDNA using transcriptor First strand cDNA synthesis kit (Roche, Indianapolis, IN, USA). mRNA expression was measured by qRT-PCR using SYBR premix Ex Taq (Takara) according to the manufacturer's instruction. All primer sets used in this study are shown in Table 2. Beta-actin was used for normalization and Relative expression of each mRNAs was calculated using ΔΔCt method.

10. Tumor infiltrated lymphocyte analysis by flow cytometry.

For subcutaneous tumor model, $5X10^5$ B16F10 cells were subcutaneously injected into C57BL/6 mice and 1ug mIFN-β was subcutaneously injected every other day and the tumor was harvested on the day after three time injection. For lung metastasis model, B16F10 $2X10^5$cells were intravenously injected and mIFN-β was also subcutaneously injected every other day and the tumor was harvested on day 14 after tumor inoculation. Tumor tissue was homogenized by using gentle max (MACS) and Tumor infiltrating lymphocytes were isolated by using lymphocyte separation media (MP, USA). Isolated TILs were stained with PE labeled anti-mouse CD8, CD4 (clone 53-6.7,RM4-4;Biolegend) and APC labeled anti-mouse CD45(clone 30-F11;Biolegend).

11. T-cell-related to chemokine in ex-vivo.

In ex-vivo for chemokine analysis, B16F10 $2X10^5$cell was intravenously injected into C57BL/6 mice. 1ug mIFN-β was subcutaneously injected every other day and the tumor was harvested on day 14 after tumor inoculation. Tumor tissue was homogenized by using gentle max(MACS) and total RNA was extracted using Hybrid-RTM kit (Geneall, Seoul, Korea) and also cDNA was synthesized using transcriptor First strand cDNA synthesis kit (Roche, Indianapolis, IN, USA). mRNA expression was measured by qRT-PCR using SYBR premix Ex Taq (Takara) according to the manufacturer's instruction. All primer sets used in this study are shown in Table 2. Beta-actin was used for normalization and Relative expression of each mRNAs was calculated using ΔΔCt method

12. Reactivated T cell in ex-vivo

B16-OVA cells were irradiated at 8000rad using Gamma irradiator. After radiation, $2X10^4$ B16-OVA cells were seeded at 24 well plates. Spleenocytes from OT-I mice were harvested and seeded $4X10^5$ and various concentration mIFN-β and anti-PD-L1-antibody were added. After 24hr, IFN-γ in culture Supernatant was collected and measured using ELISA kit (R&D system) according to manufacturer's instructions.

13. Overall survival study in syngeneic mouse model

All mouse study procedure was performed in accordance with the policies of IACUC (Institutional Animal Care and Use Committee) of Seoul National University and approved by Seoul National University IACUC (Approval No.: SNU-180305-9). Wild type female BALB/c and C57BL/6 mice were purchase from the orient bio (Republic of Korea,). $2X10^5$ B16F10 cells or $5X10^5$ RENCA cells were injected into the tail vein of C57BL/6 mice or BALB/c mice, respectively. Mice were received a subcutaneous injection of 1ug mIFN-β (Biolegend) on every other day and an intraperitoneal injection of 200ug anti-PD-L1 mAb on three times a week. Human IgG Control or PBS was used for mock treatment. According to guidelines established by IACUC, mice were sacrificed when mice body weight loss >20% compared to start of experiment. The body weights were monitored daily throughout the course of the experiment.

14. Humanized mouse model

NOD.Cg-PrkdcscidTg(hSCF/hGM-CSF/hIL3) (NOD/ SCID-SGM3) mice were obtained from the Jackson Laboratory and raised under specific pathogen-free condition at Seoul National University. 6 weeks old mice were injected with bulsulfn(MCE), and $1X10^5$ human $CD34^+$ hematopoietic cells

in 100ul PBS were injected intravenously after day. For analysis of human immune system reconstitution, Blood was collected at 4weeks, 8weeks and 12 weeks after transplantation. 8 or 12wk post-HPC transplantation, $5X10^6$ A375P human melanoma cells, suspended in serum-free media mixed with matrigel (Corning Life Sciences, Bedford, MA, USA), were subcutaneously injected in NOD/SCID-SGM3 mice. Drug treatment was started when the tumors reached $200mm^3$ in volume. Anti-PD-L1-antibody was injected intraperitoneally three times a week and R27T (hIFN-β1a) was subcutaneously injected three times a week for 3weeks. Weight of tumors were measured at the end point.

15. Statistical analysis

Graph-pad Prism software was used to determine significant difference. $P < 0.05$ was considered significant. Differences between two groups or among multiple groups were assessed by the unpaired t-test. The log-rank test was performed to assess significant difference in survival of mice on Kaplan-Meier plots among multiple groups.

Table 2. qRT-PCR primer list

mH-2Db	Forward: 5’- AGT GGT GCT GCA GAG CAT TAC AA-3
	Reverse: 5’- GGT GAC TTC ACC TTT AGA TCT GGG-3
mH-2Kb	Forward: 5’- GCT GGT GAA GCA GAG AGA CTC AG-3’
	Reverse: 5’- GGT GAC TTT ATC TTC AGG TCT GCT-3’
mCCL5	Forward: 5’- CAC CAC TCC CTG CTG CTT-3’
	Reverse: 5’- ACA CTT GGC GGT TCC TTC-3
mCXCL9	Forward: 5’- TTT TGG GCA TCA TCT TCC TGG-3’
	Reverse: 5’- GAG GTC TTT GAG GGA TTT GTA GTG G -3
mCXCL10	Forward: 5’- CTT CTG AAA GGT GAC CAG CC-3
	Reverse: 5’- GCT TCC CTA TGG CCC TCA TT-3’
mCXCL11	Forward:5’- AAC AGG AAG GTC ACA GCC ATA GC-3’
	Reverse:5’-TTT GTC GCA GCC GTT ACT CG-3’
mCCL17	Forward:5’-CAA GCT CAT CTG TGC AGA CC-3’
	Reverse: 5’-CGC CTG TAG TGC ATA AGA GTC C-3’
mCCL22	Forward:5’-AAG ACA GTA TCT GCT GCC AGG-3’
	Reverse: 5’-GAT CGG CAC AGA TAT CTC GG-3’
mFoxp3	Forward: 5’-CCT GCC TTG GTA CAT TCG TG-3’
	Reverse: 5’-TGT TGT GGG TGA GTG CTT TG-3’

mT-bet	Forward: 5’-TTC CCA TTC CTG TCC TTC AC-3’
	Reverse: 5’-CCA CAT CCA CAA ACA TCC TG-3’
mGATA3	Forward: 5’-GGA AAC TCC GTC AGG GCT A-3’
	Reverse: 5’-AGA GAT CCG TGC AGC AGA G-3’
mROR-γ	Forward: 5’-TGA GGC CAT TCA GTA TGT GG-3’
	Reverse: 5’-CTT CCA TTG CTC CTG CTT TC-3’
mBeta-actin	Forward: 5’- GTG GGC CGC TCT AGG CAC CAA-3’
	Reverse: 5’- GTC GCA CCT CCA CAT AGC TT-3’

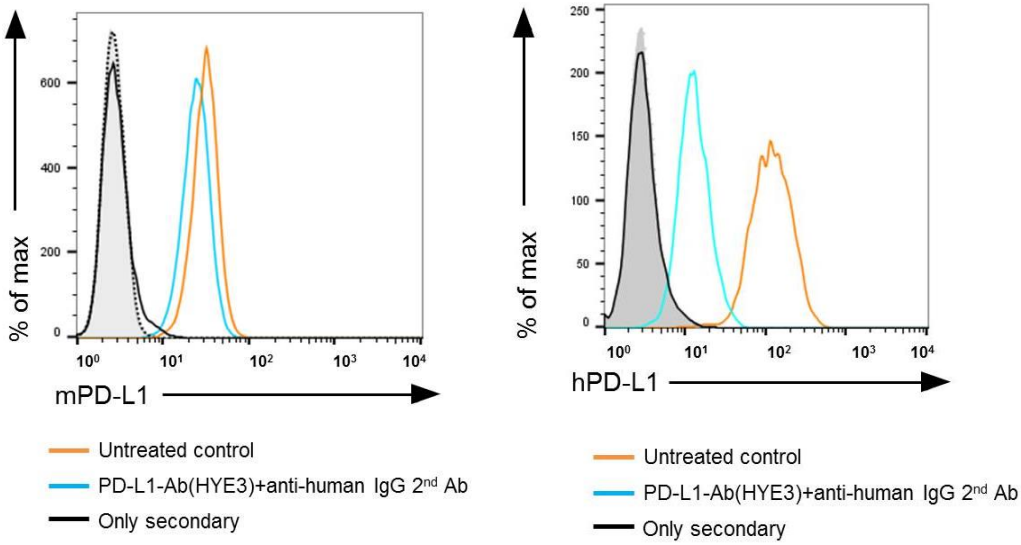
RESULT

1. Validation of anti-PD-L1-antibody (derived from atezolizumab amino acid sequence)

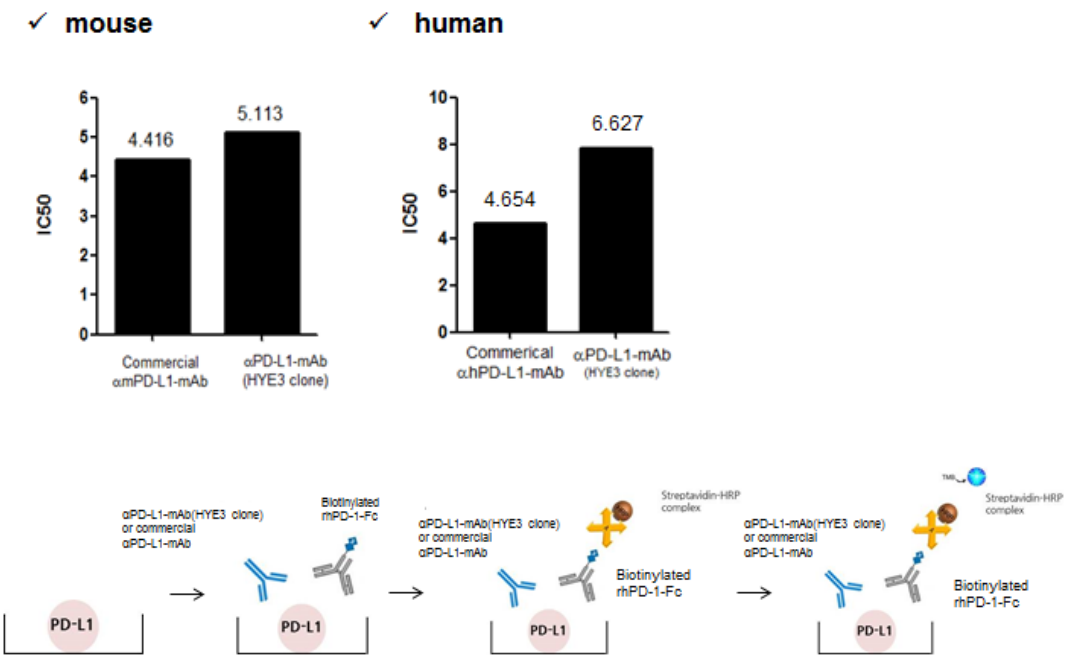
We generated anti-PD-L1-mAb that is derived from atezolizumab amino acid sequence, which is FDA approved anti-PD-L1-mAb. We designated this antibody as HYE-mAb. To verify this antibody, first we assessed their binding activity of PD-L1 and cross reactivity between mouse and human compared to commercial antibody. B16F10 mouse melanoma cell and MDA-MB-231 human TNBC cells expressing high level of PD-L1 were incubated with HYE-mAb and commercial anti-PD-L1-mAb and subsequent change in fluorescence signal was measured by flow cytometry analysis. Consequently, anti-PD-L1-antibody (clone HYE3) elicited a shift in the fluorescence signal similar to commercial antibody in mouse, not in human cell (Fig5A). Secondly, we confirmed neutralizing effect by a competitive PD-1/PD-L1 inhibition assay based on ELISA. Result of the inhibition assay showed that neutralization pattern of HYE3-mAb was similar to commercial anti-PD-L1 antibody (Fig5B). Lastly we performed T-cell activation assay to investigate the ability of HYE3-mAb to overcome PD-L1-mediated inhibition of mouse T cells. In this assay, splenocytes were co-cultured with anti-CD3 and various

concentration of antibody (HYE3 clone). HYE3-mAb was able to inhibit this activity of PD-L1 in a concentration-dependent manner similar to commercial antibody (Fig5C). Comprehensively, although binding affinity and neutralizing effect of HYE3-mAb were relatively lower compare to commercial antibody, it has strongly functional effect to overcome PD-L1-mediated inhibition of T cell. These results show that HYE3-mAb has function of anti-PD-L1-antibody.

A


% of max
mPD-L1
Untreated control
PD-L1-Ab(HYE3)+anti-human IgG 2nd Ab
Only secondary
% of max
hPD-L1
Untreated control
PD-L1-Ab(HYE3)+anti-human IgG 2nd Ab
Only secondary

B


✓ mouse
✓ human
IC50
4.416
5.113
Commercial αmPD-L1-mAb
αPD-L1-mAb (HYE3 clone)
IC50
4.654
6.627
Commerical αhPD-L1-mAb
αPD-L1-mAb (HYE3 clone)
αPD-L1-mAb(HYE3 clone) or commercial αPD-L1-mAb
Biotinylated rhPD-1-Fc
Streptavidin-HRP complex
PD-L1

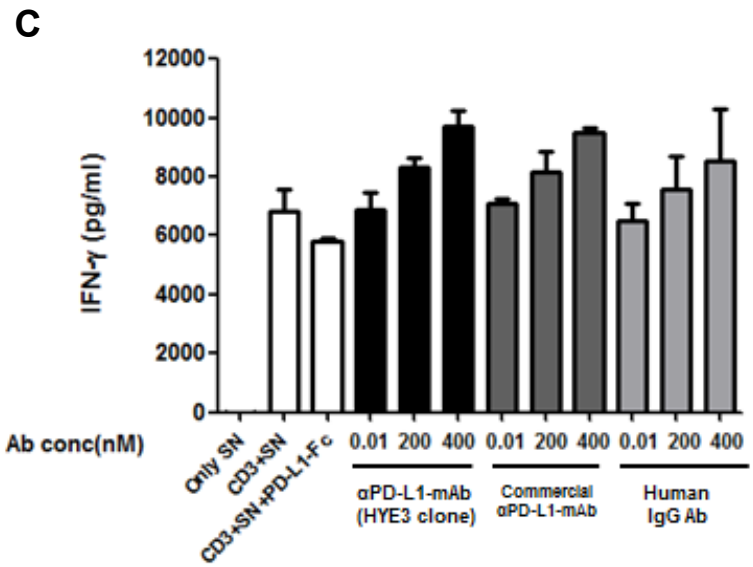


Figure 5. Anti-PD-L1-mAb (called HYE3-mAb) was identified with various experiments. Anti-PD-L1-mAb (HYE3 clone) has similar function to commercial antibody. This antibody binds to mouse or human PD-L1 and has neutralizing effect as well as overcomes PD-L1-mediated inhibition of T-cell activation similar to commercial antibody. (A) B16F10 mouse melanoma cells and MDA-MB-231 human TNBC cells were incubate with anti-PD-L1-mAb (clone HYE3) or commercial mouse or human anti-PD-L1mAb (Clone 10F.9G2, 29E.2A3;Bioxcell) and incubated with PE-conjugated goat anti-human -IgG or anti-Rat IgG secondary antibody (1ug/10^6cells). A representative histogram showing anti-PD-L1-antibody binding to both cells (blue histogram) and commercial antibody binding to both cells (orange histogram). (B) IC50 was measured by PD-1/PD-L1 competitive ELISA compare to commercial anti-PD-L1-antibody. (C) Splecnoyte was co-cultured with anti-CD3-Ab and recombinant PD-L1-fc protein. Various concentration of anti-PD-L1-mAb (HYE3-mAb or commercial mAb or human IgG Ab) was treated. After 4 days, supernatant from each well was assayed for IFN-γ by ELISA. Bars indicate mean SEM of triplicate.

2. Cell cytotoxicity function of IFN-β to induce inflammation

Interferon beta has not only direct effect in cancer cells inducing cell death and cell arrest but also immune modulatory effect using surrounding immune cells to kill the cancer. Cell apoptosis, one of the direct effects of INF-β, induce anti-inflammatory mechanism. This is important that immune cells are recruited at the tumor site by inflammation, followed by immune response. Thus, we confirmed the cell cytotoxicity function of IFN-β in each mouse cells by using WST assay. When exposed at high doses, the sensitivity of IFN-β was different from each cell (Fig6A). We designated as sensitive cell (4T1, B16F10, CT26) with 40~50% of viability and insensitive cells (RENCA, MC38) with viability above 50%. As a result, we selected the B16F10 cell, which is a type of murine melanoma cell, for future experiment Because this cell highly responds to the IFN-β (Fig6B) and has less immunogenic properties. Therefore, the cell is suitable for our study.

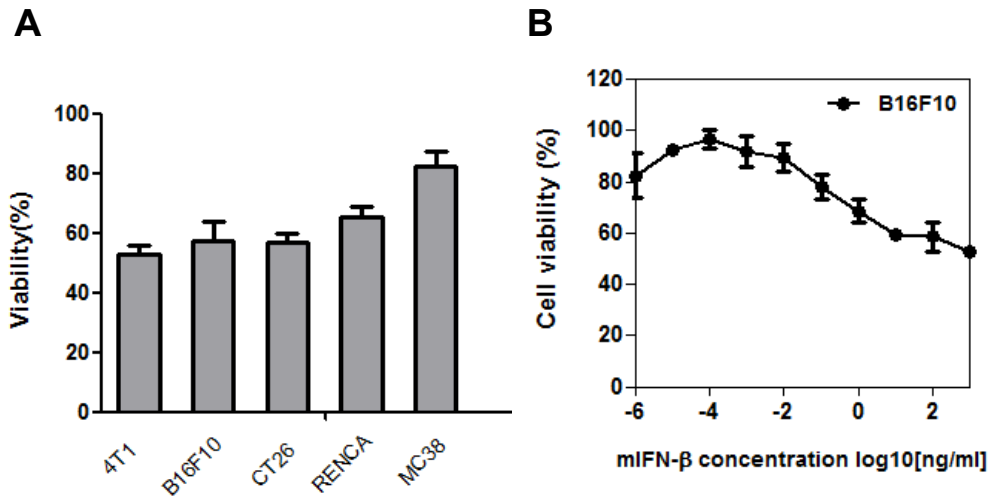


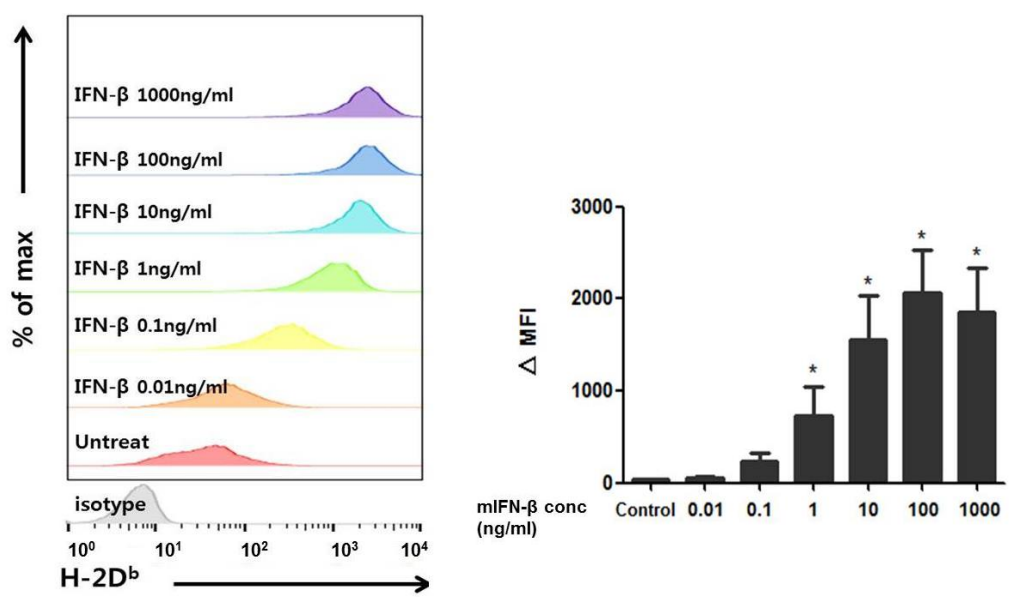
Figure6. IFN-β has cell cytotoxicity function in different mouse cell lines

Cell cytotoxicity was evaluated by using WST assay. (A) Several mouse cell lines (4T1, B16F10, CT26, RENCA, MC38) were exposure at high concentration (1000ng/ml) of mIFN-β for 48hr. (B) B16F10 cell were treated with or without varying concentration of mIFN-β for 48hr and also viability were measured using WST assay. Data are expressed as mean ± SEM of three independent experiments

3. Elevated expression level of MHC-I in cancer cell response to IFN-β

To investigate increased expression level of major histocompatibility complex-I (MHC-I), one of the immune modulatory effects of IFN-β, varying concentration of IFN-β was treated for 48hr in B16F10 cell and then expression level was measured by flow cytometry. Because the neo-antigens presented on the tumor are different depending on MHC-I isotype, we evaluated all isotype (H-2D^b, H-2K^b) expression levels. As shown in the data, the expression of MHC-I (H-2D^b) was significantly increased depending on IFN-β concentration manner (Fig7A) and the RNA expression level was significantly increased in both MHC-I isotype(H2-D^b and H-2K^b) compared to the control in vitro (Fig7B). We also found that expression level of MHC-I was elevated in ex-vivo experiment (Fig7C). But there is no significantly value. This is because exposure concentration of IFN-β in vivo was different compared to the vitro experiment. Thus, we should determine the concentration of IFN-β in vivo mouse experiment by considering the pharmacokinetic and pharmacodynamics of drug.

A


IFN-β 1000ng/ml
IFN-β 100ng/ml
IFN-β 10ng/ml
IFN-β 1ng/ml
IFN-β 0.1ng/ml
IFN-β 0.01ng/ml
Untreat
isotype
% of max
10⁰ 10¹ 10² 10³ 10⁴
H-2Dᵇ
Δ MFI
3000
2000
1000
0
Control 0.01 0.1 1 10 100 1000
mIFN-β conc (ng/ml)

B

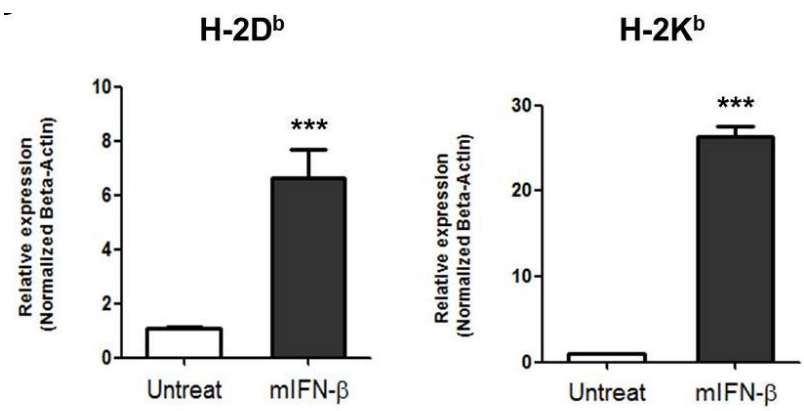

H-2Dᵇ
H-2Kᵇ
Relative expression (Normalized Beta-Actin)
***
Untreat
mIFN-β

Figure7. IFN-β increased expression level of MHC-I in B16F10 melanoma cells in vitro. (A) B16F10 cell was cultured with vehicle (PBS) or various concentration of mIFN-β for 48hr. Cells were harvested, stained APC-labeled anti- H-2D^b Ab (KH95, Biolegned) or an isotype control, and H-2D^b expression was measured by flow cytometry. The expression was evaluated average mean fluoresce intensity (MFI), which is normalized isotype control at each concentration, and the MFI value demonstrated a significant increase in H-2D^b expression on the cells, correlating with increase in concentration of mIFN-β. (B) B16F10 cells were cultured with 1000ng/ml of mIFN-β. H-2D^b and H-2K^b mRNA expression was analyzed using qRT-PCR after 48hr. Each gene was normalized by mouse beta-actin and then relative expression level of gene for the qRT-PCR data was calculated. All data represent the mean ± SEM of three independent experiments; *P<0.05, **P<0.01, ***P<0.001.

C

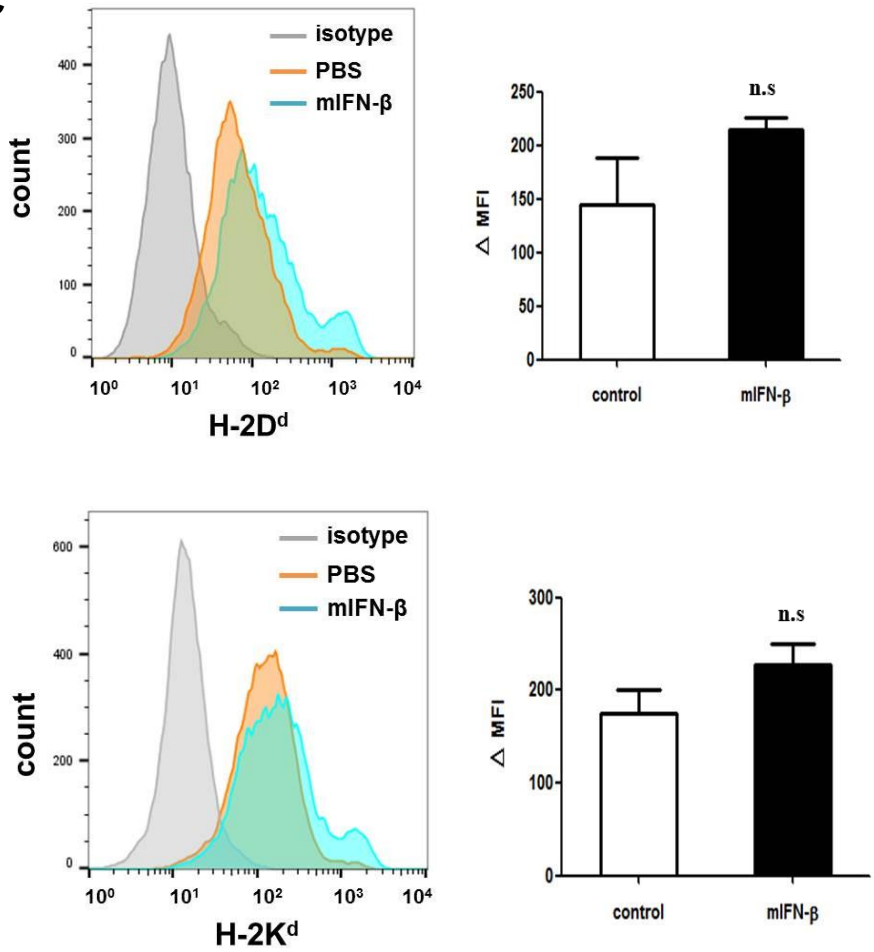

isotype
PBS
mIFN-β
count
H-2D^d
n.s
△ MFI
control
mIFN-β
isotype
PBS
mIFN-β
count
H-2K^d
n.s
△ MFI
control
mIFN-β

Figure 8. IFN-β increased expression level of MHC-I in B16F10 melanoma in ex-vivo. (A) B16F10 murine melanoma cells were intravenously injected into tail vein of C57BL/6 mice on day 0 and subcutaneously injected with IFN-β or PBS on every other day. Tumor tissues were harvested and isolated single tumor cell by lymphocyte separation medium (LSM, MP), stained APC-labeled anti H-2D^{d} and H-2K^{d}-Ab. The expression of MHC-I (H-2D/K) on B16F10 was measured using flow cytometry. ΔMFI was calculated between specific antibody and isotype control. Representative data of two independent experiments are shown (n=5 per group).

4. Increased expression of CCL5 and CXCR3 ligand (CXCL9, CXCL10, CXCL11) chemokine in response to IFN-β in-vitro

IFN-β is known for having key role in recruiting effector T cells to tumor site by inducing chemokine such as CCL5 and CXCR3 ligand (CXCL9-11). These chemokines are known as the main determinant of T-cell infiltration into tumor microenvironment (TME). Thus, we evaluated whether IFN-β promoted to these chemokine by chemokine ELISA and qRT-PCR in vitro. As shown in the data, IFN-β enhanced production of CCL5 and CXCL10 compared to control (Fig9A), and also significantly elevated mRNA expression (Fig9B).

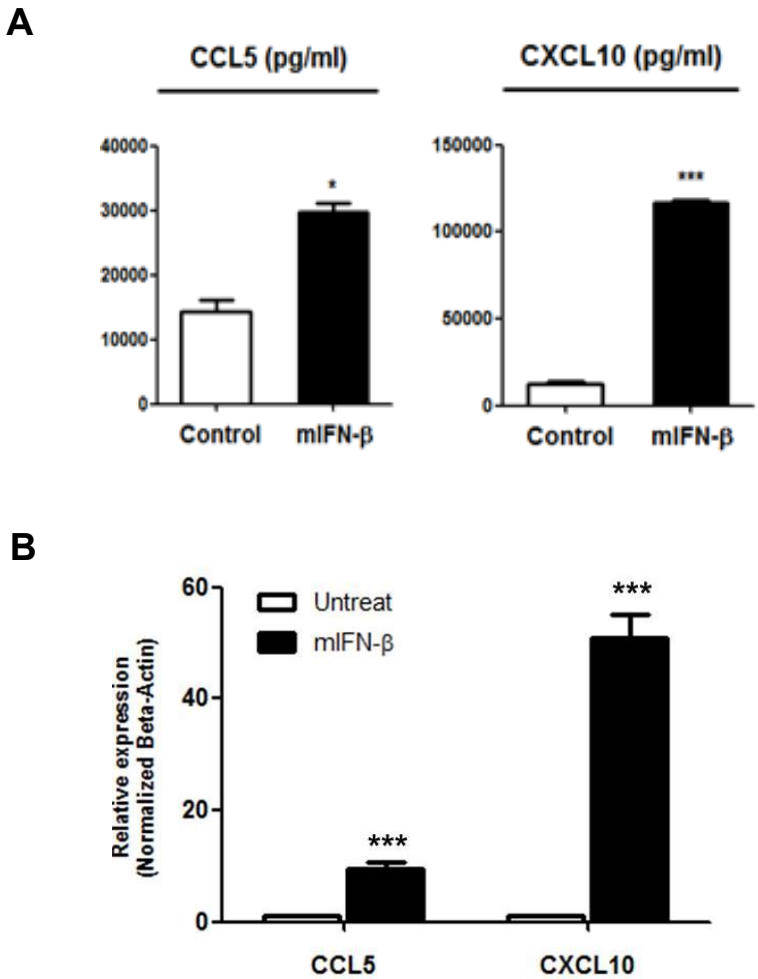


Figure 9. IFN-β induce CCL5 and CXCR3 ligand (CXCL9, CXCL10, CXCL11) in melanoma cell line. The cells were stimulated with IFN-β or PBS for 48hr (A). After 48hr, Supernatant is collected for chemokine ELISA. (B) Cell was harvested for qRT-PCR. The expression level of gene were increased in respond to IFN-β. Representative data of two or three independent experiment are shown. All data represent the mean ± SEM of two or three independent experiments; *P<0.05, ***P<0.001

5. Immune modulatory effect of IFN-β on the TME of B16F10 melanoma

To investigate the immunomodulatory effect of IFN-β on the TME, B16F10 murine melanoma cells were injected into C57BL/6 mice and IFN-β were treated with subcutaneous injection every other day for 2 weeks. Analysis of the frequency of TIL and mRNA level of chemokine in the tumor tissue showed that IFN-β treatment group enhanced the accumulation of tumor-infiltrating CD8+ and CD4+ T-cells and increased the mRNA expression of CCL5 and CXCR3 ligand (CXCL9-11) compared to control groups (Fig10A). Although no significant value was obtained in the both model, tumor infiltrating lymphocyte (TIL) showed increased tendency in lung metastasis and subcutaneous mouse model (Fig11A,B). Moreover, we evaluated the subpopulation of TIL and Th1/Th2-related chemokines by qRT-PCR. The balance of Th1 and Th2 are important. In normal circumstances, Th1 and Th2 immunity approached a balance. However, Tumor cells disrupt this balance. In the presence of tumor cell, increased Th2 immunity and decreased Th1 immunity leads to tumor progression. In addition Th2-related chemokines have a role suppressed the recruitment of Treg in TME.[16] As shown in the data, the administration of IFN-β group significantly increases the mRNA expression of Th1 chemokines (CCL9, CXCL11) and decrease the mRNA

expression of Th2 chemokines (CCL17, CCL22) (Fig10A). And the administration of IFN-β modulates the mRNA expression of transcriptional factor related TIL. In data, group stimulated IFN-β increase the expression of T-bet and decreases the expression of GATA3 and Fxp3 in the tumor microenvironment (Fig10B). We found that IFN-β have potential immunomodulatory effect in vivo.

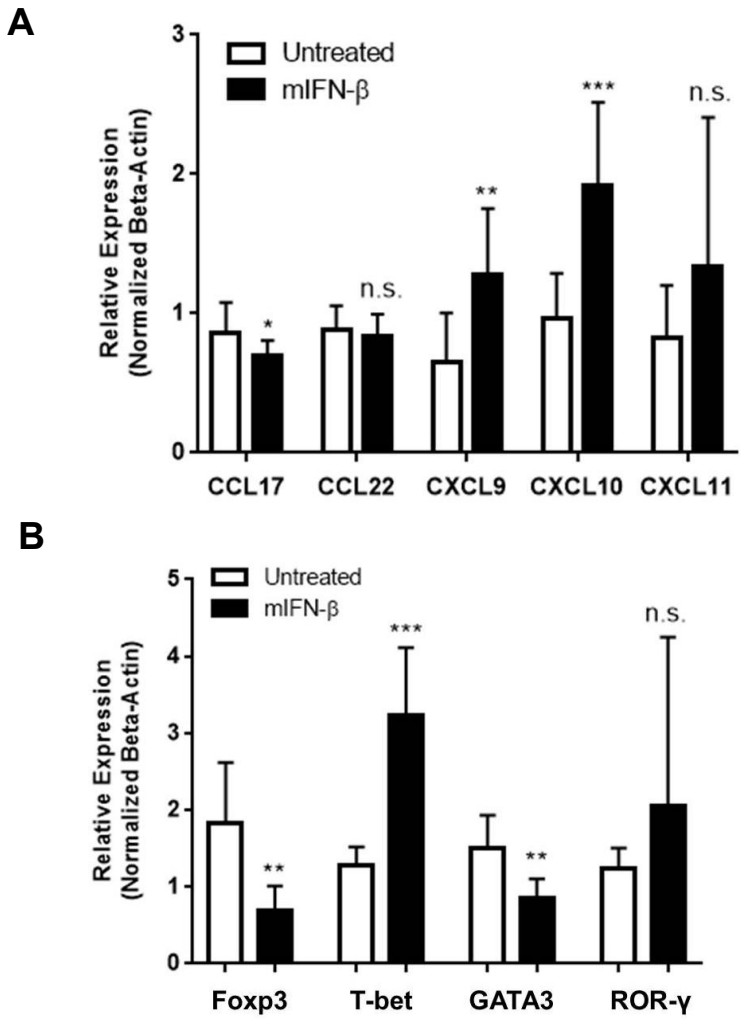


Figure10. IFN-β regulates the chemokine and transcriptional factor in B16F10 melanoma. C57BL/6 mice were intravenously injected with B16F10 melanoma cells on day 0 and subcutaneously with IFN-β or PBS on every other day. Mice are sacrificed at 14days after inoculation and tumor are harvested. The Relative expression level of chemokine (A) and transcriptional factor (Foxp3, T-bet GATA3, ROR-γ) (B) in tumor tissues were analyzed by qRT-PCR by using ΔΔCt method. Each gene was normalized by beta-actin. Error bars represent an mean +SEM, *P<0.05, **P<0.01, ***P<0.001 as determined by a student t test between indicated groups.

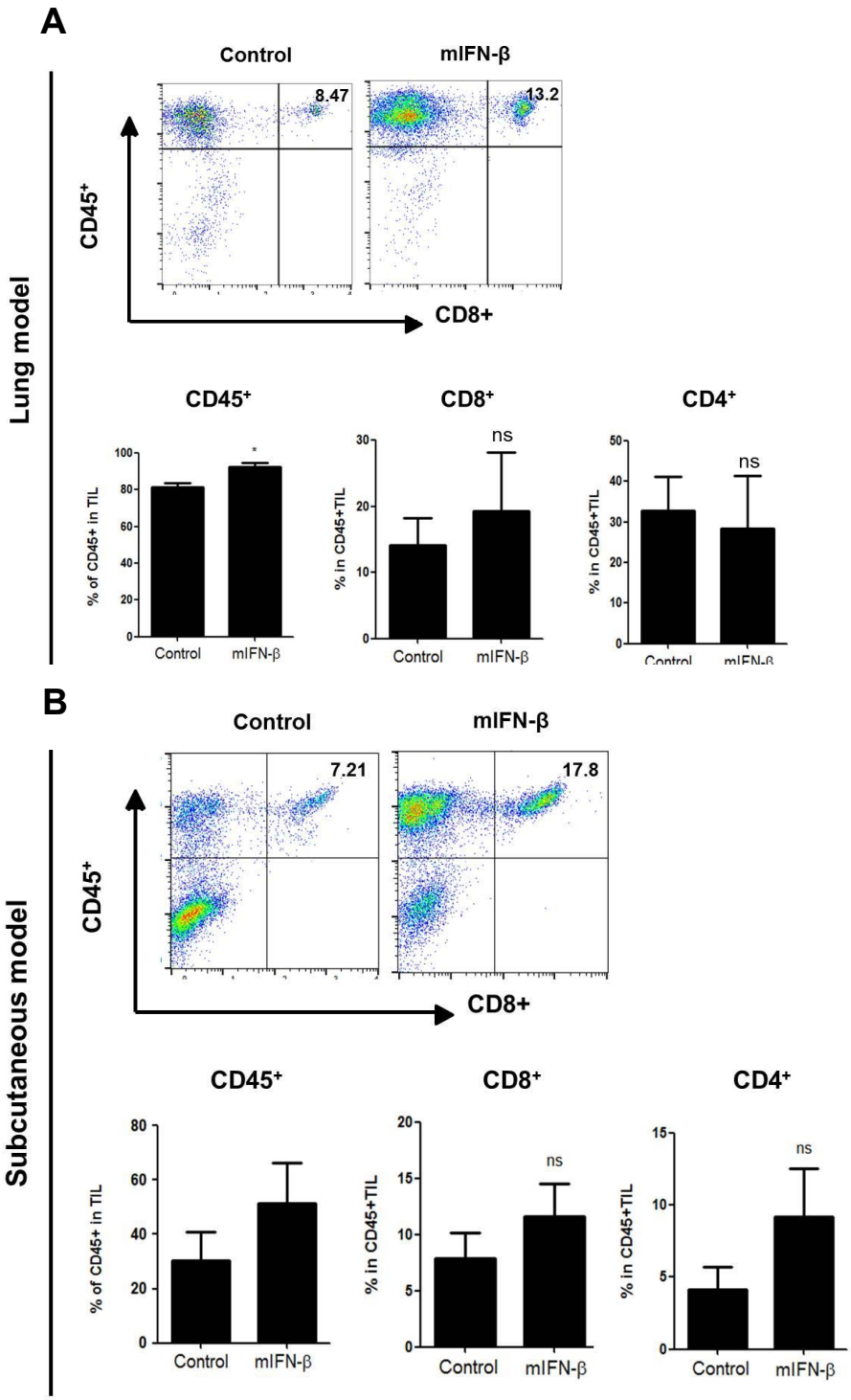

A
Control
mIFN-β
8.47
13.2
CD45+
CD8+
Lung model
CD45+
CD8+
CD4+
% of CD45+ in TIL
% in CD45+TIL
% in CD45+TIL
*
ns
ns
Control
mIFN-β
Control
mIFN-β
Control
mIFN-β
B
Control
mIFN-β
7.21
17.8
CD45+
CD8+
Subcutaneous model
CD45+
CD8+
CD4+
% of CD45+ in TIL
% in CD45+TIL
% in CD45+TIL
ns
ns
Control
mIFN-β
Control
mIFN-β
Control
mIFN-β

Figure11. IFN-β treatment resulted in accumulation of T cells in B16F10 murine melanoma tissue. (A) B16F10 melanoma cells were intravenously injected in C58BL/6 mice on day 0 and subcutaneously with IFN-β or PBS every other day for 2weeks. (B) C57BL/6 mice were subcutaneously inoculated with B16F10 cells on day 0 and subcutaneously injected with IFN-β for 1weeks. The percent of T-cells in TIL was analyzed using Flow cytometry (n=5 per group). There was no significant value.

6. Increased expression level of PD-L1 in cancer cell response to IFN-β

To investigate pro-tumorigenic function of IFN-β, we evaluate PD-L1 expression level response to IFN-β by flow cytometry. Surface PD-L1 expression significantly increasesd when IFN-β was treated in a concentration-dependent manner in vitro (Fig12A). But in ex-vivo, there was no significant difference in expression of PD-L1 (Fig12B). We thought that the exposure concentration of IFN-β in vivo was different from in vitro. As I mentioned before, we should consider the pharmacokinetic and pharmacodynamics of IFN-β in-vivo experiment.

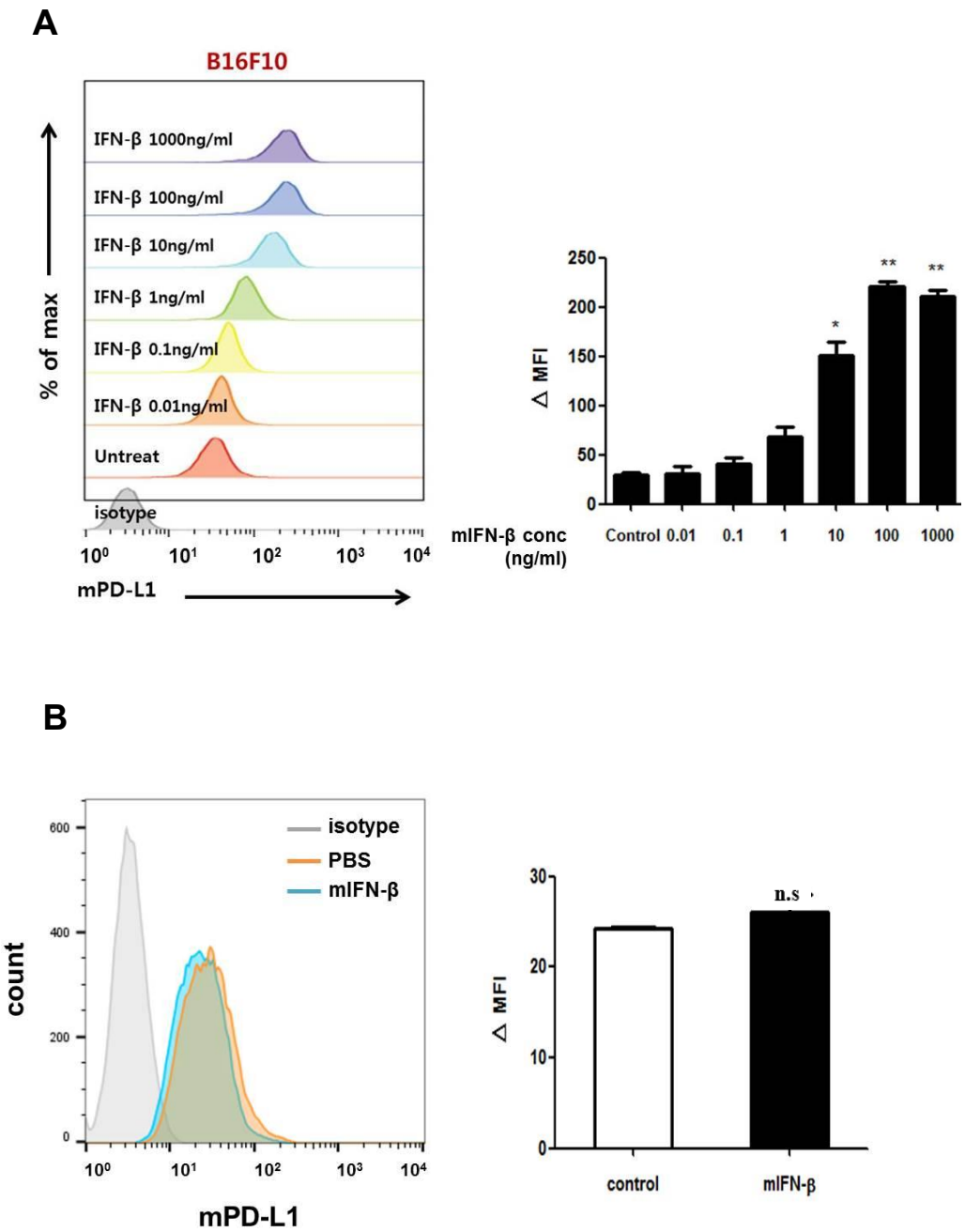

A
B16F10
IFN-β 1000ng/ml
IFN-β 100ng/ml
IFN-β 10ng/ml
IFN-β 1ng/ml
IFN-β 0.1ng/ml
IFN-β 0.01ng/ml
Untreat
isotype
% of max
mPD-L1
Δ MFI
mIFN-β conc (ng/ml)
Control 0.01 0.1 1 10 100 1000
B
isotype
PBS
mIFN-β
count
mPD-L1
Δ MFI
n.s
control
mIFN-β

Figure12. IFN-β stimulate PD-L1 expression level in murine melanoma cell and tissue.

B16F10 cells and B16F10-bearing C47BL/7 mice were treated with IFN-β in vitro and in vivo. (A) B16F10 cells were treated with various concentration of IFN-β for 48hr. (B) B16F10-bearing C57BL/6 mice were treated with IFN-β or PBS in vivo for 2 weeks. Murine cell and tumor tissue are stained with PE-labeled anti-PD-L1-Ab and mean fluorescence intensity (MFI) was calculated, which is normalized isotype control. All data represent the mean ± SEM of three independent experiments; *P<0.05, **P<0.01.

7. Reactivated T cell with anti-PD-L1-mAb and IFN-β

Elevated PD-L1 by IFN-β has many opportunities to interact with PD-1 expressed on T cell. Due to this interaction, T cell could be exhausted. Thus, we thought that PD-L1 blockade using anti-PD-L1-mAb can reactivate of T cell. To prove this, we conducted ex-vivo experiment using ovalbumin system. First, splenocyte was isolated from OT-I mouse and B16-ova cell was co-cultured with IFN-β or anti-PD-L1-mAb. After 24hr, IFN-γ in culture supernatant was analyzed by sandwich ELISA. Combination treatment with IFN-β and anti-PD-L1-antibody significantly enhanced secretion of IFN-γ, one of the activation marker of T cell. However, mono treatment with IFN-β also promote highly IFN-γ (Fig13A). We thought that this is because we experimented with splenocyte containing various immune cells instead of isolating T cell. However, we found that anti-PD-L1 antibody has an ability to reactivate exhausted T cells.

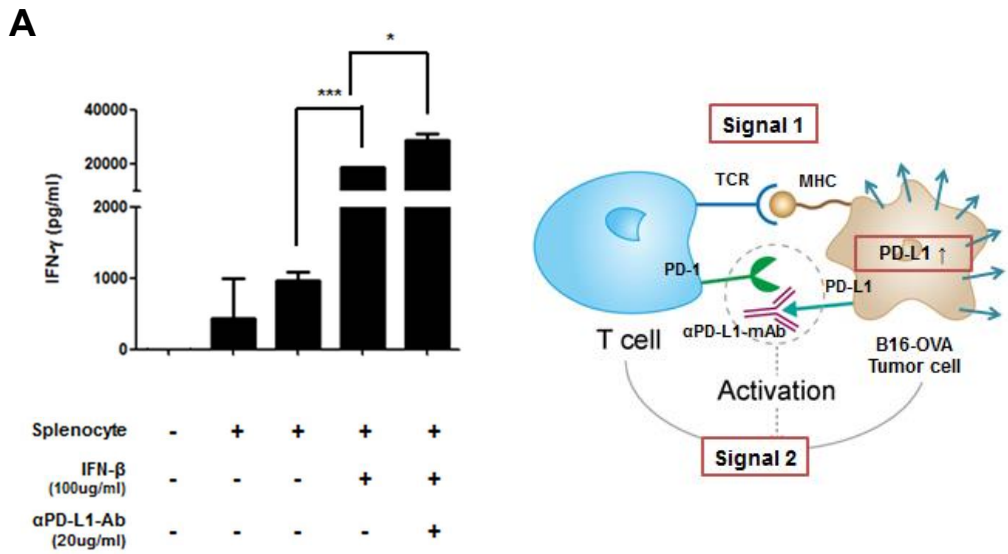


Figure13. Combination treatment with IFN-β and anti-PD-L1-mAb can reactivate T cells.

(A) Irradiated B16-OVA cells were co-cultured with splenocyte from OT-I mouse. IFN-β(100ug/ml) and anti-PD-L1-mAb(20ug/ml) were also treated. After 24hr, IFN-γ in culture supernatant was collected and measured using sandwich ELISA (R&D system). All data represent the mean ± SEM of triplicate experiment; *P<0.05, ***P<0.001.

8. Simultaneous immunotherapy with IFN-β and PD-L1 checkpoint blockade results in increase overall survival

To make lung metastasis mouse model, B16F10 murine melanoma cells were intravenously injected into tail vein of C57BL/6 mice. IFN-β was treated with subcutaneously injection every other day and anti-PD-L1-mAb was intraperitoneally injected three times a week after tumor inoculation. Mice were sacrificed when more than 20% of the weight of the mouse decrease according IACUC and we plotted the Kaplan Meier curve. The combination treatment group resulted in prolonged overall survival compared to each monotherapy and control group. However, monotherapy with IFN-β has similar anti-tumor effect to the combination (Fig14A). We thought this is due to the high concentration of IFN-β. Since B16F10 is one of the most sensitive cell lines, it was influenced by direct effect. Thus, we performed experiments using RENCA cells, one of the insensitive cell lines. In vivo mouse experiment using Renca cells, although the combination therapy group also increased overall survival compared with control or monotherapy, there was no significant difference between monotherapy with IFN-β and combination therapy group (Fig14B). We thought this was due to the exposure concentration of IFN-β in vivo. Therefore, we should consider the pharmacokinetic (PK) and pharmacodynamics (PD) of IFN-β to determine only the immune modulatory function of IFN-β

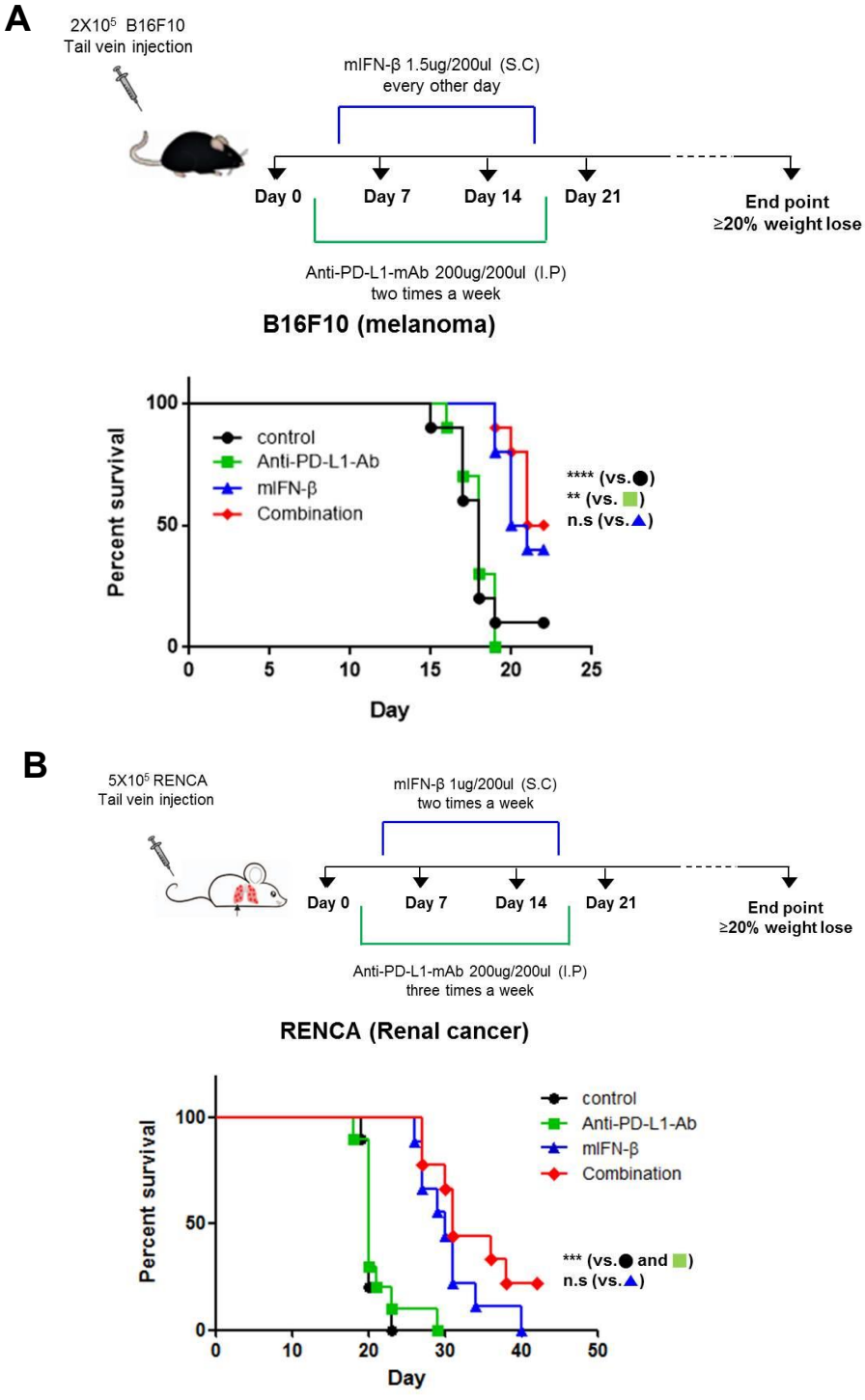

A
2X10⁵ B16F10
Tail vein injection
mIFN-β 1.5ug/200ul (S.C)
every other day
Day 0
Day 7
Day 14
Day 21
End point
≥20% weight lose
Anti-PD-L1-mAb 200ug/200ul (I.P)
two times a week
B16F10 (melanoma)
Percent survival
Day
control
Anti-PD-L1-Ab
mIFN-β
Combination
**** (vs.●)
** (vs. ■)
n.s (vs.▲)
B
5X10⁵ RENCA
Tail vein injection
mIFN-β 1ug/200ul (S.C)
two times a week
Day 0
Day 7
Day 14
Day 21
End point
≥20% weight lose
Anti-PD-L1-mAb 200ug/200ul (I.P)
three times a week
RENCA (Renal cancer)
Percent survival
Day
control
Anti-PD-L1-Ab
mIFN-β
Combination
*** (vs.● and ■)
n.s (vs.▲)

Figure14. Combination therapy group with IFN-β and anti-PD-L1-mAb prolong overall survival.

Kaplan-Meier plot of mice bearing implanted B16F10 cell or RENCA cells that were treated with vehicle(control), Anti-PD-L1-mAb, mIFN-β or combination therapy with anti-PD-L1-mAb plus mIFN-β(n=10 mice/group). All cancer cell was injected intravenously into C57BL/6 or Balb/C mice on day 0. After day (A) C57BL/6 mice bearing B16F10 was subcutaneously injected with mIFN-β every other day. Anti-PD-L1-mAb was treated with intraperitoneally injection(I.P) two times a week. (B) Balb/c mice bearing RENCA was subcutaneously injected with mIFN-βtwo times a week and Anti-PD-L1-mAb was intraperitonellay injected three times a week. All data were analyzed using a log-rank (Mantel–Cox) test (conservative) **P<0.01, ***P<0.001, ****P<0.0001.

9. Cell cytotoxicity function of R27T (glycoengineered interferon-b1a) for inflammation

Our laboratory developed a biobetter version of recombinant human rhIFN-β 1a named R27T. This is a glycoengineered version of recombinant human IFN-β 1a that improve the stability, solubility, productivity and pharmacokinetic properties compare to parent molecule. To evaluate the cell cytotoxicity function of R27T, high concentration of R27T was exposed in various types of human cancer cell lines (Fig15 A). Among several cell lines, we divided them into insensitive and sensitive cell in response to R27T (Fig 15 B). As a result, we selected the A375P human melanoma cell, one of the sensitive cell lines

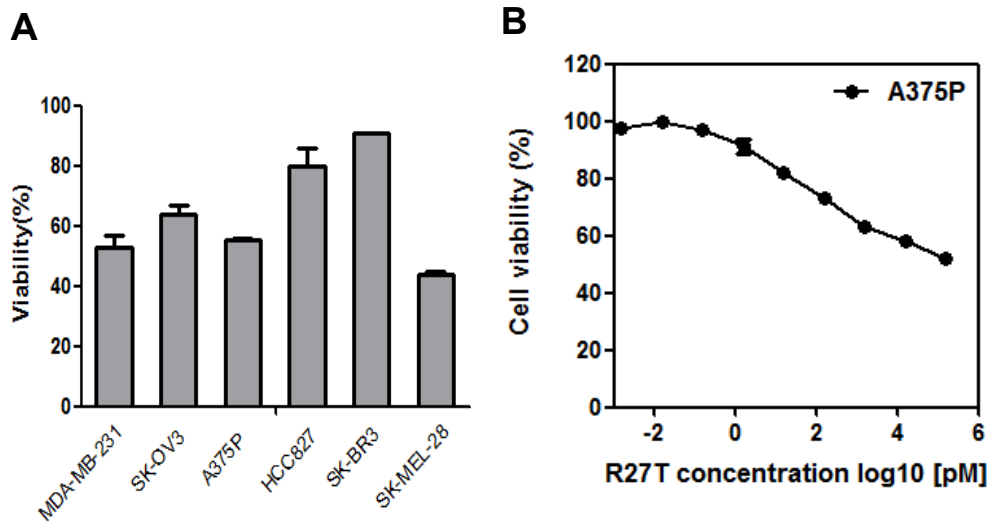


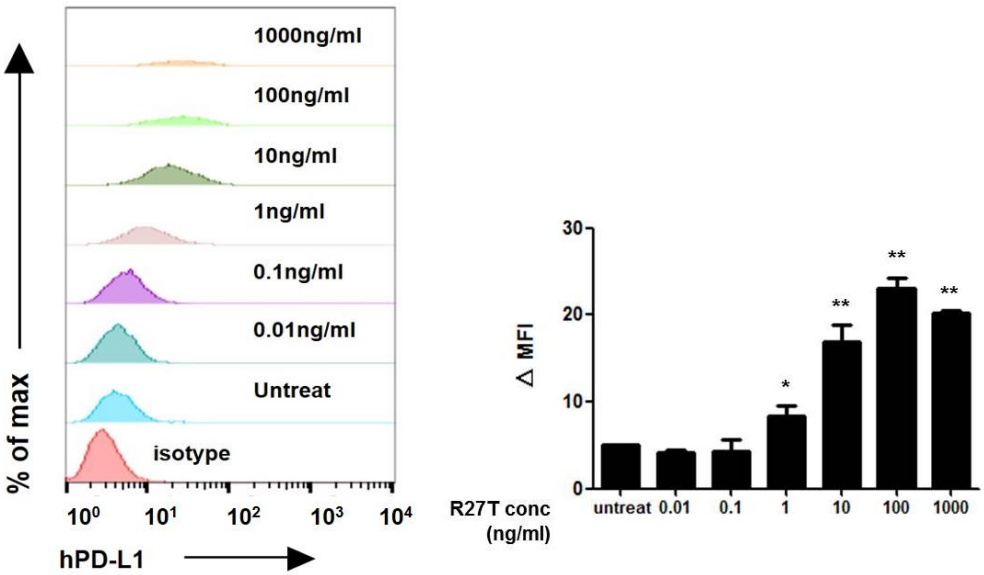
Figure15. Cell cytotoxicity of R26T in different human cell lines

Several human cell lines (MDA-MB-231, SK-OV3, A375P, HCC-827, SK-BR3, SK-MEL-28) were treated with or without various concentration of R27T. (A) The cells were exposure with high concentration (150nM) of R27T for 48hr and cell viability was measured using WST assay. (B) A375 human melanoma cells were treated with or without various concentration of R27T for 48hr and also viability was measured using WST assay. Data are expressed as mean ± SEM of three independent experiments

10. Elevated expression level of MHC-I and PD-L1 in respond to R27T

To evaluate the immune modulatory effect of Glycoengineered R27T, R27T was treated at various concentrations to confirm MHC-I and PD-L1 expression levels by FACS. We also found that expression of MHC-I and PD-L1 significantly increased depending on R27T concentration (Fig16 A, B). This also suggests that R27T is also a potential immune modulatory reagent.

A


1000ng/ml
100ng/ml
10ng/ml
1ng/ml
0.1ng/ml
0.01ng/ml
Untreat
isotype
% of max
10⁰ 10¹ 10² 10³ 10⁴
hPD-L1
Δ MFI
30
20
10
0
* ** ** **
untreat 0.01 0.1 1 10 100 1000
R27T conc (ng/ml)

B

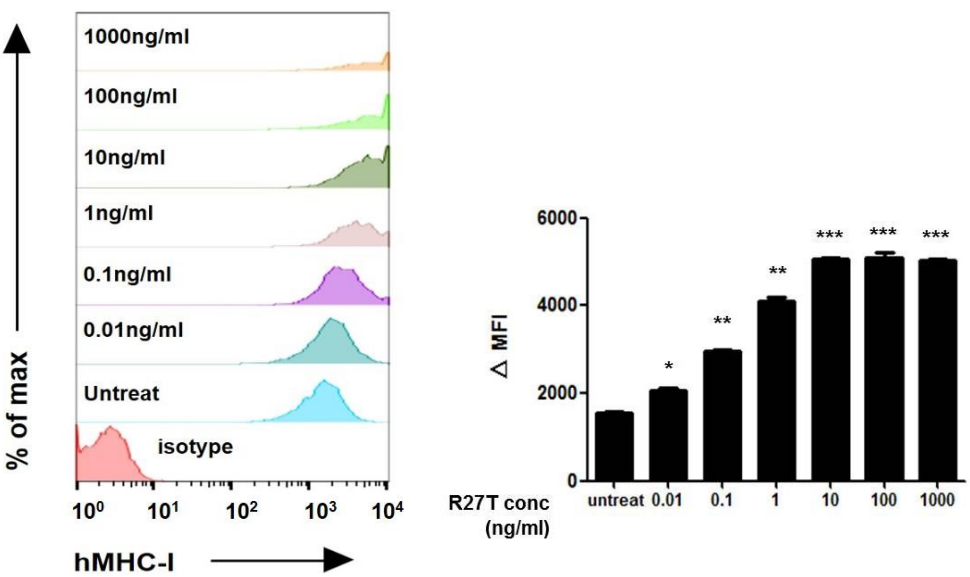

1000ng/ml
100ng/ml
10ng/ml
1ng/ml
0.1ng/ml
0.01ng/ml
Untreat
isotype
% of max
10⁰ 10¹ 10² 10³ 10⁴
hMHC-I
Δ MFI
6000
4000
2000
0
* ** ** *** *** ***
untreat 0.01 0.1 1 10 100 1000
R27T conc (ng/ml)

Figure16. R27T increased expression level of MHC-I and PD-L1 in A375P human melanoma cell. (A) A375P human melanoma cell was cultured with vehicle (R27T buffer) or various concentration of R27T for 48hr. Cells were harvested, stained APC-labeled hPD-L1(A) or hHLA-A,B,C (clone 29E.2A3, W6/32, biolegned, respectively) or an isotype control(B), and Each surface expression was measured by flow cytometry. The expression was evaluated average mean fluoresce intensity (MFI), which is normalized isotype control at each concentration, and the MFI value demonstrated a significant increase in hPD-L1 and MHC-I expression on the cells, correlating with increase in concentration of R27T. All data represent the mean ± SEM of three independent experiments; *P<0.05, **P<0.01.

11. Simultaneous immunotherapy of R27T (hIFN-β 1a) with PD-L1 checkpoin blockade lead to suppress tumor growth in humanized mouse model.

To eveluate synergistic effect of combination therapy with R27T and PD-L1 blockade in the human immune system, we construct humanized mouse using NOD/SCID-SGM3 mice. NSG-SGM3 mice are highly immunodeficient mice that lack mature T cells, B cells, and function of NK cells and are deficient in mouse cytokine signaling as well as induce human hematopoietic cytokine. Thus, these mice support the stable engraftment of myeloid liegeage and regulatory T cell population. A human immune system was constructed by injecting human hematopoietic cells (hCD34+) into this mouse. To confirm the construction of a human immune cell, hCD34, hCD3 and hCD19 set in blood at 4week and 8weeks were analyzed by flow cytometry (Fig17A). After confirming the human immune cells occurring, A375P human melanoma cells were subcutaneously injected and the R27T and anti-PD-L1-mAb were treated three times a week for 3weeks when the tumor volume reached 200mm^3. After drug treatment for 3 weeks, we measured the tumor weight at end point. As shown in the data, combination therapy group significantly inhibit the tumor growth compared to the control and monotherapy with R27T (Fig17B). Although there is a problem in this mouse

model related to cord blood volume, we were only able to use limited number of mice. Therefore, we need to repeat this experiment to confirm anti-tumor therapeutic efficacy with combination in humanized mouse model.

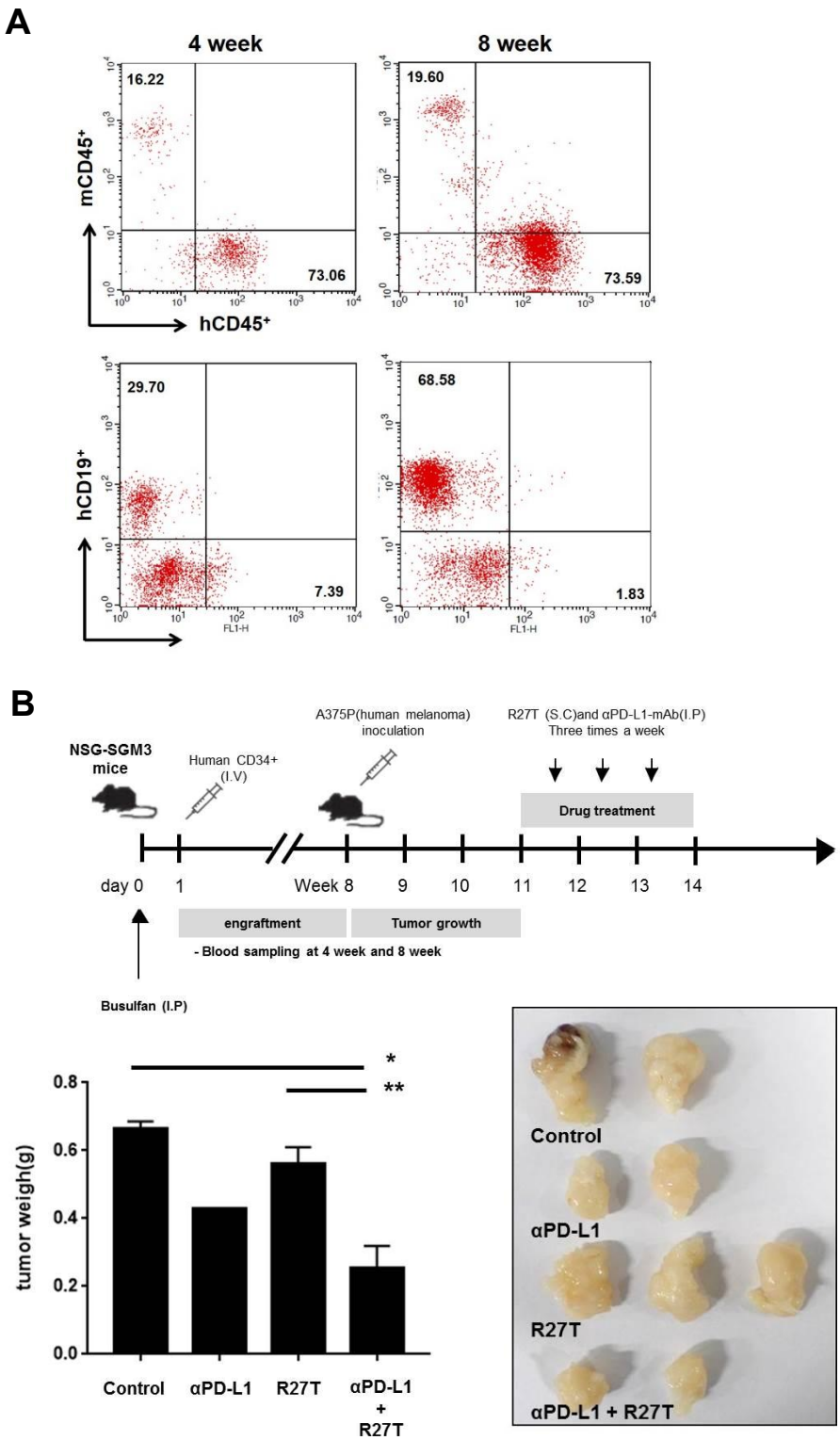

A
4 week
8 week
16.22
73.06
19.60
73.59
mCD45+
hCD45+
29.70
7.39
68.58
1.83
hCD19+
FL1-H
B
NSG-SGM3 mice
Human CD34+ (I.V)
A375P(human melanoma) inoculation
R27T (S.C)and αPD-L1-mAb(I.P) Three times a week
Drug treatment
day 0
1
Week 8
9
10
11
12
13
14
engraftment
Tumor growth
- Blood sampling at 4 week and 8 week
Busulfan (I.P)
tumor weigh(g)
0.0
0.2
0.4
0.6
0.8
*
**
Control
αPD-L1
R27T
αPD-L1 + R27T
Control
αPD-L1
R27T
αPD-L1 + R27T

Figure17. Simultaneous immunotherapy with R27T and PD-L1 blockade inhibited tumor growth in NOD/SCID-SGM3 mice.

(A) We confirmed the construction of human immune system in immunodeficiency mice by blood sampling at 4 week and 8week. FITC labeled anti- hCD45 or hCD3(clone 2D1, OKT3, respectively, biolegend) or APC-labeled anti-mCD45 or hCD19 (clone 30-F11, HIB19, respectively, biolegend) stained and analyzed by flow cytometry. (B) NOD/SCI-SGM3 mice were treated with R27T and Anti-PD-L1-mAb for 3 weeks after confirming the construction of immune system. Tumor weight was measured at end point. The significantly value were analyzed by t-test using Graphpad prism *P<0.05, **P<0.01

DISCUSSION

Currently, PD-1/PD-L1 checkpoint inhibitors, one of the cancer immunotherapy, has the remarkable clinical efficacy in various cancer types such as renal cell carcinoma, melanoma, non small cell lung cancer (NSCLC) and Hodgkin's disease. [2] However, these drugs benefit only a few patients based on their lack of pre-existing T cell and immunosuppressive tumor microenvironment.[1,3,4] Therefore, we considered the combination therapy with interferon beta for these patients who does not respond to only PD-1/PD-L1 single agent.-Our dates show that IFN-β result in increasing the expression of CCL5 and CXCR3 (CXCL9, CXCL10, CXCL11) ligands in melanoma cells, which could contribute to recruitment of effector T-cells into TME. [16,17] Because of these chemokines, TILs including $CD4^+$ and $CD8^+$ Tcells were recruited to the tumor site. Therefore, the administration of IFN-β group significantly increased the mRNA expression of Th1 chemokines (CCL9, CXCL11) and decreased the mRNA expression of Th2 chemokines (CCL17, CCL22). Th2-related chemokines have a role suppressed the recruitment of Treg in TME.[16, 18] In addition, the balance of Th1 and Th2 are important. In normal circumstances, Th1 and Th2 immunity approached a balance. However, Tumor cells disrupt this balance. In the presence of tumor cell,

increased Th2 immunity and decreased Th1 immunity leads to tumor progression[18]. But IFN-β elevated the PD-L1 expression level, which suggested an impairment of CTL function.[19]. The expression of PD-L1 has been observed in many tumors and seems to be associated with poor prognosis in several cancers including melanoma. Accordingly, we hypothesized that IFN-β might provide therapeutic benefit to combination with PD-L1 blockade. To prove our hypothesis, we used the B16F10 melanoma in syngeneic mouse model. Combination therapy increase overall survival in lung metastasis model in comparison to the control and monotherapy groups. To confirm in human system, we construct humanized mouse model using NSG/SGM3-mice.[20,21] In this model, we used gloycoengineered hIFN-β1a R27T from our laboratory. This drug R27T improve the stability, solubility, productivity and pharmacokinetic properties compare to parent molecule, IFN-β 1a. [22,23] In this humanized mouse model, combination therapy group also significantly inhibit the tumor growth compared to control and monotherapy with R27T. Since there is a problem this mouse model related to cord blood volume, we were only able to use limited number of mice. Thus, we need to repeat this experiment to confirm anti-tumor therapeutic efficacy with the combination therapy. Although our data shows significant values in-vitro, we could not show it in ex-vivo or in-vivo. This is because the exposure concentration of IFN-β in vivo was

different compared to the in vitro experiment. Therfore, for our future study, we should determine the concentration of IFN-β in vivo mouse experiment by considering the pharmacokinetic and pharmacodynamics of drug.

However, in this study, we suggest that IFN-β in combination with anti-PD-L1-mAb could be a potential and successful immune combination therapy for the treatment of melanoma.

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국문초록

PD-L1 체크포인트억제제는 기존의 항암요법과 달리 장기생존가능특성으로 다양한 암종에서 획기적인 결과를 보여주고 있다. 하지만 소수의 환자들만이 이 약물에 반응을 보이며 최근 많은 연구를 통해서 종양미세환경에서의 침윤성 림프구 부족이 무반응의 원인으로 밝혀지고 있다. 이에 따라 림프구를 종양미세환경으로 모집할 수 있는 물질과의 병용투여가 요구되어지고 있다.

이에 따라 본 연구진은 타입 I 인터페론 중 하나인 인터페론베타와 항 PD-L1 항체의 동시적 면역치료를 통한 항암치료 효능을 연구하고자 한다. 인터페론베타는 여러가지 면역조절기능을 가지고 있으나 특히 종양미세환경으로 림프구를 모집할 뿐더러 주변에 있는 면역세포의 분포 또한 조절하는 기능을 가지고 있다. 본 연구에서는 인터페론베타가 림프구를 모집하는 케모카인을 유도 할뿐더러 CD4와 CD8 T 세포를 종양미세환경으로 유도하는 것을 확인하였다. 또한 인터페론을 투여한 그룹의 종양미세환경에서 Th1과 Th2와 관련된 케모카인을 분석하였을 때 조절 T 세포의 모집을 억제시키는 Th2와 관련된 케모카인을 억제시킨 것을 확인할 수 있었다. 하지만 인터페론베타에 의해 T세포 억제시그널과 관련된 PD-L1의 발현이 증가하는 것을 확인하였고 이에 따라 항 PD-L1항체를 이용하여 PD-1/PD-L1 결합을 막음으로써 T세포의 세포독성효과를 되

살리는 것을 확인할 수 있었다. 또한 이에 대한 동시적면역치료 효과는 동종이식 마우스 모델 뿐더러 인간화마우스 모델로 확인하였으며 병용투여그룹이 단독투여 그룹보다 마우스의 전체생존율을 증가시킬뿐더러 종양의 성장을 억제하는 것을 확인할 수 있었다.

따라서 인터페론베타와 PD-L1 체크포인트 억제의 동시적치료는 면역치료제로써 가능성있는 조합임을 보여주었다.

주 요 어: 면역치료제, 항PD-L1항체, 인터페론베타, 면역조절기능, 종양침윤성림포구, 종양미세환경, PD-L1 (programed-death-ligand1),

학번: 2017-29374