약학석사학위논문

Development of Immunocytokine Based on Human PD-L1 Antibody and Interferon-β-1b Mutein

인간 PD-L1 항체와 인터페론 베타 변이체를 통한 면역 사이토카인 개발 연구

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Abstract

Development of Immunocytokine Based on Human PD-L1 Antibody and interferon-β-1b mutein

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Immunotherapy has received a great deal of attention due to its great enhancement of the long term survival rate of patients compared to conventional anti-cancer therapy. PD-L1(Programmed cell death Ligand 1), a typical target for immunotherapy, is a protein that binds to PD-1 and has various wide-reaching functions, which can cause avoiding immune destruction of cancer cells and increase regulatory T cell maturation by antigen presenting cells.

In this study, two antibody candidates selectively bound to human PD-L1 were screened using Phage display technique, which confirmed that these antibodies neutralized PD-1/PD-L1 signal at a lower concentration compared to Atezolizumab, a FDA approved PD-L1 antibody.

However, PD-L1 antibodies are known to be ineffective in more than half of the patients due to the lack of TIL(tumor infiltrating

lymphocyte cells) in the tumor micro-environment. In order to solve this problem, immunocytokines were designed by linking interferon-β-1b mutein, which is known to increase TIL, to the PD-L1 antibody and various types of immunocytokines were designed. In addition, their properties were measured after production to find the most optimal forms of immunocytokine.

From our results, we found that scfv-Fc-interferon-β-1b mutein type of immunocytokine is superior than other forms of immunocytokines in productivity, biological activity, PD-1/PD-L1 signal neutralizing ability, and PD-L1 targeting ability.

This study is significant in that it has searched for an effective human PD-L1 antibody and found an optimal form to be utilized as an immunocytokine.

keywords : **Immunotherapy, PD-L1, Atezolizumab, tumor infiltrating lymphocyte cells, antibody, interferon-β-1b mutein, immunocytokine**

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CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Word
PD-L1	Programmed cell death Ligand 1
PD-1	Programmed cell death 1
TIL	tumor infiltrating lymphocyte
IFN	interferon
Fc	fragment crystallizable
CDR	complementarity determining region
IGG	Immunoglobulin G
ELISA	enzyme linked immunosorbent assay
IC50	inhibition concentration 50
scFv	single chain Fragment variable

INTRODUCTION

Immunotherapy, the use of the immune system to induce anti-cancer effects, has received a great deal of attention due to its ability to enhance the long term survival rate of patients compared to conventional anti-cancer therapy. [1]

PD-L1, a typical target for immunotherapy, is usually overexpressed in antigen presenting cells and cancer cells. PD-L1 on cancer cells exhaust CD8+ T cell, NK cell and so on, causing immune evasion of cancer cells. PD-L1 on DC increases regulatory T cells and induces an immunosuppressive tumor micro-environment. [2] Atezolizumab, a FDA approved PD-L1 antibody, showed dramatic and long-lasting response in 20-30% patients in many types of cancer including lung, colon, head and neck, and gastric cancers in addition to melanoma and renal cell carcinoma. [3,4]

However, PD-L1 antibodies are also known to be ineffective in more than half of the patients due to the lack of TIL in the tumor micro-environment. One of the representative strategies for attracting TIL is to induce a type 1 IFN response in a tumor micro-environment. [5]

IFN-β ,one of the type 1 IFNs, has emerged as a potent anti-cancer drug with both direct apoptosis induction and indirect anti-cancer effect due to immune stimulation. In our previous study, R27T, an improved version of IFN-β, was developed by glycosylating the 25th amino acid additionally. R27T showed improved stability and solubility, less aggregation and increased half-life compared to the native IFN-β form. [6] However, when type 1 IFN was used as an anti-cancer drug, 80-85% of patients showed no response. In many

studies, PD-1 / PD-L1 signaling was identified as a cause behind lack of responsiveness, but Type 1 IFN fused with the PD-L1 antibody is expected to have synergistic effects in inducing responsiveness to a greater patient pool. [7]

In some cases, immunocytokines are used as a way to maximize the synergistic effect of cytokines and antibody drugs. Immunocytokines are antibody cytokine fusion proteins that not only have the potential to concentrate cytokines to a specific lesions but also increase the stability and half-life of cytokines, leading to powerful anti-cancer treatment. [8]

However, immunocytokines have lower productivity than conventional antibodies and have lower biological activity than natural cytokines. Recent studies also suggest that immunocytokines have a different tendency from the expected biodistribution. Thus, further studies are required to use immunocytokines as effective anti-cancer drugs. [9]

Materials and Methods

Molecular cloning and expression of PD-L1 antigen

PD-L1 antigen gene synthesis was performed by Cosmogenetech (Korea). The synthesized PD-L1 antigen gene was inserted into the pCHO 1.0 expression vector(Life Technologies) at the AvrII-Bstz17I site of the polylinker region. CHO-S cells(Life Technologies) were cultivated and transfected with the expression vector according to the manufacturer's instruction. Stable clones were selected with 200 nM of methotrexate (Sigma Aldrich) and 20 μg/ml of puromycin (Life Technologies). The culture fluid was recovered, filtered with Minisart® (sartorius stedim), and then purified using MabSelect SuRe ™ rProtein A agarose-based resin(GE Healthcare). The PD-L1 antigen were recovered using 0.1M Glycine solution(pH 3.0)

Cleavage of Fc domain from PD-L1 antigen

PD-L1 extra cellular domain and Fc domain were cleaved from PD-L1 antigen, and each domains are separated by protein A affinity chromatography after papain digestion of PD-L1 antigen (Pierce Fab Preparation Kit, Thermo Fisher Scientific). Each sample was concentrated using Amicon® Ultra (50kDa molecular weight cutoff, Merck Millipore), and Fc domain cleavage was confirmed by comparing reducing and non-reducing 10% SDS-PAGE gels stained with comassie blue (Sigma Aldrich).

Phage display

Phage library was blocked with 3% non-fat dried milk (Sigma Aldrich) in PBST (Phosphate Buffered Saline containing 0.05% Tween-20) added with Fc cleavage domain from PD-L1 antigen. The blocked phage library was bound to immunotube (Nunc) coated with PD-L1 antigen. The PD-L1 antigen was coated on the immunotube sequentially from 50μg to 6.25μg for greater screening pressure. The immunotube was rinsed several times with PBST and any phage strongly bound to PD-L1 antigen was separated from the immunotube using 100 mM TEA (Triethylamine). The selected phage library was amplified by infecting E. coli and rescued with helper phage and then the above procedure was repeated 5 times.

Expression of single chain fragment variables

E.coli (TG1 strain) infected with phage selected by phage display was induced by using IPTG (Sigma Aldrich). After that, single chain fragment variables tagged with Hemato Agglutinin was obtained by giving an osmotic shock to E.coli.

ELISA for PD-L1 candidate screening

96-well Costar Assay® plates(Corning) were coated with PD-L1 antigen or Fc cleavage domain and blocked with 3% non-fat dried milk in PBST. One plate was blocked with milk only, not coated separately to distinguish candidates binding to the milk protein. After the plates were rinsed three times, single chain fragment variables expressed from E. coli or the phage pool itself were added to each plate. Anti-Hemato Agglutinin Horseradish peroxidase antibody

(Sigma Aldrich) in 3% non-fat dried milk PBST was added and incubated at room temperature for 1 hour. The reaction was visualized by adding 30 ul of TMB substrate (surmodics inc) to the plate and incubating for 30 min. The reaction was terminated by adding 30 ul of 1M H2SO4 and the absorbance at 450 nm was measured using a microplate reader (TECAN).

PD-L1 candidates CDR sequencing

Phagemids were recovered from each E. coli infected with the selected phage using the Plus Plasmid Mini Kit (NuceloGen). CDR sequences were decoded from the recovered phagemids using DNA sequencing service from Cosmogentech (Korea).

IGG conversion and expression of PD-L1 antibody candidates

VH and VL genes were each cloned from phagemids and inserted into a pcDNA 3.3 expression vector with constant region gene of human igG1 Heavy Chain and a pOptiVEC expression vector with constant region gene of human igG1 Light Chain. To produce the complete PD-L1 antibody, Heavy Chain and Light Chain-encoding plasmids were co-transfected into the expi293 cell line according to the manufacturer's instructions.

The culture fluid of cultured transfected expi293 cell line was recovered, filtered with Minisart® (sartorius stedim), and then purified using MabSelect SuRe™ rProtein A agarose-based resin(GE Healthcare). The PD-L1 antibody candidates were recovered using 0.1M Glycine solution(pH 3.0) Antibody concentration was measured by Cedex Bio System. (Roche)

Cell culture

The CHO-K1, MKN28, MKN45, SNU216 cell line and iLite® Type 1 IFN Assay Ready Cells were cultivated in medium containing 10% fetal bovine serum (Hyclone) and 1% penicillin- streptomycin solution (GE healthcare). CHO-S cell line was cultivated with CD fortiCHO™ chemically defined meidum (Life technology) without additives. Expi-293 cell line was cultivated with Expi293™ expression medium (Life technology).

Flow cytometry

The ability of PD-L1 antibody candidates and immunocytokines to target PD-L1 was analyzed by flow cytometry. Cells were harvested using Cell dissociation buffer (Gibco) and incubated at 4 ° C for 1 h in FACS buffer (PBS containing 2% FBS) with 1 g of antibody. Cells were rinsed twice with FACS buffer and incubated with fluorescein isothiocyanate (FITC) -conjugated goat anti-human IgG secondary antibody (ThermoFisher scientific) in FACS buffer (PBS containing 2% FBS) for 1 h at 4 ° C. The fluorescence signal was obtained by using a FACSCalibur (BD Biosciences) operated by Cell Quest Pro software (BD Biosciences).

PD-1/PD-L1 competitive inhibition ELISA

96-well Costar Assay® plates(Corning) were coated with hPD-L1 Fc fusion Protein(BPS Bioscience) and blocked with 3% non-fat dried milk in PBST. The PD-L1 antibody and the biotinylated hPD-1 protein(BPS Bioscience) are sequentially added to the plate at various concentrations for competition between two proteins and incubated at room temperature for 2 hours. Horseradish peroxidase Conjugated

Streptavidin (1:500) in 3% non-fat dried milk PBST was added and incubated at room temperature for 1 hours. The reaction was visualized by adding 30 ul of TMB substrate (surmodics inc) to the plate and incubating for 30 min. The reaction was terminated by adding 30 ul of 1M H2SO4 and the absorbance at 450 nm was measured using a microplate reader (TECAN).

Type 1 IFN responsive Luciferase reporter assay

Luciferase reporter assay was performed using iLite® Type 1 IFN Assay Ready Cells (Euro Diagnostica) and Bio-Glo™ Luciferase Assay System (Promega). iLite® Type 1 IFN Assay Ready Cells were seeded in 96 well white polystyrene plate (Nunc) and cytokines or immunocytokines were treated at various concentrations. After 18 hours, the firefly luminescence signal was measured using a microplate reader (TECAN).

Molecular cloning and expression of various immunocytokines

Gene synthesis of various immunocytokines was performed by Cosmogenetech (Korea). The synthesized immunocytokines gene were inserted into the pCHO 1.0 expression vector(Life Technologies, USA) at the AvrII-Bstz17I site of the polylinker region. Expi293 cell line(Life Technologies) were cultivated and transfected with the expression vector according to the manufacturer's instruction. The culture fluid was recovered, filtered with Minisart® (sartorius stedim), and then purified using MabSelect SuRe™ rProtein A agarose-based resin(GE Healthcare). The protein of immunocytokine were recovered using 0.1M Glycine solution(pH 3.0). Antibody concentration was measured by ELISA.

RESULTS

Design and Production of PD-L1 antigen for antibody screening through phage display.

For anti-PD-L1 antibody screening, I design PD-L1 antigen was designed by linking the extra cellular domain of PD-L1 directly to the hinge region so that it could be linked to the Fc domain of IgG4 using Flexible peptide linker (GGGGSGGGGSGGGSG) (Figure 1). The PD-L1-Fc fusion protein designed was produced using the CHO Production system and purified by protien A affinity chromatography and confirmed by SDS-PAGE. Due to the high glycosylation of the Extra cellular domain of PD-L1 protein, it is smeared in the gel image and also weighs more than theoretically expected molecular weight. (Figure 2A, B) The PD-L1-Fc fusion protein was cut out using Papain enzyme. PD-L1 extra cellular domain and Fc domain were separated by protien A affinity chromatography and confirmed by SDS-PAGE (Figure 2C).

Anti-PD-L1 phage screening using phage display technology.

A high-diversity phage library was blocked with Fc domain obtained from upstream process and skim milk to remove phages binding to the Fc domain or skim milk. Blocked phage library were bound to the immunotubes coated with the the PD-L1-Fc fusion protein to isolate only the phage population binding strongly to PD-L1 extra cellular domain. (Figure 3)

ELISA was conducted to check the affinity for PD-L1 using selected

phage pools at each bio-panning step. (Figure 4A) As a result, a strong signal appeared in the third and fourth biopanning phage pools. The third and fourth biopanning phage pools were thought to contain many phages binding strongly to PD-L1. (Figure 4B) ELISA was performed with scFv expressed from a single colony of E. coli infected with the 3rd and 4th phage pools. Plates for ELISA were coated with PD-L1-Fc fusion protein, Fc domain and only skim milk to remove candidates binding to the Fc domain or skim milk (Figure 4A). As a result, many candidates binding to PD-L1 extra cellular domain could be obtained (Figure 4B), and gene sequencing of CDR regions was performed by selecting candidates with the highest 15% signal. Seven PD-L1 antibody candidates' CDR sequences were identified by next generation sequencing except overlapping sequences (Figure 4C).

IGG conversion of Anti-PD-L1 candidates and analysis of PD-L1 targeting ability and PD-1/PD-L1 neutralizing efficacy.

The selected PD-L1 candidates' scFv sequence was converted to full form IGG antibody, expressed in Expi293 production system (Figure 5A). PD-L1 antibody was isolated from the culture fluid of transfected Expi293, and confirmed by SDS-PAGE. The band was confirmed to be in the expected position; IgG : 150kDa ,HC : 50kDa ,LC : 25kDa (Figure 5B)

CHO-K1(PD-L1 negative and PD-L1 positive), MKN28 and MKN45 cell line were stained with Atezolizumab(Positive control PD-L1 antibody) and PD-L1 antibody candidates. Fluorescence-positive cells were measured by flow cytometry. 3B4 show non specific binding even in PD-L1 negative cell line. 4A2 and 4A4 showed low affinity

for PD-L1 as the peak shift in PD-L1 positive cell line was low. 3C8 and 3E5 selectively bind well to PD-L1 on cell surface (Figure 6).
The PD-1 / PD-L1 competitive inhibition ELISA was performed with Atezolizumab(a positive control) and 3C8, 3E5 and 4A2(anti-PD-L1 antibody candidates) (Figure 7A, B). PD-1 / PD-L1 signal inhibition curve and IC50 values were calculated using the GraphPad Prism® 5. PD-L1 antibody candidate 3C8 and 3E5 neutralized PD-1/PD-L1 signal at a lower concentration than Atezolizumab. The IC50 value of PD-L1 antibody candidate 4A2 could not be calculated because 4A2 did not block 100% of the signal at the highest concentration in the assay (Figure 7C, D).

Design and Production of various immunocytokine form.

Various immunocytokines are designed by fusion of interferon to the heavy chain or/and light chain of the PD-L1 antibody. Immunocytokines were also designed by replacing the upper part of the HC-cytokine of the immunocytokine hinge region with scFv (Figure 8). Various designed immunocytokines were expressed in Expi293 under the same conditions, and this was confirmed by SDS-PAGE.
Under the reducing condition, the heavy chain of the antibody was around 50 kDa and the light chain was aroud 25 kDa. Since the molecular weight of R27T is 25 kDa, the R27T (interferon- mutein) fusing to heavy chain is expected to be 75 kDa and the R27T fusing to light chain is expected to be 50 kDa (overlapping the heavy chain on the gel image). The scFv-Fc-R27T was approximately 150kDa in SDS-PAGE gel similar to the R27T fusing to heavy chain (Figure 9A, B). ELISA was performed to determine the concentration of

immunocytokine in culture medium. As a result, productivity was highest in the order of scFv-Fc-interferon form, HC-interferon form, LC-interferon form and HC / LC-interferon form (Figure 9C).

Analysis of interferon biological activity, PD-1/PD-L1 neutralizing efficacy and PD-L1 targeting ability of various immunocytokine form.

The U937 cell line transfected with the firefly luciferase gene based on the ISG promoter was treated with various concentrations and various types of immunocytokines. As a result of Type 1 IFN responsive Luciferase reporter assay, interferon biological activity was highest in the order of scFv-Fc-interferon form, HC/LC-interferon form, HC-interferon form and LC-interferon form. The biological activity of interferon did not differ according to the linker. The scFv-Fc-interferon form immunocytokines were compared with the IgG form PD-L1 antibody to determine whether the PD-1 / PD-L1 binding neutralizing efficacy differs according to the length of the linker above the hinge region. Although the neutralizing effacacy was lower than that of the IgG form PD-L1 antibody when the scFv was directly connected to the hinge region without linker(GGGGS), the neutralizing efficacy was improved compared to that of the IgG form PD-L1 antibody when (GGGGS)3 or more linkers were added. The PD-L1 targeting ability was determined by flow cytometry and was compared with the full-form IgG PD-L1 antibody by binding the same amount of immunocytokine to SNU216 and MKN45 cells, which differ in the expression level of PD-L1. The MFI ratios of the respective immunocytokines for snu216 and MKN45 were calculated and found to be superior to PD-L1 targeting ability in the order of

scfv-Fc-interferon, LC-interferon, HC-interferon and HC / LC-interferon.

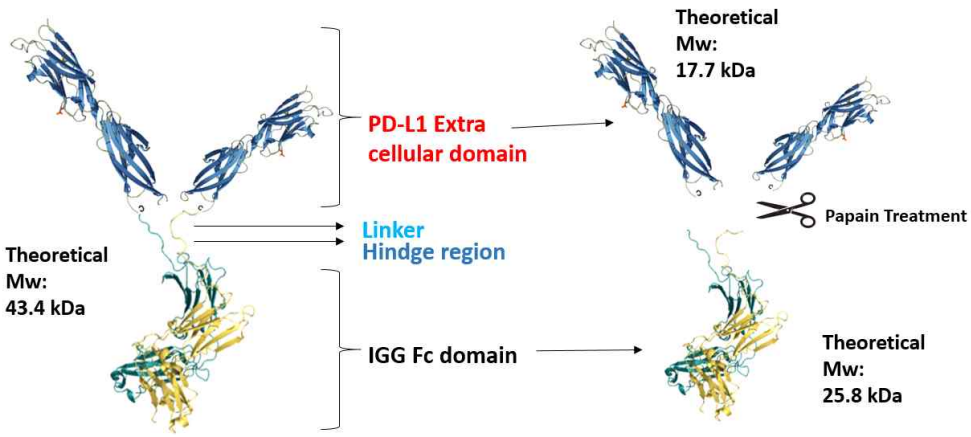


Figure 1. Structure of PD-L1 antigen. PD-L1 antigen was designed by linking the extra cellular domain of PD-L1 to the Fc domain of human Immunoglobulin G4. Flexible peptide linker (GGGGSGGGGSGGGSG) is used. PD-L1 antigen was treated with Papain to separate PD-L1 extra cellular domain and IGG Fc domain.

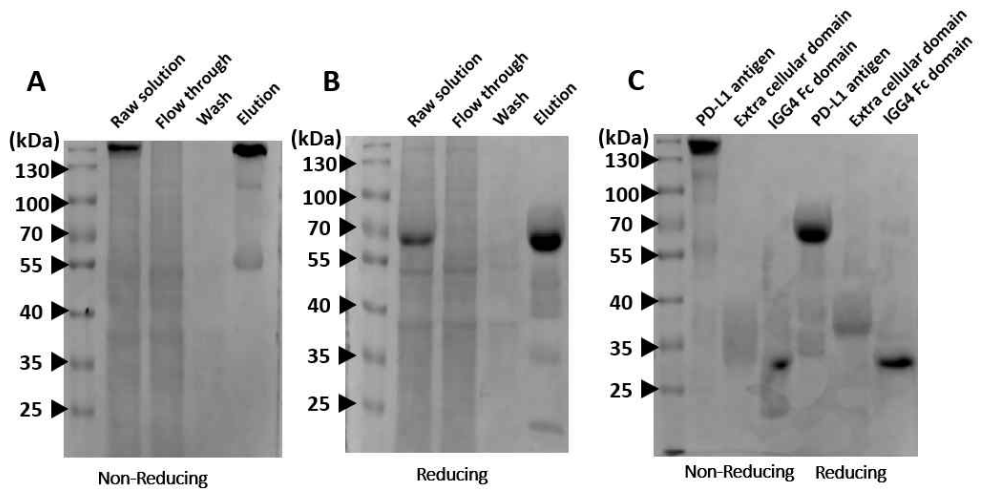


Figure 2. Purification of PD-L1 antigen, PD-L1 extra-cellular domain and IGG4 Fc domain. The PD-L1 antigen shown above was produced by using the CHO Production system and, was purified by protien A affinity chromatography (Figure 2A, B). PD-L1 extra cellular domain and Fc domain were cut out using Papain enzyme and confirmed by SDS-PAGE (Figure 2C).

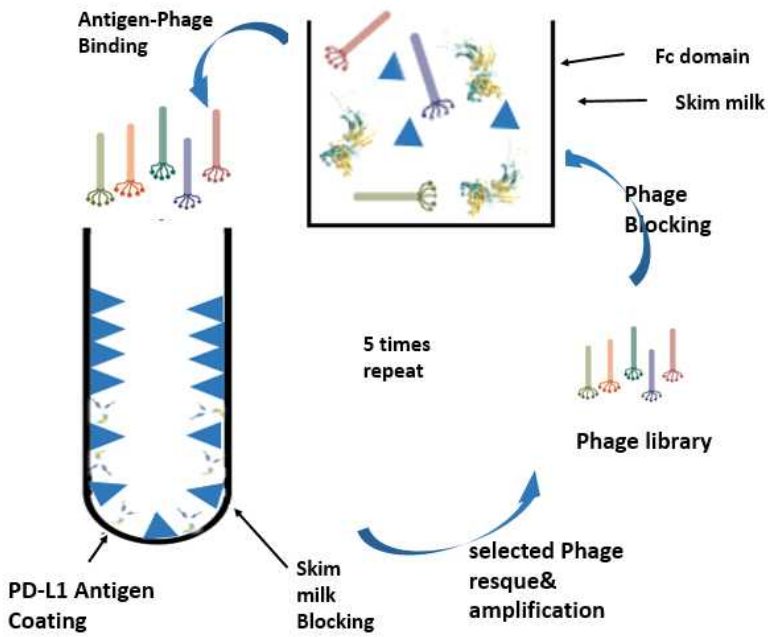
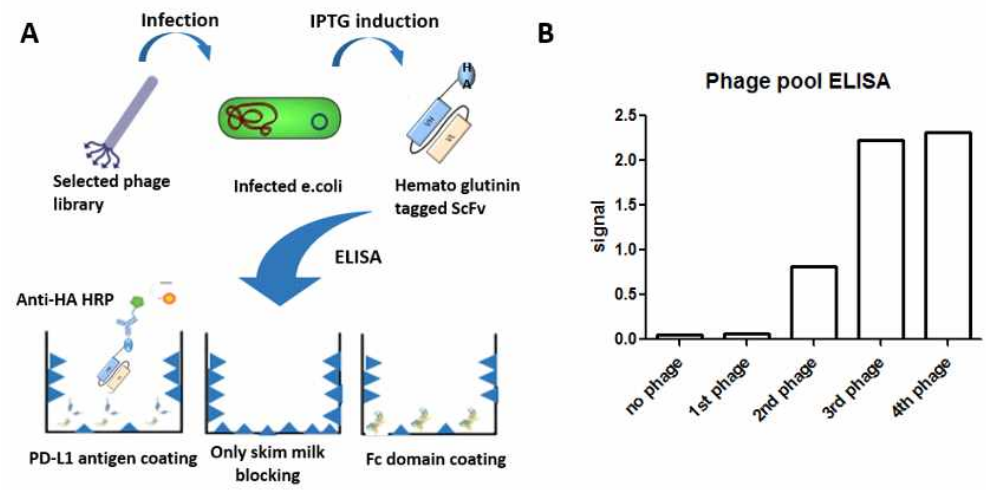
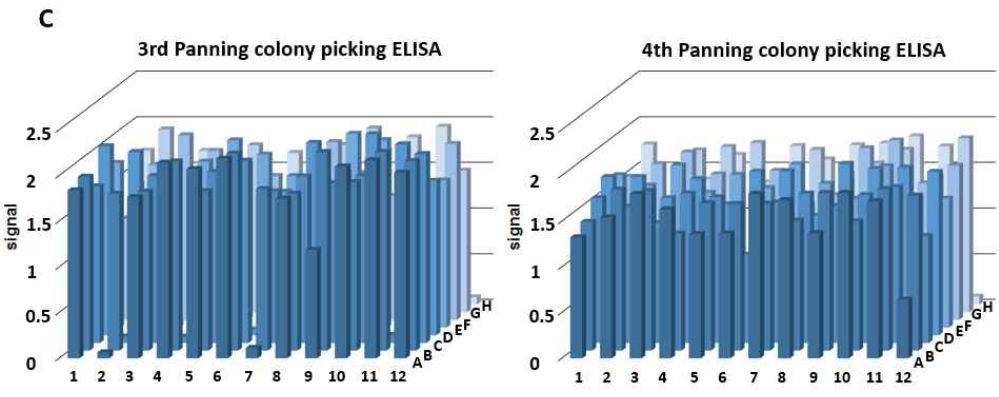
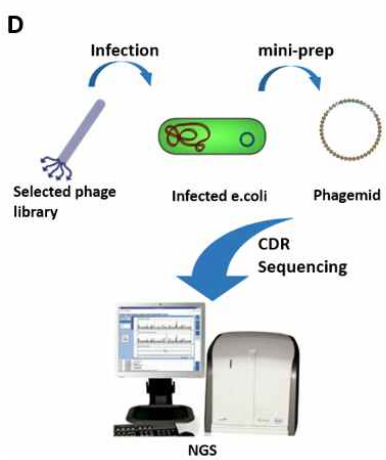


Figure 3. The graphical explanation of Phage Display Process for PD-L1 specific phage screening. A high-diversity phage library was blocked with Fc domain and skim milk which then was bound to the immunotubes coated with the PD-L1 antigen to isolate only the phage binding strongly to PD-L1.







E

scFv candidate	Heavy chain CDR sequence			Light chain CDR sequence		
	CDR1	CDR2	CDR3	CDR1	CDR2	CDR3
3E2,3A3, 3B2,3B3,4E2	NYWIA	SSVHYSGITYYNPSLKS	RGISDYLDY	RASQSISNYLA	DASNRAT	QQYGSSDWT
3E5,3E9,3A4 ,3A6,4B2, 4F2,4F3,4G4, 4A3,4D4, 4D6,4G3, 4H8	DYALT	SEIHQSGITIYNPSLKS	RSPKGGDALDY	RTSQSIGNWLA	DTSNRAT	QQYNNWPYT
3B4	DYGMH	GIIYPGVAYTRYSPSFEG	RSYLGFDS	QASQDIRNHLN	DASNRAT	QQSYITPIT
3C8	GYYMH	SVINPSGGITAYAQKFQG	RSLDTSTVAYMDV	RASQDISTYLN	KASSLQS	QQYNNWPIT
3D2,4B3	RYAMH	SSISTTSDYIYDADSVKG	RHGAMGHFDI	SGSTSNIGSKSVH	GNTNRPS	QSYDSSSWV
4A2	SYGMH	SEIHQSGITIYNPSLKS	RGRRGDSYMDV	RASQDIGNFIN	GATSRAT	QQSYSTPYT
4A4,3F3,4E3 ,4D2,4H4, 4H6	SYGMH	SEIHQSGITIYNPSLKS	RGRRGDSYFDY	RASQSVSSSYLA	DASTRAT	QQSYTP PIT

Figure 4. Evaluation of phage screening results and CDR identification.

ELISA for PD-L1 binding were carried out using selected phage pools at each bio-panning step (Figure 4A, B). Colony picking ELISA was performed with scFv from a single colony of E. coli infected with the 3rd and 4th phage pools (Figure 4C), and gene sequencing of CDR regions was performed by selecting candidates with the highest 15% signa (Figure 4D, E).

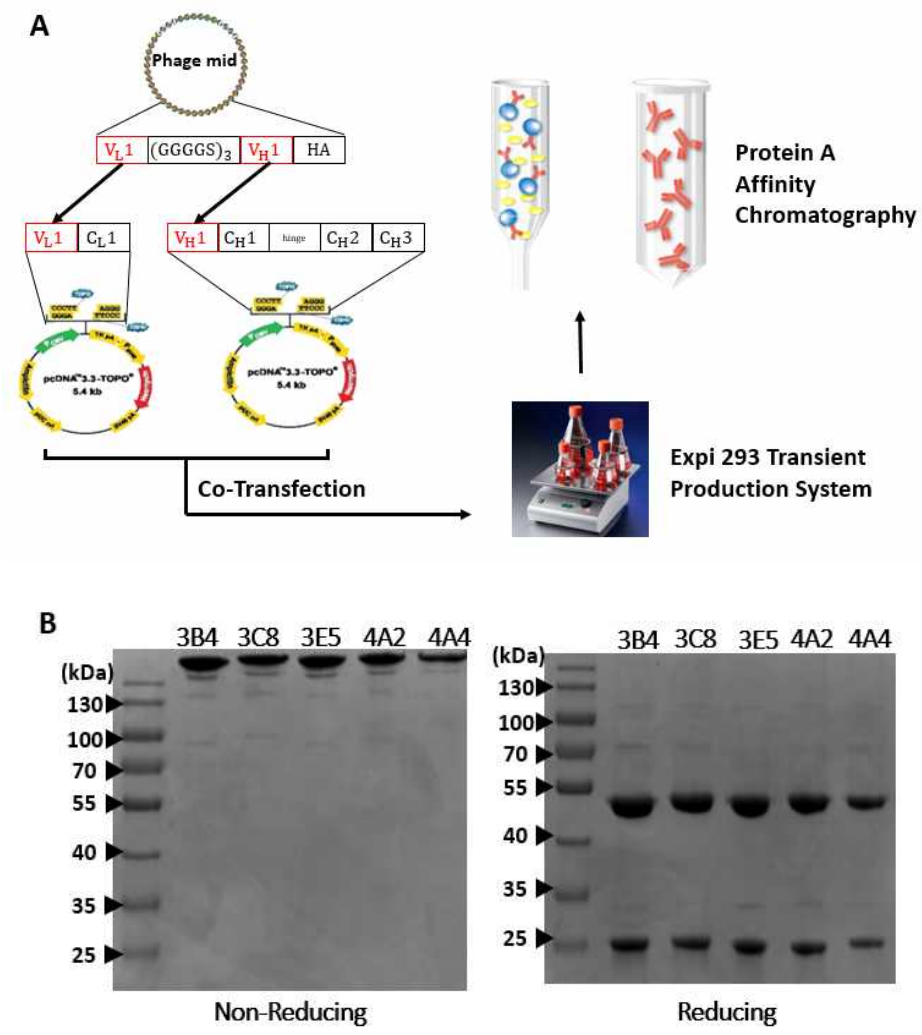


Figure 5. IGG conversion, Production and Purification of PD-L1 antibody candidates. The selected PD-L1 candidate was converted to full form IGG antibody(Figure 5A) and was expressed in Expi293 which was confirmed by SDS-PAGE(Figure 5B).

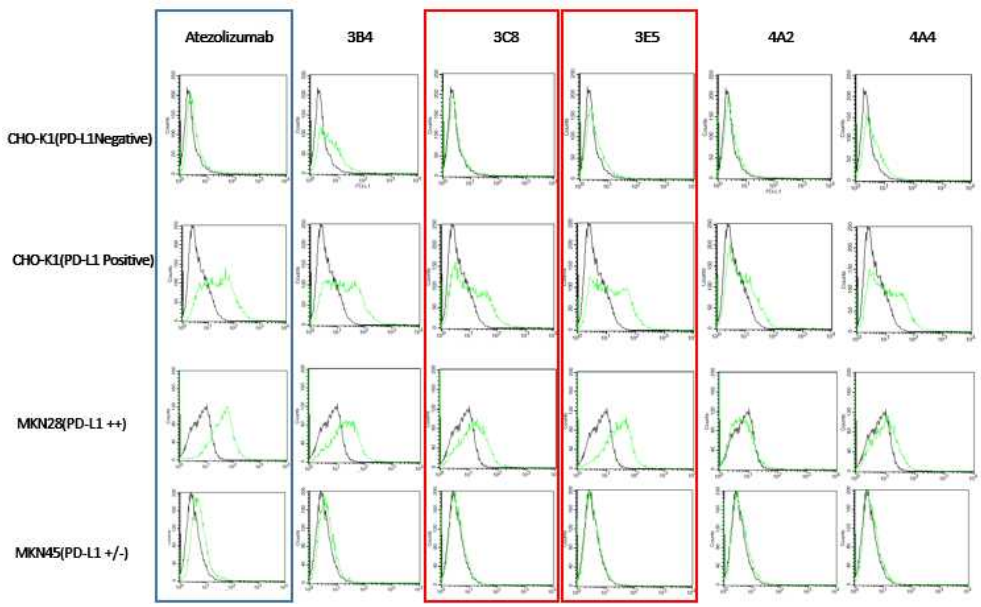


Figure 6. Analysis of PD-L1 targeting ability by Flow cytometry. Each cell line were stained with Atezolizumab(Positive control PD-L1 antibody) and PD-L1 antibody candidates. Fluorescence-positive cells were measured by flow cytometry.

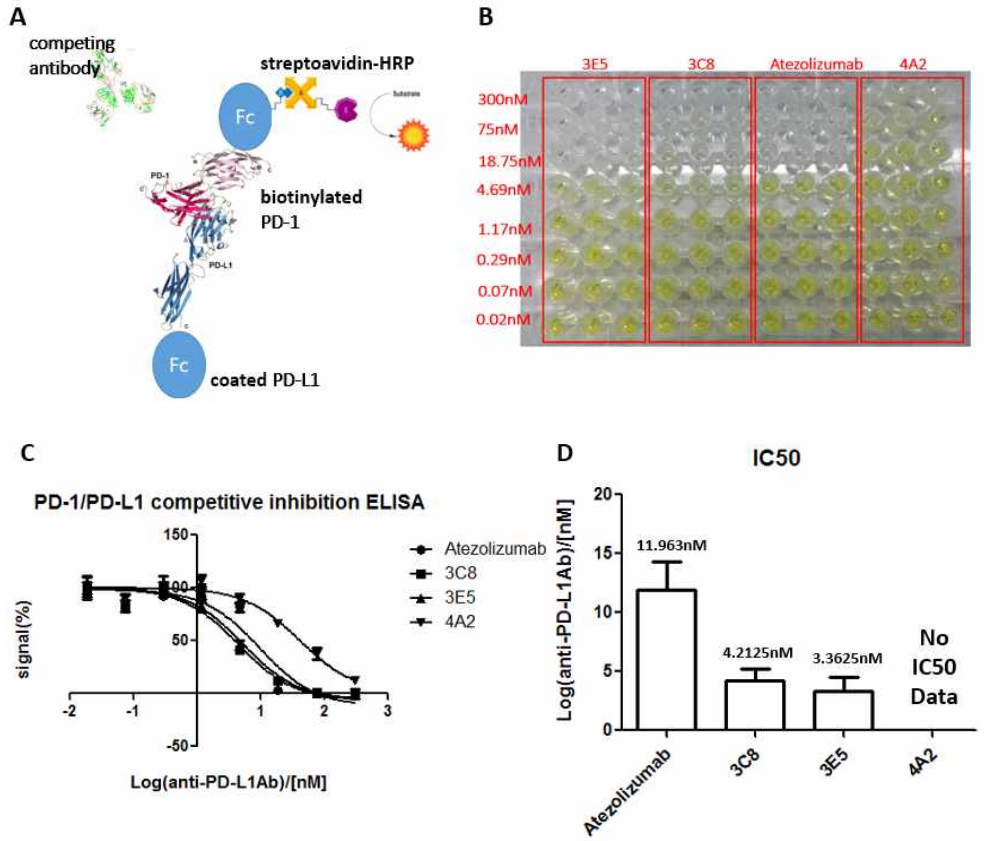


Figure 7. Measurement of PD-1/PD-L1 Neutralizing efficacy of PD-L1 candidate IGG by competitive inhibition ELISA. The graphical explanation of PD-1/PD-L1 competitive inhibition ELISA is shown (Figure 7A). Actual appearance of PD-1/PD-L1 competitive inhibition ELISA is shown (Figure 7B). PD-1/PD-L1 signal inhibition curve is displayed by using GraphPad Prism® 5 (Version 5.01, GraphPad Software Inc, USA) (Figure 7C). The IC50 value of PD-L1 antibody to neutralize PD-1/PD-L1 binding were calculated using the GraphPad Prism® 5 (Figure 7D).

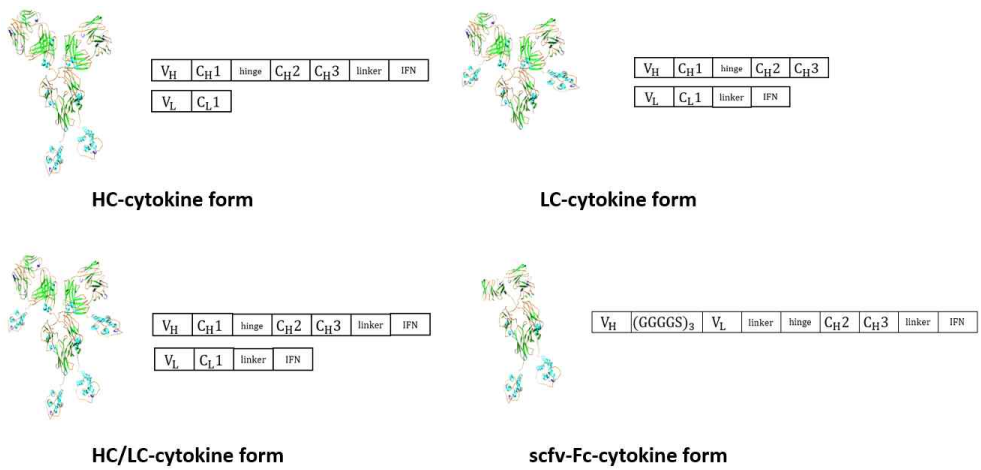


Figure 8. Design of various structure of immunocytokine. Various immunocytokines are designed by fusion of interferon to the PD-L1 antibody. Abbreviations : V, variable; C, constant; IFN, interferon

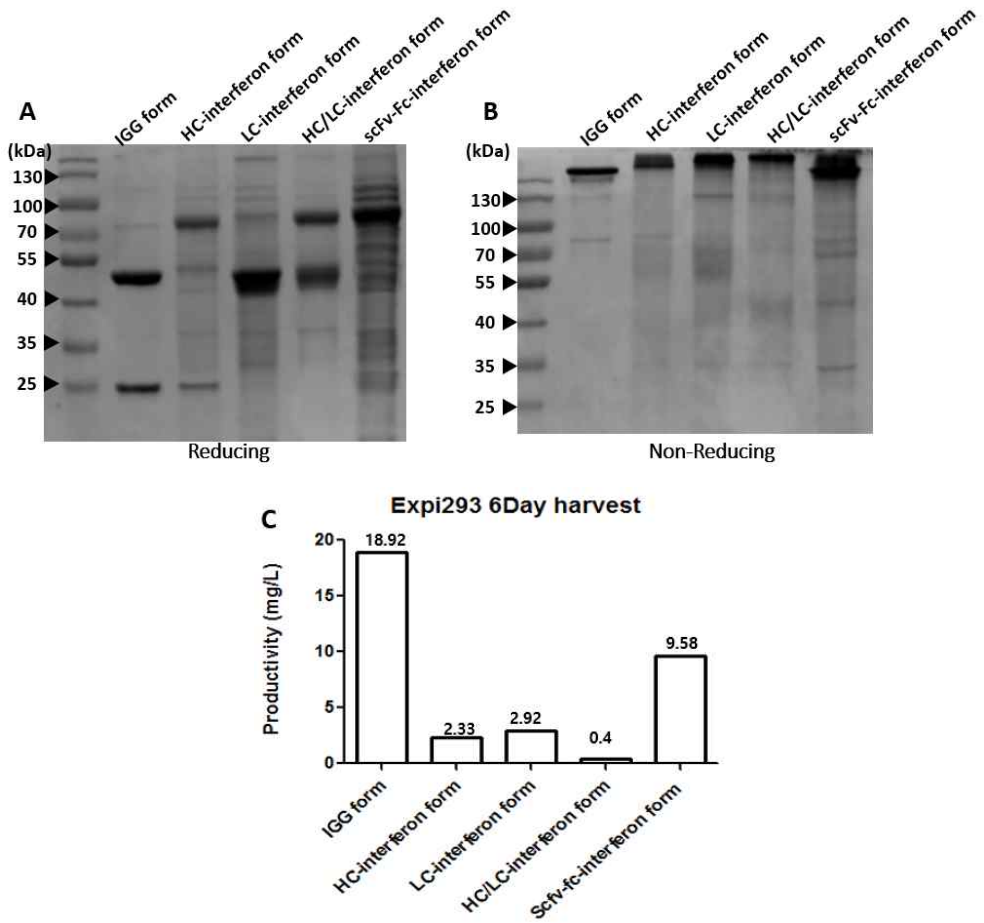


Figure 9. Production and Purification of various form of immunocytokine. Various designed immunocytokines were expressed in Expi293 and confirmed by SDS-PAGE (Figure 9A, B). Productivity was measured by ELISA(Figure 9C).

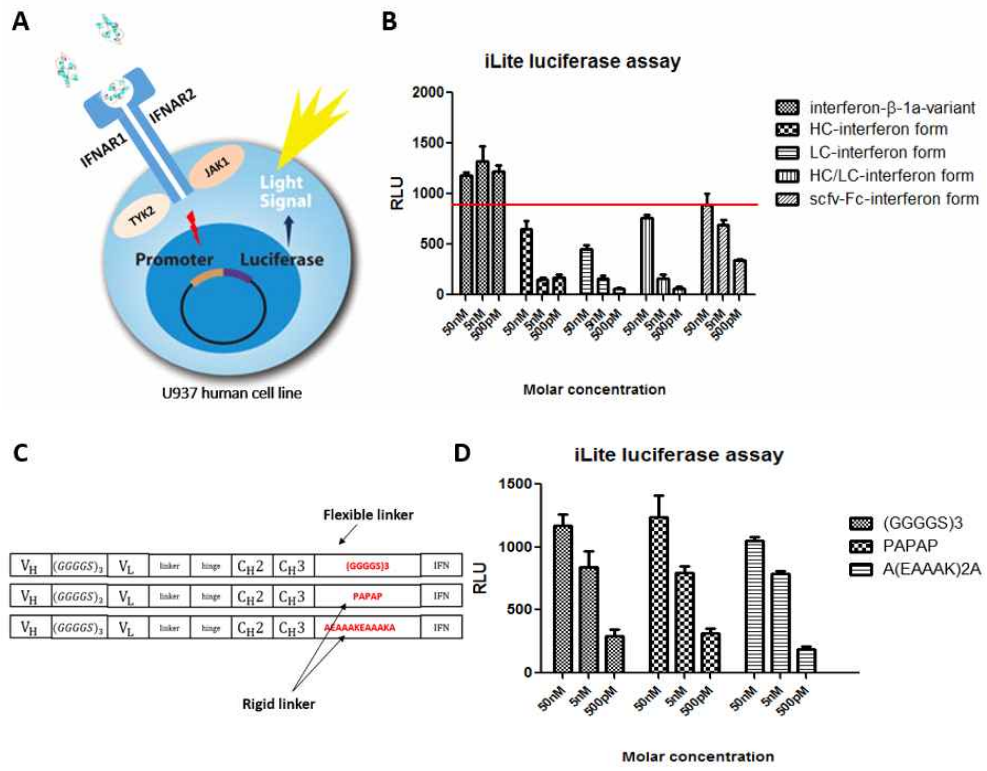


Figure 10. interferon biological activity based on various forms and linkers of immunocytokine. The graphical explanation of Type 1 IFN responsive Luciferase reporter assay is shown (Figure 10A). Type 1 IFN responsive Luciferase reporter assay was performed with various forms and various linkers of immunocytokines (Figure 10C). Luciferin signal was measured by microplate reader(TECAN) (Figure 10D).

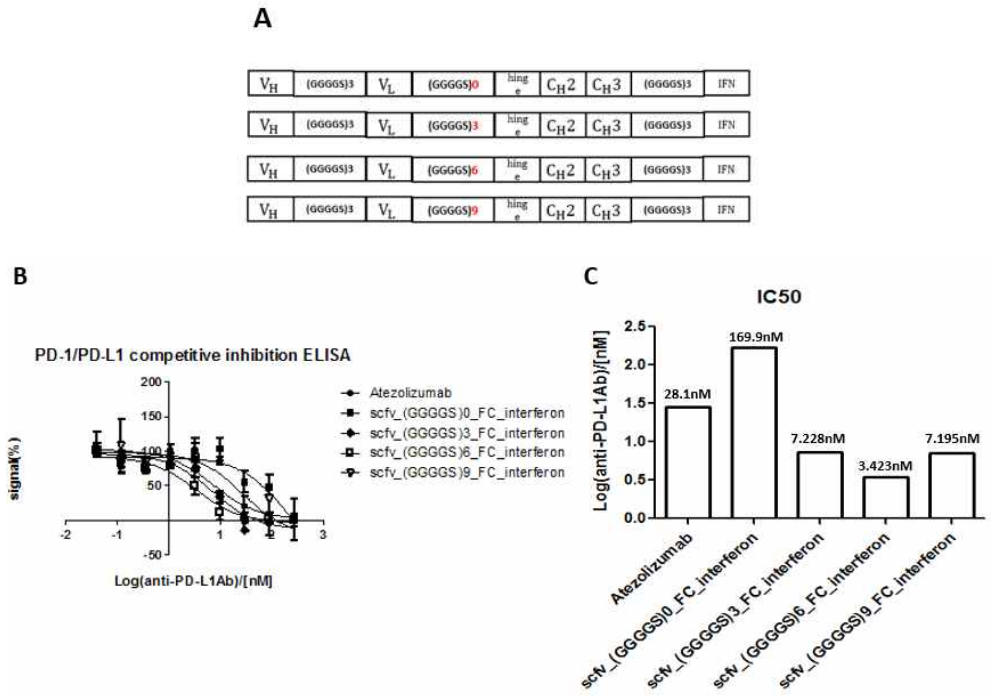


Figure 11. PD-1/PD-L1 neutralizing efficacy based on various forms of immunocytokine. PD-1/PD-L1 competitive inhibition ELISA was performed with the various scFv-Fc-interferon form immunocytokines of various lengths and IgG form PD-L1 antibody to determine whether the PD-1 / PD-L1 binding neutralizing efficacy differs according to the length of the linker above the hinge region(Figure 11A). PD-1/PD-L1 signal inhibition curve is displayed using GraphPad Prism® 5 (Version 5.01, GraphPad Software Inc, USA) (Figure 11B). The IC50 value of PD-L1 antibody to neutralize PD-1/PD-L1 binding were calculated using the GraphPad Prism® 5. (Figure 11C)

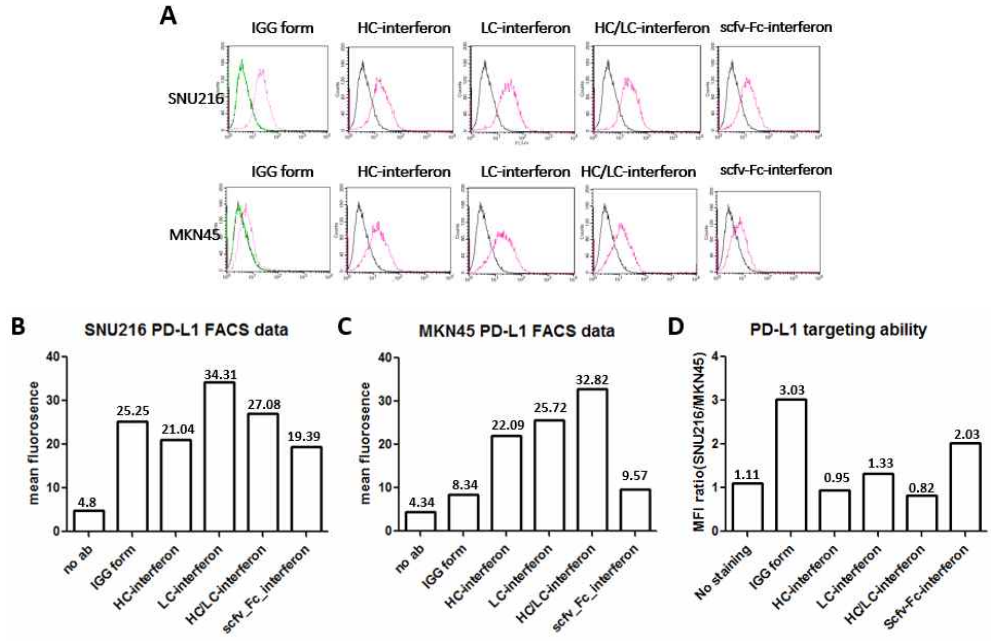


Figure 12. PD-L1 targeting ability based on various forms of immunocytokine. The same quantities of immunocytokines and full-form IGG PD-L1 antibodies were bound to the SNU216 and MKN45 cell lines of different expression levels of PD-L1 and then measured by flow cytometry(Figure 12A, B, C). The MFI ratios of the respective immunocytokines for snu216 and MKN45 were calculated(Figure 12D).

Discussion

Two years have passed since immuno oncology provoked excitement among many researchers. In the meantime, the number of clinical trials for checkpoint inhibitors targeting PD-1 and PD-L1 is nearing 1,000. Since efficacy of PD-1 / PD-L1 checkpoint inhibitors are not limited to specific indications, clinical trials are underway for almost all cancers, and licensing has already been granted for indications such as Non-small cell lung cancer, Bladder cancer, Head and Neck cancer, Renal cell cancer, Melanoma and Hodgkin's lymphoma. Therefore, the PD-1 / PD-L1 checkpoint inhibitor was established as a backbone therapy in combination with other anti-cancer drugs. [10, 11, 12]

By June 2017, a total of 66 monoclonal antibodies have been approved. 43% of these antibodies are targeted to six targets, CD20, CD3, EGFR, Her2 / neu, IL-6R and TNF-a. [13] Since monoclonal antibodies, a complex and extremely powerful therapeutic platform, have different epitopes, affinities, immunogenicity, tissue penetration and serum half-life for each clone, different antibody therapeutics for the same target have different implications. [14] In particular, recent advances in Fc engineering, antibody drug conjugate, bispecific antibody, and immunocytokine technology have shown the potential to increase the efficacy of antibody-based therapeutics on the same target. [15, 16] Two of the monoclonal antibodies targeting the promising target, PD-L1, were approved and two were undergoing Phase 3 in clinical trials, but we decided to screen for antibodies to develop PD-L1 antibody-based therapeutics. [17]

The extracellular region of PD-L1 belonging to the immuno globulin

superfamily consists of two facing beta sheets making it a highly stable protein, resulting in natural conformation without any treatment. To express N-glycosylation, which affects the function of PD-L1, mammalian cells were used to produce antigens. In addition, antibody backbone was used to facilitate purification (Figure 1, 2). [18] The anti-PD-L1 antibody candidate, 3C8 and 3E8, that effectively neutralizes PD-1 / PD-L1 binding at a lower concentration than Atezolizumab was obtained by screening antibody candidates using the PD-L1 antigen and phage display technology (Figure 6, 7).

The PD-L1 antibody has been established as a backbone therapy but is not effective in 60% 'less immunogenic' patients when used alone. Less immunogenic (low neo-antigen expression or low neo-vasculature) cancer causes less immune response and is less likely to induce invasive immune cells to the tumor-micro environment. Thus, inhibition of the checkpoint with the PD-L1 antibody does not lead to a response since there is insufficient amount of immune cell to attack the tumor. [19]

In order to solve this problem, immunocytokine were designed by linking interferon-β-1b mutein, known to increase TIL, to PD-L1 antibody. Interferon-β-1b mutein, R27T, is a bio-better with increased stability and solubility by adding glycosylation to 25th a.a. of human interferon beta 1a. It is a strong inflammatory molecule mainly acting on DC and cancer cells. When this R27T is fused to the PD-L1 antibody, the half-life of R27T is greatly increased, and R27T is concentrated on DC and cancer cell (main target of interferon-β) overexpressing PD-L1, thus making treatment effective without systemic toxicity. [6, 20]

In addition, there is a research result pointing to increased PD-1 / PD-L1 signal as a cause of no response to conventional Type 1

interferon treatment, and a synergic effect between Interferon-β-1b mutein and PD-L1 antibody is expected. [21]

However, immunocytokines have lower productivity than conventional antibodies as well as lower biological activity than natural cytokines. Recent studies also suggest that immunocytokines have a different tendency from the expected biodistribution. [9] Therefore we designed various types of immunocytokine and their properties were measured after production to find optimized forms of immunocytokine.

As a result, scfv-Fc-interferon-β-1b mutein form of immunocytokine was found to be superior to other forms of immunocytokines in productivity. Previous studies have shown that when interferon is fused to an antibody, productivity is significantly reduced compared to that of a natural form of antibody, As shown by the fact that HC / LC-interferon-β-1b mutein form of immunocytokine, which has significantly lower than other immunocytokines. However, for efficient assembly of antibodies, the ratio of heavy chain to light chain in cytosol is also an important factor affecting productivity. [22]

The scfv-Fc-interferon-β-1b mutein type of immunocytokine appears to be produced more efficiently because there is no need for an assembly step between heavy chain and light chain (Figure 9).

HC / LC-interferon-β-1b mutein form of immunocytokine was relatively high in biological activity at the same molar concentration due to the large number of interferons fused to one molecule, but the scfv-Fc-interferon-β-1b mutein form of immunocytokines showed the highest biological acitivity. It is thought that scfv-Fc-interferon-β-1b mutein form has a relatively small molecular weight compared to other immunocytokines and thus thermodynamically shows a higher

activity (Figure 10). (scfv-Fc-interferon form : 150kDa, HC-interferon form : 200kDa, LC-interferon form : 200kDa, HC/LC-interferon form : 250kDa)

Immunocytokines in the form of fusion of interferon with PD-L1 IgG antibody were equivalent in neutralizing PD-1 / PD-L1 binding when compared to PD-L1 IgG antibody (data not shown). However, the scfv-Fc-interferon-β-1b mutein type of immunocytokine was found to be dependent on the linker length between scfv and hinge region in neutralizing PD-1 / PD-L1 binding when compared to PD-L1 IgG antibody. When the scfv-Fc-interferon-β-1b mutein type of immunocytokine was directly linked to the hinge region without the linker, neutralizing efficacy was lower than PD-L1 IgG antibody. However, fusion of (GGGGS)3 or longer linkers resulted in better neutralizing efficacy than PD-L1 IgG antibody (Figure 11).

Several studies using immunocytokines have been conducted worldwide, Recently, it has been confirmed through clinical trials that immunocytokines accumulate well in tumor tissues, and it has been confirmed that CD8 T cells and NK cells are recruited into the tumor-micro environment, supporting that immunocytokine is a promising anti-cancer drug platform. [23, 24, 25]

This study is significant in that it has searched for an effective human PD-L1 antibody and found an optimal form to utilize it as an immunocytokine.

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국 문 초 록

면역 항암요법(Immunotherapy)은 기존 항암요법에 비해 암 환자의 장기 생존율을 크게 증대시켜 준다는 점에서 큰 주목을 받고 있다. 그 중 대표적인 타겟으로 알려진 PD-L1 (Programmed cell death Ligand 1)은 PD-1에 결합하여 암세포의 면역회피를 일으키고 항원제시세포의 조절 T 세포 성숙능력을 증가시키는 등 다각적으로 작용하는 단백질이다.

본 연구에서는 파지 디스플레이 기법을 활용하여 인간 PD-L1에 선택적으로 결합하는 단일 클론 항체 후보물질 두 가지를 선별하였으며, 이것들은 미FDA가 승인한 PD-L1 항체 Atezolizumab보다 더 낮은 농도에서 PD-1/PD-L1 신호를 차단함을 확인하였다.

하지만 PD-L1 항체는 미세종양환경에서의 종양 침윤 림프구 부족으로 인해 많은 환자들에서 효과를 보이지 않는다고 알려져 있다. 이를 해결하기 위하여 종양 침윤 림프구를 증가시킬 것으로 기대되는 인터페론 베타 변이체를 PD-L1 항체에 연결시킨 형태인 면역사이토카인을 구상하였고, 다양한 형태의 면역 사이토카인을 디자인하여 생산 후 각 형태별 특성을 측정하였다. 그 결과, scFv-Fc-인터페론 베타 변이체 형태의 면역사이토카인이 생산성, 생물학적 활성, PD-1/PD-L1 신호 차단 능력 그리고 PD-L1 타겟팅 능력에서 다른 형태의 면역사이토카인보다 우수한 것으로 나타났다. 본 연구는 PD-L1 항체를 선별하고 이를 면역사이토카인으로 활용하기 위한 최적의 형태를 보여주었다.

주요어 : 면역항암요법, PD-L1, Atezolizumab, 항체, 종양 침윤 림프구, interferon-β-1b mutein, 면역사이토카인

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