Development of Nivolumab Bio-Better Using Mammalian and Plant Glyco-Delete Platform

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This certifies that The Master's Thesis
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마지막으로 공부하느라 애쓴다고 비가 오나 눈이 오나 큰손녀 생각해주시는 매일 보고싶은 사랑하는 할아버지, 할머니와 항상 큰딸내미의 결정을 믿고 지지해 주시며 든든한 울타리가 되어 주시는 사랑하는 부모님, 우리 강대표님과 하여사님께 마음 깊이 존경과 감사를 표합니다. 그 동안 바쁜 언니 대신 수고해준 가은이에게도 고맙고, 이제 곧 새내기 대학생이 되는 막내 주혁이에게도 사랑한다고 말하고 싶습니다. 가족들의 배려와 뒷받침이 있었기에 이 모든 것이 가능했고, 앞으로 평생 보답할 수 있도록 열심히 노력하겠습니다.

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ABSTRACT

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The glycan in Fc region regulates therapeutic efficacy of antibody by changing various biological activities such as protein safety, mobility, protein clearance, and receptor conjugation. Also, since it has various biological activities, such as playing an important role in targeting antigen and immune response, it is a factor that determines the quality of antibody. However, since glycans are made by the action of various enzymes and the activity of each enzyme may be different depending on the production process or conditions, it is difficult to homogeneously attach to the protein.

Nivolumab is a human IgG4 monoclonal antibody that blocks PD1. It was approved for medical use in the United States in 2014 and treats several types of cancer includes melanoma, lung cancer, malignant pleural mesothelioma, renal cell carcinoma, etc. It

is mainly used as a first-line treatment for metastatic melanoma and non-small cell lung cancer, and many clinical trials are ongoing as a combination treatment with various targeted anticancer drugs. Also, Nivolumab is the type of immunotherapy works as a checkpoint inhibitor, blocking a signal that prevents activation of T cells from attacking the cancer.

Most immunomodulatory anticancer drugs including nivolumab have IgG4 backbone which has low affinity for complement and various FcγRs. However, IgG4 still has a measurable affinity for FcγRIII, so given the high drug concentrations used in therapy, the possibility of target cell ADCC cannot be completely eliminated. Therefore, a method for removing immunogenicity of the IgG4 is highly demanded.

Glyco-Delete (GD) technology is an ADCC removal platform for antibody drugs. Via glycosylation enzymes regulation to leave only one GlcNAc glycan stump, not only reduce the affinity for FcγRIII, but also provide the uniformity of antibody which is important factor for manufacturing.

In this study, GD CHO cells and GD plant systems were produced by controlling the glycosylation process of CHO cells and plant and nivolumab was used for template to prove the usefulness of GD platform. By producing nivolumab with IgG1 and IgG4 backbone in CHO cell system, we measured the affinity for PD1 and the blocking activity of PD1 signaling to check whether the GD cause any change in affinity and blocking activity of PD1 signaling. Glycan uniformity and immunogenicity were also confirmed the improved characteristics of GD nivolumab. Furthermore, by using the GD-plant system, plant-nivolumab was produced and its functions were evaluated.

This study demonstrates that GD platform of CHO cells and plant provides reduced ADCC activity to nivolumab without altering PD1 inhibiting activity. GD engineering can be contributed to as a bio-better platform for various target-masking antibodies.

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Key words: Nivolumab, Bio-Better, Glycosylation, Glyco-Delete, Antibody-Dependent Cellular Cytotoxicity (ADCC)

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I. INTRODUCTION

Antibody drugs made from CHO cells, the main antibody-producing cell line, undergo post-translational modification (PTM), the most representative of which is glycosylation[1]. The glycan constituting the Fc region is not only very important for the affinity with complement that determines the immunogenicity of the antibody or for the interaction with the antibody receptors of immune cells, but also influences the affinity with the neonatal Fc receptor (FcRn) to determine the half-life of the antibody in the blood[2]. The glycan of the antibody, which directly affects therapeutic efficacy by determining the efficacy and duration of action in the body, is a major factor determining the quality of antibody drugs[3]. However, unlike DNA, RNA, and proteins that are replicated or synthesized based on a given template, glycans are produced by the sequential action of various glycosylation enzymes without a template. Therefore,

even glycans attached to one protein are produced as a mixture of heterogeneous glycans of various types. In addition, the activity of glycosylation enzymes varies depending on the cell culture conditions, so the mixing proportion of the glycans of the protein is also variable[3]. This fact not only means that the regulation of the glycan of the antibody can change the function of the antibody, but also suggests that the form of the glycan needs to be uniform in order for the antibody to maintain a constant function.

Unlike existing 1st generation chemotherapy and 2nd generation targeted anticancer drugs, immunomodulatory anticancer drugs are classified as 3rd generation immuno-oncology drugs because they have an innovative mechanism that uses the patient's own immune system to eliminate cancer cells[4]. It has a high therapeutic effect and few side effects, so it is evaluated as an anticancer agent that not only suggested a new paradigm of anticancer mechanism but also accelerated the conquering of cancer[5].

Nivolumab is a human IgG4 monoclonal antibody that blocks PD1. It was approved for medical use in the United States in 2014 and treats several types of cancer includes melanoma, lung cancer, malignant pleural mesothelioma, renal cell carcinoma, etc. It is mainly used as a first-line treatment for metastatic melanoma and non-small cell lung cancer, and many clinical trials are ongoing as a combination treatment with various targeted anticancer drugs[6]. Also, Nivolumab is the type of immunotherapy works as a checkpoint inhibitor, blocking a signal that prevents activation of T cells from attacking the cancer. On the surface of T cells, there are proteins acting as receptors that inhibit T cell activity, such as PD1 and CTLA-4, and on the surface of cancer cells, there are PDL1 and CD80 ligand that bind to them respectively[5]. When binding occurs, the activity of T cells is suppressed, so T cells fail to inhibit the proliferation of cancer cells. Nivolumab is an anti-PD1 monoclonal antibody that binds to PD1 present on the surface of T cells. By masking PD1 from

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PDL1 interaction by cancer cell, T cell inhibition by PD1 signaling is eliminated, allowing cancer-recognizing T cells to clear the cancer[6].

Immunoglobulins of antibody molecules found in serum are divided into five isotypes: IgG, IgM, IgA, IgE, and IgD. Among them, IgG, which occupies the largest proportion, is classified into IgG1, IgG2, IgG3, and IgG4 classes according to differences in the length of the hinge region constituting the heavy chain and the number of disulfide bonds between the chains[7]. IgG1 shows the highest affinity for all FcγRs and is known as a potent activator of antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). On the other hand, IgG4 has low affinity for all receptors except FcγRI and has weak induction of Fc-mediated effector functions. Among FcγRs, the most well-characterized activating receptors are FcγRIIA and FcγRIIIA[7]. FcγRIIA is predominantly expressed in monocytes and macrophages, and FcγRIIIA is predominantly expressed in NK cells[7]. When developing anticancer antibodies, it is important to select an appropriate human IgG class that meets the expected mechanism of action. Most of the target anticancer antibodies were made from the IgG1 backbone, the most abundant form in the blood, and their main mechanism is to kill target-expressing cells by relying on high complement dependent activity and NK cell-dependent cell death[8]. Accordingly, using methods to increase target-expressing cell death such as hyper glycosylation of the Fc part glycan or fucose removal, drugs with high ADCC efficacy have been developed, which increases the binding with FcγRIII, an antibody receptor of effector cells like NK cells. However, in the case of immunomodulatory anticancer drugs such as nivolumab, the immunogenicity of complement-dependent cytotoxicity (CDC) and ADCC antibodies results in the death of T cells, so it must be removed. Most immunomodulatory anticancer drugs were designed IgG4 backbone which has low

affinity for complement and FcγRIII[7]. However, IgG4 also has an affinity for FcγRIII and cannot completely eliminate ADCC activity of IgG4 antibodies such as nivolumab given the large drug concentration used in treatment. Therefore, there is a need for a method for removing the immunogenicity of the IgG4 type of immunomodulatory anticancer antibody.

Glyco-Delete (GD) technology is an ADCC removal technology for antibody drugs that uses glycosylation enzymes to control the shape of glycan, leaving only GlcNAc glycan.[2,10]. When high-mannose glycan is bound to ASN297 of the antibody heavy chain moving from ER to Golgi, GlcNAc (N-Acetylglucosamine) is bound to the mannose residue by the GnTI (N-acetyl-glucosaminyl-transferase I) enzyme. When this GnTI is removed, the high mannose state is maintained. At this time, when EndoT (Endo-β-N-acetyl-glucosaminidase) is overexpressed from the outside, only one GlcNAc remains like a stump. Interestingly, it was reported that when this technology was applied to the anti-CD20 antibody, the binding affinity for FcγRI and FcγRIII was lowered by more than 10 times, and the ADCC immunogenicity was also reduced by about 6.6 times.[2] If this GD technology is grafted onto an anti-PD1 antibody, it can be providing a bio-betters that overcome the possibility of T cell death. In addition, applying GD to cytokine antibody, which was initially developed with IgG1 backbone, can also reduce the side effects that induce the death of non-immune cells that secrete cytokines with the intention of removing unnecessary immunogenicity in addition to the cytokine scavenging ability, the recently developed cytokine antibodies adopt the IgG4 type. Therefore, the GD technology that can remove immunogenicity from cytokine scavenging antibodies is expected to be useful. Moreover, in addition to providing the homogeneity of the antibody, the remaining N-glycan stumps provide better structural stability than removal of all glycans by amino

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acid substitution.

Plants are considered attractive hosts for the mass production of recombinant protein drugs due to their economic efficiency, stability, and convenience[11-18]. Successful production of antibodies in plant systems will not only lower the production costs, but also eliminate the risks from viruses in the mammalian host systems[13]. The success of the plant-produced antibody cocktail, ZMapp, in the 2014 Ebola outbreak supported the superiority of the plant protein production system, especially for antibody production[9,19]. This is an example of the successful use of plant antibodies as clinical therapeutics. In addition, Elelyso®, sold by Protalix BioTherapeutics Inc., is a β-Glucocerebrosidase produced from carrot callus and is being used as a treatment for Gaucher's disease, a rare disease[15,20]. Antibody production through plant system not only suggested superiority compared to existing anti-infection antibodies through the case of ZMapp, but also proves safety when used clinically with the case of Elelyso. When GD technology is applied to a plant system with such superiority, the advantage of removing plant-specific glycans, β-1,2-Xylose (core xylose) and core α-1,3-Fucose, is additionally provided. Whether it induces an acute allergy in the human body to plant-specific glycans or not is still controversial, it seems to be related to reports that 50% of non-allergic people have antibodies specific for β-1,2-Xylose, and 25% of them have antibodies against core α-1,3-Fucose in their sera[21]. According to these findings, it is considered safe to remove plant-specific glycans. In addition, when GD technology is applied to plant systems, the uniformity of glycans is greatly increased, and the problem that N-glycan of plants containing terminal mannose residues reduces the circulating half-life of therapeutic proteins is also known as an advantage.

In order to determine the effectiveness of applying GD technology to immunomodulatory anti-cancer antibodies, GD technology of CHO and plant system

were established and GD nivolumab was produced to verify the GD effect. CHO and plant GD nivolumab not only showed antibody binding and PD1 signal inhibition comparable to that of the prototype, but also showed a significant decrease in ADCC. This GD platform is expected to provide a bio-better platform that provides uniformity and alleviates the side effects of targeting antibodies such as under immunomodulatory cancer.

II. MATERIALS AND METHODS

1. Construction of GnTI knockout CHO cell

The HITI CRISPR/Cas9 method was used to construct GD CHO cells[16]. GD technology is a technology that reduces ADCC of an antibody by controlling the shape of antibody glycans using enzymes related to protein glycosylation. HITI CRISPR Cas9 is a method in which the Mgat1 (NCBI ENSG00000131446) gene encoding the GnTI (N-acetyl-glucosaminyl-transferase) enzyme is first knocked out to remove the GnTI effect of binding GlcNAc (N-Acetylglucosamine) to the Mannose residue, and a reporter gene that can select cells with mutations is inserted into the target position cut with Cas9. The LentiVirus system is used to overexpress the EndoT (Endo-β-N-acetyl-glucosaminidase, NCBI LOC6044791) that cuts the glycan and leaves only one GlcNAc after removal of the desired gene to construct the final CHO-GnTI (-) cell. CHO-GnTI (-) cells were used for experiment after hygromycin selection. The success of the knockout was confirmed by PCR and sequencing results.

2. Generation and purification of GD nivolumab with IgG1 and IgG4 backbone

Light and heavy chain sequences of nivolumab were obtained from GenBank (MC034325) and long chain cDNAs were synthesized by Bioneer (Daejeon, Korea) and plant codon-optimized according to the manufacturer's protocol (Bioneer, http://eng. bioneer.com, Daejeon, Korea). To produce various types of nivolumab in CHO cells, the light and heavy chains of nivolumab with IgG1 and IgG4 backbone were inserted into the pLVX vector. Then each clone was transfected into HEK cells

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to generate lentiviruses. The HEK cells media containing lentiviruses for light and heavy chains were treated after mixed for infection of CHO WT and CHO GnTI (-) cells. After selection of each chain expressed-CHO cells by puromycin and bleomycin resistance, the cells were amplified. Nivolumab producing cells grown to 80% confluency in RPMI-1640 medium containing 10% FBS were washed twice with PBS and refreshed with EX-CELL® CD CHO Serum-Free medium(sigma) containing 1 mM sodium butyrate. Conditioned media containing monoclonal antibody was obtained by further incubation for 14 days at 30 ℃ in 5% CO_2, 95% air. Then, the nivolumab released from cells to media was collected and purified using the protein A column (ThermoScientific). Buffer change in and concentration was performed by ultrafiltration with Amicon® Ultra-2 (Millipore, UFC801024) before filter sterilization and storage. The antibodies were analyzed by SDS-PAGE and coomassie blue staining, and their concentration was quantified relative to BSA band intensity for 0.5, 1.0, and 2.0 µg of BSA used as standard.

3. Construction of Nivolumab expression vectors in *Nicotiana benthamiana*

Original and plant codon-optimized light and heavy chains were inserted into the pCAMBIA 1300 binary vector (p35S:BiP:nivolumab light chain/heavy chain-HDEL). For ER localization, signal sequence of the luminal binding protein, BiP, and ER retention signal sequence, HDEL, were ligated into the N-terminal and C-terminal regions, respectively. The resulting constructs (35S:BiP-pco light chain nivolumab, 35S:BiP-pco heavy chain nivolumab, 35S:BiP-pco heavy chain nivolumab-HDEL, 35S:BiP-original light chain nivolumab, 35S:BiP-original heavy chain nivolumab, and 35S:BiP-original heavy chain nivolumab-HDEL) were transformed into

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Agrobacterium tumefaciens GV3101 competent cells using the freeze-thaw method into *N.benthamiana* plants [17,18]. PCR followed by sequencing of products was performed to verify the constructs. Transformed Agrobacteria were incubated in YEB medium containing 50 mg/ml kanamycin and 50 mg/ml rifampicin at 28°C for 2 days. Agrobacteria were re-suspended in infiltration solution (10 mM MES, pH 5.7, 10 mM MgCl$_2$, and 500 μM acetosyringone) and infiltrated into the abaxial side of leaves using a syringe as described previously[19]. Four-week-old tobacco (*N.benthamiana L.*) plants, grown on the soil at 25 ± 0.5 ℃ under long-day conditions (16 h light and 8 h dark), were used for infiltration. After infiltration, tobacco plants were further incubated for 3–4 days in the 16 h light/8 h dark cycle at 25 ± 0.5 °C. Subsequently, infiltrated leaves were harvested for all experiments.

4. Plant Nivolumab Purification from *Nicotiana benthamiana* leaf crude extract

Infiltrated leaves were harvested after 3–4 days for all experiments. Wild-type and infiltrated tobacco leaves were ground with a mortar and pestle under liquid nitrogen to extract crude protein. The powder was suspended, and the total soluble proteins were extracted with two volumes of protein extraction buffer containing 50 mM Tris–HCl (pH 7.2), 150 mM NaCl, and protease inhibitor cocktail (Sigma-Aldrich) followed by incubation for 15 min at 4°C. The protein suspensions were centrifuged twice at 16,000 g for 30 min at 4°C. The supernatant was filtered through two layers of Miracloth to remove insoluble material then recentrifuged at 16,000 ×g for an additional 30 min. Total protein concentration of supernatant (water-soluble protein) was quantified using Bradford reagent with BSA standard. The clarified extract was filtered through 0.22 μm pore filters (Sartorius Stedim Biotech GMBH, Gottingen,

Germany) and then loaded onto a protein A affinity chromatography column (Pierce, GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada). The column was washed with extraction buffer, and antibodies were eluted using 100 mM glycine pH 3.0. Elution fractions were immediately neutralized with 2.0 M Tris–HCl (pH 7.4) and analyzed using Bradford assay and western blot to determine antibody quantity and purity. Antibodies were stored at -80 °C until used. Antibody amount and concentration was measured by Human IgG ELISA Kit (E88-104, Bethyl Laboratories) and equal amounts of antibody were confirmed by molecular mass normalized the band intensity calculation after coomassie gel staining of antibodies.

5. Generation of the Jurkat-PD1 cell and Jurkat-PD1-NFAT cell lines

The Jurkat-PD1 cell line was created by transfecting Jurkat cells with pLVX vector expressing PD1, then selected with 1 µg/mL puromycin. Puromycin-resistant cells were screened by FACS for high-level PD1 expression. The Jurkat-PD1-NFAT cell line was generated by transfecting the Jurkat-PD1 cells with pGL3 vector expressing luciferase under the control of the NFAT response elements from the IL-2 promoter (Addgene #17870) followed by selection with 10 µg/mL Blasticidin. Blasticidin-resistant cells were screened for high-level luciferase expression with the 5 µl anti-CD3/anti-CD28 activator (25 IU, #10971, STEMCELL).

6. SDS-PAGE and Immunoblot Analysis

Total leaf protein extracts (12–20 µg protein) were analyzed via SDS-PAGE under reducing conditions in 12% polyacrylamide gels. After SDS-PAGE, the gels were

transferred onto PVDF membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked for 30 min with 5% (m/v) skim milk in T-TBS buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.01% Tween 20. For immune detection of expressed nivolumab antibodies, anti-human Ig Fc-specific HRP-conjugated antibody (1:2000 dilution; ab97225, Abcam) was used. Protein signals were detected and quantified using chemiluminescence detector LAS-4000 (GE Healthcare, Little Chalfont, UK) and the ImageQuant program (GE Healthcare), respectively. To measure the yield of nivolumab expressed in tobacco leaf cells, purified CHO K1 cell-expressed nivolumab (50, 100, 200, and 300 ng) was used as a positive control. The immunoblot signals were quantified using the Bio-Imaging Analyzer (Image Gauge 3.0; Fujifilm, Tokyo, Japan).

7. Protein mass analysis

The molecular mass of antibodies were determined with Reverse Phase (RP) separation using Waters Acquity Iclass UPLC system (Milford, MA, USA) The separation was performed with a Thermo MabPac™ RP column (2.1 mm . 50 mm, 4 µm particle size) at a flow rate of 0.2 mL/min. The mobile phases were 0.1% formic acid in water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B) applied in a gradient mode. The gradient was 0–2 min, fixed at 25% Eluent B, 2–20 min, linear increase from 25 to 45% Eluent B. The effluent was injected to Thermo-Fisher LTQ Orbitrap mass spectrometry (Thousand oaks, CA, USA). The Fourier Transform Mass Spectrometry (FTMS) resolution and mass range were 120,000 and 400–4000 m/z, respectively. The mass spectra were deconvoluted with Protein Deconvolution 2.0 using isotopically unresolved mode.

8. Binding affinity test using Flow Cytometry analysis

To compare the binding affinities of the antibodies, flow cytometry (FACS, BD Biosciences, Franklin Lakes, NJ, USA) was used. Each antibody concentration was calculated by non-reducing coomassie staining of SDS-PAGE gel then the result was confirmed by ELISA (Human IgG ELISA Quantitation Set, E80-104, Bethyl laboratory). 1.5×10^5 Jurkat PD1 cells were treated with nivolumab, rituximab, nivolumab IgG1 WT, nivolumab IgG1 GD, nivolumab IgG4 WT, nivolumab IgG4 GD, pNiv-no HDEL, pNiv-HDEL, pNiv-HDEL with Endo H at concentrations of 0.1, 0.3, 1, 3, and 10 µg/mL for 30 min, followed by incubation with anti-human Ig Fc-specific FITC-conjugated secondary antibody for 30 min at 4℃. Rituximab (Mabthera®, Roche, commercially available rituximab) was used as negative control. The cells were washed twice with PBS and then analyzed. A sample treated only with anti-human Ig Fc-specific FITC-conjugated antibody was used for subtracting the background fluorescence. The binding was measured as the Geomean fluorescence intensity of each sample by FACS Verse (BD biosciences) and calculated by the Flow Jo software.

9. Human PD1 binding specificity test by ELISA

The binding ability of anti-PD1 was evaluated by ELISA. Briefly, the MaxiSorp high protein-binding-capacity 96-well ELISA plate was coated with 10 ng/well (100 µl) of recombinant human PD1 His tag protein (#8986-PD-100, R&D Systems) at 4℃ overnight. The coated ELISA plate was washed 3 times and blocked with PBST. The

two-fold serial dilution of monoclonal antibodies in PBS (starting from 200 ng/well, 100 µl) was added to the plate, incubated at 37°C for 1 h, and then washed 3 times with PBST. The goat anti-human IgG-HRP was added (100 µL/well at a 1:10,000 dilutions in PBST), incubated at 37°C for 1 h, and washed 3 times with PBST. The TMB substrate solution (100 µL/well) was added to the plate and incubated in the dark at RT for 20 min. The stop solution (50 µL/well, 2 M H_2SO_4) was added to the plate to stop the reaction, and the absorbance at 450 nm was determined using a Cytation™ cell imaging multi-mode reader.

10. Cytokine production test by ELISA

A total of 1×10^5 cancer cells were seeded per a well in their respective growth medium for 30 min and then 1×10^5 Jurkat PD1 cells were co-cultured in a 96-well round-bottomed plate (ThermoScientific™) using 200 µL RPMI-1640. After 10 min, cells were stimulated with anti-CD3/anti-CD28 activator (25 IU, #10971, STEM CELL) 5 µl and treated with each antibody (10 µg/ml). Then plates were incubated 24 h at 37°C in the presence of 5% CO_2. The supernatant was collected by test plate centrifugation at 1500 rpm for 20 min. ELISA plates (MultiSciences Lianke Biotech Co., Ltd. Hangzhou, China) were used to evaluate IL-2 (ELISA MAX™ Standard Set Human IL-2 #431081, Biolegend) and INF-γ (ELISA MAX™ Standard Set Human IFN-γ #430101, Biolegend) levels.

11. NFAT-Luciferase reporter cell system

Cancer cells were seeded in a white 96-well assay plate with 5×10^5 cells per well, and cultured at 37°C, 5% CO_2 for 12–14 h. After removing the medium, 1×10^6 Jurkat-PD1-NFAT luciferase cells in 50 μL assay medium (RPMI1640 medium with 2% FBS) was added into each well. Cells were stimulated with anti-CD3/anti-CD28 activator (25 IU, #10971, STEM CELL) 5 μl and treated with each antibody (30 μg/ml) After adding antibody into each well, the assay plate was incubated at 37°C, 5% CO_2 for 6 h. Then 100 μL luminescence substrate (Promega Bio-Glo™ Luciferase Assay) was added into each well and relative luciferase units (RLU) were scored using the luminometer SpectraMax®M5.

12. Purification of PBMC cells

PBMC was purified using blood from healthy donors who voluntarily participated in our study according to IRB procedure (#4-2016-0600) approved by the committee of Yonsei IRB board. Briefly, 6 mL of blood with 6 mL PBS loaded onto 6 mL Ficoll (Sigma-Aldrich, Histopaque-1077) and centrifuged at 400 ×g for 30 min at 20°C to separate white blood cells from red blood cells. The white blood cell layer was collected in fresh tubes and washed three times (centrifuged at 300 ×g for 10 min) with RPMI-1640 medium to completely remove the platelet. Purified PBMC cells were counted and incubated in RPMI-1640 medium until use.

13. Antibody-Dependent Cell Cytotoxicity analysis

To measure the Antibody-dependent cell death, healthy 1×10^5 cells/well Jurkat-PD1 cell were stained with 0.5 µM Calcein-AM for 30 min at 37°C for staining viable cells. Cells were washed with PBS and re-suspended in 50 µL of serum-free RPMI media and then 10 µg/mL of each antibody was added and incubated for 10 min in 37°C CO_2 incubator. After antibody treatment, purified peripheral blood mononuclear cells (PBMC) (PBMC:Jurkat PD1 = 3:1) were added and incubated at 37°C in CO_2 incubator for 4 h. The % of cell lysis (% of cells losing fluorescence among 1×10^4 counted total cells) was calculated by FACS Verse (BD Biosciences) and FlowJo software.

14. Complement Dependent Cell Cytotoxicity analysis

To measure the complement-dependent cell death, healthy 1×10^5 cells/well Jurkat-PD1 cell were stained with 0.5 µM Calcein-AM (Invitrogen, C3100MP) for 30 min at 37°C. Cells were then washed with PBS and re-suspended in 50 µL of serum-free RPMI media and then 10 µg/mL of each antibody was added and incubated for 10 min in 37°C CO_2 incubator. After adding 6 µL of rabbit complement MA (CEDARLANE, CL3221), the cells were incubated further 2 h in 37°C CO_2 incubator. Then, 150 µL of cold PBS was supplemented to stop further reaction and to make the appropriate volume for the FACS analysis (FACS Verse, BD biosciences). Flow Jo software was used for the calculations.

15. Statistical analysis

All the statistical analyses were calculated using GraphPad Prism (version 5.0). For the analysis of the dose–response curves from the binding assay, the antibody concentrations were log-transformed and binding affinity from mean fluorescence intensities were normalized then analyzed with a 4-parameter non-linear regression analysis (log(agonist) vs. normalized response—Variable slope). CDC and ADCC data are presented as the means $\pm$ standard error of the mean. Statistical analysis was performed with Student's t-tests, analysis of variance (ANOVA), followed by Tukey's multiple comparison, or one-way ANOVA using GraphPad Prism, as appropriate. P values < 0.05 were considered statistically significant.

III. RESULTS

1. **Successful production of GD IgG1 and IgG4 nivolumab produced in GnTI (-) CHO cells**

To generate GD CHO cells, The HITI CRISPR/Cas9 method was firstly used to delete Mgat1 (NCBI ENSG00000131446) encoding GnTI (N-acetyl-glucosaminyl-transferase I) enzyme by inserting hygromycin gene in site of Cas9 cleavage[22]. GnTI is critical enzyme for glycan elongation at Golgi by trimming mannose and attaching several GlcNAc to high mannose residue of glycan chain. Hygromycin selected GnTI KO cells were transfected with EndoT (Figure 1A). The LentiVirus system is used to overexpres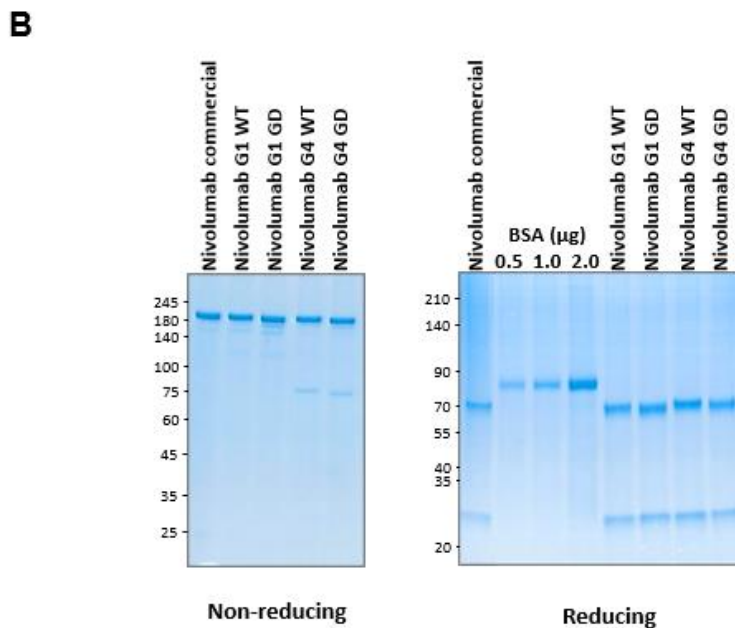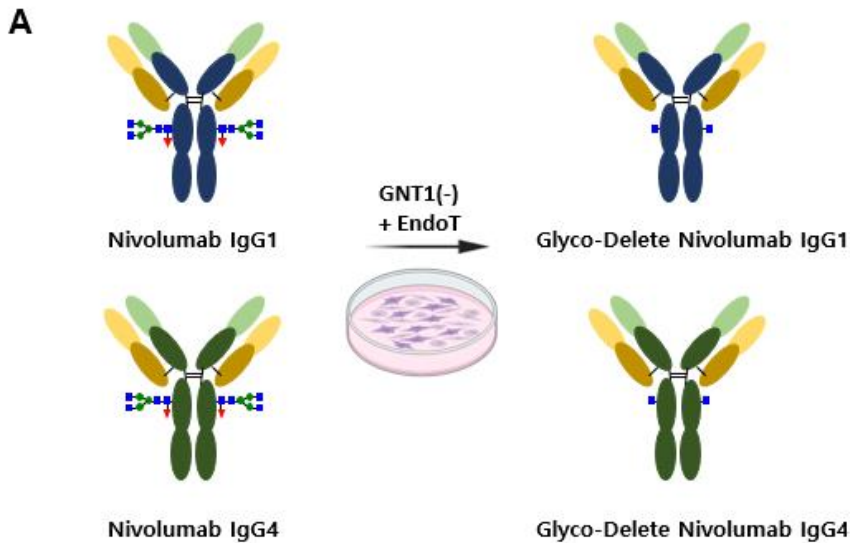s the EndoT (Endo-β-N-acetyl-glucosaminidase, NCBI LOC6044791) that cuts the glycan and leaves only one GlcNAc after removal of the desired gene to construct the final CHO-GnTI (-) cell. The success of the knockout was confirmed by PCR and sequencing results (Figure 1B and 1C). We chose EndoT from the fungus *Hypocrea jecorina* as the Endo-β-N-acetylglucosaminidase to target to the Golgi because optimum pH for EndoT function (pH 6.0) is close to that in the mammalian trans-Golgi apparatus. After hygromycin selection for successful production of CHO-GnTI (-) cell, IgG1 and IgG4 DNA are subcloned in the completed cells to make IgG1, IgG4 Nivolumab WT, and GD in two types (Figure 2A). The biochemical properties of each completed antibody were determined through non-reducing and reducing SDS-PAGE, respectively, and compared with commercial Nivolumab produced from CHO cells as a control (Figure 2B). As a result, it was confirmed that the band of the GD nivolumab samples of the non-reducing gel was lower than the band of the nivolumab WT samples. The same result was confirmed when the heavy chain band of the reducing gel was observed.

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Also, Whether or not the GD technology was applied well was confirmed through mass spectrometry after treatment with IdeS (Immunoglobulin degrading enzyme of *Streptococcus pyogenes*), a cysteine proteinase which cleaves IgG with a unique degree of specificity in the hinge region to yield F(ab')2 and Fc fragments. As a result of the Fc fragment analysis, it was confirmed that the high mannose form and the GD form coexist in a similar ratio, contrary to the expectation that it is composed of only the GD form (Figure 3). Through this, it could be seen that GD was not completely applied, and that the two forms constitute an Fc fragment. In general, it is known that the CHO system does not perform homogenously when manufacturing antibodies through engineering[3].

A Wild Type Genomic DNA of CHOK1 cell

Donor DNA
IRES | HygroR | pA

: cleavage site by CRISPR/Cas9

Genomic DNA after HITI

B M D7 E2 H7 H11

[Annealing Temp : 64℃]

C · HITI -donor(pIBla-crigri MGAT1) : 305~4507(4203bp) / U2~IRES-R2 : 36~779(744bp)

D

CHO GnTI(-) cell → Endo T OX & Puromycin selection → CHO GnTI(-)- Endo T stable cell → Niv IgG1/IgG4 HC & Niv LC Subcloning → Purification

Figure 1. Generation of CHO GnTI (-) cells. (A) Schematic graphic of HITI CRISPR/Cas9 method. The success of the knockout was confirmed by PCR (B) and DNA sequencing (C) of targeted region. (B) hygromycin incorporation results of H11 genome at HITI target region of Mgat1. Arrow indicates the starting point of variable sequences caused by genome modification. (D) Schematic flowchart of process from GD cell generation to GD antibody purification

Figure 2. Successful production of GD nivolumabs with IgG1 and IgG4 backbone. (A) Schematic graphic of GD process of nivolumab. (B) Nivolumab commercial, nivolumab G1 WT, nivolumab G1 GD, nivolumab G4 WT, and nivolumab G4 GD were subjected to SDS-PAGE under non-reducing and reducing conditions. BSA (0.5, 1.0, and 2.0 μg) was used as the standard.

CHOK1-Glycodeleted-H11-Nivol IgG4 _Ides treated

Total Ion Chromatogram

NL:
1.11E9
TIC MS
190109_6I_17

Deconvoluted Spectrum

Mass analysis summary

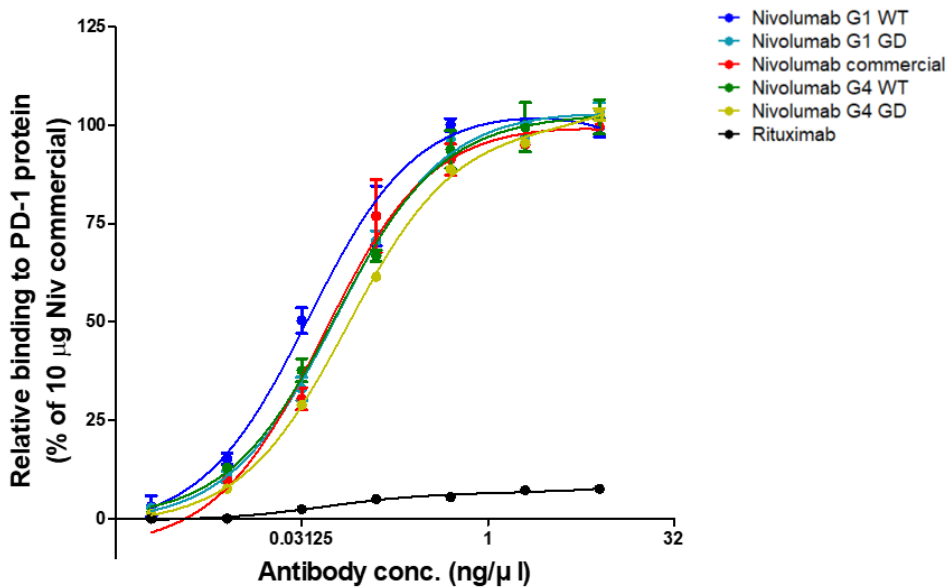|		CHOK1-Glycodeleted-H11-Nivol IgG4				
I		Theoretical mass G0F-2Lys	Theoretical mass drug bank G0F-2Lys	Observed mass	△ mass	△ mass drug bank
1.Native		146235.126	146255.206	145752.2	482.926	503.006
		Theoretical mass G0F-1Lys	Theoretical mass drug bank G0F-1Lys	Observed mass	△ mass	△ mass drug bank
2.Ides treate	Fc/2	25216.099	25216.099	24989.8	226.299	226.299
		25216.1	25216.099	23975.8	1240.3	1240.299
	F(ab')2	95838.968	95859.048	95815.7	23.268	43.348

Figure 3. Glycan analysis of IgG4 CHO GD nivolumab. Total ion chromatogram and deconvoluted spectrum of the IgG4 CHO GD nivolumab after treated IdeS protease. Main peak indicated the calculated molecular mass of F(ab')$_2$ and Fc fragments. High mannose form (A) and GD form (B) exist together to form Fc fragments.

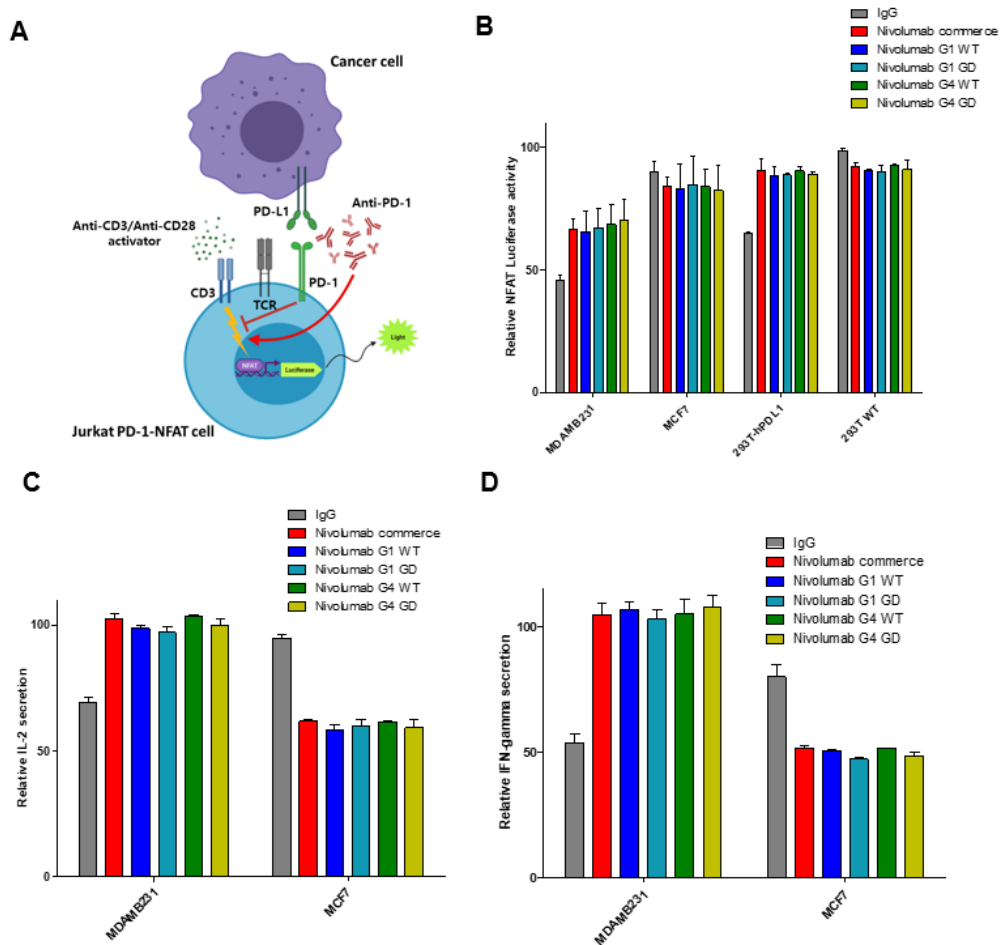## 2. GD nivolumab has PD1-blocking activity of comparable to nivolumab WT

To determine the binding equivalence of the antibody manufactured with a different backbone and commercial nivolumab, an ELISA assay was performed on a plate coated with PD1 protein (Figure 4). As a result of the experiment, it was confirmed that the antibody had the same binding affinity for PD1 regardless of the backbone. Furthermore, we were able to find that GD nivolumab had similar PD1-blocking activity compared to WT nivolumab. To confirm PD1 blockade affinity, Jurkat PD1-NFAT Luciferase reporter cell system was manufactured[6,26] (Figure 5A). Jurkat-PD1-NFAT Luciferase stable cells were prepared by using HEK 293T cells to make lentivirus, and then infecting the previously prepared Jurkat PD1 cells with the virus and undergoing blasticidin selection. As cancer cells, breast cancer cell lines MDAMB231 and MCF7 cells were used as PDL1 (+) and PDL1 (-), respectively. In addition to these breast cancer cells, human PDL1 was overexpressed in HEK 293T and stable cells were prepared through puromycin selection and used in the experiment as PDL1 (+). When Jurkat PD1-NFAT cells are treated with anti CD3/CD28 activator, signal transduction occurs, and NFAT-Luciferase is activated. When PD1 expressed on the surface of Jurkat PD1-NFAT cells binds to PDL-1 of cancer cells, NFAT signal transduction is inhibited. At this time, when anti-PD1 is treated, this inhibition is reverse-blocked, so that NFAT signal transduction proceeds as it is, and luminescence measurement is possible accordingly. This confirms the blockade ability of anti-PD1. As a result of the sample treated with IgG, which serves as a negative control, PDL1 (+) cells showed higher luciferase activity due to PD1-PDL1 binding than PDL1 (-) cells (Figure 5B). Upon treatment with the nivolumab

antibody, it was observed that the luciferase activity was restored by reverse-blocking, and the recovery showed a similar pattern regardless of the type of antibody treated. Interestingly, in the case of PDL1(-) cells, a pattern in which luciferase activity was not restored but rather decreased when treated with nivolumab antibody was observed in both cell lines.

When PD1 on the surface of T cells does not bind to PDL1 on the surface of cancer cells, but binds to an antibody such as anti-PD1 and the activity continues, T cells secrete cytokines such as IL-2, IFN-γ and granzyme. To determine the difference in cytokine secretion according to the type of antibody in these immune responses, cytokine secretion was measured by ELISA assay after each antibody was treated[22-25] (Figure 5C and 5D). Breast cancer cell lines were used as PDL1 (+) and PDL1 (-). As a result, similar as the Luciferase activity assay results, when PDL1 (+) cells were treated with IgG as a negative control, low IL-2 and IFN-γ secretion due to high PD1-PDL1 binding was observed. It was found that the cytokine secretion was recovered when the nivolumab antibody was treated. As for the degree of recovery, a slight difference in secretion could be observed for each type of antibody treated, but both cytokines showed a similar secretion pattern overall. Through these experiments, it was confirmed that the PD1 blockade ability of each antibody was similar.

Figure 4. Binding affinity of GD nivolumabs to PD1. (A) Specific binding to human PD1 protein is confirmed by ELISA. 0.0019, 0.0078, 0.3125, 0.125, 0.5, 2.0, and 8.0 ng/µl antibodies (nivolumab G1 WT, nivolumab G1 GD, nivolumab commercial, nivolumab G4 WT, nivolumab G4 GD, Rituximab) were incubated on plates coated with human PD1 protein and detected with HRP-conjugated anti-human IgG antibody. Rituximab was used as negative control. Binding activity was calculated using absorbance value at 492 nm (mean±SD) from triplicates and the relative binding force was calculated by taking the maximum value as 100%.

Figure 5. Comparable PD1 blockade activity of GD nivolumabs. (A) Schematic graphic of Jurkat PD1-NFAT reporter cell system. (B) PD1 blockade activity of the GD nivolumabs was confirmed by NFAT Luciferase assay. Jurkat PD1-NFAT cells were co-cultured with PDL1 (+) or PDL1 (-) cells and stimulated with anti-CD3/anti-CD28 activator. Each of the different nivolumabs was treated at 30 µg/mL to observe PD1 blocking effect. Luciferase activity was measured 6 h after stimulation. IL-2 (C) and IFN-γ (D) secretion activity of T cell. Jurkat PD1 cells were co-cultured with breast cancer cell lines and treatment with anti-CD3/anti-CD28 activator and 30 µg/mL of antibodies. After 24 h, supernatants were collected and amount of secreted cytokine determined using an ELISA assay. IgG was used as negative control and the absorbance at 492 nm (mean±SD) from triplicates are presented.

3. GD nivolumab has reduced CDC and ADCC efficacy

After confirming binding affinity and cytokine secretion ability, the complement-dependent cytotoxicity (CDC) activity (Figure 6A) and ADCC activity (Figure 6B) of each antibody were finally confirmed using Jurkat PD1 cells. IgG was used as a control to compare the degree of death among antibodies with different backbones. As a result, when the antibody concentration was treated at 10 µg/mL, about 3 times higher CDC was observed in the IgG1 backbone antibody than IgG4 backbone. Also, CDC by GD antibody was reduced compared to that of wild type antibody. In the case of ADCC assay, antibody-dependent cell cytotoxicity was measured using human PBMC as effector cells. At this time, it was found that ADCC was about two times higher in the IgG1 backbone nivolumab than in the commercial nivolumab. Similarly, the death of GD antibody was reduced compared to wild type antibody and it was confirmed that the pattern of death was almost consistent with the CDC assay performed previously.

A

B

Figure 6. Reduced CDC and ADCC efficacy of IgG4 GD nivolumabs

(A) The CDC efficacy of each antibody were analyzed using calcein-loaded Jurkat PD1 cells. Cell death rate was subtracted from the % of live cell. 10 µg/ml dose of each antibody were used to the CDC test. Nivolumab G1 WT was used as positive control. (B) ADCC assay performed with PBMCs from healthy donor. 10 µg/ml dose of each antibody were used to ADCC test. The effector cell: target cell ratio was 3:1 and Nivolumab G1 WT and IgG were used as positive and negative control, respectively.

4. Successful production of GD nivolumab in *Nicotiana benthamiana*

In the previous experiments in CHO system, it was confirmed that the CDC and ADCC results were lower in the IgG4 backbone than in the IgG1 backbone. Therefore, plant Nivolumab was produced as an IgG4 backbone. The heavy chain was manufactured in two forms with or without HDEL, an ER retention signal sequence, at the C-terminus of the heavy chain (Figure 7A). The two heavy chain and light chain constructs were stably incorporated into the tobacco genome via agrobacterium-mediated infiltration. HDEL sequence tag, used localize proteins in the ER, is one method to eliminate plant-specific glycosylation[31,32,41]. HDEL-tagged Ig produced in plants was more than 90% high mannose, which was similar to antibodies produced in mammalian systems[32]. ER retention increased the yields of monoclonal antibodies and prevented cleavage of proteins in the apoplasts of *N. benthamiana* leaves[35,36]. Antibodies made from tobacco were treated with Endoglycosidase H (Endo H) to produce GD form, and their biochemical properties were confirmed through non-reducing, reducing SDS-PAGE (Figure 7B). Endo H is a recombinant glycosidase which cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. Commercial Nivolumab produced from CHO cells was used as a control. Plant antibody showed higher heavy chain and light chain molecular weight than Nivolumab produced in CHO under reducing conditions regardless of HDEL attachment. Even under non-reducing conditions, the plant antibody showed a higher molecular weight than the CHO-produced nivolumab, and among them, the HDEL sequence-attached antibody showed an increased molecular weight due to the HDEL tag. When the Endo H treated sample was loaded, it was confirmed that the height of the band of the antibody to which the HDEL

sequence was attached was lowered, whereas the height of the band of the antibody to which the HDEL sequence was not attached didn't change. This proved that Endo H acts on high mannose form of N-glycans and confirmed the successful working of Endo H. In addition, by checking the molecular weight size of Endo H, it was also possible to confirm the presence of Endo H in samples.

Figure 7. Expression of IgG4 nivolumab in *Nicotiana benthamiana*. (A) Schematic graphic of GD process of nivolumab in *Nicotiana benthamiana* leaves. Transient infiltration method was used for genome integration and pNiv-no HDEL (ER retention signal) and pNiv-HDEL was produced. Endo H was used to cleave the high mannose formed glycan in pNiv-HDEL. (B) Nivolumab commercial, pNiv-no HDEL with or without Endo H, pNiv-HDEL with or without Endo H were subjected to SDS-PAGE under non-reducing and reducing conditions. BSA (0.5, 1.0, and 2.0 µg) was used as the standard.

5. Confirmation of binding affinity to PD1 and blocking activity of plant GD nivolumab

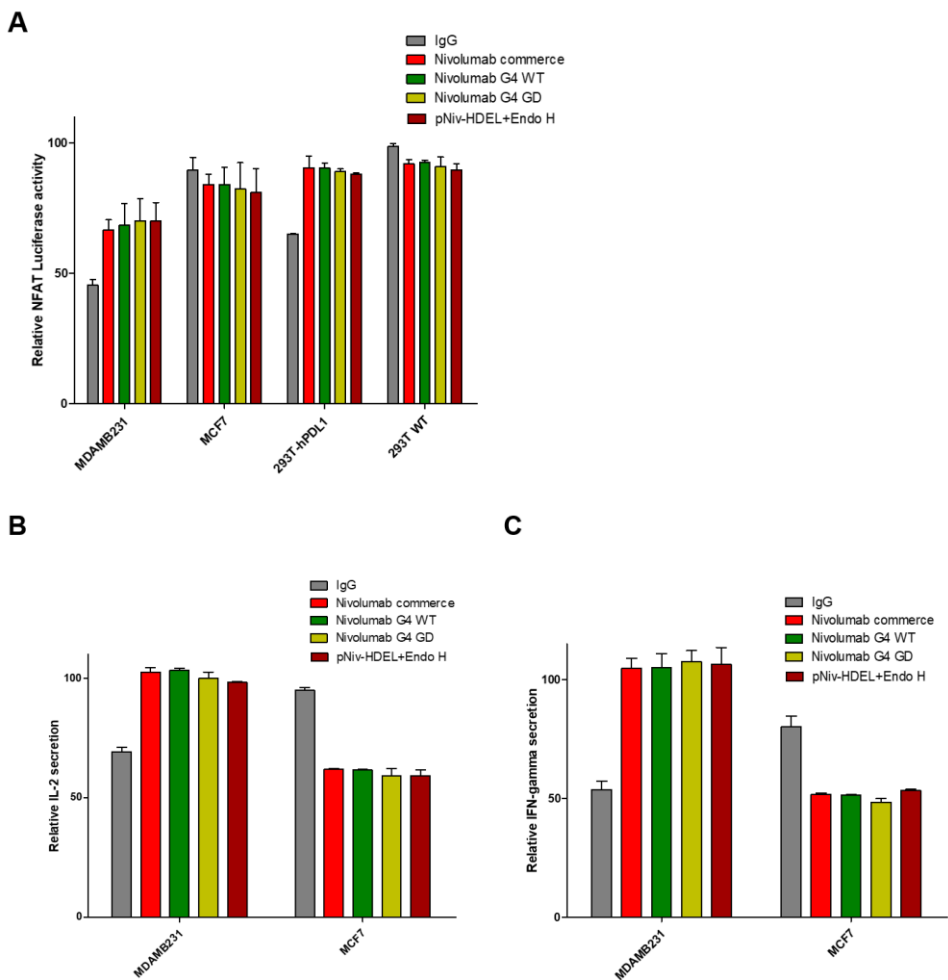To compare the binding affinity between the antibody extracted from CHO and the plant antibody, ELISA assay was performed on a plate coated with PD1 Protein (Figure 8A). Rituximab was used as a negative control. As a result of the experiment, it was confirmed that both the antibody produced from mammalian cells and plants showed the same binding affinity for PD1. There are studies that have shown similar cases in the past[36].The study stopped there at that time, but we went further and measured the binding affinity in cells expressing PD1 on the surface (Figure 8B). For the experiment, stable cells were prepared by expressing PD1 in CHO-K1 cells. After making Lentivirus using HEK 293T cells, CHO-K1 cells were infected with the virus and subjected to puromycin selection. Binding affinity was measured using FACS and rituximab was used as a negative control. After the cell-based binding assay using CHOK1-PD1 cells, it was observed that the binding affinity of the antibody produced in mammalian cells was about 30% higher than that of the antibody produced in plants. The binding affinity between plant antibodies was similar. This pattern of binding affinity was similar to that previously seen in various plant-based antibodies previously studied in our laboratory. We produced a total of three different antibodies from plants and used them in previous studies[37-39]. Except for one case[32], it showed a lower binding affinity than the antibody made from mammalian cells, and a difference in binding affinity was also observed depending on plant species[39]. PD1 blockade ability of plant antibody was measured using the previously generated Jurkat PD1-NFAT Luciferase reporter cell system (Figure 5A). As cancer cells, breast cancer cell lines MDAMB231 and MCF7 cells were used as

PDL1 (+) and PDL1 (-), respectively. In addition to these breast cancer cells, human PDL1 was overexpressed in HEK 293T and through puromycin selection, stable cells were prepared for the experiment as PDL1 (+). As a result of the sample treated with IgG, which serves as a negative control, PDL1 (+) cells showed higher luciferase activity due to PD1-PDL1 binding than PDL1 (-) cells (Figure 9A). Upon treatment with the nivolumab antibody, it was observed that the luciferase activity was restored by reverse-blocking, and the recovery showed a similar pattern regardless of the type of antibody treated. Surprisingly, in the case of PDL1 (-) cells, a pattern in which luciferase activity was not restored but rather decreased when treated with nivolumab antibody was observed in both cell lines as in the CHO system.

Like the previous experiment, cytokine secretion was measured by ELISA assay after each antibody was treated (Figure 9B and 9C) to determine the difference in cytokine secretion according to the type of antibody in these immune responses. Breast cancer cell lines were used as PDL1 (+) and PDL1 (-). As a result, similar as the Luciferase activity assay results, when PDL1 (+) cells were treated with IgG as a negative control, low IL-2 and IFN-γ secretion due to high PD1-PDL1 binding was observed. It was found that the cytokine secretion was recovered when the nivolumab antibody was treated. As for the degree of recovery, a slight difference in secretion could be observed for each type of antibody treated, but both cytokines showed a similar secretion pattern overall as in the CHO system.

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Figure 8. Confirmation of binding affinity to PD1 of the produced plant GD antibody. (A) Binding affinity to human PD1 protein by ELISA. Two-fold dilutions of antibodies (nivolumab commercial, pNiv-HDEL, pNiv-HDEL with Endo H treatment, and rituximab) were incubated on plates coated with human PD1 protein and detected with HRP-conjugated anti-human IgG antibody. Rituximab was used as negative control. The absorbance value at 492 nm (mean±SD) from triplicates are presented. (B) Binding affinity to human PD1 expressing on CHO cell by FACS analysis. PD1 expressed in CHO cell was used as antigen for each antibody. Dose-dependent binding capacity of nivolumab commercial, pNiv-HDEL, and pNiv-HDEL with Endo H from *Nicotiana benthamiana* (0.1, 0.3, 1, 3, and 10 µg/ml) measured by flow cytometry. 10 µg/ml dose of each antibody and 2nd anti-human IgG-FITC was used to detect each attached antibody. FITC intensities of 10 µg/ml of each antibody bound to cells are depicted by normalized mean fluorescence intensity (MFI). Rituximab was used as negative control.
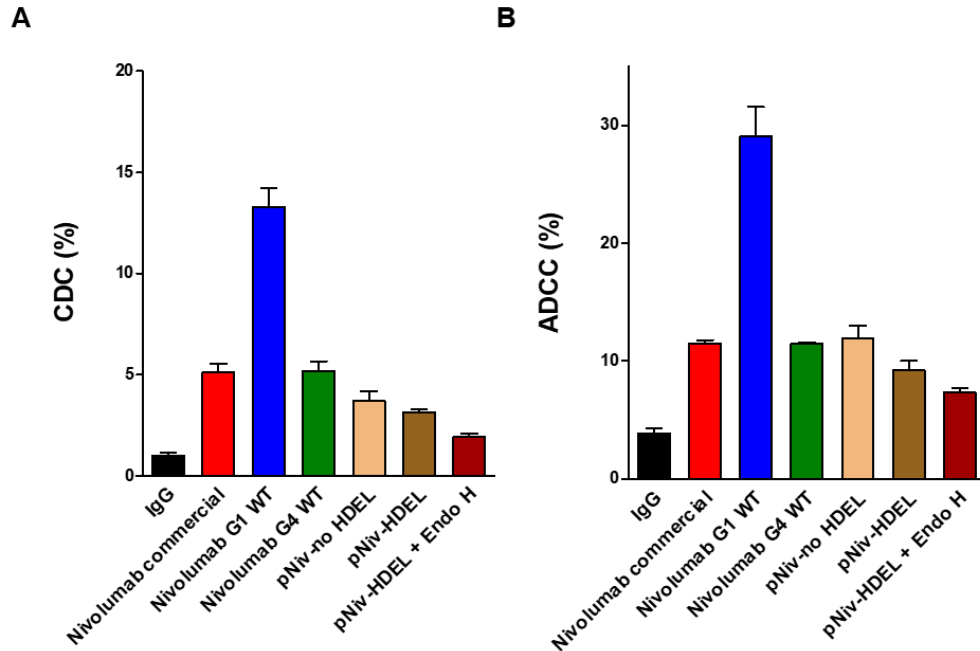
Figure 9. Confirm blockade activity of Plant GD nivolumab. (A) PD1 blockade activity of the produced plant GD antibody was confirmed by NFAT Luciferase assay. Jurkat PD1-NFAT cells were co-cultured with PDL1 (+) or PDL1 (-) cancer cells and stimulated with anti-CD3/anti-CD28 activator. Each of the different nivolumabs was treated at 30 µg/mL to observe PD1 blocking effect. Luciferase activity was measured 6 h after stimulation. IL-2(B) and IFN-γ(C) secretion activity of T cell. Jurkat PD1 cells were co-cultured with breast cancer cell lines and treatment with anti-CD3/anti-CD28 activator and 30 µg/mL of antibodies. After 24 h, supernatants were collected and amount of secreted cytokine determined using an ELISA assay. IgG was used as negative control and the absorbance at 492 nm (mean±SD) from triplicates are presented.

6. Plant GD nivolumab has reduced CDC and ADCC efficacy

After checking the PD1 blockade ability, the CDC (Figure 10A) and ADCC activity (Figure 10B) of each antibody were confirmed using Jurkat PD1 cells. IgG was used as a negative control and each antibody was treated at a concentration of 10 µg/ml. As a result of the experiment, when comparing antibodies with an IgG4 backbone, it was confirmed that the CDC and ADCC caused by antibody produced in plants were reduced in common. Especially, we found that the death resulting from the treatment of plant GD antibody was lower than that caused by the wild type of antibody from CHO system.

Figure 10. Reduced CDC and ADCC efficacy of Plant GD nivolumab. (A) The CDC efficacy of plant-GD nivolumab were analyzed using calcein-loaded Jurkat PD1 cells and FACS. Cell death rate was subtracted from the % of live rate which was calculated by calcein-AM-contained cell after 10 µg/ml dose of each antibody were treated. (B) ADCC assay was performed using PBMCs from healthy donor. 10 µg/ml dose of each antibody were used to the ADCC test. The effector cell: target cell ratio was 3:1. IgG was used as negative control.

Figure 11. Graphic summary. GD CHO cell system and GD tobacco systems were produced by controlling the glycosylation process of CHO cells and plant to produce a form in which only one GlcNAc molecule was attached to ASN297 of the antibody. After purifying the IgG1 and IgG4 types of nivolumab antibodies, the maintenance of intrinsic nivolumab ability was confirmed and the improvement of immunogenicity was observed. As a result, it was found that both ADCC of GD nivolumab from CHO cell and plant were lowered than that of original nivolumab. Accordingly, GD platform can be a bio-better platform for signaling regulating antibody.

IV. DISCUSSION

In this study, GD CHO cell system and GD plant systems were produced by controlling the glycosylation process of CHO cells and plant to produce a form in which only one GlcNAc molecule was attached to ASN297 of the antibody. After purifying the IgG1 and IgG4 types of nivolumab antibodies, the maintenance of intrinsic nivolumab ability was confirmed and the improvement of immunogenicity was observed. It was found that both ADCC of GD nivolumab from CHO cell and plant were lowered than that of original nivolumab.

Producing any antibody using the constructed GD cell line can abolish the ADCC activity of the antibody. However, as a result of mass spectrometry of the glycans after GD, endo T did not completely remove the high mannose residue in nivolumab. In general, it is known that the CHO system does not produce homogenous output when modifying glycan of antibodies through glyco-engineering[3]. In this point, transgenic plants can be a useful platform for GD antibody production, because it is known that the final glycan structure of engineering by transgenic plant is pretty homogeneous in compared to CHO system[44]. Although we failed to produce transgenic Arabidopsis thaliana due to the lethal effects of endo T over-expression, it seems that GD transgenic plant resistant to endo T over-expression can probably provide uniform GD antibody. In addition, the GD nivolumab from CHO cell can be uniformed by endo H treatment after purification, as we did plant nivolumab.

Plants are considered attractive hosts for the mass production of recombinant protein drugs due to their economic efficiency, stability, and convenience. But Plant-specific glycosylation has been regarded as a major obstacle in the industrialization of plant-produced biopharmaceuticals because of its possible immunogenicity and

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hypersensitivity. Whether it induces an acute allergy in the human body to plant-specific glycans or not is still controversial. About 50% of non-allergic people have antibodies specific for β-1,2-Xylose, and 25% of allergic patients have antibodies against core α-1,3-Fucose in their sera[21]. It is considered safe to remove plant-specific glycan just in case. In this respect, GD technology can not only remove ADCC of antibodies, but also efficiently remove plant-specific glycans. If the entire process of GD technology can be established in plant species, it can provide the industrialization platform for biopharmaceuticals using plant system.

Meanwhile, the IgG4 antibody produced using a CHO system showed a lower ADCC efficacy than the IgG1 backbone antibody in wild type and GD type. We not only checked whether the antibody's intrinsic ability was maintained, but also ultimately observed changes in glycan uniformity, protein stability, and immunogenicity after antibody purification. In the process, the binding affinity to PD1 coated on the plate was determined by ELISA assay and as a result, it was confirmed that there was no difference in binding affinity between CHO and plant antibodies. These results are consistent with the results of previous studies that the binding affinity of nivolumab produced in tobacco is the same[36]. However, in the subsequent cell-based binding assay, the binding affinity of the plant antibody was significantly lower than that of the mammalian antibody. This pattern of binding affinity was similar to that previously seen in various plant-based antibodies, such as ofatumumab and rituximab previously reported in our laboratory[37-39]. Except for obinutuzumab[37], ofatumumab[38] and rituximab[39] showed a lower binding affinity than the antibody made from CHO cells, and a difference in binding affinity was also observed depending on plant species in case of rituximab[39]. Through several experiments using rituximab produced from plants at the time, it was revealed that

plant-specific glycosylation was not the cause of the low binding affinity of the plant antibody. Thus, the reason for the low binding affinity of plant antibodies seems to be related to other types of post-translational modifications that have not yet been identified.

The antibody produced by applying the GD technology in the CHO system, no change was found in the PD1 binding site. Through ELISA and luciferase assay that confirmed the binding affinity for PD1, it was confirmed that the Fab and Fc functions of nivolumab were completely separated. Therefore, it can be seen that the GD technology can be applied to all types of IgG4 antibodies as long as the antibody does not have a glycan in the Fab portion that affects binding affinity. These results also prove that GD technology is a suitable platform for making bio-betters.

V. CONCLUSION

This study reports that nivolumab was successfully produced in CHO cell system and also in *Nicotiana benthamiana* leaves with the HDEL-tagged and GD form. Our finding is that the ADCC activity of GD nivolumab from CHO cell and plant were lowered than the original antibody. Through these results, we demonstrate that GD nivolumabs produced in CHO cells and plants have low ADCC activity without any changes in PD1 signaling inhibition. We also proved GD technology can be applied to various target-masking antibodies to improved therapeutic effect.

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ABSTRACT (IN KOREAN)

동물 및 식물 Glyco-Delete 플랫폼을 이용한 니볼루맵 바이오베터의 개발

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강 초 은

항체에 부착된 당사슬은 다양한 생물학적 활성, 즉 단백 안전성, 이동성, 단백 제거율, 수용체 접합성 등을 변화시켜 치료 효능, 체내 지속성, 타겟팅 및 면역반응 등에 중요한 역할을 수행하기 때문에 항체의약품의 품질을 결정하는 주요인자이다. 그러나 당사슬은 다양한 효소의 작용으로 만들어지고 생산공정 과정이나 조건에 따라 각 효소의 활성이 다를 수 있으므로 단백질에 균일하지 않게 부착될 수 있다. 이러한 당사슬을 균일하게 조절할 수 있다면 항체의 품질을 지속적으로 유지할 수 있다. 특히 표적가림용 항체의약품의 경우 항체부착에 의해 표적발현 세포의 사멸을 초래할 수 있는데, 당사슬 조절을 통해 그러한 표적발현세포의 사멸 능력을 제거할 수 있다.

본 연구는 CHO 세포와 식물인 담배의 당화과정을 조절하여, 항체의 ASN297에 한 개의 GlcNAc 분자만 부착된 형태를 생산하는 당사슬제거

(Glyco-Delete, GD) CHO세포와 당사슬제거 담배시스템을 구축하고 이를 통해 생산된 항체의 기능을 평가하는 연구이다. IgG1과 IgG4 형태의 니볼루맵들을 생산하여, 고유능력인 항원결합력, PD1 활성화 예방능력 및 싸이토카인 분비 등의 T 세포 활성조절능력을 확인할 뿐 아니라, 궁극적으로 PD1 발현 세포에 대한 면역원성의 감소를 관찰하는 것을 목적으로 했다. 또한 당사슬제거 식물 시스템을 이용하여 마찬가지로 식물 니볼루맵을 생산하여 그 기능을 함께 평가하고자 했다.

본 연구는 CHO 세포 및 식물에서 생산된 당사슬제거 니볼루맵들의 면역원성이 낮아짐을 증명했다. 이를 통해 니볼루맵의 타겟인 T세포의 사멸을 줄여 개선된 안정성을 규명함과 동시에, 항체 적용 시 면역활동에 참여하여 효과를 일으키는 T세포의 사멸을 줄임으로써 궁극적으로 향상된 항체의 효능 또한 규명하였다. 이러한 결과는 니볼루맵과 같은 표적가림용 항체의 성능을 향상시켜 동·식물 항체 플랫폼의 개선에 기여할 수 있을 것이다.