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Production of Human Fc Gamma Receptors With Efficient Disulfide Formation, Glycosylation, and *in vivo* Biotinylation

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ABSTRACT OF THE THESIS

Production of Human Fc Gamma Receptors With Efficient Disulfide Formation, Glycosylation, and *in vivo* Biotinylation

by

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Bridging effector cells to immunoglobulin G (IgG) antibodies, Fc gamma receptors (FcγRs) initiate and regulate humoral immunity and thus their ectodomains are important as therapeutics and biomedical reagents. This study aims to produce fully biotinylated, N-glycosylated, and disulfide bond formed FcγRs in mammalian cells. Several low-affinity FcγRs were the targets, including FcγRIIA and FcγRIIIA of activation functions and FcγRIIB of inhibition functions. *In vivo* biotinylation in 293 cells was achieved by co-expression of AviTag fused FcγRs and *E. coli* biotin ligase (BirA). To facilitate biotinylation, endoplasmic reticulum retention signal DYKDEL was fused with BirA. Both stable and transient transfections were studied and the ratio of FcγR and BirA plasmids during co-transfection was optimized as well. Produced FcγRs were tested by SDS-PAGE, streptavidin pulldown assays, and binding kinetic analysis. Results indicated that FcγRIIIA(F176), FcγRIIIA(V176) and FcγRIIB produced from the associated stably

transfected cell lines exhibited expected disulfide and glycosylation profiles with complete biotinylation at yields of 15.3, 9.1, and 25.7 μg purified receptor ectodomains per mL culture. And transient transfection led production of 7.0 and 3.6 μg purified FcγRIIA(H131) and FcγRIIA(R131) per mL culture with high quality in terms of biotinylation efficiency, disulfide bond formation, and glycosylation. Produced FcγRs demonstrated their expected binding affinities towards IgG1 trastuzumab consistent with values reported in literatures. This work provided high quality reagents for engineering endeavor of antibody Fc fragments, and the methods demonstrated here can be applied for *in vivo* biotinylation of other important glycoproteins in mammalian cells.

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Introduction

Importance of FcγRs in immunology

Human Fc gamma receptors (FcγRs) are important components of the humoral immune system along with antibodies or immunoglobulins (Igs). Among various types of Igs, IgGs are the most studied and best characterized Igs [1]. IgGs are divided into four subclasses as IgG1, IgG2, IgG3, and IgG4. Each subclass of the IgGs is different in sequence, structure, binding properties to cellular FcγRs as well as in terms of effector functions [2], [3]. IgG antibodies play a significant role as the first line of defense against invading pathogens [1].

While IgG antibodies have significant roles in terms of the immune response against invading pathogens, it is crucial for IgGs to interact with FcγRs in order to effectively combat against the invading microorganisms. FcγRs belong to the large Ig superfamily and are type 1 transmembrane glycoproteins [4]. They are found almost every cell throughout the body. FcγRs are categorized into three main groups: FcγRI, FcγRIIA/B, and FcγRIIIA/B [5] according to each group's own characteristics such as its structure, function, glycosylation, and affinity for IgG [5], [6], [7], [8], [9], [10].

FcγR family and its immunological function

FcγRI (also termed CD64) has the highest level of affinity to IgG antibodies, while FcγRII (CD32) and FcγRIII (CD16) have lower affinities to IgGs [6]. In human, only FcγRIIB of the FcγR family is known as the inhibitory FcγR. All the other FcγRs function as activating FcγRs. As IgG antibodies function as the bridge between antigen and FcγRs,

the FcγRs interacted with IgG antibodies can initiate the signaling pathway throughout the cell for the proper immune response. Therefore, FcγRs are indispensable parts of the IgG biological activities as the adaptive immune system [11].

FcγRs production in mammalian cells

FcγRs are glycoproteins which their glycosylation can substantially alter their function. Thus, protein glycosylation is significantly important subject especially in terms of producing therapeutic glycoproteins [12]. Mammalian cell expression systems are the preferred method for the production of glycoproteins due to their innate protein processing machinery, including that of protein glycosylation, is very similar to that in human [12]. To date, there were numerous attempts to express (glyco)proteins in prokaryotic based expression system. Yet, many of them failed to render those proteins in the correct structures. As a result, various types of eukaryotic based expression systems have been developed [13]. It is rather intuitive that proteins would be best expressed in their native cell types under the ideal physiological conditions, where numerous molecular systems work together for efficient production and quality control at various stages, including synthesis and folding, post-translational modifications and subcellular targeting [13]. As already mentioned, a mammalian expression system would be ideal for expressing human therapeutic glycoproteins because of their similar innate protein processing machinery. It is a very important factor since the efficient post-translational processing in the secretory pathway is critical for the correct folding of the proteins of interest [13].

Biotinylation of protein

Biotin, or vitamin H, is a naturally occurring cofactor for carboxylase enzymes, present in all living organisms [14], [15]. Any biotinylated substrate can be bound very tightly by the (strept)avidin proteins with $K_D = 10^{-14} – 10^{-15}$ M [16], [17], [18], [19], [20]. Due to its strong interaction, biotin-(strept)avidin interaction has been exploited in many biotechnological applications including purification, immunohistochemistry, drug targeting, protein targeting, gene therapy, and vector targeting [21], [22], [23].

Before the work of Cronan et al., biotin labeling of a drug, protein, or virus has initially been performed *in vitro* by using chemical reagents [22], [23]. However, Cronan et al. demonstrated biotinylation by fusing biotin acceptor domains to the C-terminal of the protein of interest [24], [25], [26]. This approach is more advantageous than chemical biotinylation which often generates random and heterogenous products that have impaired functions including binding properties and loss of affinity (especially if the desired proteins are antibodies) [26], [27]. It was found that utilizing *E. coli* biotin ligase enzyme (BirA) can successfully biotinylate target substrates with 100% efficiency using AviTag. AviTag is also known as Acceptor Peptide, or AP, which is only 15-amino acid in length [16], [28], [29], [30], [31], [32], [33], [34].

Endoplasmic Reticulum retention signal

It is a complicated process to secret proteins from eukaryotic cell system [35]. During the protein synthesis, newly generated both secretory and membrane proteins enter the endoplasmic reticulum (ER) in unfolded state [35]. In the ER, the newly generated proteins undergo post-translational modification including glycosylation, disulfide bond formation,

and assembly into oligomers [35]. Post-translational modification of the protein in the ER is possible because ER contains a number of soluble proteins, which many of them help the maturation of the newly synthesized secretory proteins, despite the continual flow of material through the secretory pathway [36]. Lys-Asp-Glu-Leu (KDEL) is a carboxy-terminal tetrapeptide which is common in animal cells and retains the resident proteins that contains this sequence [37].

Objective

In this experiment, we initially produced human Fc gamma receptors (FcγRs) via stable transfection. Stably transfected and produced FcγRs posed a couple of problems in terms of disulfide bond formation, glycosylation, and *in vivo* biotinylation efficiency especially FcγRIIA(H131) and FcγRIIA(R131). These listed problems were significantly improved after transiently transfected and produced FcγRIIA(H131) and FcγRIIA(R131). The results were verified by SDS-PAGE.

2. Materials and Methods

2.1 Cloning FcγR and BirA plasmids

To construct the target FcγR plasmid, AviTag and His_6tag was inserted to the downstream of the gene encoding FcγRIIIA(F176) by using NsiI and EcoRI. Then, the FcγRIIIA(F176)-AviTag-His_6tag was inserted downstream from SP and replaced Herceptin-HC region in the mammalian expression vector, pcDNA-Intron-WPRE-SP-Herceptin-HC-IRES-zeo, in order to generate pcDNA-Intron-WPRE-SP-FcγRIIIA(F176)-IRES-zeo, or IIIA(F176)[pFcγR] in short. The vector plasmid was from the lab stock. FcγRIIIA(F176) was replaced by other target FcγR with EcoRV and NsiI sites for constructing the other target FcγR plasmid. [pFcγR] was subjected for cloning into *E. coli* by chemical transformation. Transformed *E. coli* were subjected to mini-prep to prepare the high quality and sterile plasmid DNA for transfection.

To construct BirA expression plasmid, the gene of *E. coli* biotin ligase (BirA) was amplified from pET21a-BirA (Addgene, USA), fused with an endoplasmic reticulum (ER) retention signal DYKDEL [26] (DYKD is the truncated form of the FLAG epitope tag) at its C-terminus by extension PCR. It was then cloned via XhoI and HpaI to the lab stock plasmid, pcDNA-Intron-WPRE-SP-XhoI-3A2-IgL-IRES-Hy, to generate pcDNA-Intro-WPRE-SP-BirA-IRES-Hy, or [pBirA] in short. The zeocin resistance gene was replaced with hygromycin resistance gene by overlapping PCR. [pBirA] was subjected cloning into *E. coli* by chemical transformation. Transformed *E. coli* were subjected to miniprep to prepare the high quality and sterile plasmid DNA for transfection.

2.2 Cell culture and transfection

Expi293F™ cells was cultured in Expi293 media (Gibco, USA) and used for transfection throughout the entire experiment. All transfections were performed with high-quality plasmid DNA and polyethyleneimine MAX 40K (PEI) (Polysciences, USA) at a ratio of 1: 3.5 (w/w). The PEI stock was prepared in ddH2O at a concentration of 2 mg/ml, filtered via 0.22 μm filters and stored at -20 ˚C up to one month. The final concentration of plasmid DNA in transfected cell culture was 1 μg/ml.

2.2.1 Stable transfection

The cells were grown to 10 ml in Expi 293 media to a density of 2-2.5 × 10^6 cells ml^{-1}. Then, the media was changed into the same, but fresh Expi 293 media. The cell suspension was transferred to sterile a 15ml centrifuge tube. It was centrifuged at 1500 ×g at room temperature for 4 minutes. The supernatant was aspirated. 9 ml of Expi 293 medium was added. After cells were gently resuspended with a serological pipette, each 1.8 ml of cell culture were transferred to a sterile test tube with a cap. The cap was not tightly closed for aeration. The cell suspension in the tubes were placed into a 37 ˚C incubator shaker set at 8% CO_2, with shaking at 160 rpm to prevent the settlement of the cell.

For transfecting 1.8 ml of cell culture, 7 μg of PEI was diluted into a total volume of 200 μl of fresh Expi 293 media. For the co-transfection of FcγR and BirA DNA, 1 μg of [pFcγR] and 1 μg of [pBirA] were also diluted together in a total volume of 200 μl of fresh Expi 293 media. For the transfection of FcγR only, 2 μg of [pFcγR] was diluted in a total

of 200 µl of fresh Expi 293 media. The diluted solutions were incubated at room temperature for 4 minutes. Then, the diluted PEI solution was added into the diluted DNA solution. The dilution mixture was incubated at room temperature for 10 minutes. After the incubation, the PEI:DNA mixture was carefully added to the suspension cells in the test tube from the incubator shaker. The cells were returned to 37 ˚C incubator shaker set at 8% CO_2, with shaking at 160 rpm.

2.2.2 Selection of transfected mammalian cells

Selection process is a necessary part in stable transfection and maintaining the transfected cell line. In this experiment, positive selection was employed. For transfected cells with [pFcγR] only, zeocin was utilized. For co-transfected cells with [pFcγR] and [pBirA], zeocin and hygromycin-B were utilized.

From the next day of transfection, the Expi 293 media was changed with fresh Expi 293 media with antibiotics. 700 µg ml^{-1} of zeocin was added while 250 µg ml^{-1} of hygromycin-B was added. The process was repeated every day for four days from the date of transfection. It is also highly recommended to check cell viability and number by using a hemocytometer and trypan blue.

After 5^{th} day of changing media, the amount of zeocin and hygromycin-B should be reduced to 100 µg ml^{-1} and 50 µg ml^{-1}, respectively. Started from 5^{th} day, the media can be changed or passaged every two to three days as if cell number exceeds 2×10^6 cells ml^{-1}.

2.2.3 Transient transfection

First, it was required to grow 30 ml of cells in the Expi 293 solution medium to a density of 2-2.5 × 10^6 cells ml^{-1}. Then, the cell suspension was transferred to a sterile 50 ml centrifuge tube. The cell suspension was centrifuged at 1500 ×g at room temperature for 4 minutes. The supernatant was aspirated. 27 ml of the fresh Expi 293 medium was added. After cells were gently resuspended with a serological pipette, they were transferred to a sterile 125 ml flask with a cap with ventilation membrane. The suspension cell in the flask were placed into a 37 ˚C incubator shaker set at 8% CO_2, with shaking at 130 rpm to prevent the settlement of cells.

105 μg of PEI was diluted into a total volume of 1.5 ml of fresh Expi 293 media. For co-transfection, 15 μg of [pFcγR] and 15 μg of [pBirA] were also diluted together in a total volume of 1.5 ml of fresh Expi 293 media. For transfection of FcγR only, 30 μg of [pFcγR] was diluted in a total volume of 1.5 ml of fresh Expi 293 media. The diluted solutions were incubated at room temperature for 4 minutes. Then, the diluted PEI was added to the diluted DNA solution. After incubating the diluted mixture at room temperature for 10 minutes, the PEI:DNA mixture was carefully added to the suspension cells from the incubator shaker. The cells were returned to the 37 ˚C incubator shaker set at 8% CO_2, with shaking at 130 rpm.

2.2.4. Expression in mammalian cells

For the stable transfection, which was done in 2 ml, the volume of stable cell line was gradually expanded to 30 ml. When the cell density reached to 1-1.5 × 10^6 cells ml^{-1}

after the volume was expanded to 30 ml, the media was changed into fresh media without any antibiotics. Only for the cells co-transfected with [pBirA], it was required to add biotin with the final concentration of 100 μM. After 5-7 days the culture was centrifuged, and the supernatant was saved for protein purification.

For the transient transfection, which was done in 30 ml, no antibiotics should be involved in any steps of the procedure. For the cells co-transfected with [pBirA], biotin was supplemented with the final concentration of 100 μM the next day the expression began. The cell culture was centrifuged, and its supernatant was saved for protein purification.

2.3.1 Human Fc gamma receptor purification on Ni-NTA

The harvested cell culture was centrifuged at 1000 ×g for 10 minutes at 4 ˚C. The supernatant was centrifuged again at 15000 ×g for 30 minutes at 4 ˚C. 1.5 ml of Ni-NTA resin (ThermoFisher, USA) was pre-equilibrated in His-tag binding buffer (20 mM Imidazole, 20 mM Tris-base, 500 mM NaCl, pH 7.9) with the volume of 30-35 ml. The collected supernatant was fully loaded onto the Ni-NTA resin and washed with 30-35 ml of His-tag binding buffer.

His-tag binding buffer and His-tag elution buffer (1 M Imidazole, 500 mM NaCl, 20 mM Tris-base, pH 7.9) were mixed in a ratio of 4:1 to make His-tag elution buffer mixture. The protein was eluted with the his-tag elution buffer mixture in 1 ml aliquots. The first, elute-through aliquot was discarded. Total of five more consecutive aliquots of the His-tag elution buffer mixture were used to elute the protein. The eluted protein was

collected into each centrifugal filter with 3000 MWCO (Amicon, USA). The elute-through proteins were centrifuged 4000 rpm at 4 ˚C until the protein concentration was between 500-1000 μg ml^{-1}.

The concentrated purified protein was subjected to dialysis against 1000-fold excess PBS at 4 ˚C for overnight. Then, the dialyzed protein was dialyzed again against 1000-fold excess PBS at 4 ˚C overnight before they were collected. The concentration of the collected dialyzed protein was measured on a plate spectrophotometer (BioTek, USA) with PBS as the blank.

2.3.2 Antibody purification with Protein A resin

The harvested cell culture was centrifuged at 1000 ×g for 10 minutes at 4 ˚C. The supernatant was centrifuged again at 15000 ×g for 30 minutes at 4 ˚C. 1.5 ml of Protein A resin (Genscript, USA) was pre-equilibrated in Protein A binding buffer (0.15 M NaCl, 20 mM Na_2HPO_4, pH 7.0) with the volume of 30-35 ml. The collected supernatant was fully loaded onto the Protein A resin with controlled flow rate and washed with 30-35 ml of Protein A binding buffer.

The purified antibody was eluted using 15 ml of Protein A elution buffer (0.1 M Citric Acid, pH 3.0) and collected in 50 ml conical tube which contains 5 ml of neutralizing buffer (1 M Tris-HCl, pH 9.0) to neutralize the eluted antibody to the final pH of 7.0. The neutralized antibody was subjected to a series of dialyses using 30K MWCO (Amicon, USA) against PBS. The neutralized antibody was initially concentrated into the approximate volume of 1000 μl. Then, the concentrated antibody was dialyzed against 10-

20 fold excess PBS and mixed well by gently shaking. It was centrifuged to concentrate its volume to approximately 1000 µl. This step was repeated until the antibody was eventually dialyzed against total of at least 10000-fold excess PBS. Its final concentration was measured on a plate spectrophotometer (BioTek, USA) using PBS as the blank.

2.4.1 SDS-PAGE characterization of FcγRs

For the SDS-PAGE characterization of FcγRs, a PCR tube was prepared to contain 15 µl of 8 µM target FcγR and add 5 µl of 4 × laemmli buffer and mixed well. Then, the sample was heated at 95 ˚C for 5 minutes in a PCR block with a heated lid at 105 ˚C. After the boiling and cooling, a 5 µl of Color Protein Molecular Standard (New England BioLabs, USA) was added onto the marker lane. 20 µl of the samples were loaded and run on the hand-casted 12% SDS-PAGE gel prepared prior to the experiment. Initially, electrophoretic separation was proceeded at 400 mA, 80 V for minimum 30 minutes. At the constant current, the voltage was then increased to 100 V until the tracking dye front reaches the bottom of the gel. The gel was then removed from the casting assembly and stained with Coomassie Blue. The sufficiently destained gel was visualized on an imager (Bio-Rad Laboratories, USA).

For streptavidin pulldown assay, a PCR tube was prepared to contain 15 µl of 8 µM biotinylated target FcγR and add 10 µl of 4 × laemmli buffer. Then, the sample was heated at 95 ˚C for 5 minutes in a PCR block with a heated lid at 105 ˚C. After the boiling and cooling, 15 µl of streptavidin monomer was added with appropriate molar amount. 5 µl of Color Protein Standard (New England BioLabs, USA) was added onto the marker lane.

The biotinylated FcγR which was not subjected to streptavidin treatment, 15 µl of PBS was added. Samples were rested on the bench for 10 minutes as the sufficient interaction was necessary between biotinylated FcγR and streptavidin monomer. 20 µl of the samples were loaded and run on the hand-casted 12% SDS-PAGE gel prepared prior to the experiment. Initially, electrophoretic separation was proceeded at 400 mA, 80 V for at least 30 minutes. At the constant current, the voltage was then increased to 100 V until the tracking dye front reaches the bottom of the gel. The gel was then removed from the casting assembly and stained with Coomassie Blue. The sufficiently destained gel was visualized on an imager (Bio-Rad Laboratories, USA).

The stock concentration of streptavidin monomer was 75 µM. Streptavidin monomer was diluted in PBS based on that the concentration of biotinylated FcγR was 8 µM. The molar ratio between streptavidin monomer and biotinylated FcγR ranged from 0-3, with 3 at most.

SDS-PAGE experiment in reducing conditions involved using β-mercaptoethanol (β-ME) (Sigma-Aldrich, USA). β-ME as the reducing agent, 100 µl of β-ME was diluted and mixed with 900 µl of 4 × laemmli buffer to making reducing laemmli buffer. The reducing laemmli buffer was used in the same manner as 4 × laemmli buffer was used.

2.4.2 BLI Characterization of FcγRs

Out of many instruments based on bio-layer interferometry (BLI), advanced kinetics feature from BLItz (ForteBio, USA) was employed for the experiment. PBS was used as the general BLItz kinetics buffer throughout this experiment. In addition, all the

steps of experiments were based on the standard tube position which means the hydrated streptavidin sensor was dipped into the 0.5 ml black tube containing PBS buffer or appropriate solution for each step. All the PBS buffer and solutions were carefully pipetted into each 0.5 ml black microcentrifuge tube and equilibrated to the room temperature prior to the experiment. No bubble was present in any of the buffer and solutions. Once used streptavidin sensors were discarded immediately after each run.

Each streptavidin biosensor was placed and hydrated in a well of a 96-well plate containing 250 µl of PBS for 30 minutes. Biotinylated FcγRs was prepared at the mass concentration of 160 nM in PBS. Herceptin IgG WT, G236A (GA), P238D (PD) and (LPL) were prepared at various dilutions in PBS such as at 125 nM, 250 nM, and 500 nM and/or at 250 nM, 500 nM, and 1000 nM.

For initial baseline, 250 µl of PBS buffer was pipetted into a 0.5 ml black microcentrifuge tube and loaded into the system. Hydrated streptavidin biosensor was attached to the BLI system. Initial baseline measurement time duration was 20 seconds. To immobilize ligand or loading the bait, 250 µl prepared target biotinylated FcγR was loaded into the system for 120 seconds. Then, baseline was measured in 250 µl PBS buffer for 20 seconds. BLI signal from the association step was measured for 60 seconds. During the step, a target IgG molecule at one specific concentration was associated. Dissociation step was lasted for 120 seconds in 250 µl of PBS buffer. Table 6 shows time spent for each step.

Table 1. Advanced BLItz kinetics experimental design for tube position

Step	Time (sec)
Hydration of sensors	1800
Initial baseline	20
Loading of FcγR	120
Baseline	20
Association with the target IgG	60
Dissociation	120

Analyzing the measured data required functions of BLI system. During the BLI experiment, it is very important to also run for reference controls for both FcγR and IgG at 0 mg ml^{-1}. It is to normalize the data by subtracting the signal from the reference curve from that of the analyte [38]. Also, it was highly recommended to set up inter-step corrections to correct the baseline differences between two steps when the same buffer was used for the baseline and dissociation step. This was done by selecting both the 'start of association' and 'start of dissociation' prior to data analyses. Between local and global analysis options, global analysis option was chosen. Global analysis calculates the kinetic constants and binding affinities based on the entire set of analyte concentrations which is more suitable for this experiment. It was to evaluate the kinetic constant values and equilibrium binding constant of an IgG clone, not to evaluate them at the individual concentration of the same IgG clone. Also, global analysis is more generally used. It is also

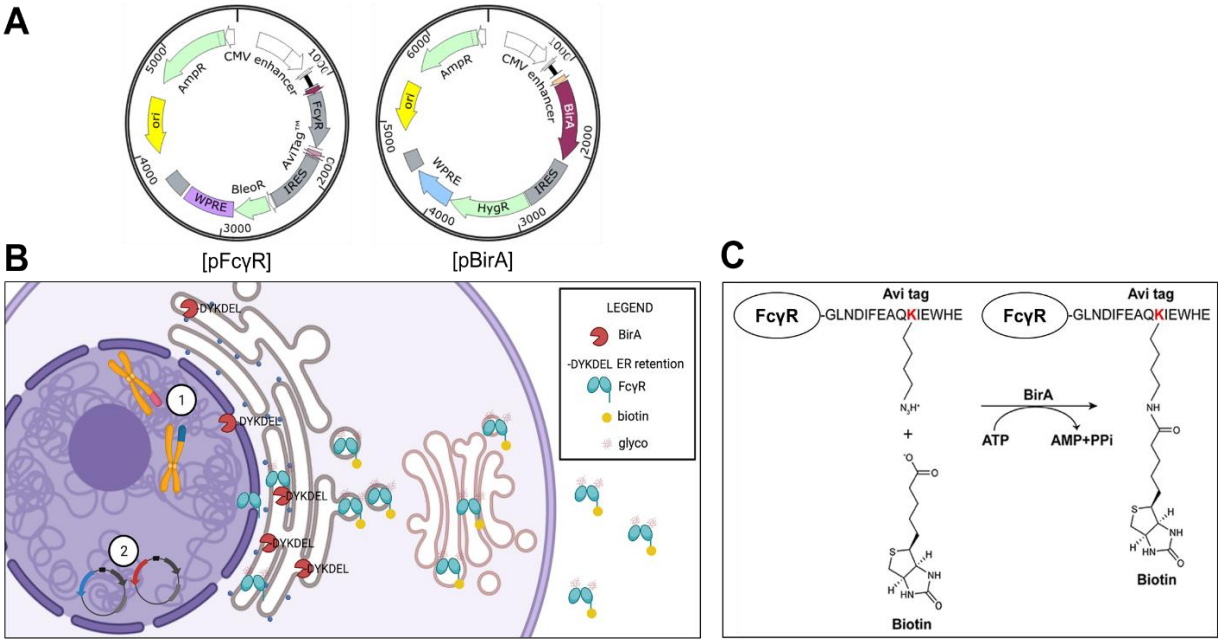
strongly recommended to evaluate the quality of fit both by visually and by R^2 values which should be above 95% [38].

3. Result and Discussion

3.1 Design of Experiment

Plasmids, [pFcγR] and [pBirA] were constructed (Figure 1A) and employed for both stable and transient transfections (Figure 1B). Initially, FcγRs were stably transfected for better yields. Then, FcγRIIA were transiently transfected for easier comparison to address the problems associated with the biotinylation efficiency, disulfide bond formation, and glycosylation. With AviTag, enzymatic biotinylation was done by utilizing BirA fused with endoplasmic reticulum retention signal, DYKDEL (Figure 1C). FcγRIIIA(F176), FcγRIIIA(V176) and FcγRIIB produced from the associated stably transfected cell lines at yields of 15.3, 9.1, and 25.7 μg purified receptor ectodomains per mL culture. And transient transfection led production of 7.0 and 3.6 μg purified FcγRIIA(H131) and FcγRIIA(R131) per mL culture.

Figure 1. Cartoon figures of the design of the experiment



(A) Plasmid constructions. (B) In vivo biotinylation at ER. (C) BirA mediated biotinylation at AviTag.

3.2 SDS-PAGE gel results of human Fc gamma receptors in reducing and non-reducing conditions show problem regarding glycosylation efficiency

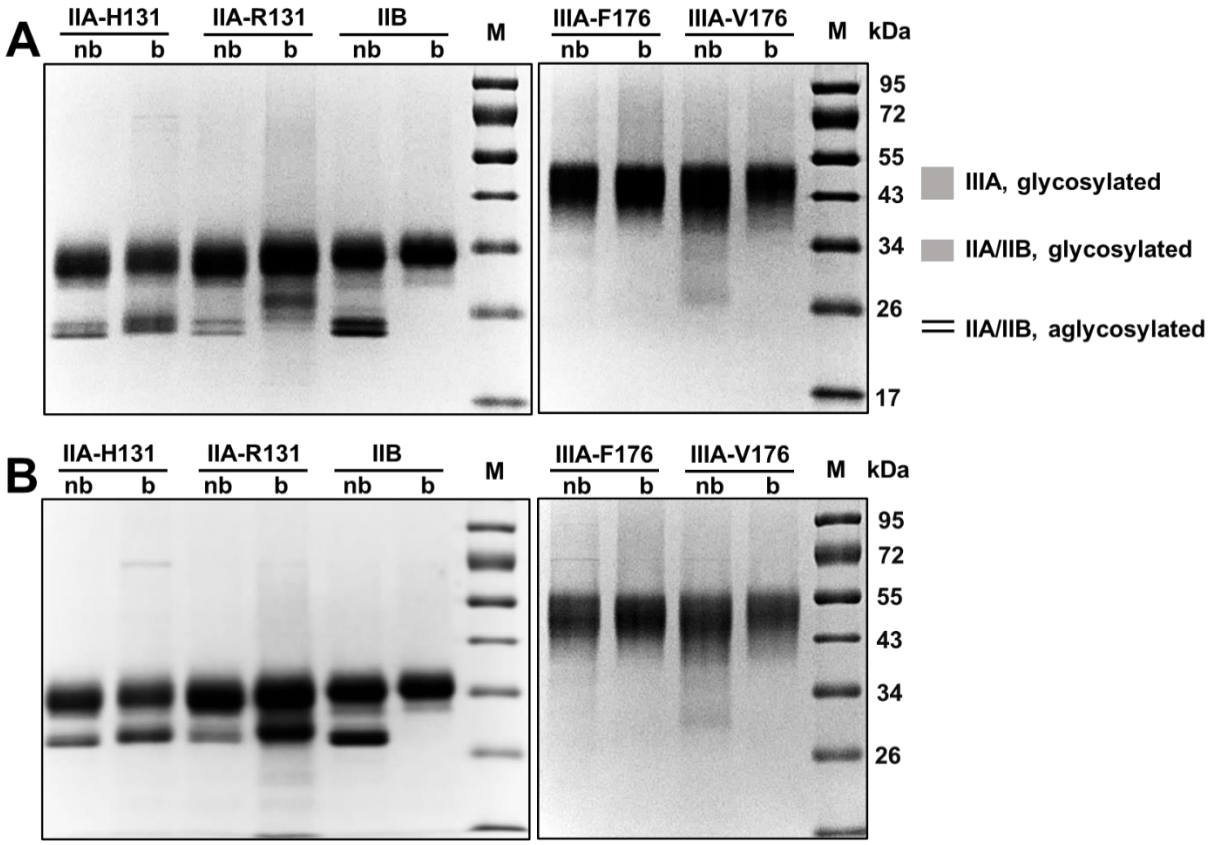
After producing both non-biotinylated and biotinylated human Fc gamma receptors (FcγRs), it was decided to examine their validity as a functional, structural protein. In order to do so, all the five pairs of FcγRs with and without biotinylation were ran on 12% SDS-PAGE gels under the non-reducing conditions (Figure 2A). Surprisingly, two apparent bands instead of a single band were observed on five FcγRs: FcγRIIA(H131) with and without biotinylation, FcγRIIA(R131) with and without biotinylation and non-biotinylated FcγRIIB. All the mentioned five FcγRs have an intense and relatively bigger band at 32

kDa while another but relatively smaller band at 24 kDa. Meanwhile, all the FcγRIIIAs had one broad band which is ranged from 40 to 50 kDa.

Before proceeding further, there was a strong desire to verify if the obtained result was a natural phenomenon for the five mentioned FcγRs to have two bands. Thus, all the five pairs of FcγRs were ran on an SDS-PAGE gel under the same condition except introducing β-mercaptoethanol (β-ME) which can reduce the disulfide bonds in FcγRs. The SDS-PAGE gel under reducing condition showed the similar result (Figure 2B). The mentioned five FcγRs still had two bands even in reducing condition while the rest of the FcγRs show one band. Therefore, SDS-PAGE gel results in both non-reducing and reducing conditions proved the validity of the structures of the FcγRs.

It is clearly visible that non-biotinylated FcγRIIA(H131), biotinylated FcγRIIA(H131), non-biotinylated FcγRIIA(R131), biotinylated FcγRIIA(R131), and biotinylated FcγRIIB have two apparent bands at 32 kDa and 24 kDa. FcγRs are glycosylated as part of their post-translational modification process in the endoplasmic reticulum (ER), therefore FcγRs are glycoproteins. Since the molecular weight of FcγRs without glycosylation are approximately 24 kDa, the bands at 24 kDa are presumably thought as un-glycosylated FcγRs. Likewise, the other bands at 32 kDa are thought as glycosylated FcγRs. Therefore, they are bigger and heavier in term of structure and weight, respectively. This explains the reason the glycosylated FcγR parts are positioned higher than the un-glycosylated FcγRs are. In other words, those already mentioned five FcγRs have problem regarding glycosylation efficiency as some portion of them are not glycosylated.

Figure 2. Non-reducing and reducing SDS-PAGE gels for all FcγRs



FcγRs produced from stable cell lines with (b) or without (nb) *in vivo* biotinylation. Purified FcγRs were analyzed at **(A)** non-reducing and **(B)** reducing conditions.

Comparing the gel in both non-reducing and reducing conditions, it is observed that the un-glycosylated bands are positioned higher in the reducing condition than that of in the non-reducing condition. This is due to β-mercaptoethanol (β-ME), the reducing agent, breaks down the disulfide bonds of FcγRs. Therefore, the FcγR protein sizes become larger which lead to bigger and more intense bands sizes (therefore positioned higher) on the gel with the reducing condition. However, biotinylated FcγRIIA(R131) is an exception from this. The un-glycosylated band of it stays at the same band size at 28 kDa. In fact, un-glycosylated FcγRIIA(R131) in non-reducing condition was at 28 kDa while all other un-

glycosylated FcγRs were at 24 kDa. In reducing condition, all other un-glycosylated FcγRs became bigger in size due to their disulfide bonds were broken by the reducing conditions. Thus, the bands of the un-glycosylated FcγRs were at 28 kDa. This different phenomena of FcγRIIA(R131) from other FcγRs leads to a thought that there may be a problem regarding disulfide bonds with biotinylated FcγRIIA(R131) and needs a further investigation.

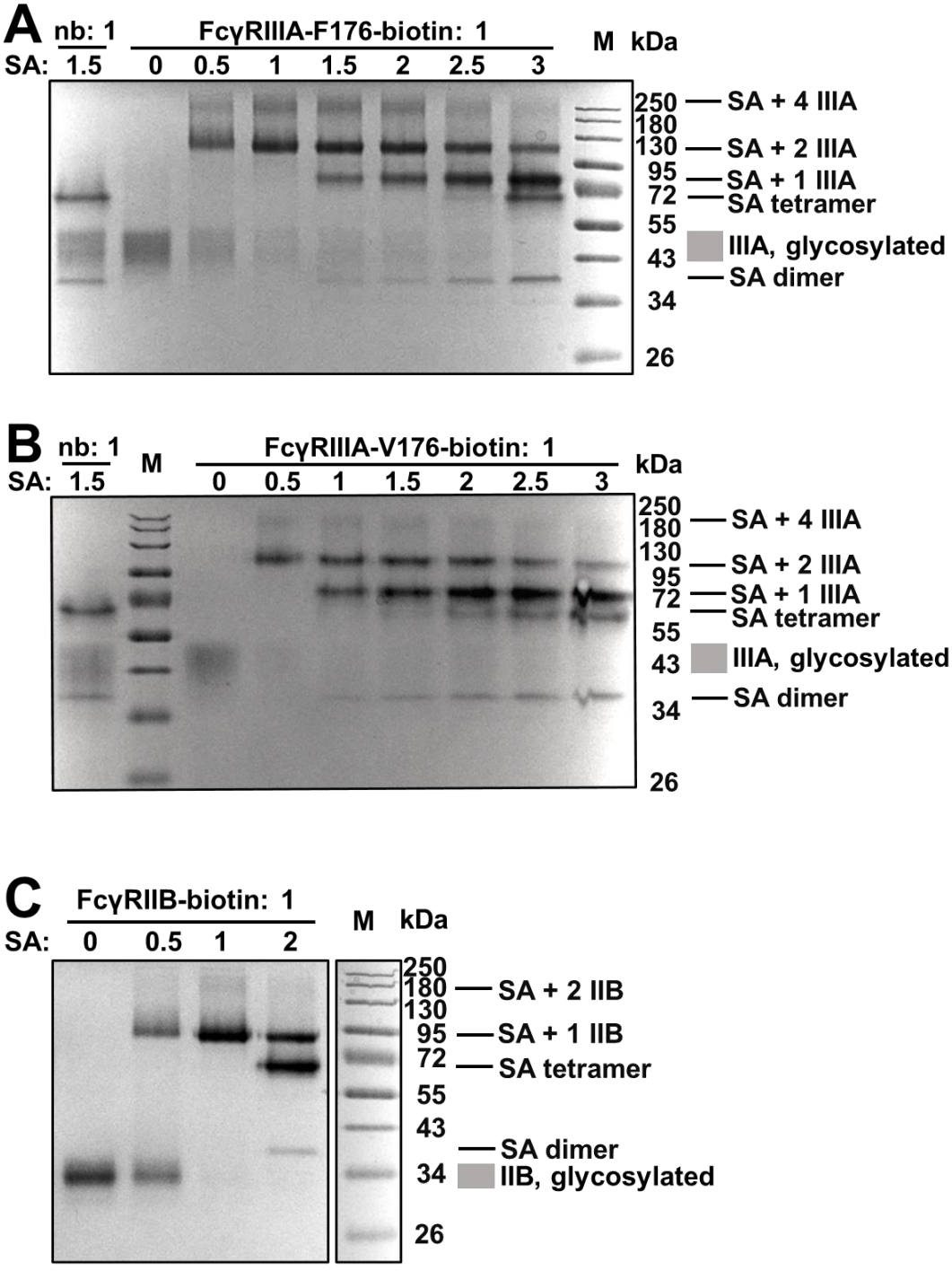
According to the SDS-PAGE gel results (Figure 2), it is obvious that FcγRIIIAs have higher glycosylation efficiency than FcγRIIA/B. For instance, FcγRIIA(H131), regardless of its biotinylation, has each band at 32 kDa and 24 kDa representing glycosylated and aglycosylated parts, respectively. Meanwhile, FcγRIIIA(F176) and FcγRIIIA(V176) have only one band ranging from 40 to 50 kDa regardless of their biotinylation. Their higher efficiency of glycosylation can be explained by their N-linked sites. FcγRIIIA has 5 N-linked sites while FcγRIIA has 2 and FcγRIIB has 3 [1]. As a result, FcγRIIIAs possess more glycans. This means FcγRIIIAs have to spend more time to be processed in the endoplasmic reticulum (ER) and therefore resulting better glycosylation than FcγRIIA/B.

After verifying the validity of produced FcγRs, streptavidin probing was conducted on all the biotinylated FcγRs to examine their biotinylation efficiency. In this section, the main foci are FcγRIIB, FcγRIIIA(F176), and FcγR(V176). The rest of FcγRs, FcγRIIA(H131) and FcγRIIA(R131), will be discussed in the later section.

3.3 SDS-PAGE gel results of FcγRIIB, FcγRIIIA(F176), and FcγRIIIA(V176) shows high biotinylation efficiency

A pair of both non-biotinylated FcγRIIIA(F176) and biotinylated FcγRIIIA(F176) and that of FcγRIIIA(V176) were subjected to streptavidin probing. Each FcγRIIIA of both pairs showed one broad band at ranging from 40 to 50 kDa regardless of biotinylation. According to the streptavidin probed SDS-PAGE gel results, both pairs of FcγRIIIA(F176) and FcγRIII(V176) showed relatively high biotinylation efficiency (Figure 3A and B). One of the means to determine the biotinylation efficiency was to observe the intensity of the remaining band at 40-50 kDa where the lanes have the interaction between streptavidin and biotinylated FcγRs. As the molar ratio of streptavidin to the biotinylated FcγR is increasing, the intensity of the band ranging between 40 and 50 kDa was fading. For a 1:3 molar ratio of FcγRIIIA and streptavidin, it can be said that there is no remaining band at 40 to 50 kDa which means all the target biotinylated FcγRIIIA are truly undergone successful biotinylation and completely interacted with streptavidin. Therefore, it could be said that both biotinylated FcγRIIIA(F176) and FcγRIIIA(V176) have high biotinylation efficiency.

Figure 3. Streptavidin pulldown assay on FcγRIIIA(F176), FcγRIIIA(V176) and FcγRIIB.



Pulldown assays of **(A)** FcγRIIIA-F176, **(B)** FcγRIIIA-V176 and **(C)** FcγRIIB with streptavidin at 0-3 molar ratios between SA monomer and receptors. Non-biotinylated (nb)

receptors were used as controls. All receptors were produced by using the cell line stably expressing BirA.

The possible reason for FcγRIIIA(F176) and FcγRIIIA(V176) having high biotinylation efficiency can be their relatively longer time in ER. As already mentioned, FcγRIIIAs have more N-linked sites than FcγRIIA/B [1]. FcγRIIIAs therefore have to go through longer post-translational modification in the ER than FcγRIIA/B do. Moreover, co-transfected BirA plasmid is ER-retained due to its ER-retaining DYKDEL sequence. All these factors combined together, FcγRIIIAs are also biotinylated with high efficiency as well as their high glycosylation efficiency while they stay in the ER for their relatively longer post-translational modification process.

Non-biotinylated FcγRIIB showed two bands, each different in intensity, on both SDS-PAGE gels in non-reducing and reducing conditions while biotinylated FcγRIIB showed one band at 32 kDa (Figure 2). Streptavidin (SA) probing was also done on biotinylated FcγRIIB with increasing molar ratio of SA (Figure 3C). As expected, non-biotinylated FcγRIIB showed two bands at 32 kDa and 24 kDa. However, biotinylated FcγRIIB showed single band at 34 kDa. According to its SDS-PAGE gel result, FcγRIIB shows high biotinylation efficiency (Figure 3C). When more amount of streptavidin presents than that of biotinylated FcγRIIB, it is completely reacted with streptavidin and the excessive streptavidin was shown on its bands at 70 kDa as its tetramer and 40 kDa as its dimer.

Despite non-biotinylated FcγRIIB simultaneously consists of both glycosylated and un-glycosylated portions, biotinylated FcγRIIB is almost entirely glycosylated, if not 100% glycosylated, as well as it shows high biotinylation efficiency. Even though the only

difference between the two FcγRIIB is biotinylation, it brought a different and meaningful outcome. FcγRIIB has 3 N-linked sites [1]. This number is more than that of FcγRIIAs while less than that of FcγRIIIA. In addition, BirA plasmid utilized in this experiment was ER-retained with DYKDEL sequence at its C terminal as already mentioned. Therefore, the reason non-biotinylated FcγRIIB is poorly glycosylated while biotinylated FcγRIIB is efficiently glycosylated and biotinylated at the same time can be said as followed. The non-biotinylated FcγRIIB has shorter post-translational modification compared to biotinylated FcγRIIB. The biotinylated FcγRIIB can be said it is retained in ER relatively longer due to BirA possessing ER-retaining sequence, DYKDEL. Therefore, there are higher possibility that biotinylated FcγRIIB underwent its post-translational modification with higher quality.

3.4 SDS-PAGE gel results of streptavidin probing on FcγRIIA(H131) and FcγRIIA(R131) shows poor biotinylation efficiency

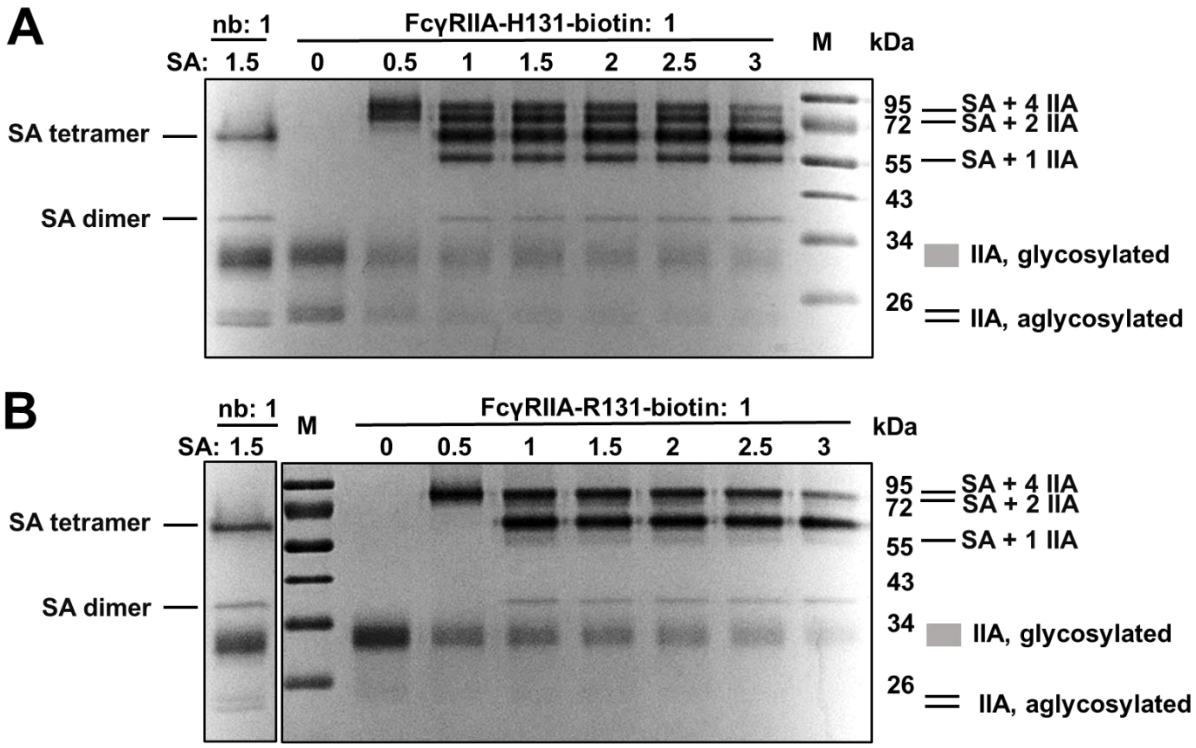
Streptavidin probing was also conducted on both biotinylated FcγRIIA(H131) and FcγRIIA(R131) as well (Figure 4). Unlike biotinylated FcγRIIB, FcγRIIIA(F176), and FcγR(V176), the two different FcγRIIA groups show relatively inadequate quality in terms of glycosylation (Figure 2). FcγRIIA groups also show inadequate quality in terms of biotinylation efficiency compared to other FcγRs mentioned in this experiment.

Non-biotinylated FcγRIIA(H131) shows one intense and larger band at 32 kDa and another band, but relatively small, at 24 kDa (lane 1 from Figure 4A). Streptavidin has its bands at 70 kDa as its tetramer and 40 kDa as its dimer while biotinylated FcγRIIA(H131) also has the same bands at 32 kDa and another band at 24 kDa (lane 2 from Figure 4A).

From lanes 4 to 9, the molar amount of FcγRIIA(H131) is constant although the molar amount of streptavidin is increasing. Despite the increasing molar amount of streptavidin, there are still two faint bands remaining at 32 kDa and 24 kDa (Figure 4A). This means that the biotinylation of FcγRIIA(H131) was not efficient. In other words, some of FcγRIIA(H131) was not biotinylated at all and did not interact with streptavidin even though excessive amount of streptavidin was present.

FcγRIIA(R131) also poses the same problem. The non-biotinylated FcγRIIA(R131) has two bands at 32 kDa and 24 kDa. Biotinylated FcγRIIA(R131) has also two bands at 32 kDa and 24 kDa while streptavidin has its bands at 70 kDa and 40 kDa. By observing its SDS-PAGE gel (Figure 4B), it is apparently visible its biotinylation is not as efficient as FcγRIIB or FcγRIIIAs despite the molar ratio of streptavidin is increasing. In fact, there are excessive streptavidin presents on lane 10, however, still un-biotinylated FcγRIIA(R131) is shown at 32 kDa and 24 kDa (Figure 4B). Therefore, the excessive streptavidin could not interact with un-biotinylated FcγRIIA(R131).

Figure 4. Streptavidin pulldown assay on FcγRIIA(H131) and FcγRIIA(R131)



Pulldown assays of **(A)** FcγRIIA-H131 and **(B)** FcγRIIA-R131 with streptavidin at 0-3 molar ratios between SA monomer and IIA. Non-biotinylated (nb) FcγRIIAs were used as controls. FcγRIIAs were produced by using the cell line stably expressing BirA.

The putative reason of both biotinylated FcγRIIA(H131) and biotinylated FcγRIIA(R131) has relatively poor biotinylation efficiency compared to biotinylated FcγRIIB as well as biotinylated FcγRIIIA(F176) and FcγRIIIA(V176) may be explained as followed. As already mentioned, FcγRIIA has 2 N-linked sites, which is the least compared to that of FcγRIIB and FcγRIIIA. FcγRIIIA has the most N-linked sites, which are 5, compared to the other FcγRs mentioned in this experiment [1]. In the case of FcγRIIAs, their number of N-linked sites was not enough to yield their glycosylation and biotinylation with as in high efficiency as biotinylated FcγRIIB or FcγRIIIAs. Their

relatively small number of N-linked sites presumably led the FcγRIIAs to undergo comparably short post-translational modification. Therefore, it can be said the post-translational modification of FcγRIIA was not sufficient to yield glycosylation and biotinylation of high quality.

3.5 Transient transfection to improve the overall quality of FcγRIIA(H131) and FcγRIIA(R131)

According to the overall gel images and comparing their results, FcγRIIA groups have problems regarding glycosylation, biotinylation efficiency, and disulfide bonds. Thus far, all biotinylated FcγRs were stably co-transfected with the associated [pFcγR] and [pBirA] in 1:1 mass ratio. In order to address these mentioned problems and improve the overall quality of FcγRIIA(H131) and FcγRIIA(R131), the FcγRIIAs were transiently co-transfected with [pBirA] in 1:2 mass ratio as an experimental group. Meanwhile, a 1:1 mass ratio of the associated IIA[pFcγR] and [pBirA] was also transiently co-transfected as a control. The main purpose was to first improve the biotinylation efficiency by increasing the amount of [pBirA]. Transient transfection was employed for easier comparison. Duplicate of each biotinylated FcγRIIA as the control and the experiment was generated.

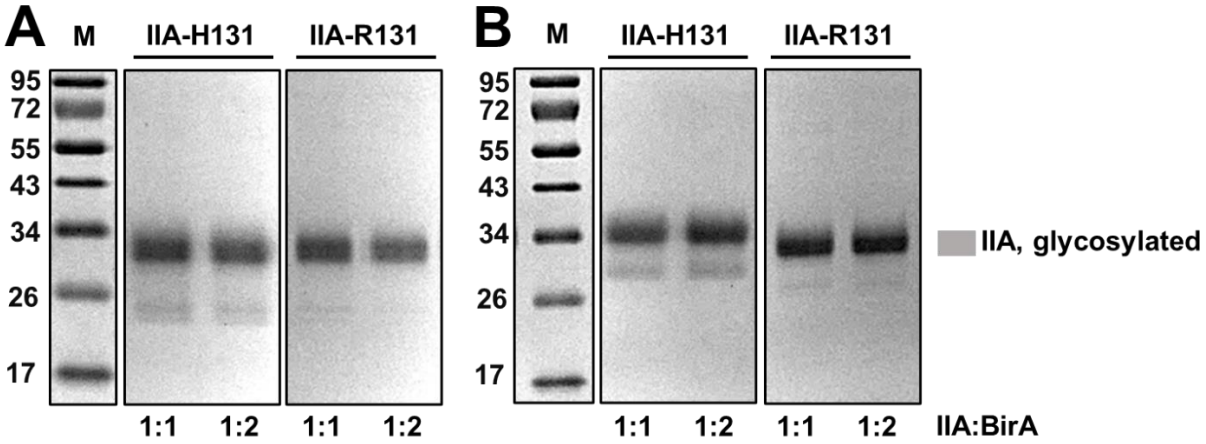
Initially, the duplicates of both 1:1 and 1:2 mass ratios of FcγRIIA(H131) and FcγRIIA(R131) were run on SDS-PAGE gels in non-reducing and reducing conditions. For the reducing condition, β-mercaptoethanol (β-ME) was used as the reducing agent. Surprisingly, there was no difference in outcomes of the different ratios of IIA(H131) [pFcγR] and [pBirA]. In non-reducing condition, both 1:1 and 1:2 ratio of IIA(H131)

[pFcγR] and [pBirA] had the comparable band sizes with the comparable intensity at 32 kDa, however, much less intense bands at 24 kDa. Although it was a surprise that the bands of different mass ratios of IIA(H131) [pFcγR] and [pBirA] show similar result, it was observed that the amount of aglycosylated FcγRIIA(H131) was clearly reduced (Figure 5A) compared to stably transfected FcγRIIA(H131) in non-reducing and condition (Figure 2). This was also observed with FcγRIIA(R131) in non-reducing condition.

For the further verification of these conditions, both FcγRIIA(H131) and FcγRIIA(R131) were also ran in the reducing condition. The results are very similar to that of non-reducing conditions which was discussed. While the bands of different mass ratios of IIA(H131) [pFcγR] and [pBirA] showed very comparable intensity and sizes of bands to each other, the aglycosylated FcγRIIA(H131) was noticeably reduced (Figure 5B) compared to that of stably transfected FcγRIIA(H131) in reducing condition (Figure 2). This observation is also able to be applied to FcγRIIA(R131).

Moreover, there is a significant improvement in terms of disulfide bond of FcγRIIA(R131). Comparing stably transfected both non-biotinylated FcγRIIA(R131) and biotinylated FcγRIIA(R131) in non-reducing condition (Figure 2), the stably transfected biotinylated FcγRIIA(R131) has abnormal disulfide bond which was already mentioned earlier. The substantial improvement of the abnormal disulfide bond of the stably transfected, biotinylated and aglycosylated FcγRIIA(R131) could be observed in transiently transfected, biotinylated and aglycosylated FcγRIIA(R131) (Figure 5).

Figure 5. Non-reducing and reducing SDS-PAGE gels for FcγRIIA(H131) and FcγRIIA(R131)



FcγRIIAs produced with transient co-transfection of both the receptor and *BirA* plasmids for *in viv*o biotinylation. For 2-2.5x10^6 cells, 15 µg (IIA) [pFcγR] and 15 µg [pBirA] (1:1) or 10 µg (IIA) [pFcγR] and 20 µg [pBirA] (1:2) were used for co-transfection. Purified receptors were analyzed at **(A)** non-reducing and **(B)** reducing conditions.

After verifying FcγRIIAs, streptavidin (SA) probing assay was conducted to test their biotinylation efficiency. The bands of streptavidin were shown at 70 kDa as its tetramer and 40 kDa as its dimer. It is shown that biotinylated FcγRIIA(H131) with the different mass ratios of BirA was probed with increasing molar ratio of streptavidin.

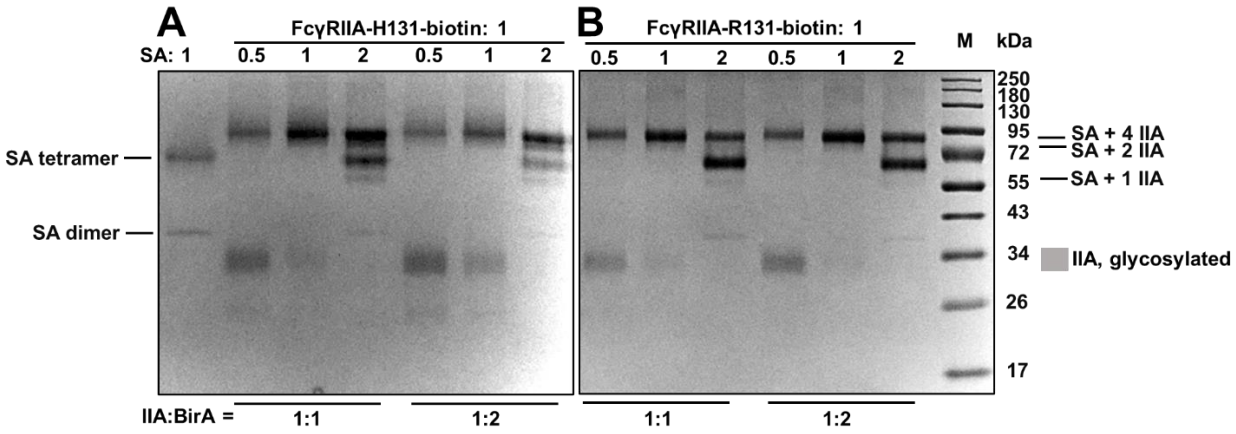
The ratio 1:1 of IIA(H131) [pFcγR] and [pBirA] underwent streptavidin probing with increasing molar ratio from 0.5, 1, to 2 to the constant molar amount of FcγRIIA(H131), which set to 1. It was visible that its improved biotinylation efficiency (Figure 6) compared to that of stably transfected FcγRIIA(H131) (Figure 4A). For stably transfected FcγRIIA(H131), there was unbiotinylated FcγRIIA(H131) which did not interact with streptavidin even though the biotinylated FcγRIIA(H131) to streptavidin ratio was 1:3 and there was excessive amount of streptavidin present. In contrast, almost all

transiently transfected biotinylated FcγRIIA(H131) interacted with streptavidin when the ratio of biotinylated FcγRIIA(H131) and streptavidin was only 1:2.

This observation is similar for the ratio of 1:2 of IIA(H131) [pFcγR] and [pBirA]. The streptavidin probing was conducted in the same manner with that of 1:1 ratio of IIA(H131) [pFcγR] and [pBirA]. There is no difference between the control and experiment groups in terms of biotinylation efficiency. The ratio of 1:2 IIA(H131) [pFcγR] and [pBirA] showed almost all transiently transfected FcγRIIA(H131) was biotinylated. Therefore, almost all biotinylated FcγRIIA(H131) was able to interact with streptavidin.

The aforementioned phenomenon also applies to FcγRIIA(R131). Similarly, there are two different mass ratios of IIA(R131) [pFcγR] and [pBirA] in 1:1 and 1:2 which were undergone streptavidin probing. The molar ratio of biotinylated FcγRIIA(R131) to streptavidin increased from 1 to 0.5, 1, to 2 (Figure 6B). Similar to the result of biotinylated FcγRIIA(H131), both group of biotinylated FcγRIIA(R131) showed significantly improved biotinylation efficiency.

Figure 6. Streptavidin pulldown assay on transiently transfected FcγRIIA(H131) and FcγRIIA(R131)



Pulldown assays of **(A)** FcγRIIA-H131 and **(B)** FcγRIIA-R131 with streptavidin at 0-2 molar ratios between SA monomer and the receptors. FcγRIIAs were produced with transient co-transfection of IIA and BirA at DNA mass ratio of 1:1 or 1:2.

Combining the results, it is clear that there were meaningful improvements in terms of aglycosylation, abnormal disulfide bond, and weak biotinylation efficiency. However, it is difficult to say those mentioned problems were improved due to change of the mass ratio of IIA [pFcγR] and [pBirA]. Both the ratios 1:1 and 1:2 of IIA [pFcγR] and [pBirA] show almost similar results. Other difference besides the different mass ratio of the associated [pFcγR] and [pBirA] would be that they are transiently transfected. Transient transfection produces proteins of better quality, though, with low yield. In contrast, stable transfection produced proteins of relatively worse quality with high yield. Thus, it can be said stable transfection produces proteins including those which are not sufficiently went through their post-translational modification process in ER, therefore, it has high yield. This may be possible due to longer protein production time and the plasmid integration

into the genome of the cells by the selection process. For transient transfection, it can be said most of the produced proteins underwent post-translational modification in ER although it has relatively low yield due to shorter production time and the absence of the plasmid integration into genome of the cell.

3.6 BLItz characterization

BLItz experiments were conducted in order to further validate if the produced FcγRs in this project have a good affinity. Advanced kinetic feature with streptavidin biosensor was employed to assess this function of the FcγRs. Table 7 shows the average K_D values with standard deviations of each different FcγR with IgG WT, IgG mutants G236A (GA), P238D (PD), F243L R292P Y300L (LPL).

Table 2. Binding affinities of produced FcγRs and comparison with reported values (nM)

	IgG wt		IgG carrying Fc mutation		
	This study	Literature (Ref)	Mutant	This study	Literature (Ref)
IIA-H131	288 ± 67	292[1] – 800[2]	GA	121 ± 35	130 ± 10[3]
IIA-R131	608 ± 260	203[1] – 800[2]	GA	208 ± 109	161 ± 3[3]
IIB	359 ± 53	1274[1] – 3100[2]	PD	307 ± 42	N/A
IIIA-F176	468	554[1] – 850[2]	LPL	245 ± 57	N/A
IIIA-V176	608 ± 39	502[1] – 850[2]	LPL	113	N/A

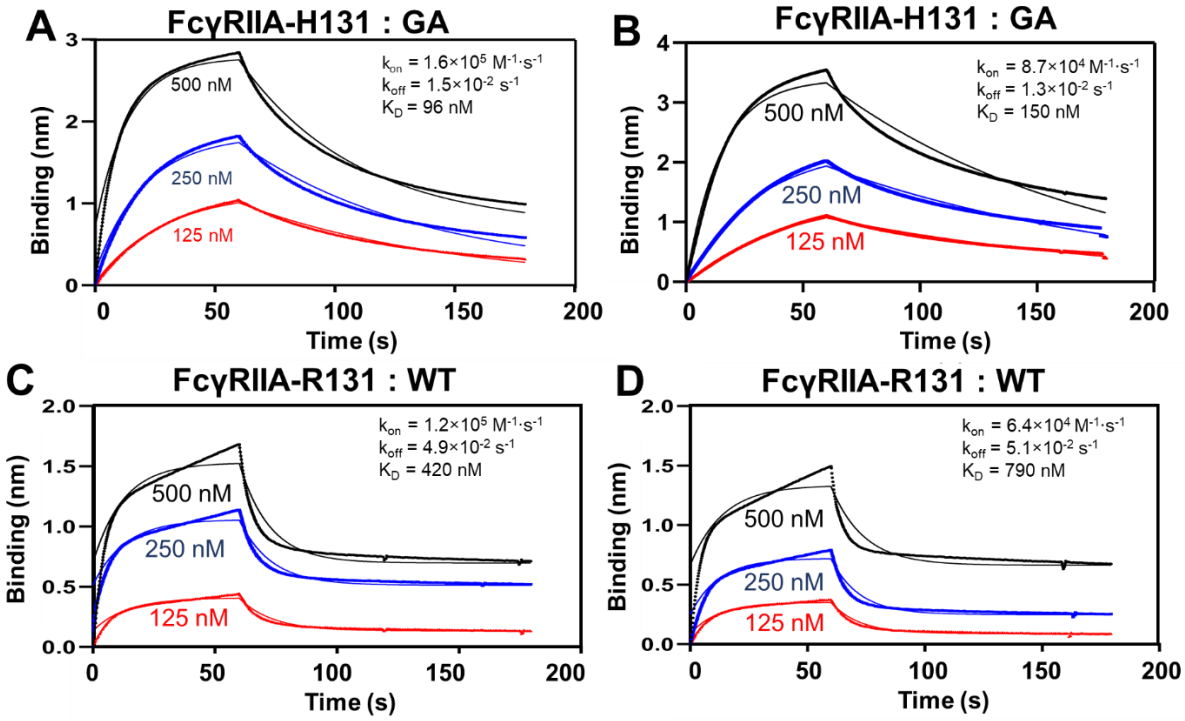
According to Table 7, the K_D values of the FcγRs experimented with IgG WTs roughly fall into the reported literature values. While the majority of the produced FcγRs (FcγRIIA(H131), FcγRIIA(R131), and FcγRIIIA(V176)) show their K_D values align with that of the literature values, the K_D values of FcγRIIB and FcγRIIIA(F176) deviate from the reported K_D values. Although those two FcγRs show their K_D value deviates from that of reported values, K_D values of produced FcγRIIIA(F176) is rather in the proximity of the

literature values and the deviation is not significant. However, FcγRIIB has K_D value that is roughly four-times less than that of the reference values.

In cases of IgG mutant GA, a positive control for FcγRIIA, the produced FcγRIIA also showed their K_D values fall within the range of the literature values. For other IgG mutants, PD and LPL, the corresponding literature values could not be found.

FcγRIIA(H131) and FcγRIIA(R131) produced from transient transfection were characterized by BLItz with the concentration from 125 to 500 nM (Figure 7A and B). The results of that FcγRIIA(H131) was tested with IgG mutant GA and FcγRIIA(R131) was tested with WT were shown (Figure 7). The BLItz result of IIA(H131) [pFcγR] and [pBirA] mass ratio of 1:1 shows k_{on} value is 1.6×10^5 $M^{-1}s^{-1}$, k_{off} is 1.5×10^{-2} s^{-1}, and K_D is 96 nM (Figure 7A). Meanwhile, IIA(H131) [pFcγR] and [pBirA] mass ratio of 1:2 shows k_{on} value is 8.7×10^4 $M^{-1}s^{-1}$, k_{off} is 1.3×10^{-2} s^{-1}, and its K_D value is 150 nM. These results shows that there is not a meaningful difference between the different plasmid mass ratio of FcγRIIA(H131) and BirA.

Figure 7. BLItz results of transiently transfected FcγRIIA



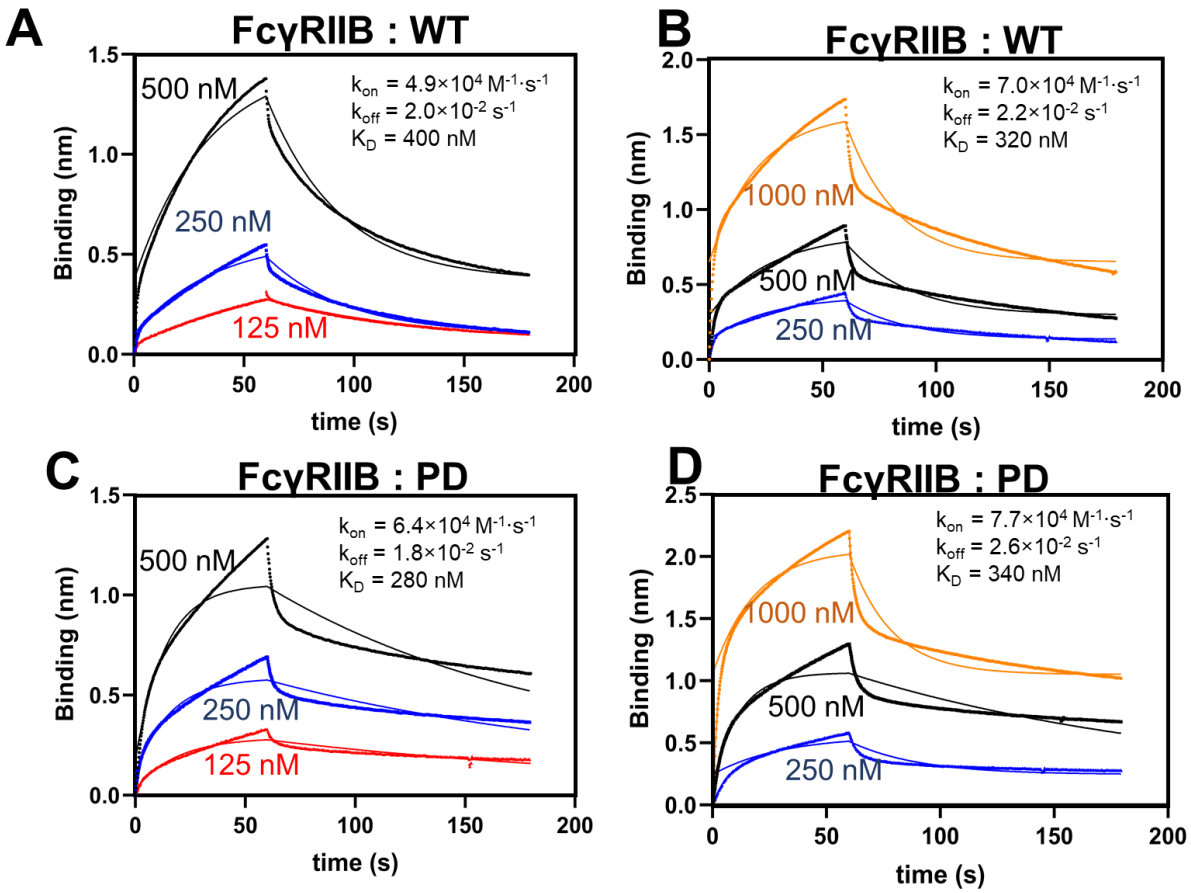
BLItz results of transiently transfected FcγRIIA. **(A)** IIA(H131) [pFcγR] and [pBirA] mass ratio of 1:1, **(B)** IIA(H131) [pFcγR] and [pBirA] mass ratio of 1:2, **(C)** IIA(R131) [pFcγR] and [pBirA] mass ratio of 1:1, and **(D)** IIA(R131) [pFcγR] and [pBirA] mass ratio of 1:2. Presented graphical data show FcγRIIA(H131) was tested with IgG mutant GA while FcγRIIA(R131) was tested with IgG WT.

The similar phenomenon happens for FcγRIIA(R131) (Figure 7C and D). As already mentioned, FcγRIIA(R131) was tested with IgG WT. The BLItz result of IIA(R131) [pFcγR] and [pBirA] mass ratio of 1:1 shows k_{on} values at 1.2 × 10^5 M^{-1}s^{-1}, k_{off} is at 4.9 × 10^2 s^{-1}, and K_D value at 420 nM. These values are little lower than those of IIA(R131) [pFcγR] and [pBirA] mass ratio of 1:2 (Figure 7C). The BLItz result of IIA(R131) [pFcγR] and [pBirA] mass ratio of 1:2 is showing that k_{on} 6.4 × 10^4 M^{-1}s^{-1}, k_{off} is 5.1 ×10^{-2} s^{-1}, and K_D is 790 nM (Figure 7D). Although there are differences of the values

between the different mass ratio of IIAR131 [pFcγR] and [pBirA], they are not apparent to be meaningful differences.

Stably transfected FcγRIIB was tested its binding affinity via BLItz as well. Two different concentration ranges of FcγRIIB were tested with IgG WT and IgG mutant PD. PD is a negative control for FcγRIIA and FcγRIIIA but positive control for FcγRIIB. Comparing the two different concentration ranges of FcγRIIB that were tested with WT, there is no meaningful differences in terms of the constant values. This is also observed when FcγRIIB was tested with PD; there is no obvious differences between each constant values (Figure 8).

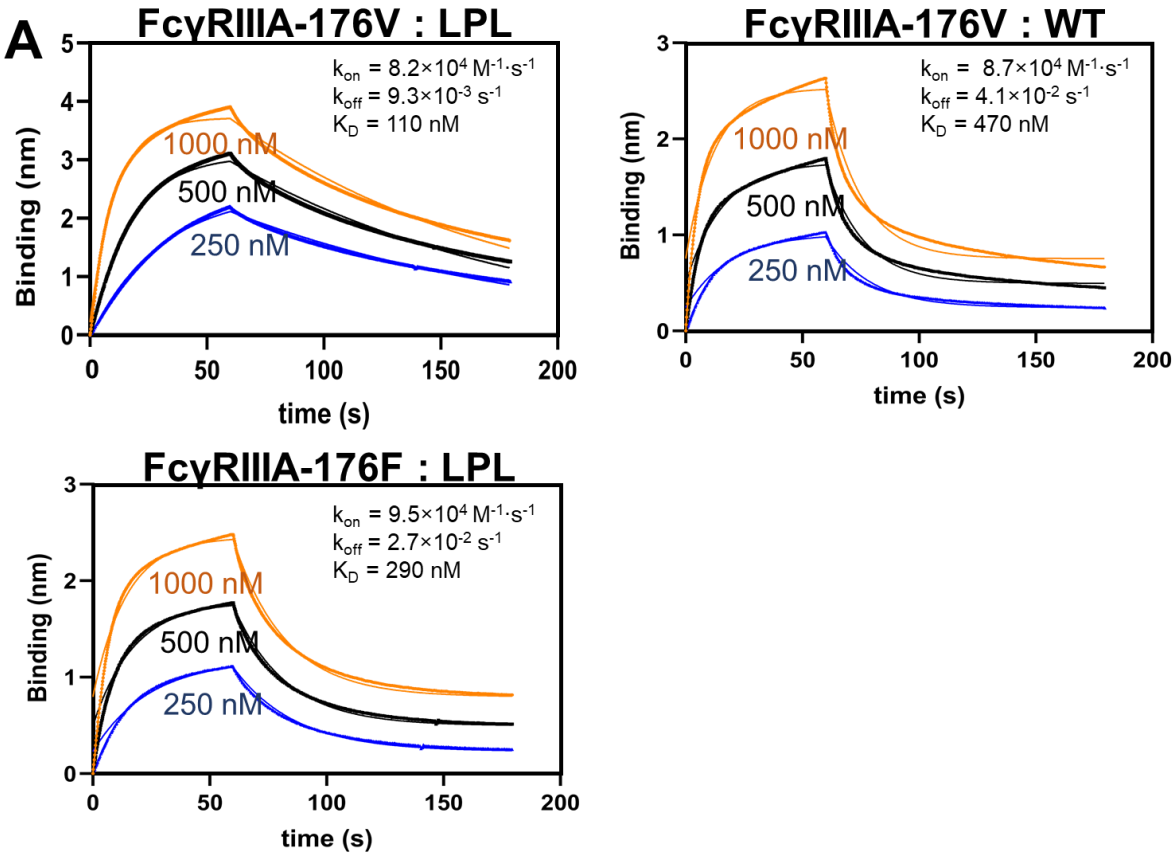
Figure 8. BLItz result of stably transfected FcγRIIB



BLItz results of stably transfected FcγRIIB. FcγRIIB tested with **(A)** and **(B)** IgG WT while **(C)** and **(D)** IgG mutant PD.

Stably transfected both FcγRIIIA(F176) and FcγRIIIA(V176) were tested with LPL. FcγRIIIA(V176) was tested with WT in addition. Notably, K_D value of FcγRIIIA(V176) to WT was higher than that of FcγRIIIA(V176) to LPL even though LPL is a positive control to FcγRIIIA (Figure 9).

Figure 9. BLItz result of stably transfected FcγRIIIA



BLItz results for stably transfected FcγRIIIA. FcγRIIIA(V176) were tested with **(A)** IgG mutant LPL and **(B)** IgG WT. FcγRIIIA(F176) were tested with **(C)** IgG mutant LPL.

As already mentioned, most of the K_D values of produced FcγRs fall within the range of the reported values (Table 2). Only FcγRIIB shows significant deviation from the literature values. It is not known why only FcγRIIB shows such deviation while all other FcγRs do not deviate. The table shows FcγRs except FcγRIIB have reasonable binding affinities. This result therefore successfully validates the produced FcγRs have a good affinity.

4. Conclusion

In this study, it was attempted to produce high quality human Fc gamma receptors (FcγRs) which are fully biotinylated, N-glycosylated, and disulfide bond formed. Initially, all of the FcγRIIA(H131), FcγRIIA(R131), FcγRIIB, FcγRIIIA(F176), and FcγRIIIA(V176) were stably transfected by *in vivo* biotinylation in 293 mammalian cell line. *In vivo* biotinylation was achieved by co-expressed AviTag fused FcγRs and *E.coli* biotin ligase (BirA) with endoplasmic reticulum retention signal, DYKDEL. Stably transfected and purified FcγRs were subjected to SDS-PAGE experiment as well as streptavidin pulldown assay. Following SDS-PAGE results demonstrated that both FcγRIIA(H131) and FcγRIIA(R131) had a few problems in terms of biotinylation efficiency, disulfide bond formation, and glycosylation. The other FcγRs, FcγRIIIA(F176), FcγRIIIA(V176) and FcγRIIB showed expected disulfide bond formation, glycosylation, and complete biotinylation efficiency at 15.3, 9.1, and 25.7 µg purified receptor per mL culture, respectively. To address the mentioned problems of FcγRIIA, FcγRIIA(H131) and FcγRIIA(R131) were transiently transfected and co-expressed with BirA with DYKDEL sequence at the optimized plasmid mass ratio (1:2) as well as the original plasmid mass ratio (1:1). The transiently transfected FcγRIIA(H131) and FcγRIIA(R131) were produced with yield of 7.0 and 3.6 µg purified receptor per mL culture, respectively. There was no difference in SDS-PAGE results when different plasmid ratios were compared. However, meaningful improvements were observed when transiently transfected FcγRIIA(H131) and FcγRIIA(R131) are compared to stably transfected FcγRIIA(H131) and FcγRIIA(R131), respectively. From SDS-PAGE

streptavidin pulldown assay results, the transiently transfected both FcγRIIA(H131) and FcγRIIA(R131) demonstrated expected disulfide bond formation, improved glycosylation, and complete biotinylation efficiency. Although transient transfection produced relatively low yield, it produced proteins of high quality. To further validate the function of the produced FcγRs, transiently transfected FcγRIIA and stably transfected the other FcγRs were undergone BLItz with advanced kinetic feature. By comparing the reported literature values of binding affinities, the produced FcγRs demonstrated their binding affinities are consistent with the literature values. This work provided high quality reagents for engineering endeavor of antibody Fc fragments, and the methods demonstrated here can be applied for *in vivo* biotinylation of other important glycoproteins in mammalian cells.

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