Discovery of a Novel Therapeutic Target for Enhancing Anti-CD20 Antibody-Induced Direct Cell Death in B Cell Lymphoma Using APEX2-Based Proximity Labeling

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Directed by Professor Joo Young Kim

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

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December 2023

This certifies that the Master's Thesis
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The Graduate School
Yonsei University

December 2023

ACKNOWLEDGEMENTS

First of all, I would like to give a great gratitude to professor Joo Young Kim for advising and supporting me during entire of my graduate school life. Last 2 years I spent in professor Kim's laboratory was awesome experience for me to elicit my enthusiasm and for deciding my future career. It is a profound honor to have completed my master's degree at Yonsei University under Professor Kim's supervision.

I would like to extend the gratitude to professor Jin Seok Kim and professor Jinu Lee for being thesis committee member. Plenty of advises and feedbacks I've got from them was very helpful for me to be more matured scientist.

The most admirable personnel, Dr. Dong Hyuk Lee, was a great advisor and co-worker. His generous mentorship and guidance have enriched my academic journey, for which I am immensely grateful.

Thanks to Jeong Ryeol, Yerim, Do Hyun, Geun Ah, Beom Jun and Jinkyung for sharing their thoughts and being wonderful teammate in our laboratory.

Lastly, but certainly not least, I wish to express my deepest gratitude to my parents for their boundless love and unwavering support throughout my life. Your love has been my foundation, and I am profoundly grateful for all that you have done for me.

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ABSTRACT

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Non-Hodgkin's lymphoma is a clonal tumor originating from mature and immature B cells, accounting for over 500,000 annual cases worldwide. Obinutuzumab, the anti-CD20 antibody for treatment of BNHL, is recognized as biobetter of rituximab due to the enhanced antibody-dependent cell-mediated cytotoxicity (ADCC), as well as direct cell death (DCD) through antibody binding. However, the molecular biology mechanisms underlying this direct cell death induction remain poorly understood.

In this study, our objective was to identify proteins that regulate cell death mediated by obinutuzumab binding to CD20. By fusing engineered ascorbate peroxidase 2 (APEX2) to the Fc terminus of obinutuzumab, we employed liquid chromatography-mass spectrometry to identify biotin-labeled proteins within a 20 nm radius of CD20 bound by obinutuzumab, comparing it to rituximab. Out of the 482 proteins, we selected protein candidates based on the genes where at least 2 peptides were detected, and the protein exhibited an intensity surpassing the value stated in the negative control. Final 14 protein candidates were based on the highest obinutuzumab/rituximab intensity ratio.

To assess the impact of candidate proteins on obinutuzumab-induced lymphoma cell death and differentiate them from rituximab, we utilized shRNA-mediated knockdown cells and CRISPR-Cas9 overexpression cells. Among the candidates, MPZL1

demonstrated the most significant influence, and this effect was dependent on the quantity of MPZL1 but unrelated to phosphatase-related signal transduction. Concabody, the anti-CD20 antibody fused with the MPZL1 lignad, concanavalin, exhibited a remarkable increase in DCD for both obinutuzumab and rituximab, with this tendency was effectively recapitulated in the cell death of CD19-expressing B-lymphoma cells derived from the bone marrow in chronic lymphocytic leukemia patients.

In this study, we propose MPZL1 as a protein associated with DCD through the identification of obinutuzumab binding CD20-associated proteins and suggest concabody as a biobetter of obinutuzumab based on MPZL1 dependent DCD mechanisms.

Key words : non-hodgkin lymphoma, obinutuzumab, rituximab, direct cell death, ascorbate peroxidase 2, myelin protein zero like protein 1, concanavalin A

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I. INTRODUCTION

Non-Hodgkin lymphoma (NHL) is a prevalent hematological malignancy which accounts about 3% of cancer diagnoses and deaths[1-2]. Most of NHL is majorly constituted with B cell non-Hodgkin's lymphomas (BNHLs) which are clonal tumours of mature and immature B cells[3-4]. According to the World Cancer Research Fund International, BNHL occurs in more than half a million people worldwide annually[5].

The standard frontline treatment for BNHL remains chemo-immunotherapy with R-CHOP+/- radiation based on disease stage and clinical risk factor[6]. However, to enhance upfront therapy, anti-CD20 monoclonal antibodies (mAb) such as rituximab, obinutuzumab, or ofatumumab are introduced[7]. Since the anti-CD20 antibody has advantage in effective depletion of B cells, recently, it became attractive treatment of antoimmune diseases which involves B cells[8]. CD20's molecular function has been linked to the signaling tendency of the B cell receptor (BCR) and its interactions with numerous surface proteins on B cells, such as CD40, MHCII, CD53, CD81, and CD82[9]. Combining anti-CD20 mAb with BCR signaling inhibitors requires a better understanding of the mechanism of regulation[10].

Anti-CD20 mAb function through several mechanisms, including complement-dependent cytotoxicity (CDC), complement-dependent cellular cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP)[11], and DCD induction[10]. Although rituximab has considerably improved clinical outcomes for patients, many patients experience relapse or fail to respond[12]. Obinutuzumab, a humanized, glyco-engineered type II anti- CD20 mAb, can efficiently induce DCD depending on homotypic adhesion by actin reorganization, Bcl-2 overexpression, and caspase activation[13-16]. This approach potentially leads to improved clearance of B cell malignancies compared to rituximab with increased ADCC and DCD[13]. Obinutuzumab-induced DCD triggers rapid cell death, plasma membrane damage, lysosome membrane permeabilization (LMP), and cathepsin-mediated cell death, bearing the morphologic features of necrosis[14]. DCD and LMP dependent cell death are presumed to be a strong therapeutic target[13-14].

Enzyme catalyzed proximity labeling (PL) in combination with mass spectrometry analysis is emerging as an approach to reveal protein-protein interaction networks, dissect complex biological processes, and characterize the subcellular proteome[15]. APEX2, one of the PL enzymes[16-19], facilitates rapid biotinylation of proteins located within approximately 20 nm by catalyzing biotin phenol radicals in living cells in the presence of hydrogen peroxide[20]. These catalyzed biotin phenoxyl radicals covalently react with electron-rich amino acids like tyrosine, tryptophan, histidine, and cysteine[21].
This approach allows the study of the mechanism of DCD induced by obinutuzumab binding to CD20 by elucidating the involved protein complexes compared to rituximab binding without disrupting the cell membrane complex.

MPZL1 is widely expressed member of the immunoglobulin superfamily and also known as hyperphosphrylated transmembrane glycoprotein involved in extracellular matric-induced signal transduction[22]. MPZL1 is also known as PZR (protein zero receptor) and highly expressed in different cancer cells[23-24]. MPZL1 dependent signaling is also known

to be inducing metastasis and invasion in non-small cell lung cancer[24]. This receptor is known to be major receptor of with plant lectin family, concanavalin A (ConA)[28], and has an important role in cell signaling through inducing Src family tyrosine-protein kinases signalings[25]. In recent times, ConA internalization based autophagy cell death in hepatoma cells[26] and apoptosis induction in varios cancer cell types[28] were reported. Similarly, ConA also known to induce LMP dependent cell death[27].

This study aimed to identify proteins around CD20 binding to obinutuzumab, selectively determine significant proteins related to obinutuzumab-specific LMP and DCD, elucidate the associated molecular mechanisms, and contrive a method to induce enhanced DCD using this information. The identified protein, MPZL1, discovered through this process, led to the development of a ConA-bound anti-CD20 antibody, concabody, exhibiting higher DCD and ADCC compared to obinutuzumab.

II. MATERIALS AND METHODS

1. Cell lines

CHO-K1 and HEK293T were purchased from the Korean Cell Line Bank. Raji cell line was gifted from Prof. Seong Hwan Kim (Chungnam National University, Daejeon). CHO-K1 and Raji Cells were maintained in RPMI1640 media supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO2. HEK293T cells were maintained in DMEM high glucose media supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2. For cell cultures, DMEM culture medium (Dulbecco's modified Eagle medium, 11995-065), RPMI 1640 (Roswell Park Memorial Institute medium, 11875-093), Fetal bovine serum (FBS) (26140-079), Penicillin-streptomycin (15140-122, Gibco, Life technologiesTM, Carlsbad, CA, USA); Trypsin-EDTA 0.05% solution (25300-062, Gibco, Life technologiesTM, Carlsbad, CA, USA) were used.

2. Cloning

Sequences of rituximab (MabThera) was obtained by patent publication (US2015O141620A1). Sequence of obinutuzumab (GA101) was obtained by patent publication (US011110087B2). Anti-CD20 antibodies heavy and light chains were cloned into pLVX recipient vector to produce lentivirus. For APEX2, pcDNA3 Connexin43-GFP-APEX2 was a gift from Alice Ting (Addgene plasmid, #49385)[28]. Concanavalin A sequence was synthesized by Bionics (Seoul, Korea) based on concanavalin A sequence observed by Nonis et. al[29].

3. Lentivirus production

293T cells 8×10^5 per well were seeded in 6 well plates, and after 24 hours, total 3 μg of target DNA and lentivirus component DNA clone pMD2G and psPAX2 were co-incubated in 100 μL of PBS with PEI (polyethylenimine) for 15 minutes and treated to the cells. Media (DMEM full media) was changed after 6 hours incubation. The cell

cultured media was harvested after 48 hours and centrifuged at 4°C for 1000 RCF for 5 minutes. The supernatant was filtered using 0.45 μm PES syringe filter and transferred to a clean tube to remove debris. Store it at −70°C.

4. Antibody production

A. CHO-K1 based antibody production

For anti-CD20 antibodies production, 5×10^4 CHO-K1 cells per well were seeded in a 12 well plate and cultured. After 24 hours, polybrene (ant-pr-1, Invivogen, 10 mg/ml) is pretreated 3 hours before infection. The cells were washed, and lentivirus containing culture media and a fresh culture solution were mixed and treated at a ratio of 1:1 and cultured for 24 hr. The media was changed and selected antibody producing cells by treating puromycin 10 μg/ml for 72 hours. After selection, remaining cells were expanded for antibody production. Obtained cells were incubated in EX-CELL CD CHO media (10743011, Thermofisher) for 2 weeks cells in a 30°C CO_2 incubator. The culture solution was collected and centrifuge at 4°C for 1000 RCF for 3 min to remove the mixed cells by transferring only the supernatant to a clean tube. Store it at 4°C.

B. HEK 293T cell-based antibody production

For APEX2 fused antibodies production, HEK 293T cells 3×10^6 were seeded in 100π culture dish. After 24 hours, 10 μg of APEX2 fused antibody DNA were co-incubated in 200 μl of PBS with PEI for 15 minutes and treated to the cells. Media (DMEM full media) was changed after 24 hours incubation. The cell cultured media was harvested after 120 hours and centrifuged at 4°C for 1000 RCF for 5 minutes. Store it at 4°C.

C. Freestyle 293F cell-based antibody production

For concabody production, freestyle 293F cells 3×10^6 were seeded in erlenmeyer flask. After 24 hours, 20 μg of ConA fused antibody DNA were co-incubated in 500ul of FreeStyle™ 293 Expression Medium (Invitrogen) with PEI for 15 minutes and treated to the cells. The cell cultured media was harvested after 120 hours and centrifuged at 4°C

for 1000 RCF for 5 minutes. Store it at 4°C.

5. Antibody purification

The culture medium containing the antibody was treated with ProA™(rProtein A Agarose Resin, Amicogen, South Korea) and reacted at 4°C for 24 hr. Media was centrifuged at 2000 RPM for 5 minutes. Beads were collected into prepared activated column and wash beads using cold PBS for 3 times. Antibodies were eluted from the beads by elution buffer (75 μL of 0.1 M Citric acid solution) into tubes 25 μL of 1 M Tris for neutralization. Eluted antibodies were dialyzed twice in PBS for 6 hours and concentrated by Amincon® Ultra-15 Centrifugal Filter Units (Merck KGaA, Darmstadt, Germany). Total protein was quantified by NanoDropTM Lite Spectrophotometer.

6. Functional assays

A. Cell binding assay

B lymphoma Raji cells were resuspended in 50 μL of PBS with 7, 21, 35 and 70 nM of anti- CD20 antibodies (obinutuzumab, rituximab), anti-CD20-APEX2 fused antibodies (OA, RA), control antibodies (trastuzumab, TA) or concabodies (Obi-ConA, Rit-ConA) and incubated at 4°C for 30 minutes. After rinsing cells with PBS, cell pellets were resuspended with 50 μL PBS with an anti-human Ig Fc-specific FITC-conjugated secondary antibody (109- 095-008, 1:200 dilution; Jackson Laboratories) at 1:500 dilution and incubated at 4°C for 30 minutes. After washing twice with PBS, a total of 10,000 cells were counted by flow cytometry (FACS LSR II SORP system, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software. For ConA binding assay, 1 μg/ml of concanavalin A (C2272, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was treated for 30 minutes. Anti-streptavidin APC was 1:500 treated for 30 minutes.

B. Direct cell death (DCD) assay

For measurement of DCD, Raji or Ramos cells per well were resuspended into 100 μL of

RPMI full media and then indicated dose of antibody (7, 21, 35 and 70 nM) were treated for 6 hr. For ConA DCD assay, ConA (C0412, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were treated to the cells as indicated concentration. Then, cells were stained with 1 μl of 1 mg/ml PI or 0.5 μl of 0.25 μg/ml Calcein AM for 30 min in 37°C. The PE positive or FITC negative cells were used as a readout of cell death (% of fluorescence exposing cell number among 10,000 counted total cells) was calculated by flow cytometry (FACS LSR II SORP system, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software.

C. Lysosomal membrane permeabilization (LMP) assay

Raji cells 1×10^5 per well were prepared and resuspended into 100 μL of RPMI full media with 7 or 21 nM of obinutuzumab or trastuzumab. After 4 hours incubation, cells were stained with LysoTrackerTM Deep Red (InvitrogenTM, cat. L12492) for 30 minutes in 37°C. The geometric mean change of lysotracker was calculated by flowcytometry (FACS LSR II SORP system, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software.

D. Complement dependent cytotoxicity assay (CDC) assay

Raji cells 1×10^5 per well were prepared and resuspended into 100 μL of RPMI full media with indicated dose of antibodies for 10 minutes. Rabbit complement MA (CL3221, Cedarlane) 1:100 was treated to the cells and incubated for 1 hr at 37°C CO_2 incubator. The percentage of cell lysis was analyzed by FACS Verse (FACS, BD Bioscience, Franklin Lakes, NJ, USA) and calculated by % of PI positive cells among 1x104 total cells counted.

E. Antibody dependent cellular cyototoxicity (ADCC) assay

Peripheral blood mononuclear cells (PBMC) were purified from healthy donors who voluntarily participated in this study, for which informed consent was obtained to the study contents. All of these processes were conducted in accordance with the IRP

procedure (#4-2016-0600) approved by the Yonsei University Institutional Review Committee. Briefly, 6 mL of blood and 6 mL PBS were loaded onto 6 mL Ficoll (Histopaque-1077, Sigma-Aldrich) and centrifuged at 400×g for 30 min at 20 °C to separate white blood cells. The white blood cell layer was collected and washed three times with RPMI-1640 medium to completely remove the platelets. Raji cells were washed with PBS and stained with 0.25 μM calcein-AM. Cells were incubated for 30 minutes and rinsed twice with RPMI full media. Purified PBMC (effector: target = 4: 1) were added and cells were treated with the indicated dose of antibodies and incubated at 37°C in 5% CO_2 for 4 hours. The % of $FITC^+$ cells among total 60,000 PBMC was calculated by flowcytometry (FACSymphony A5, BD Biosciences, Franklin Lakes, NJ, USA) and analysed by FlowJo software. Normalization was done by ADCC % = 100 – (% of Treated/Untreated live cell population) ×100.

F. Chronic Lymphocytic Leukemia (CLL) patient B cell depletion assay

CLL patient cells 1×10^5 per well were prepared and resuspended into 100 μL of RPMI full media with 2.1 nM of antibodies for 6 hours at 37°C CO_2 incubator. B cells were stained using allophycocyanin (APC)-anti-human CD19 antibody (392504, BioLegend, San Diego, USA). The percentage of cell lysis was analyzed by flowcytometry (FACSymphony A5, BD Biosciences, Franklin Lakes, NJ, USA) and analysed by FlowJo software. Normalization was done by B cell depletion % = 100 – (% of treated/untreated live cell population) ×100.

7. APEX2 dependent screening

A. APEX2 proximity labeling optimization

Antibody, biotin phenol, H2O2 treatment concentration and incubation time for APEX2 proximity labeling were optimized using 2×10^5 B cell lymphoma Raji cells. Reaction was conducted in 50 μL of RPMI full media. For antibody concentration optimization, 7, 21, 35, 70 nM of each APEX2 fused antibodies were treated with 0.5 mM of biotin phenol and H_2O_2. For incubation time optimization, 35 nM APEX2 fused antibodies were treated

for 15, 30 minutes and 1 hour. For biotinylation, 0, 0.25, 0.5, 2.5, 5 mM of biotin phenol or H_2O_2 were treated with 0.5 mM of H_2O_2 or biotin phenol to check efficiency of APEX2 depending on reagents. Western blot was used for analysis of biotinylated protein.

B. Protein biotinylation using APEX2 fused antibodies

Based on optimization of APEX2 dependent biotinylation, samples for HPLC-MS/MS were prepared. In 1ml of RPMI full media containing conical tubes, 1×10^7 of Raji cells was prepared, and each antibody (Obi LC-Tra HC-APEX2, Rit-APEX2, Obi-APEX2) were treated to each tube for 30 minutes in 37°C. incubator. During incubation, 2× and 1× quenching solution (0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Trolox, 10 mM Sodium azide, 10 mM Sodium ascorbate, PBS in DW) and 9.8 mM H_2O_2 solution in PBS were prepared. Cells were centrifuged 1000 RCF for 5 minutes and removed supernatant of the cells. The cells were washed twice and resuspended with 1ml of prewarmed RPMI full media with 0.5 mM of biotin phenol. After 1 minute, 0.5 mM of H_2O_2 solution were treated to each tube and incubated for 1 minute. Immediately, 1 ml of 2× quenching solution was put to stop biotinylation reaction. The biotinylated samples were centrifuge at 1000 RCF for 5 minutes, remove supernatant and mix gently with 1ml of 1× quenching solution. After another centrifugation, supernatant was discarded and 1 ml of RIPA buffer with quenching reagents were suspended with cells and incubated for 20 minutes at 4°C. Protein lysates were centrifuged at 18000 RCF for 10 minutes at 4°C, and supernatants with biotinylated proteins were carefully transferred to new tubes.

C. Biotinylated proteins enrichment and purification

Streptavidin agarose beads were used for biotinylated protein enrichment. Supernatant with biotinylated proteins was incubated with beads at 4°C under end-to-end rotation. After 2 hours incubation, tubes were centrifuged for 2 minutes at 500 RCF and washed with 1ml of washing buffer (1 M KCL, 0.1 M Na_2CO_3, 2 M Urea in 10 mM Tris-HCl pH 8 in 1 ml of RIPA buffer). Repeatedly, beads were washed with 1ml RIPA buffer without any detergents (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA). On bead

digestion was done to elute biotinylated proteins. Beads were incubated with 25 μL of elution buffer 1 (50 mM Tris-HCl pH 7.5, 2 M Urea, 5 μg/ml trypsin, 1 mM DTT) in a thermomixer at 30 deg. at 400 RPM. After 30 minutes, beads were centrifuge at 2500 RCF for 2 minutes at 4°C and transferred supernatant to new tube. Additionally, 25 μL of elution buffer 2 (50 mM Tris-HCl pH 7.5, 2 M urea, 5 mM iodoacetamide) were resuspended and centrifuge at 2500 RCF for 2 minutes at 4°C for twice. Elution 1 and 2 were mixed and incubated in thermomixer at 32 °C at 400 RPM overnight to continue digestion (protected samples from light). After 12 hours, reaction was stopped by adding 1 μL trifluoroacetic acid.

8. Western blotting

Obtained lysates samples from same number of cells in RIPA buffer were mixed with 6× lammeli sample buffer and 20 μL of each sample were loaded on acrylamide gel to separate the protein depending on size by electrophoresis. Gel with separated proteins was transferred to a nitrocellulose (NC) membrane. The proteins electrically delivered to the NC membrane was blocked with TBS-T containing 5% non-fat milk and 0.1% Tween 20. After 1 hour incubation at room temperature, primary antibody was reacted for another 1 hour. After washing three times with TBS-T for 10 minutes each, the secondary antibody was reacted for 1 hour at room temperature. After washing three times with TBS-T for 10 min each, proteins were detected by using ECL solution.

9. Silver staining

Obtained lysates samples from same number of cells in RIPA buffer were mixed with 6× lammeli sample buffer and 20 μL of each sample were separated on Pre-cast 10% SDS-PAGE gels in both reducing and non-reducing conditions. Gel was stained by PierceTM Silver Stain Kit (ThermoFisher Scientific, cat. 24612)

10. SDS-PAGE gel electrophoresis

After quantification, 3 μg of antibodies or protein samples were mixed with 6×laemmli

sample buffer and separated on 10% SDS-PAGE gels in both reducing and non-reducing conditions. Gel was stained by Coomassie blue.

11. High performance liquid chromatography – Mass spectrometer analysis

Protein samples digested from streptavidin agarose beads were isolated to new tube for HPLC- MS/MS (Bruker, timsTOF Pro 2). HPLC-MS/MS analysis was conducted by Korea Basic Science Institute (KBSI, Seoul, South Korea). For qualitative analysis, final protein target lists were filtered out from analyzed data based on following strategies:

1. Intensity of each detected protein was normalized by total intensity acquired by analysis. Lists were ordered in intensity descending manner.
2. Normalized intensity from obintuzumab-APEX2 and rituximab-APEX2 were divided to calculate fold changes between two anti-CD20 antibodies.
3. Protein detected by more than 2 unique peptides were selected. Protein lists overlapping with control-APEX2 were discarded.
4. Proteins showing larger than >1.4-fold changes were selected.
5. Plasma membrane proteins were selected as final protein targets.

12. Knockdown and overexpression system development

A. Doxycycline induced shRNA dependent knockdown system development

Doxycycline induced shRNA dependent knockdown system was developed by cloning lentiviral vectors with target shRNA antisense guides. Antisense guide RNA sequences targeting UTR acquired by SplashRNA website (http://splashrna.mskcc.org/) were amplified by PCR and amplification products. In LT3GEPIR plasmid with pRRL backbone (Addgene, #111177)[31], amplified products were XhoI/EcoRI cloned. Cloned vector was transfected with psPAX2 and pMD2G to 8×10^5 of 293T cells for lentivirus production. Obtained lentivirus was transduced to 5×10^4 of Raji cells and selected by 5 μg/ml of puromycin. After selection, 1 μg/ml of doxycycline was treated to 1×10^6 of shRNA inducing Raji cells, and GFP positive cells were isolated by sorter (FACS Aria III, BD Biosciences, Franklin Lakes, NJ, USA).

B. Target protein CRISPR-Cas9 based overexpression system development

For overexpression system development, lentiMPH v2 (Addgene plasmid, #89308)[32], and lentiSAMv2 (Addgene plasmid, #75112) was a gift from Feng Zhang[32]. For the guide RNA sequence, sgRNA sequences were designed by CRISPick (Broad Institute, https://portals.broadinstitute.org/gppx/crispick/public).

C. MPZL1 Y241F dominant mutant cells development

The MPZL1 clone (hMU008848) was provided from Korea Human Gene Bank, Medical Genomics Research center, KRIBB, Korea. MPZL1 Y241F domoinant mutation was generated by PCR amplification using following primers:

XbaI_MPZL1_For: TATATATCTAGAATGGCAGCGTCCG,

Esp3I_MPZL1 Y241F_Rev: TATATACGTCTCTGGGCCCTGGTGAGATCC,

Esp3I_MPZL1 Y241F_For: TATATACGTCTCGGCCCAGTCATATTTGCACAG,

NotI_3XFLAG stop_Rev: TATATAGCGGCCGCTTACTTGTCATC.

13. RNA extraction and Real time PCR

Total 3×10^6 shRNA inducing Raji cells were prepared by treating 0.1 μg/ml of doxycycline for 3 days. RNA was isolated by suspending 500 μL of Trizol with cells and incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. After incubation, 100 μL of chloroform was added, vortexed vigorously and incubated at room temperature. After 3 minutes, samples were centrifuged at 13000 RCF for 15 minutes at 4°C. Colorless upper aqueous phase was carefully transferred to new tube by without disturbing the interphase. For RNA precipitation, 250 μL of isopropanol was mixed and incubated for 10 minutes at room temperature. Solution was centrifuged at 13000 RCF for 10 minutes at 4°Cm and RNA pellet was washed twice using 500 μL of cold 75% ethanol. RNA pellet was centrifuged at 7500 RCF for 5 minutes and removed all leftover ethanol for 5 minutes drying in vacuum. RNA pellet was dissolved with 50 μL of DW and quantified by NanoDropTM Lite Spectrophotometer.

Extracted RNA was reverse transcribed by using SuperiorScript III cDNA Synthesis Kit (Enzynomics, cat. EZ405S). Acquired cDNA was used to quantify knockdown efficiency by using QuantStudio 3 Real-time PCR instrument (Applied Biosystems) with AccuPower® 2×Greenstar qPCR master mix (Bioneer, K-6251). The PCR program conditions are following: 95°C, 2 mins. (initial denaturation) / 95°C, 10 sec; 58°C, 30 sec (Denaturation, anneal, elongate, measure) / melt curve step was followed default setting of the machine manual. Data was normalized by calculating ΔΔCT values between GABDH and target genes.

14. Gene ontology

Gene information was acquired by Genecards.org, and protein information was acquired by Uniprot.org. Gene ontology and classification were analyzed by Metascape.org.

15. Statistical analysis

Statistical analyses were performed using Prism v9 (GraphPad Software). Data are presented as the means ± standard deviation as indicated in the figure legends. Statistical significance was assessed by Welch's multiple unpaired t test. Significance is indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

III. RESULTS

1. APEX2 fusion antibodies development and optimization

Biotinylation of proteins around CD20 in which anti-CD20 antibodies bind was conducted by developing and optimizing antibody design and proximity labeling conditions. First design was APEX2 directly fused form at the end of Fc region of each antibodies (Fig. 1A). As a negative control antibody (Obi^{m}-A), obinutuzumab light chain (LC) N97A mutation was generated[33]. Successfully purified antibodies were all qualified by SDS PAGE, but binding affinity of APEX2 fused antibodies (OA, RA) was decreased when it is compared to wild type antibodies (Obi, Rit). Since negative control (Obi^{m}-A) unexpectedly showed remained binding affinity to the CD20s (Fig 1B), we generated second generation of APEX2 fusion antibodies with 4×G4S linker between Fc region and APEX2 (Fig. 1C). Purified antibodies were successfully purified (Fig. 1D), and functionality in both binding and death were all maintained as wild type antibodies (Fig. 1E).

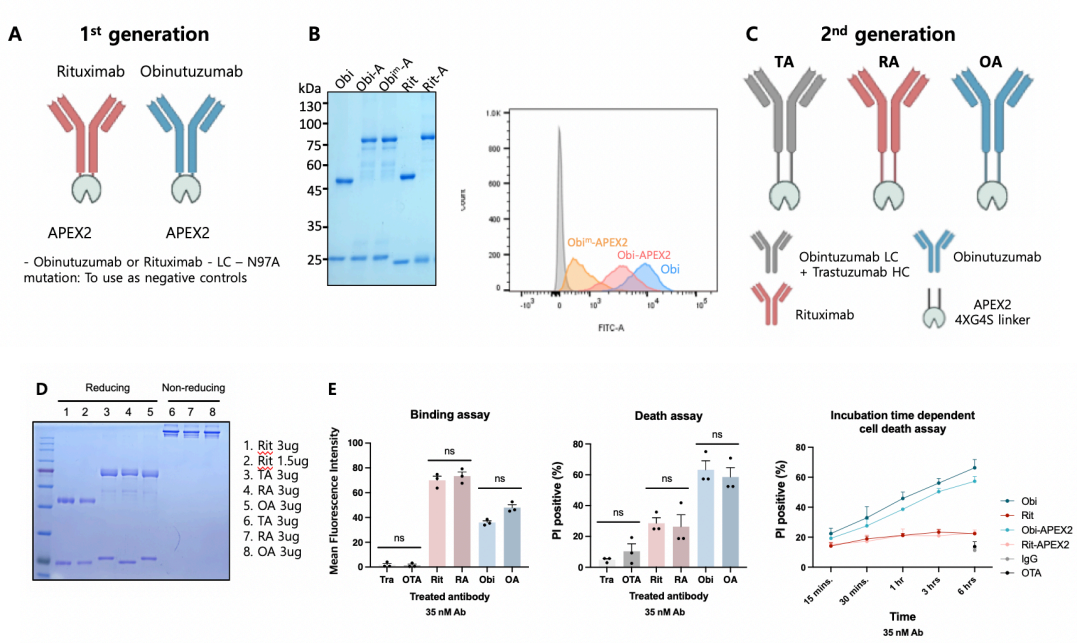

Figure 1. Development of APEX2 fused anti-CD20 antibodies for proximity labeling. (A) Developed first design was APEX2 directly fused form at the end of Fc region of each antibodies. Negative control antibody (Obi^{m}-A), obinutuzumab light chain (LC) N97A mutation was generated. (B) Purified antibodies validation by SDS PAGE coomassie blue staining was done in reducing condition. Binding toward CD20 was measured by flowcytometry. (C) Development of second generation APEX2 fused antibodies with 4×G4S linker. (D) Purified antibodies validation by SDS PAGE coomassie blue staining was done in both reducing and non-reducing conditions. (E) Functionality in both binding and death were analyzed by flowcytometry. Geometric fluorescence intensity of FITC conjugated secondary antibodies of each antibodies were analyzed for binding assay. Calcein-AM staining was used for cell death assay. All experiments were conducted using B cell lymphoma Raji cells with indicated antibody concentrations.

2. APEX2 dependent proximity labeling and HPLC-MS/MS analysis

Next, we conducted proximity labeling condition optimization. Protein biotinylation efficiency was analyzed in different biotin-phenol, H_2O_2, antibody concentration and treatment time. As a result, 0.5 mM of biotin-phenol and H_2O_2 (Fig. 2A), 5 μg/ml (35 nM) of each APEX2 fused antibodies (Fig. 2B), and 30 minutes of antibody incubation time before biotinylation (Fig. 2C) were fixed, and biotinylation and different patterns of eluted proteins of each antibody were analyzed by western blotting (Fig. 2D) and silver staining (Fig. 2E). On-bead digestion of enriched samples was conducted for further HPLC-MS/MS analysis. The protein lists of each antibodies were acquired (Table 1), and obinutuzumab dominant 14 candidates were selected by qualitative strategies (Fig. 3A, B). Subcellular localization of detected proteins was mainly analyzed as membrane proteins, especially plasma membrane as we designed (Fig. 3B).

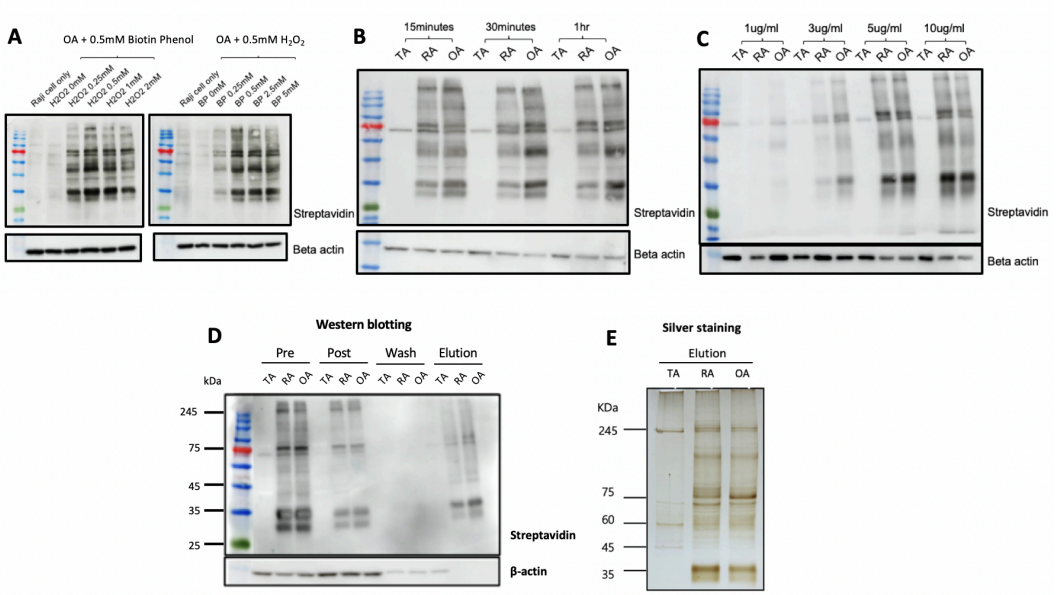

Figure 2. Proximity labeling condition optimization. (A)-(C) Biotinylation efficiency and patterns were analyzed by western blot. (A) Titration of biotin phenol (BP) and H_2O_2. OA antibodies were pre-incubated for 30 min with the indicated concentrations of BP or H_2O_2 (0, 0.25, 0.5, 1, 2 mM for H_2O_2, 0, 0.25, 0.5, 2.5, 5 mM for biotin phenol), followed by the addition of 0.5 mM H_2O_2 or BP for 1 min. (B) Incubation time and (C) antibody concentrations dependent biotinylation efficiency analyzed by adding 0.5 mM of BP and H_2O_2. 5×10^5 Raji cells per sample were used for biotinylation. Cell lysates were probed with Streptavidin-HRP. (D) 5×10^6 Raji cells with 5 μg/ml of each antibody, 0.5 mM of biotin phenol and H_2O_2 were used for MS/MS sample preparation and shows enriched samples by western blot and silver staining (E). Streptavidin agarose bead was used to enrich biotinylated proteins. On-bead digestion of enriched samples was conducted for further HPLC-MS/MS analysis.

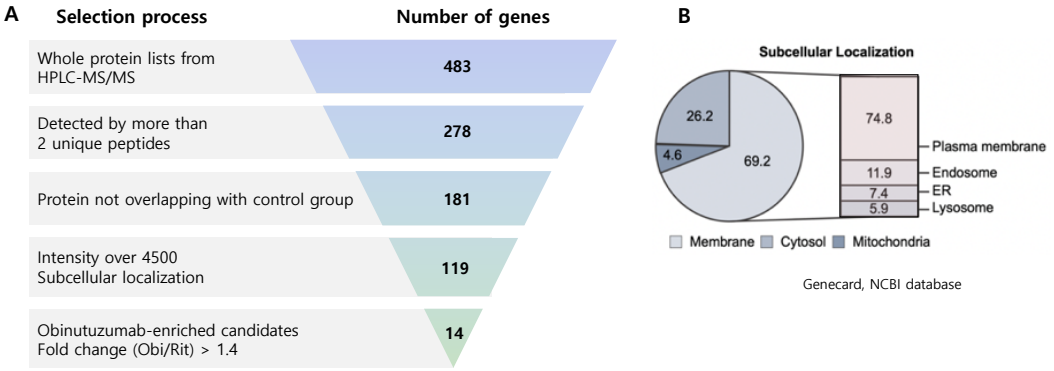

Figure 3. HPLC-MS/MS results and candidate selection. The protein lists captured by each antibody were acquired (Table 1) (A) Obinutuzumab-dominant 14 candidates were selected by following qualitative strategies: 1. Intensity of each detected protein was normalized by total intensity acquired by analysis. Lists were ordered in intensity descending manner. 2. Normalized intensity from OA and RA were divided to calculate fold changes between two anti-CD20 antibodies. 3. Protein detected by more than 2 unique peptides were selected. Protein lists overlapping with TA were discarded. 4. Proteins showing larger than >1.4-fold changes were selected. 5. Plasma membrane proteins were selected as final protein targets. (B) Subcellular localization of detected proteins was analyzed by Genecards, NCBI databases.

3. Therapeutic target selection and functional validation in knockdown and overexpression systems

Direct cell death involvement of each protein candidates was validated by LMP and DCD assays in knockdown (KD) and overexpression (OX) systems. However, among 14 candidates, two samples sh4 and sh11 were not able to developed as knockdown cells, so these two were unfortunately discarded from our candidates. Doxycycline treatment for 6 days induced shRNA and lowered expression level was quantified by real-time PCR (Fig. 4A). Including CD20 KD cells, 6 proteins showed significant difference in LMP dependent death level between doxycycline untreated to treated cells when obinutuzumab was treated to each cell lines (Fig. 4B). DCD assay was conducted to confirm if those proteins were really involved to DCD and the possibilities to be our new therapeutic target. As a result, sh3 which is MPZL1, showed consistency with LMP results and most significant difference in KD systems (Fig. 4C). The reduced DCD level was maintained when doxycycline was treated for longer days (Fig. 4D). Since CD20 binding had no difference between normal and KD cells, these LMP and DCD level changes were not because of antibody function itself but MPZL1 expression and functions (Fig. 4E). Reversely, in MPZL1 overexpressed cells by CRISPR-Cas9 validated by real-time PCR and western blot (Fig. 4F), DCD level was significantly increased (Fig. 4G) as we expected. Thus, we selected MPZL1 as our new therapeutic target for further studies to enhance DCD function in anti-CD20 antibodies.

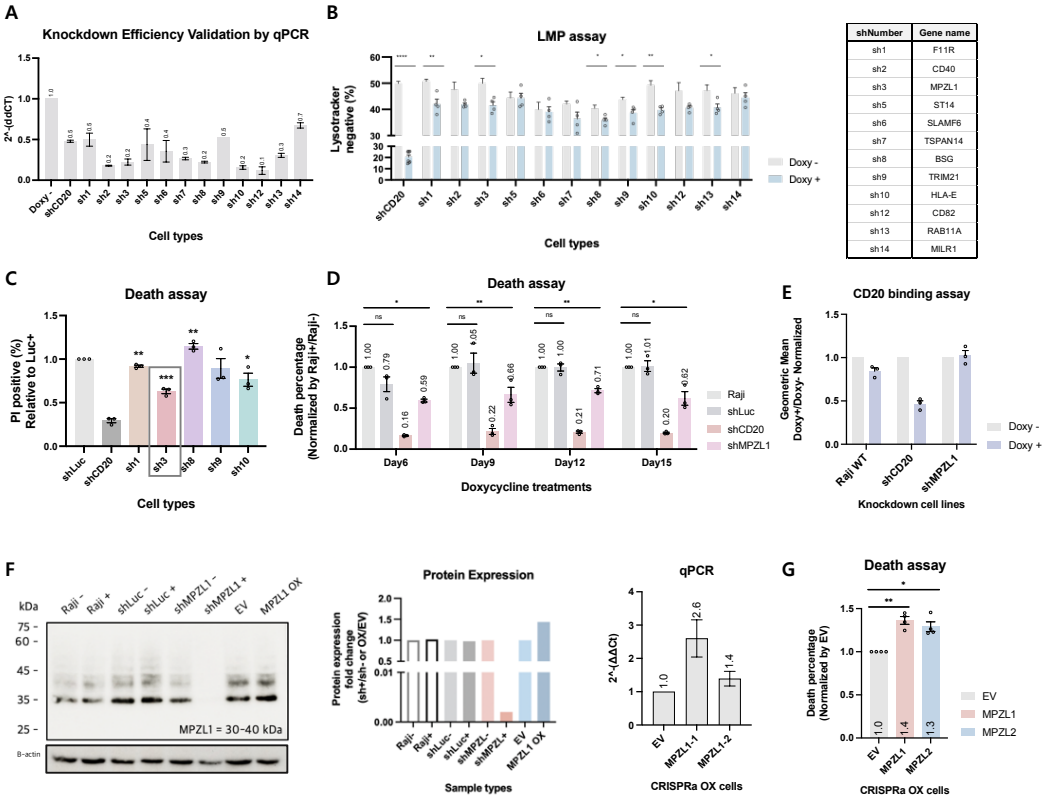

Figure 4. Final therapeutic target selection in different cell systems. (A) Knockdown efficiency of each targets was analyzed by real-time PCR quantification. Knockdown cell lines were developed by introducing doxycycline induced shRNA system to Raji cells. (B) LMP assay was conducted by lysotracker deep red staining after treating obinutuzumab to each target knockdown systems. (C) DCD assay was conducted only with genes that showed significant differences between doxycycline – and + samples. PI positive staining after obinutuzumab was detected. (D) Final selected target DCD analysis in knockdown system depending on doxycycline treatment days. (E) CD20 binding assay in MPZL1 knockdown system. (F) MPZL1 overexpressed cells by CRISPR-Cas9 validated by real-time PCR and western blot. (G) DCD assay in MPZL1 overexpression system. Binding, LMP, DCD assays were all conducted in 1×10^5 Raji cells and analyzed by flowcytometry.

4. Contribution of concanavalin A and development of Concabody for improving DCD effect of the anti-CD20 antibodies

Based on research reports, we found that MPZL1 is the major receptor of lectin concanavalin A (ConA) which causes autophagic and lysosomal rupture dependent cell death by internalization. We tested if ConA can induce LMP dependent cell death also in B cell lymphoma Raji cells, and it caused LMP dependent cell death in dose dependent manner (Fig. 5A). In addition, this death percentage was decreased in MPZL1 knockdown cells (Fig. 5B), and this infers that ConA can work as simulator of MPZL1 in B cell lymphoma cells. By this result, we hypothesized that ConA fused anti-CD20 antibodies can work as DCD booster while non-specific toxicity of ConA can be reduced by antibody affinity to CD20s of lymphoma cells. In order to access functional assays, ConA was fused at the end of Fc region of anti-CD20 antibodies and purified (Fig. 5C). Single ConA or anti-CD20 antibodies, ConA-fused antibodies treatments were compared to ConA and antibody co-treated DCD level, and in both anti-CD20 antibodies. ConA fused anti-CD20 antibodies showed significantly increased DCD effects in Raji cells, while ConA fused negative control does not showed toxicity (Fig. 5D). ConA fused antibodies functional dependency on MPZL1 was analyzed in MPZL1 knockdown cells which resulted that ConA fused antibodies showed decreased death level than both wild type antibodies or fused antibodies in control cells (Fig. 5E, F). This infers that ConA fused antibodies increase direct cell death function through MPZL1 dependently. Additionally, ConA fused antibody working mechanism was analyzed using MPZL1 Y241F mutation[35], which is dominant negative mutation of phosphorylation that inhibits further signalings of MPZL1 (Fig. 5G). However, compared to EV (empty vector) control samples, MPZL1 mutant cells do not showed significant difference by ConA fused treatment which means this ConA fused Abs are working MPZL1 signaling independent manner.

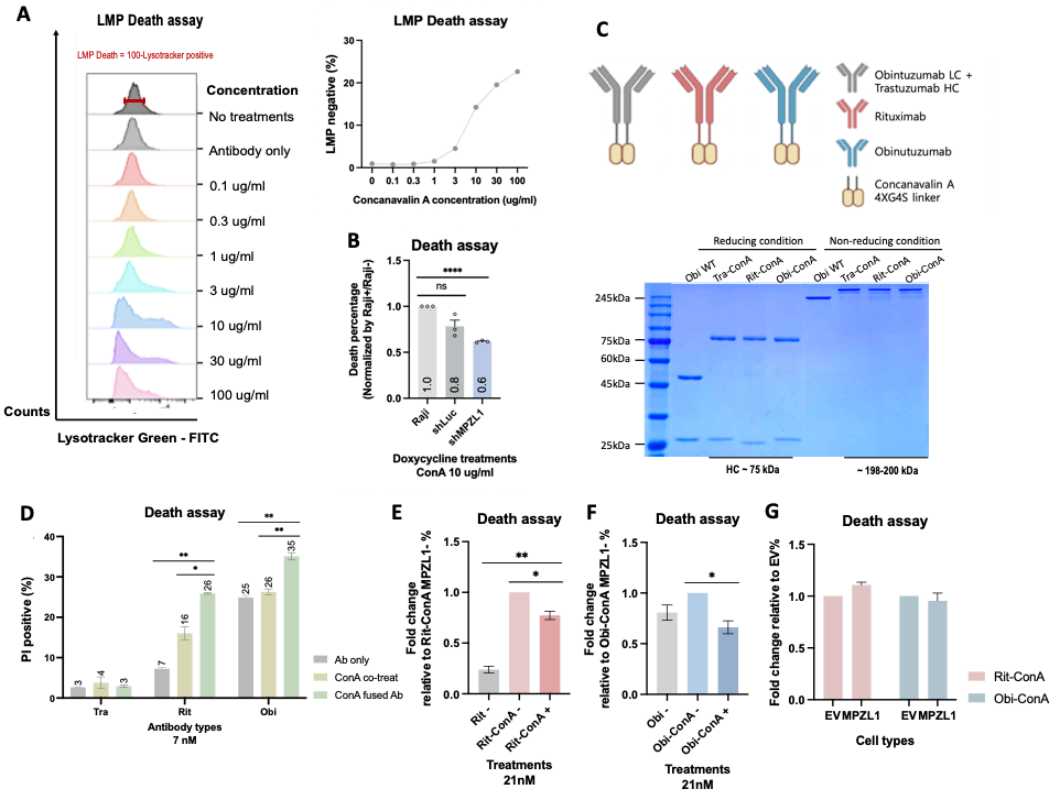

Figure 5. Concabody, the concanavalin A fused anti-CD20 antibody. (A) Concanavalin A was treated in Raji cells, and LMP assays were conducted in dose dependent manner. (B) DCD assay in MPZL1 knockdown system was done by treating 10 μg/ml of ConA. (C) Development of concabody, and purified antibodies validation by SDS PAGE coomassie blue staining was done in both reducing and non-reducing conditions. (D) DCD assay was conducted with anti-CD20 antibodies only, ConA and anti-CD20 antibodies co-treat, and ConA fused antibody treatments. (E) ConA fused antibodies (E) Rit-ConA Ab and (F) Obi-ConA Ab functional relationship with MPZL1 were analyzed by death assay in control cells (−) MPZL1 knockdown cells (+). (G) ConA fused antibody working mechanism was analyzed by death assay in MPZL1 Y241F mutant cells which contains phosphorylation dependent signaling inhibition mutation.

5. Concabody for improving DCD effect of the anti-CD20 antibodies

As ConA fused Abs can induce both direct cell death and LMP dependent death, it was analyzed in 2 different B cell lymphoma Raji cells (Fig. 6A) and Ramos cells (Fig.6B). Additionally, antibody dependent cellular cytotoxicity (ADCC) function was increased as direct cell death in B cell lymphoma Raji cells (Fig. 6C). However, even though death ability in ConA fused Ab was increased in both Raji and Ramos cells, B cell depletion ability in CLL patient cells showed no significant difference in Rit and Rit-ConA antibodies (Fig. 6D). Thus, this infer that ConA can increased LMP dependent DCD effects of no DCD inducing antibodies and can be suggested as new therapeutic tool to overcome limitation of wild type antibodies..

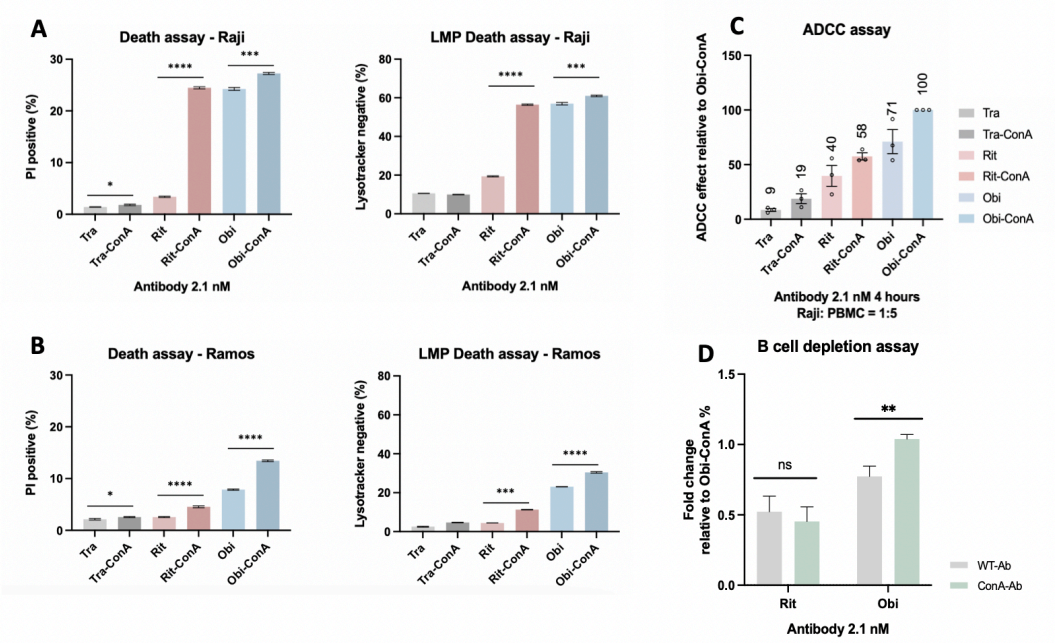

Figure 6. Concabody death functional assays in different B cell lymphoma. ConA fused antibodies' LMP and DCD assays were conducted in 2 different B cell lymphoma, 1×10^5 Raji cells (A) and Ramos cells (B). (C) Antibody dependent cellular cytotoxicity ability was analyzed by co-incubating 1×10^4 Raji cells with 5×10^4 of healthy donor PBMC cells. (D) Additional B cell depletion ability was analyzed in CLL patient cells. B cell population was counted by CD19-APC positive cells.

IV. DISCUSSION

Non-Hodgkin lymphoma (NHL) is a prevalent hematological malignancy which accounts about 3% of cancer diagnoses and deaths[1-2]. Most of NHL is majorly constituted with B cell non-Hodgkin's lymphomas (BNHLs) which occurs in more than half a million people worldwide annually[5]. The standard treatment for BNHL remains chemo-immunotherapy with R-CHOP +/− radiation[5], but anti-CD20 monoclonal antibodies (mAb) such as rituximab, obinutuzumab, or ofatumumab are introduced[6] to enhance therapeutic effects. Obinutuzumab, is a humanized type II anti-CD20 monoclonal antibody showing increased antibody-dependent cell-mediated cytotoxicity (ADCC) and induces direct cell death (DCD) relative to type I anti-CD20 antibodies. However, molecular biological mechanisms and regulatory proteins related to induced DCD are poorly understood. In this study, through APEX2 proximity labeling, it allowed the study of the mechanism of DCD induced by obinutuzumab binding to CD20 by elucidating the involved protein complexes compared to rituximab binding without disrupting the cell membrane complex.

Construction and optimization of anti-CD20-APEX2 antibodies allowed biotinylation of previously unidentified protein complexes around CD20s that cooperate with DCD condition (Fig. 1C, E). APEX2-fused rituximab and obinutuzumab maintained the antibody characteristics such as CD20 affinity, DCD pattern and biotinylation activity of APEX2, but the biotinylation patterns were markedly different (Fig. 2D, E). Obinutuzumab dominant proteins relative to rituximab were selected by qualitative strategies (Fig. 3A, B). Total 14 protein candidates' DCD involvement was validated through analyzing lysosome membrane permeability (LMP) dependent or direct cell death effects in doxycycline inducible shRNA knockdown (Fig. 4A) and CRISPR-Cas9 based overexpression (Fig.4G) system. In knockdown system, F11R, MPZL1, HLA-E genes showed lowered level in both LMP and DCD, while other genes did not come up with consistent results in both assays. Among those three candidates, we chose myelin protein zero like protein 1, MPZL1, which diplayed most significantly decreased DCD effect in knockdown system as our new

therapeutic target. This result was additionally validated in overexpression system which appeared to increase DCD effects as we expected.

Based on research reports, we hypothesized that MPZL1 major binding ligand conacanavalin A (ConA) might increase DCD function by stimulating MPZL1 dependent down signalings or inducing cell death by internalization of ConA. It was validated by treating ConA to the B cell lymphoma Raji cells and showed LMP dependent DCD (Fig. 5A). This result was amplified when ConA was co-treated with anti-CD20 antibodies, especially rituximab which does not showed DCD (Fig. 5D). Since ConA binds to glycosylated proteins, non-specific binding death induction was concerned. To minimized toxicity due to non-specific binding, we fused ConA to the Fc region of anti-CD20 antibodies (concabody) as we did in development of APEX2 (Fig. 5C). Interestingly, concabody showed synergistic effect compared to ConA and anti-CD20 antibodies co-treatment while toxicity was extremely low (Fig. 5D). Concabody MPZL1 dependency and working mechanism was analyzed in knockdown cells (Fig. 5E, F) and dominant negative MPZL1 Y241F mutant cells (Fig. 5G). These results showed that concabody works as death indubcer in MPZL1 dependent manner but not depending on MPZL1 phosphorylation related death signalings. Since ConA and obinutzumab both are known to act by endocytosis, concabody internalization dependent death mechanism should be more discussed in further studies.

The possibility of application of concabody in different B cell lymphoma was analyzed through death and B cell depletion assay in Raji (Fig. 6A), Ramos (Fig. 6B), CLL patient cells (Fig. 6C). Profound DCD increase were observed in both Raji and Ramos treated with Rit-ConA and Obi-ConA and Obi-ConA also effective in in CLL patient B cell depletion, although Rit-ConA Ab showed no significant difference in CLL patient B cell. In addition to direct cell death, ADCC effects were also increased in concabodies compared to wild type antibodies. In previous our study, we have suggested that anti-TNFα/CD20 antibodies which showed higher direct cell death boosted ADCC effects34 which is consistent with

this results. As a result, we infer that ConA can increased LMP dependent DCD effects also in no DCD inducing antibodies and also can induce ADCC effects which can be more powerful therapeutic tool to overcome limitation of wild type antibodies, even this could be depending on B cell lymphoma types.

V. CONCLUSION

In this study, we developed anti-CD20-APEX2 antibodies to allow biotinylation of previously unidentified protein complexes around CD20 that cooperate with DCD condition. Both of rituximab or obinutuzumab fused APEX2 still remained the antibody characteristics such as CD20 affinity, DCD pattern and biotinylation activity of APEX2, but the biotinylation patterns were markedly different. Different patterns of biotinylated proteins were analyzed through HPLC-MS/MS. Obinutuzumab dominant 14 protein candidates were validated to figure out most significantly involving protein component in LMP dependent DCD effects. Through two different systems, doxycycline inducible shRNA knockdown system and CRISPR-Cas9 based overexpression system, candidates' influences in DCD were validated. As a result, MPLZ1 was selected as out new therapeutic target, and its major ligand, ConA, treatment to B cell lymphoma cells showed enhanced LMP dependent DCD level when it is co-treated with anti-CD20 antibodies. This effect was synergistically increased by fusing ConA to the antibodies (concabody), especially rituximab. As a result, this study suggests that concabody might be applied as B cell lymphoma treating therapeutics which is able to increase death level in both rituximab and obinutuzumab.

This study suggests that the amount of MPZL1 is important for the molecular mechanisms involved in the DCD process. Based on this mechanism, we propose concabody, a ConA-binding anti-CD20 antibody, as a biobetter that exhibits higher DCD and ADCC than obinutuzumab.

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APPENDICES

Table 1. Analyzed APEX2 fused antibody dependent proximity labeled protein lists through HPLC-MS/MS

Rituximab-APEX2 lists				Obinutuzumab-APEX2 lists			
Intensity Fold	**Gene**	**Intensity Fold**	**Gene**	**Intensity Fold**	**Gene**	**Intensity Fold**	**Gene**
Rit Exclusive	FCRL3	0.62	SLC39A10	Obi Exclusive	IGHV1-69	1.54	TSPAN14
Rit Exclusive	ATP11C	0.61	PLXNA1	Obi Exclusive	HNRNPH1	1.51	SH2D1A
Rit Exclusive	MFGE8	0.61	PTPRC	Obi Exclusive	CORO1A	1.5	CD40
0.94	ALCAM	0.61	CD46	Obi Exclusive	ACLY	1.47	ST14
0.91	CD22	0.6	CD19	Obi Exclusive	RAB11A	1.45	CD82
0.88	SLC3A2	0.59	PLXNB2	7.66	LDHB	1.44	F11R
0.84	ITGA4	0.57	MET	6.21	RPS25	1.36	HSP90AA1
0.84	ATP1A1	0.51	CD83	4.8	TRIM21	1.34	UBC
0.78	ITGB1	0.45	SLC39A6	4.43	BSG	1.26	B2M
0.78	ADGRE5	0.37	PTPRJ	2.88	HLA-E	1.26	MPZL1
0.76	ITGB2	0.36	NOTCH2	2.5	CCT7	1.21	HLA-DQA1
0.75	ICAM1	0.36	NEO1	2.22	CCT8	1.16	HLA-C
0.74	ADAM17	0.34	ATP1B3	2.13	PTBP1	1.15	CD37
0.73	CR2	0.32	DAG1	2.08	SLAMF6	1.15	CD38
0.72	NRP2	0.2	CANX	2.08	RPS18	1.12	HLA-DRA
0.72	ADAM10	0.16	TMEM30A	1.86	CCT2	1.12	HLA-DRB3
0.68	TFRC	0.14	IL21R	1.77	MILR1	1.07	HLA-A
0.67	IGSF8	0.09	LNPEP	1.75	MPZL1	1.07	FCGR2B
0.66	CD72	0.06	PODXL	1.62	YWHAZ	1.06	SLC2A5
0.64	ECE1	0.02	HLA-DQA2	1.6	VCP	1.05	SEMA4A

ABSTRACT(IN KOREAN)

항 CD20 항체 세포 직접 사멸 기능 증진을 위한 APEX2 에 의한 근거리 표지 기반 치료용 타겟 발굴

<지도교수 김주영>

연세대학교 대학원 의과학과

최아연

비호치킨성 림포마는 성숙 및 미성숙 B 세포의 클론성 종양으로 세계적으로 매년 50만명 이상에서 발병하는 림프종이다. 최근 개발된 항암항체인 오비누투주맙은 특징적으로 리툭시맙과 같은 type I항 CD20항체와 다르게 항체결합에 의한 직접 세포 사멸을 유도한다. 그러나 직접 세포 사멸 유도와 관련된 분자 생물학적 메커니즘은 라이소좀의 붕괴를 통한 다는 것 외에는 잘 알려져 있지 않다.

본 연구에서는 오비누투쥬맙 결합 CD20주변 단백을 탐색하여 이 결합에 의한 세포사멸을 조절할 수 있는 단백을 발굴하고자 오비누투쥬맙의 Fc 부분 말단에 ascorbate peroxidase (APEX2)를 융합한 항체를 이용하여 리툭시맙과 오비누투쥬맙이 결합한 CD20의 20 nm 반경 내로 biotin 표지된 단백들을 액체 크로마토그래피-질량분석을 이용하여 동정하였다. 동정된 482가지 단백중에서 오비누투쥬맙에 더 근거리로 존재하는 후보군을 구축하기 위해 대조군 그룹과 동일하게 검출된 단백들은 제거한 후, 해당 단백절편이 2개 이상이 검출된 최종 세포막 단백질 중 Obi/Rit Intensity 비율이 높은 순으로 단백질 14개를 선정하였다. 후보유전자군들의 오비누투주맙 라이소좀 붕괴와 직접 결합에 의한 세포사멸에 미치는 영향을 확인하기 위해 각 shRNA에 의한 발현 저하 세포와 CRISPR-Cas9 시스탬을 이용한 과발현 세포를 구축하여 관련성을 분석하였다. 그 중 MPZL1이 가장 유의한 영향을 미치는 것으로 확인되었고, 이러한 영향은 MPZL1의 양에 의존적이였으나 인산화관련 신호단전달과는 무관하였다.

MPZL1 ligand로 알려진 concanavalin A를 항 CD20 항체에 결합한 형태인 concabody는 오비누투주맙뿐 아니라 리툭시맙의 직겁 결합에 의한 세포사멸을 매우 극적으로 증가시켰을 뿐 아니라 이러한 경향은 항체 의존적 세포 염증 반응에서도 증가된 효과를 보였다. 특히 Obi-ConA의 경우, CLL환자 골수에서 유래한 CD19발현 B림프암세포사멸에서도 증가된 효과를 나타내었다.

본 연구는 오비누튜주맙과 결합하는 CD20주변단백의 탐색을 통해 DCD와 관련된 기전단백으로 MPZL1을 제시할 뿐 아니라 이 기전을 이용한 concabody를 오비누투주맙의 바이오베터로 제안한다.

핵심되는 말 : B-세포 비호지킨 림프종, 오비누투주맙, 리툭시맙, 직접 세포 사멸, APEX2, MPZL1, Concanavalin A