Anti-CD20 / TNFR1 bispecific and fusion antibodies for improving treatment of B cell Non-Hodgkin's lymphoma

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Directed by Professor Joo Young Kim

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ABSTRACT

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BNHL are clonal tumors of mature and immature B cells. BNHL occurs more than half a million people worldwide each year. Rituximab has greatly improved the outcome for patients with BNHL. Nevertheless, disease resistance or relapse occur in all BNHL subtype patients. Obinutuzumab was developed for these rituximab resistant or relapsed patients. Obinutuzumab has a distinct mode of action in that working primarily by LMP and DCD. the mechanism of LMP and DCD by obinutuzumab remains unclear. We are interested in the specific mechanisms of LMP dependent DCD, and especially increasing the LMP dependent DCD are expected to be a strong therapeutic option. In clinical studies, TNFR1 expression shows poorer prognosis in Many BNHL subtypes. And similar to obinutuzumab, TFNR1 also induces LMP dependent cell death. Based on this evidences, we hypothesize that there would be synergistic effects when the mechanism of obinutuzumab and TNFR1 is achieved simultaneously, and we developed bispecific antibody and fusion antibodies against CD20 and TNFR1 and confirmed the effects.

Here, we confirmed the colocalization of CD20 and TNFR1 after obinutuzumab binding. Based on this results, we developed an anti-CD20 / TNFR1 bispecific and obinutuzumab × TNFα fusion antibodies (Obi × TNF WT and Obi × TNF Mutant) capable of binding to both CD20 and TNFR1 to strengthen obinutuzumab induced DCD. Bispecific antibody showed lower DCD but Obi × TNF WT

and Mutant fusion antibodies increased DCD compared to obinutuzumab. Based on the previous results that increased DCD by antibody could enhance ADCC, we tested that Obi × TNF fusion antibodies would enhance ADCC more than obinutuzumab. Obi × TNF Mutant fusion antibody induced ADCC almost two fold than obinutuzumab. Finally, we demonstrated that this enhanced DCD and ADCC are induced by TNFR1 in the TNFR1 overexpressed cell model. Besides, Obi × TNF Mutant not only does not cause, but also block NF-κB signaling induced by TNFα, thereby implying that it also works as a blocker of NF-κB signaling.

Accordingly, our study suggests the possibility that CD20 and TNFR1 dual targeting can be a good therapeutic strategy for improving treatment of BNHL, and our Obi × TNF Mutant fusion antibody with enhanced DCD and ADCC will provide bio-better therapeutic for BNHL patients.

Key words: BNHL, obinutuzumab, CD20, TNFR1, TNFα, bispecific and fusion antibodies, LMP, DCD, ADCC

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I. INTRODUCTION

B-cell Non-Hodgkin's lymphoma (BNHL) are clonal tumors of mature and immature B cells that constitute the majority (80-85%) of non-Hodgkin lymphomas (NHLs).[1,2] According to World Cancer Research Fund International, BNHL occurs more than half a million people worldwide each year.[3] Although BNHL occurs mostly in the West, the number of patients with BNHL is gradually increasing in Korea as well.[4] Therefore, research and development of treatments for BNHL have been done for a long time and are needed even more in the future.

The appearance of the anti-CD20 antibody rituximab in 1997 has greatly improved the outcome for patients with BNHL.[5] Nevertheless, disease resistance or relapse occur in virtually all BNHL subtype patients with follicular lymphoma and chronic lymphocytic leukemia (CLL) , and about half of patients with aggressive BNHL, for example, diffuse large B cell lymphoma (DLBCL).[3,6] In order to overcome rituximab resistance, many associated studies have been conducted,[7] and 2nd generation anti-CD20 antibodies,[8] combined treatment with chemotherapy,[9] and resistance avoidance drugs[10] have been developed.

Obinutuzumab, 2nd generation anti-CD20 antibody was developed for these rituximab resistant or

relapsed patients.[11] Obinutuzumab is a novel type II anti-CD20 antibody that has been glyco-engineered to enhanced affinity for the human FcγRIIIa receptor on effector cells and enhanced antibody-dependent cell-mediated cytotoxicity (ADCC). Obinutuzumab has a distinct mode of action relative to type I anti-CD20 antibodies working primarily by inducing direct cell death (DCD) and ADCC. Unlike apoptosis and other pronounced programmed cell deaths, the mechanism of rapid DCD by obinutuzumab remains unclear. The features of DCD include rapid cell death, plasma membrane damage and lysosomal swelling. According to research,[13,14] obinutuzumab induced DCD was associated with homotypic adhesion and the rearrangement of actin cytoskeleton, which in turn triggered lysosome membrane permeabilization (LMP) and cathepsin-mediated cell death bearing the morphologic features of necrosis. We are interested in the specific mechanisms of LMP dependent DCD, and especially increasing the LMP dependent DCD are expected to be a strong therapeutic option.

In clinical studies, many BNHL subtypes accelerate their proliferation through Tumor necrosis factor receptor1 (TNFR1) induced NF-κB signaling. Tumor necrosis factor α (TNFα) and its receptors TNFR1 have been identified in the sera of CLL patients in increased concentration. Also, membrane TNFR1 are increased in CLL patients of reactive lymph node, indicative for an aggressive disease, thus suggesting a role in CLL progression.[15] In addition, there is a result that DLBCL patients with TNFα and TNFR1 expression show poorer prognosis than those who do not.[16, 17]

Previous studies have implicated endocytosis and LMP as early steps of TNFα induced apoptosis.[18, 19] TNFα / CHX increase lysosomal ceramide that is subsequently converted into sphingosine.[20] Although ceramide accumulation does not significantly alter the acidic compartment, the sphingosine therein generated causes LMP followed by relocation of lysosomal cathepsin to the cytoplasm.[21] Interestingly, obinutuzumab, not rituximab colocalized with TNFR1 after CD20 binding and obinutuzumab DCD was decreased by TNFα scavenger, humira.[22] It is not known exactly what similarity between the LMP mechanism of obinutuzumab and TNFR1 signaling, but there might be a synergistic effect that can induce LMP when the mechanism is achieved at the same time.[23]

The term bispecific antibody is used to describe a large family of molecules designed to recognize two different epitopes or antigens. Bispecific antibodies are attractive in that they can show two effects at once, but many researchers expect to have more synergic effects through bispecific antibodies that that have both antibodies.[24, 25] Several types of bispecific antibody have been developed but the most well-known of these are bispecific T cell engager antibodies (BiTE). BiTE shows additional cancer therapeutic effect of the bispecific antibodies in that it attracts T cells and induces the activation of the immune system.[26] The target of bispecific antibody can exist in various forms such as two membrane

proteins of the same cell or the protein of the cytosol through the ones in the membrane sequentially.

Fusion antibodies are engineered antibodies conjugated with cytokines, enzymes and receptors. Fusion antibodies can also produce synergistic effects that go beyond the function of antibodies alone, like bispecific antibody. Fusion proteins with cytokine that can increase ADCC are also being developed. Several fusion antibodies with both cytokine and non-cytokine payloads have earned FDA approval or are currently being investigated in clinical trials.[27] In particular, there was a fusion antibody that could induce WNT signaling without ligands by placing the two receptors close together.[28] Thus, we are interested in developing bispecific and fusion antibodies that can bind to two antigens in one cell and produce synergic effects.

Here, first we elucidate association between CD20 and TNFR1 when obinutuzumab induced DCD occurs. We develop an anti-CD20 / TNFR1 bispecific and obinutuzumab × TNFα fusion antibody capable of binding to both CD20 and TNFR1 to strengthen obinutuzumab induced DCD. We show that the fusion antibodies induce a higher DCD than that of obinutuzumab, so DCD occurs efficiently when both CD20 and TNFR1 proteins are captured at the same time. Our study will suggest the possibility that CD20 and TNFR1 dual targeting can be a good therapeutic option for improving treatment of BNHL, and will provide bio-better therapeutic for BNHL patients.

II. MATERIALS AND METHODS

1. Cells, reagents and solutions

Human Ramos, HEK293T and HeLa cell line were purchased from the Korean Cell Line Bank. Raji cell line was gifted from Prof. Seong Hwan Kim (Chungnam National University, Daejeon). Ramos and Raji Cells were maintained in RPMI1640 media supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C, 5% CO_2. HEK293T and HeLa cells were maintained in DMEM high glucose media supplemented with 10% FCS 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 technologies™, Carlsbad, CA, USA); Trypsin-EDTA 0.05% solution (25300-062, Gibco, Life technologies™, Carlsbad, CA, USA) were used. For immunoblotting, NaCl (S7653), Triton X-100 (T8787), Glycerol (G5516, Sigma Aldrich); EDTA (15694, Usb, USB Corporation, Cleveland, OH, USA); Tris Ultrapure (T1501, Duchefa, Haarlem, The Netherlands), Complete proteinase inhibitors (Roche Applied Science, Mannheim, Germany), HCl (084-05425), NaOH (196-05375, Wako, Osaka, Japan); BCA Protein Assay Kit (23227, Pierce™, ThermoScientific, Rockford, IL, USA), 5x sample buffer, pre-made 10% SDS-PAGE gels (KG7040) (KOMA Biotech, Seoul, Korea) were used. For immunocytochemistry, 4% Paraformaldehyde (19943), BSA (10857, Affymetrix, Cleveland, Ohio, USA), Phosphate-buffered saline (PBS) (P5493, Sigma Aldrich) were used.

2. Antibodies

Antibodies used were mouse anti-TNFR1 (sc-8436, Santa Cruz Biotechnology), mouse anti-CD20 (sc-393894, Santa Cruz Biotechnology), anti-GAPDH antibody (sc-47724, Santa Cruz Biotechnology), a horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (115-035-003, Jackson Laboratories, PA, USA), FITC conjugated goat anti-Human IgG antibody (109-095-003, Jackson Laboratories), Alexa Fluor-647 conjugated goat anti-mouse IgG antibody (115-605-006, Jackson Laboratories), Alexa Fluor-680 conjugated goat anti-mouse IgG antibody (115-625-146, Jackson Laboratories), rituximab (Mabthera, Roche, Basel, Switzerland). Obinutuzumab, anti-TNFR1 antibody (ATROSAB), anti-CD20 / TNFR1 bispecific antibody, Obi × TNFα WT and Mutant fusion antibodies were produced in our

laboratory.

3. Immunoblotting

Cells were rinsed 3 times with cold PBS and lysed in lysis buffer (150 mM NaCl, 5 mM Na-EDTA, 10% glycerol, 20 mM Tris-HCl (pH 8.0), 0.5% triton X-100 and proteinase inhibitor (Complete, Roche Applied Science)) at 4°C for 20 min. Cell lysates were cleared by centrifugation in centrifuge at 17,000 ×g for 10 min at 4 °C. Transfer only the supernatant and quantify total protein by Bradford quantification. Cell lysate samples were prepared by addition of 5×sample buffer, heated at 95 °C for 5 min, resolved by either 10% or SDS–PAGE gels and then transfer to a nitrocellulose membrane. The part other than the protein electrically delivered to the NC membrane was blocked with TBS-T containing 5% non-fat milk for 1 hr at room temperature. The primary antibodies, mouse anti-TNFR1 (sc-8436, Santa Cruz Biotechnology), mouse anti-CD20 (sc-393894, Santa Cruz Biotechnology), anti-GAPDH antibody (sc-47724, Santa Cruz Biotechnology) diluted in TBS-T containing 2% BSA were added and incubated at 4 °C overnight. The membrane was then rinsed and incubated for 1 h at room temperature with a horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (115-035-003, JACKSON Lab, PA, USA). After rinsing, protein bands were visualized with Super Signal West Femto Luminol/enhancer solution (TC263618, Thermoscientific) and captured digitally using the Chemi-luminescent Image Analyzer (LAS 4000 mini, Fujifilm).

4. Measurement of membrane protein expression

Ramos and Raji cell line were resuspended in 100 μl of PBS, and primary antibodies, mouse anti-TNFR1 (sc-8436, Santa Cruz Biotechnology) and human IgG (I4506-10MG, Sigma) were treated with 70 nM and Incubate at 4°C for 30 min. After rinsing once with cold PBS, add a FITC-conjugated goat anti-human IgG Fc-specific secondary antibody (109-095-003, Jackson Laboratories) at 1:500 dilution in PBS and Incubate at for 30 min. After washing, a total of 10,000 cells were counted by flow cytometry (FACS, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software.

5. Immunocytochemistry

After or before Ramos cells were treated with obinutuzumab treatment, fixed with 4%

paraformaldehyde in PBS for 10 minutes at room temperature then rinsed with PBS for three times. Cells were incubated with blocking solution (5% horse serum, 1% BSA, 0.1% gelatin, 0.001% sodium azide in PBS pH7.4) at room temperature for 30 minutes. After rinsing twice with PBS, cells were incubated with mouse anti-TNFR1 antibody (sc-8436, Santa Cruz Biotechnology) in 1% BSA at room temperature for 1 hr, rinsed 3 times with PBS and incubated with the FITC-conjugated goat anti-human IgG Fc-specific secondary antibody and Alexa680 conjugated goat anti-mouse Fc specific secondary antibody for 30 min at room temperature in the dark. Cells were rinsed 3 times, mounted with fluorescent mounting medium (S3023, Dako, Glostrup, Denmark), and cells were visualized on a confocal microscope (LSM 780 controlled with Zen software; Carl Zeiss, Jena, Germany).

6. Cloning of Antibodies Plasmid

For the sequence of anti-TNFR1 antibody (ATROSAB), refer to patent #US8859739B2. The variable region of the heavy chain and light chain uses DNA synthesis from Genscript. Light chain is inserted to pLVX viral vector by Xba1, heavy chain variable region to pLVX viral vector that already have heavy chain constant region by Xba1 and Nhe1. For anti-CD20 / TNFR1 bispecific antibody construct, anti-TNFR1 antibody heavy chain including Q39K, K62E, H172A, F174G, T366S, L368A, Y407V and light chain including D1R, Q38D, L135Y, S176W were synthesized from Genscript. In obinutuzumab region, heavy (Q39Y, T366W) and light chain (G38R) mutations were mutated by site-directed mutagenesis. Mutation primer is determined using primer X program. For heavy chain Q39Y mutation, primer TTGTCCAGGGGCGTACCGCACCCA and AACTGGGTGCGGTACGCCCCTGGA, for heavy chain T366W mutation, primer GAAGCCTTTGACCAGGCACCACAG and CAAGAACCAGGT CAGCCTGTGGTG, for light chain G38R mutation, primer CTGCCCTGGCTTTCTCAGGTACCA and GTA TTG GTA CCT GAG AAA GCC AGG were used. DNA sequences were validated by sequencing. For Obi × TNF WT, 2 inserts (obinutuzumab HC, GSGSG linker + soluble TNFα (aa77-233)) were ligated with BamH1 and ligated into pLVX-CIP vector with Xba1 and Not1. GSGSG linker + sTNFα was constructed by extension PCR. Primer CGGGATCCGGCAGCGGCGTCAGATCATC TTCTCGAAC and GTTCGAGAAGATGATCTGACGCCGCTGCCGGATCCCG, CGGGATCCGGCA GCGGCGGATCTGGTAGCGGCGTCAGATCATCTTCTCGAACC and GGTTCGAGAAGATGATC TGACGCCGCTACCAGATCCGCCGCTGCCGGATCCCG, CGGGATCCGGCAGCGGCGGATCTG GTAGCGGCGGGAGCGGGTCAGGCGTCAGATCATCTTCTCGAACC, GGTTCGAGAAGATGAT CTGACGCCTGACCCGCTCCCGCCGCTACCAGATCCGCCGCTGCCGGATCCCG were used. For

Obi × TNF Mutant, sTNFα Mutant (A210S, V211T, S212T, Y213H, Q214N, T215Q) was mutated by site-direct mutagenesis using Obi × TNF WT template. Primer AGCCGCATCAGCACCACCCACAAC CAGAAG GTCAAC and GTTGACCTTCTGGTTGTGGGTG GTGCTGATGCGGCT were used.

7. Lentivirus production

All proteins and antibodies were stably expressed through lentiviral infection. Lentiviruses were produced by co-transfecting HEK293T cells with a lentiviral expression cassette and packaging plasmids (pMD2.5G (12259, Addgene) and psPAX2 (12260, Addgene)) using PEI (polyethylenimine) (23966-1, Polysciences, PA, USA) transfection reagent. Viral supernatant was collected 24 hr after transfection, centrifuged at 1,000 ×g for 5 min and filtered with a 0.45 μm filter.

8. Cloning, Lentivirus production and stable cell lines

Human MS4A1 and TNFRSF1A cDNA in pCNS vector were purchased from Korean gen bank. MS4A1 and TNFRSF1A cDNA full length was cloned into pLVX lentiviral vector with Xba1. TNFRSF1A Δ CD (lack cytoplasmic domain (aa233-455)) was clone into pLVX lentiviral vector with Nhe1 and Not1. Primer CCCGCTAGCATGGGCCTCTCCACC and GGGCGGCCGCTTAGTAGAGCTTGGACTTCC ACC were used. Lentiviruses were produced as previously described. To generate HeLa cell stably overexpressing CD20 or TNFR1ΔCD and Raij cell stably overexpressing empty vector and TNFR1. HeLa and Raji cells were plated in 12-well plates with 10 μg/ml polybrene (TR-1003-G, Sigma Aldrich) and incubated with virus-containing medium for 24 hr. After removal of viruses, cells were supplemented with fresh medium containing 1 μg/ml puromycin (ant-pr-1, Invivogen) for 48 hr. Protein expression was confirmed by flow cytometry and immunoblotting.

9. Generation of antibody producing CHO cells

CHO-K1 cells were plated in a 12 well plates at 5×10^4 cells/well and incubated for 24 hr. To generate obinutuzumab, anti-TNFR1, Obi × TNF WT and Mutant fusion antibodies, cells were incubated with virus containing antibody HC, LC vectors in a ratio of 3:2 with 10 μg/ml polybrene (TR-1003-G, Sigma Aldrich) for 24 hr. After removal of viruses, cells were selected by incubating fresh medium containing 10 μg/ml puromycin (ant-pr-1, Invivogen), 20 μg/ml blasticidin S (ant-bl-05, Invivogen) for 72 hr. To

generate anti-CD20 / TNFR1 bispecific antibody, cells were incubated with virus containing obinutuzumab HC Mutant, obinutuzumab LC Mutant, anti-TNFR1 HC Mutant, anti-TNFR1 LC Mutant vectors in a ratio of 3:2:3:2 with 10 μg/ml polybrene (TR-1003-G, Sigma Aldrich) for 24 hr. After removal of viruses, cells were selected by incubating fresh medium containing 10 μg/ml puromycin (ant-pr-1, Invivogen), 20 μg/ml blasticidin S (ant-bl-05, Invivogen), 200 μg/ml hygromycin B (H0192, Duchefa Biochemie), 400 μg/ml G418 (ant-gn-1, Invivogen) for 72 hr. After selection, enough stable cells are secured by proliferating the remaining cells.

10. Production and Purification of Antibodies

Antibodies producing cells grown to 80% confluence in RPMI containing 10% FBS and 1% penicillin/streptomycin were rinsed twice with PBS and refreshed with CD CHO Medium (10743029, Gibco, Life technologies™, Carlsbad, CA, USA) containing 8 nM L-glutamine (25030081, Gibco, Life technologies™) and 1 mM sodium butyrate (LS 033-01, WELGENE, Daegu, Korea). Conditioned media containing Antibodies were obtained by further incubation for 2 weeks at 30°C in 5% CO_2/95% air. Antibodies were purified via affinity chromatography using Pierce Protein A Agarose (20333, Thermofisher). Media containing the antibody was incubated with protein A agarose bead at 4°C for 24 hr. Remove the supernatant by centrifugation, and wash 3 times with a washing buffer (0.1 M $NaPO_4$, 0.15 M NaCl, pH 7.4). Antibodies were eluted by 6 fractions with elution buffer (0.2 M glycine, pH 3). Dialysis of the eluted antibody was performed in 2 L PBS for 6 hr at 4°C, a total of 2 times and concentrated with Amicon® Ultra-15 Centrifugal Filter Units (UFC900324, Merck, 3K). Total antibodies were quantified by NanoDrop™ Lite Spectrophotometer. After quantification, 1 μg antibodies were added with 5× laemmli sample buffer and separated by Pre-cast 10% SDS-PAGE gels and stained with Coomassie blue.

11. Antibodies Binding affinity assay

Ramos and HeLa-CD20, TNFR1 ΔCD cells are resuspended in 100 μl of PBS, and antibodies are treated with increasing dose or 70 nM and incubated at 4°C for 30 min. After rinsing twice with PBS, cells were treated with a FITC-conjugated anti-human Ig Fc-specific secondary antibody (109-095-003, Jackson Laboratories) at 1:500 dilutions and incubated at 4°C for 30 min. After rinsing twice with PBS, a total of 10,000 cells are counted by flow cytometry (FACS, BD Biosciences, Franklin Lakes, NJ, USA) and

analyzed with FlowJo software.

12. TNFR1 signaling reporter assay

NF-κB promoter dependent GFP reporter HEK293 cells are gifted from Prof. Jinu lee (Yonsei University College of Pharmacy, Korea). Stable HEK 293 cells are rinsed once, seeded 1×10^5 cells per well in 48 well plate and incubated for 24 hr. In antibody induced NF-κB signaling activation assay, Cells were incubated with indicated dose of antibodies (0, 0.1, 0.3, 1, 3, 10 nM) for 24 hr. In antibody dependent NF-κB signaling blockage assay, cells were pretreated with indicated dose of antibodies (70 nM) before 30 min, and then incubated with 20 ng/ml TNFα in CO_2 incubator at 37°C for 24 hr. cells were rinsed once with PBS and attached with Versene solution (15040066, Gibco Life technologies™). cells were plated to 96 well opaque plate and centrifuged and rinsed twice with PBS. The fluorescence intensity of GFP per well was measured using a microplate reader (Flexstation 3, Molecular Devices) with an excitation/emission of 488 nm/520 nm. For normalization, cells were lysed with Triton X 2% and stained with PI staining. Plates were Incubated on shake plate and The fluorescence intensity of PI per well was measured using a microplate reader (Flexstation 3, Molecular Devices) with an excitation/emission of 518 nm/620 nm.

13. Antibody induced direct cell death assay

To measure direct cell death assay, 1×10^5 cells/well cells were resuspended into 100 μl of RPMI full media and then incubated with indicated dose of antibodies (7, 21, and 70 nM) for 6 hr. Then, cells were stained with PI (Propidium iodide) for 20 min in 37°C. Fluorescence of PI was used as a readout and % of cell lysis (% of fluorescence losing cells number among 10,000 counted total cells) was calculated by FACS Verse (FACS, BD Biosciences, Franklin Lakes, NJ, USA) and Flow Jo software.

14. PBMC isolation

Heathy donor blood is mixed with PBS 1:1 ratio and piled up a layer on the tube where ficoll (HISTOPAQUE-1077, Sigma, 10771) was put in advance. the white blood cell and the red blood cell were Separated by centrifugation at 25°C for 400 ×g and 30 min, and then only the white blood cell is

collected and transferred it to a new tube. After rinsed with PBS, centrifuged at 300 ×g for 10 min at room temperature to remove the supernatant. Repeated this rinsing process twice to completely remove the platelet. Then, PBMC were resuspended in RPMI full media to be counted and used.

15. Antibody dependent cellular cytotoxicity assay

For Antibody dependent cellular cytotoxicity (ADCC) assay, Ramos and Raji cells were washed with PBS and stained with 0.25 μM calcein-AM. Cells were Incubated for 30 min and rinsed twice with PBS. Purified peripheral blood mononuclear cells (PBMC) (effector : target= 5 : 1) were added and treated with the indicated dose of antibodies (7, 70 nM) and then incubated at 37°C in CO_2 for 4 hr. The % of live cell among 60,000 counted total PBMC cells was calculated by FACS Verse (FACS, BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software. Normalization was done by ADCC % = 100 – (% of live cell / % of target cell population) *100

16. Statistical analysis

Data are presented as the means ± standard error of the mean. Statistical analysis was performed with Student's t-test, followed by Tukey's multiple comparison using the GraphPad Prism software package (version 9.0), as appropriate. $P<0.05$ was considered statistically significant.

III. RESULTS

1. CD20 & TNFR1 colocalization after obinutuzumab treatment

It was known that TNFR1 is involved in DCD induced by obinutuzumab in B cell lymphoma.[23] We showed that TNFR1 expression on Ramos and Raji B lymphoma cells by immunoblotting (Fig.1A). TNFR1 was expressed in Ramos not Raji cells and experiments were conducted with Ramos cells. and then we analyzed membrane expression of TNFR1 (Fig.1B). It was confirmed that TNFR1 was also expressed in the Ramos cell membrane. Then we compared CD20 and TNFR1 localization between obinutuzumab treatment after and before cell fixation (Fig.1C). When cells were treated with obinutuzumab after fixation, CD20 and TNFR1 were less colocalized. But when cells were treated with obinutuzumab and incubated for 10 min then fixed, they were more colocalized. These data indicate that when obinutuzumab bound to CD20, there was a change in the B cell lymphoma membrane, and it was confirmed that membrane CD20 and TNFR1 were located close together. Based on this result, we decided to develop anti-CD20 / TNFR1 bispecific and fusion antibodies.

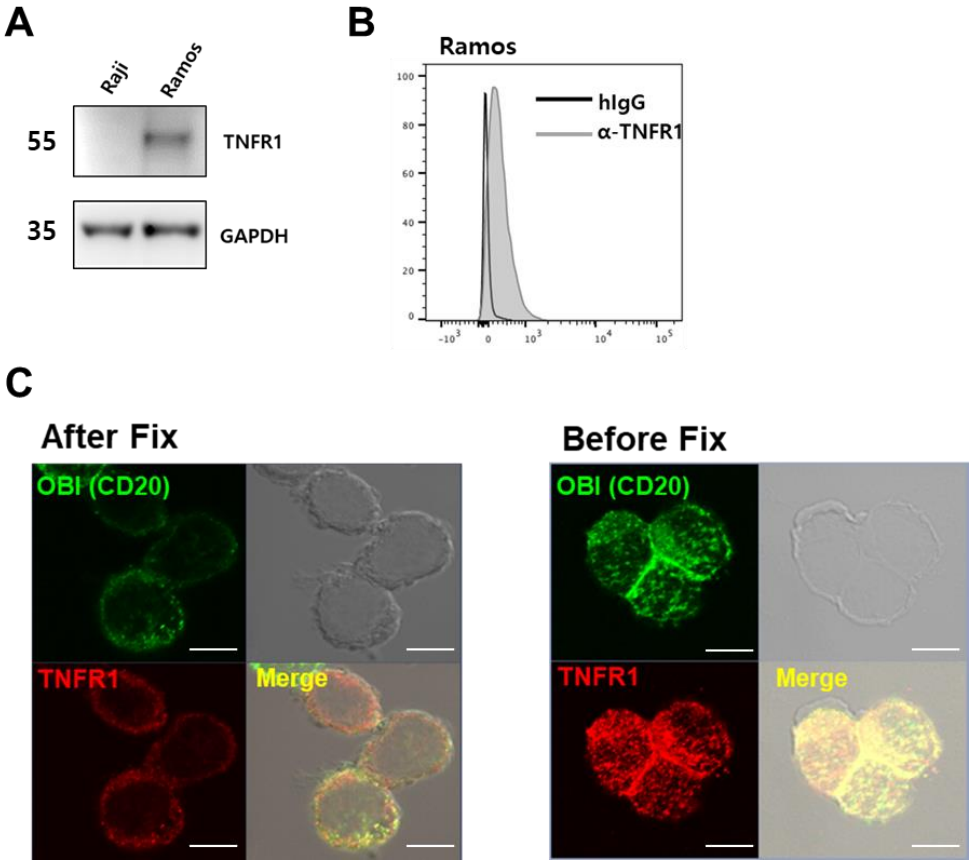

Figure 1. CD20 & TNFR1 colocalization after obinutuzumab treatment. (A) Immunoblotting with anti-TNFR1 antibody for TNFR1 expression in Raji and Ramos B lymphoma cell lines. GAPDH was used for control for protein loading amount. (B) Flow cytometry histogram of membrane TNFR1 expression in Ramos cell line. Mouse anti-TNFR1 antibody and FITC conjugated goat anti-mouse IgG antibody was used. (C) Immunofluorescence of CD20 & TNFR1 on Ramos cell after obinutuzumab treatment. (Left) cells were fixed and treated with obinutuzumab and mouse anti-TNFR1 antibody. (Right) cells were pretreated with obinutuzumab for 10 min, fixed and treated with mouse anti-TNFR1 antibody. Obinutuzumab were labeled FITC-conjugated anti-human IgG secondary antibody (green), TNFR1 was labeled with Alexa680-conjugated anti-mouse IgG secondary antibody (red) and Merged images show colocalization (yellow). Scale bars, 5 μm.

2. Anti-CD20 / TNFR1 Bispecific antibody and fusion antibodies

We developed anti-CD20 / TNFR1 bispecific antibody (Bispecific antibody), obinutuzumab × soluble TNFα WT (Obi × TNF WT) and obinutuzumab × soluble TNFα Mutant (Obi × TNF Mutant) (Fig.2). Bispecific antibody contains 'knobs into holes' mutations in the Fc region to be antibody heterodimerization.[30] Knobs mutations in obinutuzumab Fc contains T366W, and holes mutation in anti-TNFR1 Fc contains T366S, L368A, Y407V. Antibody Fab region contains 'orthogonal Fab interfaces' mutations which pair each antibody's heavy and light chain precisely (Fig.2A).[31] Obinutuzumab heavy chain Fab contains Q39Y and light chain contains G38R. Anti-TNFR1 antibody heavy chain Fab contains Q39K, K62E, H172A, F174G and light chain contains D1R, Q38D, L135Y, S176W. Soluble TNFα WT and TNFα Mutant[32] (A210S, V211T, S212T, Y213H, Q214N, T215Q) that has lost the ability to activate TNFR1 are conjugated in c-terminal obinutuzumab of heavy chain with GSGSG linker (Fig.2B,C). We validated these antibodies using SDS-PAGE gel. Bispecific antibody's size positions between obinutuzumab and anti-TNFR1 antibody in non-reducing condition (Fig.3A). Obi × TNF WT and Obi × TNF Mutant heavy chains were located almost 15 kDa higher than obinutuzumab in reducing condition. To precisely validate their binding to both targets, we tested these antibodies' affinity using CD20 or TNFR1 ΔCD overexpressed HeLa stable cell lines. All antibodies bound to HeLa-CD20 stable cells as much as obinutuzumab, but bispecific antibody showed reduced affinity due to one Fab region for CD20 (Fig.3B). Bispecific, Obi × TNF WT, and Obi × TNF Mutant bound HeLa-TNFR1 ΔCD stable cells, but only Obi × TNF WT showed equivalent affinity to anti-TNFR1 antibody (Fig.3C). Thus, it was confirmed that the antibodies were produced properly and that the binding ability was maintained.

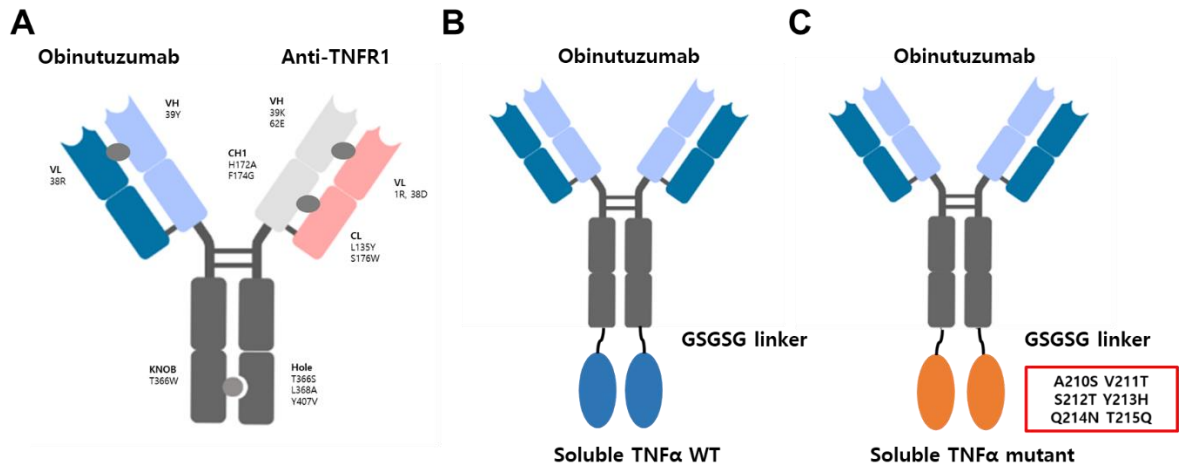

Figure 2. Constructs of Anti-CD20×TNFR1 bispecific and fusion antibodies. Scheme of anti-CD20 / anti-TNFR1 bispecific and Obinutuzumab × sTNFα fusion antibodies. (A) Anti-CD20 / anti-TNFR1 bispecific antibody. Bispecific antibody Fc region contains 'knobs into holes' mutations (knobs mutations in obinutuzumab Fc contains T366W, and holes mutation in anti-TNFR1 Fc contains T366S, L368A, Y407V) to be antibody heterodimerization. Fab region contains 'orthogonal Fab interfaces' mutations (obinutuzumab heavy chain Fab contains Q39Y and light chain contains G38R. Anti-TNFR1 antibody heavy chain Fab contains Q39K, K62E, H172A, F174G and light chain contains D1R, Q38D, L135Y, S176W) which pair each antibody's heavy and light chain precisely. (B) Obi × TNF WT fusion antibody. Soluble TNFα (aa77-233) WT are conjugated in c-terminal obinutuzumab of heavy chain with GSGSG linker. (C) Obi × TNF Mutant fusion antibody. Soluble TNFα (aa77-233) Mutant (A210S, V211T, S212T, Y213H, Q214N, T215Q) that has lost the ability to activate TNFR1 are conjugated in c-terminal obinutuzumab of heavy chain with GSGSG linker.

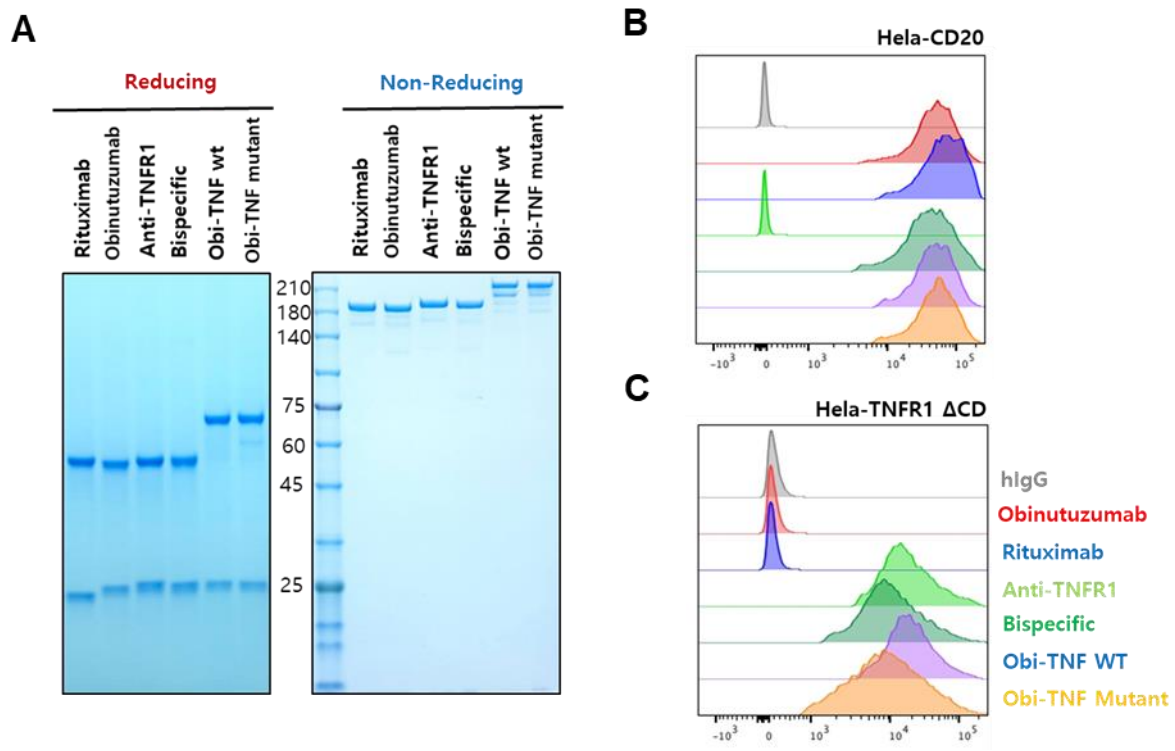

Figure 3. Validation of Anti-CD20/TNFR1 bispecific and fusion antibodies. (A) Validation of integrity of each antibodies using SDS-PAGE in reducing and non-reducing conditions. Total antibodies were quantified by NanoDrop™ Lite Spectrophotometer. Antibody 1 μg antibodies were loaded in reducing condition and in non-reducing condition. Samples were separated by 10% SDS-PAGE gels and stained with coomassie blue. (B, C) Flow cytometry histograms showing binding affinity of antibodies to (B) CD20 and (C) TNFR1 using HeLa cells stably over-expressing CD20 and TNFR1 ΔCD (lack cytoplasmic domain (aa233-455)). Stable cells were incubated with 70 nM each antibody. Antibodies were stained with FITC conjugated anti-human IgG antibody. a total of 10,000 cells are counted by flow cytometry and analyzed with FlowJo software.

3. Comparison of NF-κB mediated TNFα signaling activation capacity of anti-CD20 / TNFR1 bispecific and fusion antibodies

We compared NF-κB mediated TNFα signaling activation capacity of anti-CD20 / TNFR1 bispecific and fusion antibodies. We used NF-κB promoter dependent GFP fluorescence HEK293 reporter cell system. The strength of TNFα signaling was visualized by GFP fluorescence responsive to NF-κB promoter (Fig.4A). TNFα was used for positive condition and other antibodies were compared. In representative images, TNFα fluorescenses more GFP than Obi × TNF WT, but when normalized to the number of cells, Obi × TNF WT activated more than TNFα. At the same molar concentration, Obi × TNF WT had two TNFαs and it was assumed that two fold amount of TNFα could affect cell's viability. Obi × TNF Mutant could not activate TNFα signaling at 3 nM or less, but slightly at 10 nM. Other antibodies didn't activate TNFα signaling at all up to 10 nM. (Fig.4B,C).

Because NF-κB signaling by TNFα is known to have many side effects related to inflammatory responses, etc, we showed if antibodies could block this NF-κB signaling by TNFα (Fig.5A). After pretreatment with the antibodies, TNFα was added to the HEK reporter system. Enbrel was used for positive condition to block TNFα signaling and confirmed that Enbrel totally block. We expected that anti-TNFR1 would block TNFα signaling as much as Enbrel, but it blocked only half of the blockage of Enbrel, suggesting that this system can also be affected by TNFR2. When other antibodies were compared with anti-TNFR1, bispecific antibody blocked TNFα signaling less than anti-TNFR1 but Obi-TNF Mutant blocked much as anti-TNFR1 antibody (Fig.5B, C). Other antibodies were not shown to be able to block TNFα signaling. Taken together, it is resulted that bispecific antibody and Obi × TNF Mutant induces little TNFα signaling and can also block excessive TNFα effect, resulting in few side effects.

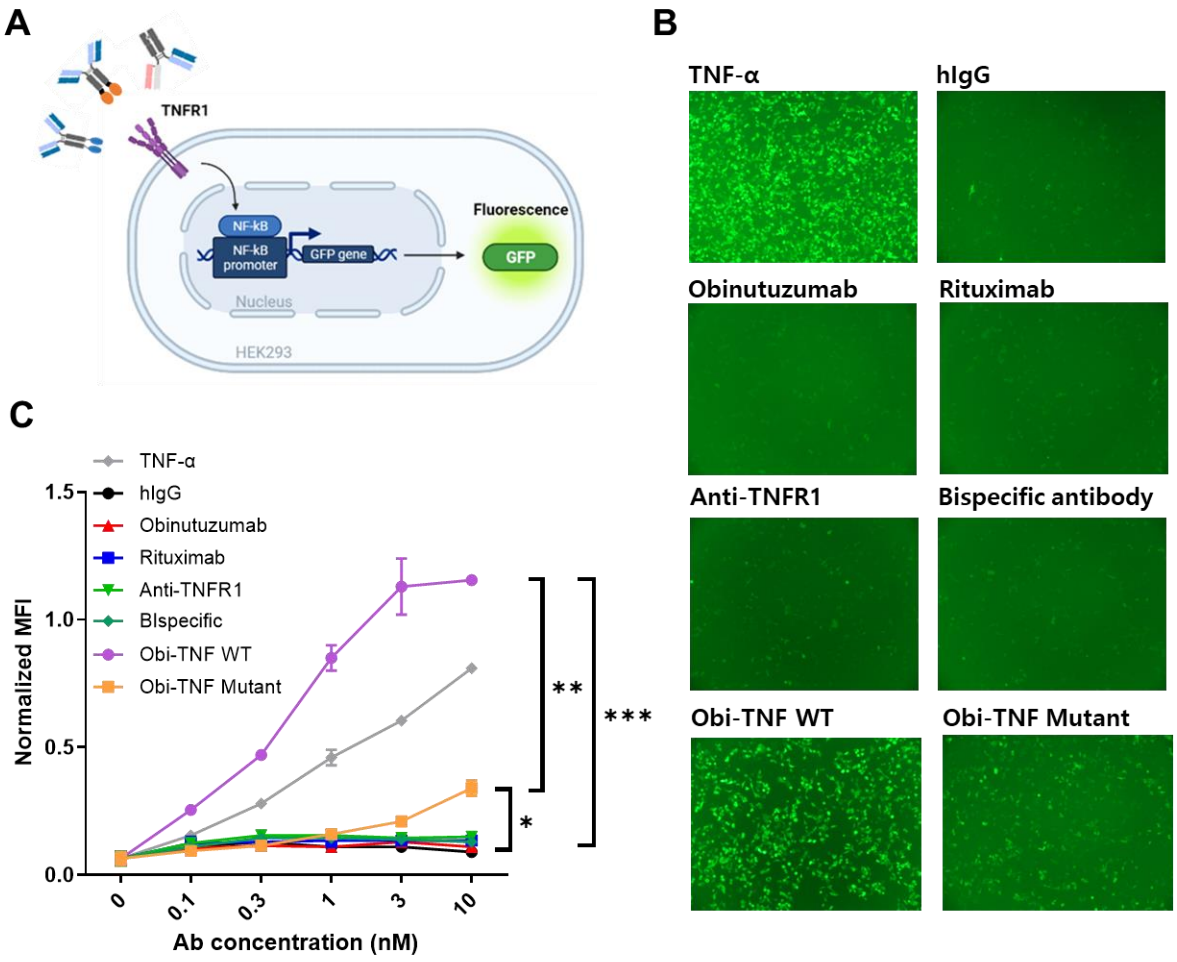

Figure 4. Activation of TNFα signaling by anti-CD20 / TNFR1 bispecific and fusion antibodies. (A) Schematic of Antibody induced TNFα signaling activation assay. HEK293 reporter cells stably express NF-κB promoter dependent GFP gene. When NF-κB signaling is induced by TNFα or antibodies, GFP is expressed in cytosol and cells become fluorescent. Thus strength of TNFα signaling was visualized by GFP fluorescence. (B) Representative of images showing GFP fluorescence in HEK293 reporter cells induced by TNFα and antibodies. Cells were incubated with 10 nM of TNFα and antibodies for 24 hr. TNFα used for positive condition. (C) Normalized quantification of GFP fluorescence intensity. Cells were incubated with indicated dose of antibodies (0, 0.1, 0.3, 1, 3, 10 nM) for 24 hr. TNFα used for positive condition. GFP fluorescence in HEK293 reporter cells induced by TNFα and antibodies was measured using a microplate reader with an excitation/emission of 488 nm/520 nm. For normalization, cells were lysed with 2% Triton X and stained with PI staining. The fluorescence intensity of PI was measured using a microplate reader with an excitation/emission of 518 nm/620 nm. Normalization is done by GFP mean fluorescence intensity/PI mean fluorescence intensity of lysed cells. Statistical analysis was performed with t-test, followed by Tukey's multiple comparison *p>0.05, **P>0.01, ***p>0.001.

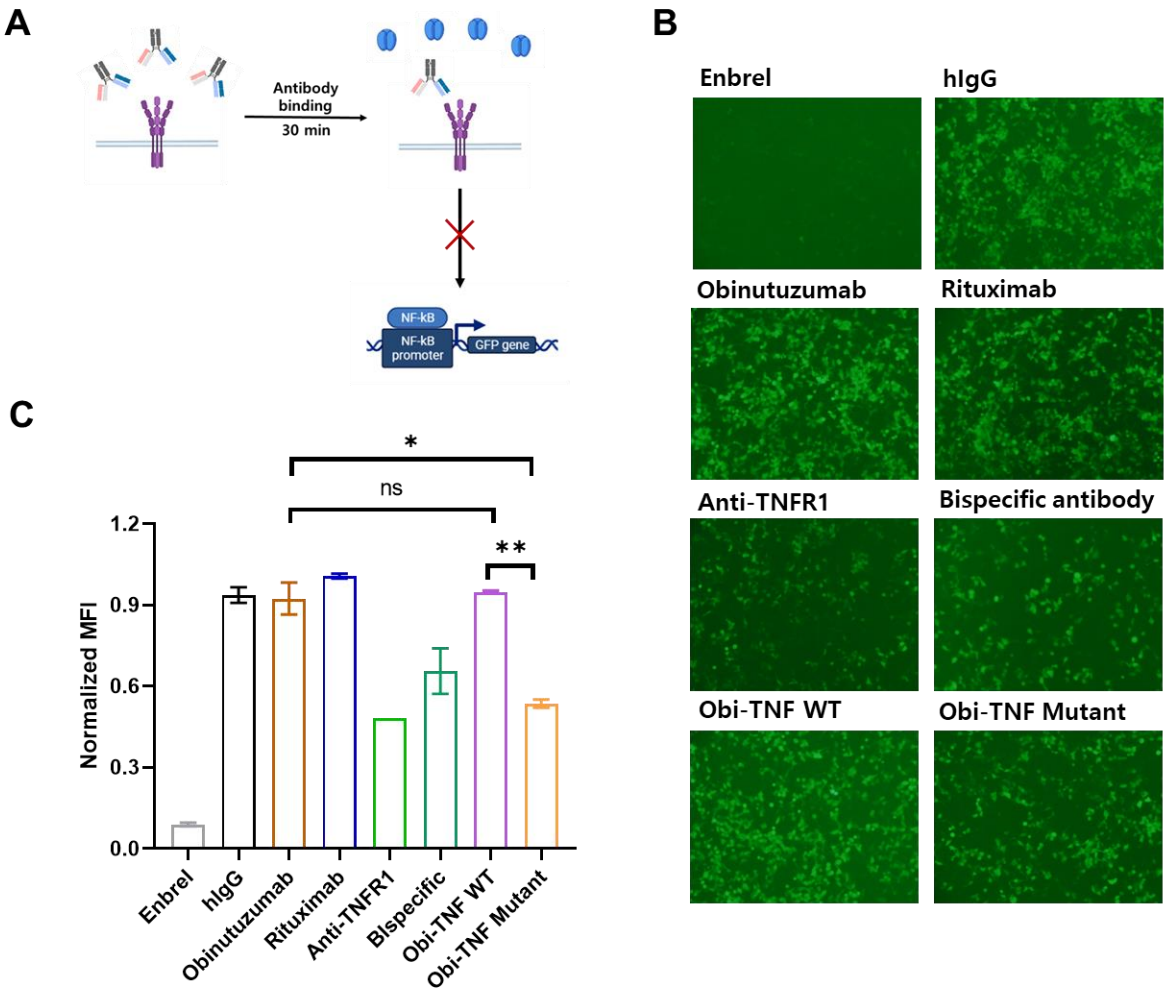

Figure 5. TNFα signaling blockade by anti-CD20 / TNFR1 bispecific and fusion antibodies. (A) Schematic of antibodies mediated TNFα signaling blockage assay. HEK293 reporter cells stably expressing NF-κB promoter dependent GFP gene were pretreated with indicated dose of antibodies (70 nM) before 30 min, and then incubated with 20 ng/ml TNFα for 24 hr. (B) Representative of images showing GFP fluorescence in HEK293 reporter cells blocked by antibodies. Enbrel was used for positive condition. (C) Normalized quantification of GFP fluorescence intensity. Enbrel was used for positive condition. GFP fluorescence in HEK293 reporter cells blocked by Enbrel and antibodies was measured using a microplate reader with an excitation/emission of 488 nm/520 nm. For normalization, cells were lysed with 2% Triton X and stained with PI staining. The fluorescence intensity of PI was measured using a microplate fluorescence reader with an excitation/emission of 518 nm/620 nm. Normalization is done by GFP Mean fluorescence intensity/PI Mean fluorescence intensity of lysed cells. Statistical analysis was performed with Student's t-test, followed by Tukey's multiple comparison *p>0.05, **P>0.01

4. Obi × TNF Mutant fusion antibody induce higher DCD and ADCC compared to obinutuzumab in TNFR1 dependent manner

To show if these antibodies are actually effective in BNHL expressing CD20 and TNFR1, we compared binding affinity, DCD and ADCC of bispecific and fusion antibodies in endogenously TNFR1-expressing Ramos cell lines (Fig.6A). Binding affinity was determined by cell binding assay with increasing antibodies' concentrations (Fig.6B). Consistent with previous data,[32] rituximab bound almost twice than obinutuzumab. Obi × TNF WT and Mutant bound almost equally with obinutuzumab. Bispecific antibody bound less than obinutuzumab. It was difficult to compare the binding differences by TNFR1 because the expression of CD20 was too high compared to that of TNFR1. But the difference between anti-TNFR1 antibody and hIgG was clearly seen, suggesting that TNFR1 affects affinity. Based on affinity result, we compared antibody dependent DCD. Bispecific antibody induces DCD less than obinutuzumab (Fig.6C). This is probably because the binding affinity of the bispecific antibody was lower than that of obinutuzumab. Obi × TNF WT and Obi × TNF Mutant induced higher cell death compared to obinutuzumab. There was no significant difference in DCD between the fusion antibodies. It proved that DCD occurs efficiently when both CD20 and TNFR1 proteins are captured at the same time. Finally, we previously had results that increased DCD by antibody could enhance ADCC, and predicted that DCD increased by fusion antibodies could enhance ADCC. We performed ADCC assay with effector and target cells at a ratio of 5:1 (Fig.6D). Obi × TNF Mutant induced ADCC almost two fold than obinutuzumab. Here we resulted that Obi × TNF Mutant fusion antibody induce higher DCD and ADCC compared to obinutuzumab in TNFR1 dependent manner.

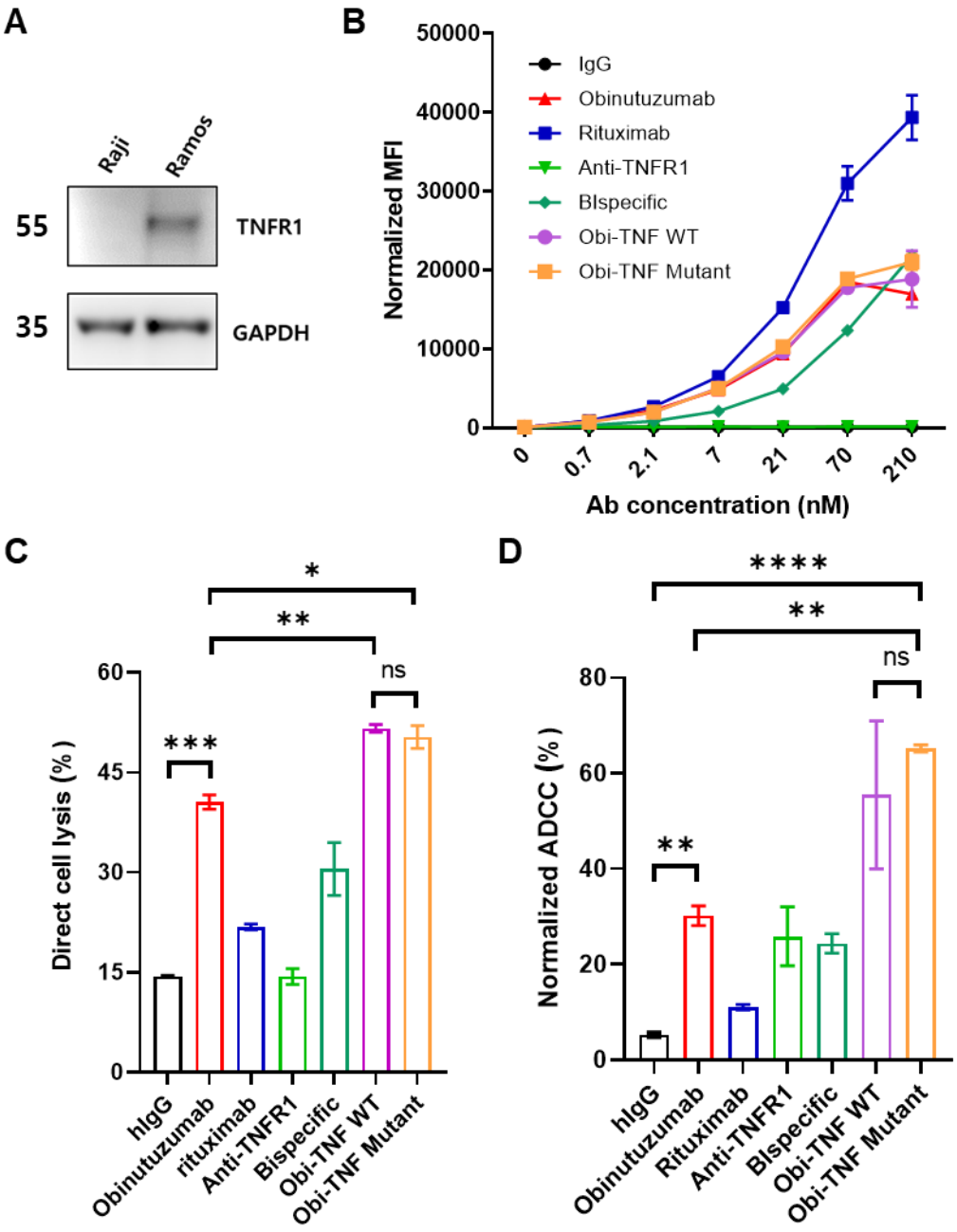

Figure 6. Obi × TNFα Mutant fusion antibody induce higher DCD and ADCC compared to Obinutuzumab in TNFR1 dependent manner. (A) Immunoblotting of TNFR1 expression in Raji and Ramos B lymphoma cell lines. GAPDH was used for control for protein loading amount. (B) Flow cytometry analyses of antibodies dose (0, 0.7. 2.1. 7. 21. 70. 210 nM) dependent Ramos cell biding affinity assay. Antibodies were stained with FITC conjugated anti-human IgG antibody. (C) Quantification of antibody dependent DCD with Ramos cells. Ramos cells were treated with 7 and 70 nM antibodies for 6 hr. Quantificational result of 7 nM antibodies is represented. (D) Quantification of

ADCC with Ramos cells. PBMC (effector) and Ramos cells (target) were co-cultured at a ratio of 5:1. Mixed cells were treated with 7 and 70 nM antibodies for 4 hr. Quantificational result of 7 nM antibodies is represented. Statistical analysis was performed with student's t-test, followed by Tukey's multiple comparison *p>0.05, **P>0.01, ***p>0.001.

5. Enhanced DCD and ADCC are induced by TNFR1

To confirm that the enhanced DCD and ADCC is caused by TNFR1, we tested DCD and ADCC with Raji cells stably overexpressing TNFR1. We generated Raji cells that stably express empty vector (EV) and TNFR1 using lentivirus. We confirmed Raji cells overexpressing EV and TNFR1 by immunoblotting (Fig.7A). In the Raji cells overexpressing TNFR1, it was seen that lots of TNFR1 was expressed, and that there was little in EV. It was also confirmed that the expression of CD20 was not changed even when TNFR1 was overexpressed. And in this Raji stable cell lines, membrane TNFR1 was analyzed by flow cytometry (Fig.7B). Membrane TNFR1 is only slightly increased in TNFR1 overexpressing Raji cell compared to EV, and it seems that cells expressing too much membrane TNFR1 died in the process of generating Raji cell stably overexpressing TNFR1. First, we performed DCD in these stable cell lines (Fig.7C). At low concentration, Obi × TNF WT and Mutant fusion antibodies induced higher DCD compared to obinutuzumab only in TNFR1 Raji cells. And at high concentrations, fusion antibodies and obinutuzumab similarly induced DCD in TNFR1 overexpressing Raji cells but higher DCD in TNFR1-overexpressing Raji cells compared to EV. Finally, we tested ADCC assay in these stable cell lines (Fig.7D). Similar to the previous result in Ramos cell (Fig.6D), Obi × TNF Mutant induced higher ADCC compared to obinutuzumab significantly. Here, these data indicated that DCD occurs efficiently when both CD20 and TNFR1 proteins are captured at the same time and DCD and ADCC was enhanced by TNFR1.

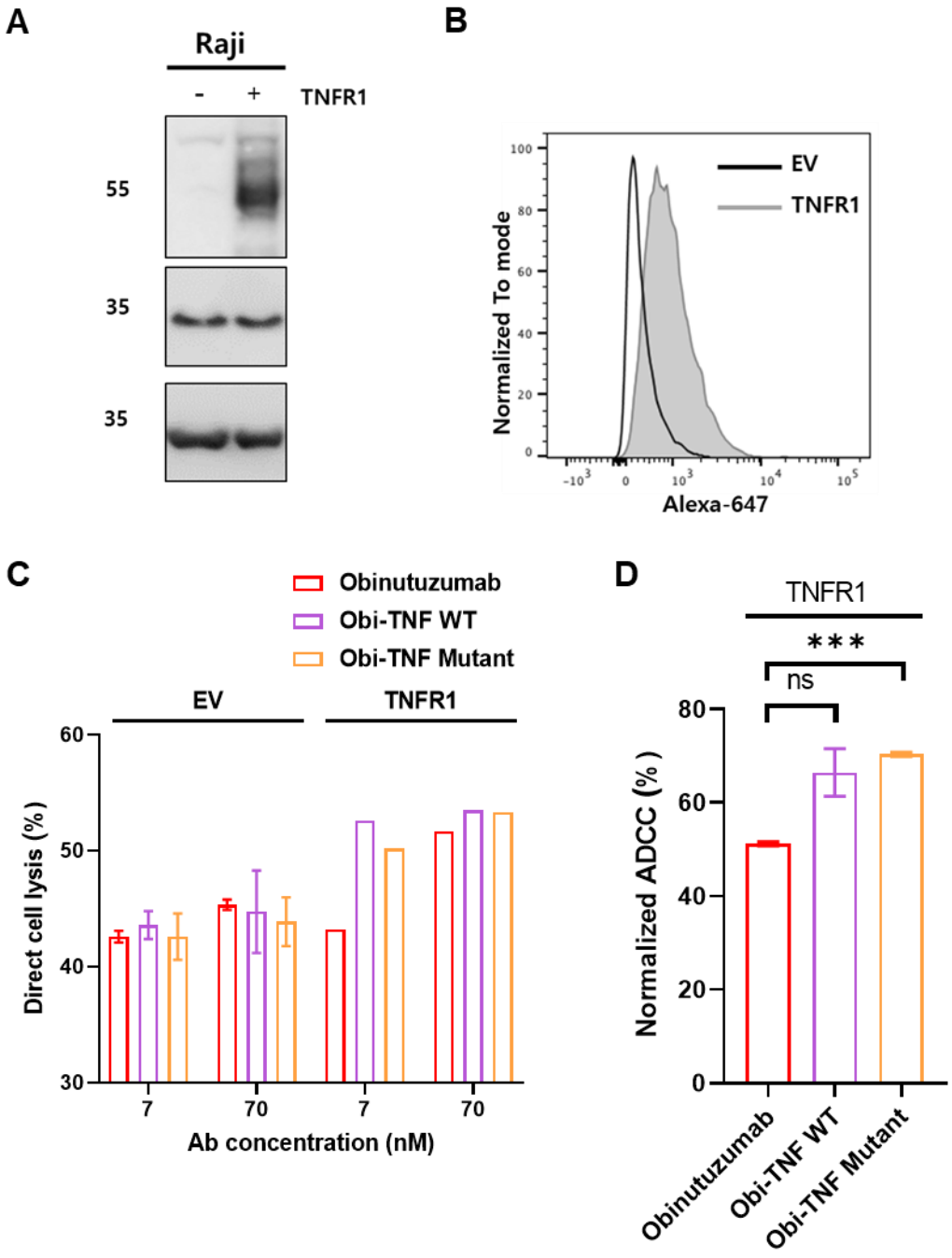

Figure 7. Enhanced DCD and ADCC are induced by TNFR1 (A) Immunoblotting with anti-TNFR1 antibody for TNFR1 expression in Raji cells stably over-expressing empty-vector (EV) and TNFR1. CD20 was used to show that there is no change in CD20 expression by TNFR1 overexpression. GAPDH was used for control for protein loading amount. (B) Flow cytometry histogram of membrane TNFR1 expression in Raji cells stably over-expressing EV and TNFR1. Mouse anti-TNFR1 antibody and FITC conjugated goat anti-mouse IgG antibody was used. (C) Quantification of antibody dependent DCD

with Raji cells stably over-expressing EV and TNFR1. Stable Raji cells were treated with 7 and 70 nM antibodies for 6 hr. (D) Quantification of ADCC with Ramos cells. PBMC (effector) and stable Raji cells (target) were co-cultured at a ratio of 5:1. Mixed cells were treated with 7 nM antibodies for 4 hr. Statistical analysis was performed with t-test, followed by Tukey's multiple comparison *p>0.05, **P>0.01, ***p>0.001.

IV. DISCUSSION

Although there have been results suggesting that obinutuzumab and TNFR1 are related, we particularly confirmed the colocalization of CD20 and TNFR1 after obinutuzumab binding. Based on this results, we developed anti-CD20 / TNFR1 bispecific and obinutuzumab × sTNFα fusion antibodies to strengthen the obinutuzumab induced DCD. First, we compared whether these antibodies could induce or block NF-κB mediated TNFα signaling. Bispecific antibody and Obi × TNF Mutant not only does not cause, but also block TNFα signaling, thereby implying that reducing side effects. The binding affinity of the bispecific antibody was slightly lower than that of obinutuzumab but Obi × TNFα fusion antibodies showed similar binding affinity to obinutuzumab. The bispecific antibody showed lower DCD compared to obinutuzumab, which is probably due to the lower affinity. All of the Obi × TNF Mutant fusion antibodies increased DCD compared to obinutuzumab suggesting that DCD can be caused without NF-κB signaling. Based on the previous results that increased DCD by antibody could enhance ADCC, we predicted that Obi × TNF fusion antibody would enhance ADCC more than obinutuzumab. Obi × TNF Mutant induced ADCC almost two fold than obinutuzumab, but Obi × TNF WT did not. Although it is difficult to explain clearly, we confirmed that Obi × TNF WT has a higher affinity for TNFR1 than Obi × TNF Mutant. This result suggest that a moderate affinity is required enhanced ADCC and provide another proof that enhanced ADCC is not related to NF-κB signaling. Finally, we confirmed that whether this increased DCD and ADCC was really caused by TNFR1 in the TNFR1 overexpressed cell model. It was also confirmed that Obi × TNF fusion antibodies caused higher DCD than obinutuzumab and Obi × TNF Mutant caused higher ADCC in TNFR1 overexpressed cells. It was confirmed DCD occurs efficiently when both CD20 and TNFR1 proteins are captured at the same time. Thus, DCD and ADCC were induced by TNFR1.

The Obi × TNF fusion antibodies showed a higher DCD than obinutuzumab. However, it is not clear how the DCD induced by Obi × TNFα fusion antibodies is increased. With our previous results, it is expected that LMP and endocytosis are probably related. As described in the introduction, it seems that DCD increases by combined LMP and endocytosis by TNFR1 and obinutuzumab. Especially, we found that TRPML2 in the lysosome is inhibited when obinutuzumab bind to CD20. We presume that TRPML2 inhibition is bound by endocytosis of sphingomyelin on the plasma membrane.[33] When TNFR1 is physically engaged with CD20 by Obi × TNF fusion antibodies, endocytosis occurs more strongly and TRPML2 is inhibited more, which leads to an increase in LMP. These are subjects that needs to be proved experimentally.

Also, we expect that the cell death of this Obi × TNF Mutant can be further increased by antibody engineering. First, considering the distance between CD20 and TNFR1, it can be applied by varying the linker lengths between the antibody and TNFα Mutant.[34] Second, according to recent research[35], activating of T cells with anti-CD3 antibody was effective in treatment of various cancers. so we can apply anti-CD3 scFv to obinutuzumab light chain C-terminal to activate T cell. If we increase this antibody induced DCD by optimizing linkers and T cell activation by anti-CD3 scFv further in this way, we will be able to enhance the ADCC and treatment of BNHL even more.

Additionally, we would like to see if the Obi × TNF Mutant is effective clinically. So, we apply these antibodies to samples of patients with CLL, which is one of the subtypes of BNHL. If efficacy is demonstrated in patient samples, it will become a practically applicable therapeutic agent. In addition, rituximab is already used in the treatment of autoimmune diseases, and obinutuzumab is also in clinical trials.[36, 37] The Obi × TNF Mutant has been shown to have less side effects caused by TNFα, so we presumed that it can be applied as a more effective treatment for autoimmune diseases.

Finally, our study suggests the possibility that CD20 and TNFR1 dual targeting can be a good therapeutic option for improving treatment of BNHL, and our Obi × TNF Mutant fusion antibody with enhanced DCD and ADCC will provide bio-better therapeutic for BNHL patients.

V. CONCLUSION

In this study, first we confirmed that CD20 and TNFR1 colocalized when obinutuzumab induced DCD occurs. Based on this result, we developed anti-CD20 / TNFR1 bispecific and obinutuzumab × sTNFα fusion antibodies capable of binding to both CD20 and TNFR1 to strengthen the obinutuzumab induced DCD. The Obi × TNFα fusion antibodies showed higher DCD than obinutuzumab. Consistent with our hypothesis, increased DCD by Obi × TNF Mutant enhanced ADCC almost two fold than obinutuzumab. Besides, Obi × TNFα Mutant showed decreased TNFR1 signaling imply that it has less side effects. Finally, we demonstrated the enhanced DCD and ADCC by Obi × Mutant is induced by TNFR1 in TNFR1 overexpressed cell models.

Accordingly, our study suggests the possibility that CD20 and TNFR1 dual targeting can be a good therapeutic option for improving treatment of BNHL, and our Obi × TNF Mutant fusion antibody with enhanced DCD and ADCC will provide bio-better therapeutic for BNHL patients.

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ABSTRACT (in Korean)

B세포 비호치킨 림프종의 치료를 높이는 항 CD20 / TNFR1이중항체 및 융합 항체의 개발과 적용

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B세포 비호지킨 림프종은 성숙 및 미성숙 B 세포의 클론성 종양으로 매년 전 세계적으로 50만 명 이상에서 발병한다. 리툭시맙은 항 세포표면항원무리20 (CD20) 단클론 항체의약품치료제로서 B세포 비호지킨 림프종 치료를 크게 개선하였으나 리툭시맙에 대한 내성과 재발이 많이 발생하는 문제가 있었다. 유형 2 항 CD20 항체인 오비누투주맙은 이러한 리툭시맙 내성 또는 재발 환자를 위해 개발되었다. 오비누투쥬맙은 CD20에 결합만으로도 리소좀 투과성 증가에 의한 세포 사멸을 유도하는 특징적인 작용 방식을 가지고 있다. 하지만 오비누투주맙 결합에 의한 리소좀 막 투과성 증가 및 직접적인 세포 사멸의 메커니즘은 아직 명확하지 않다. 한편, 임상 연구에서 종양 괴사 인자 수용체1 (TNFR1) 발현은 많은 B세포 비호지킨 림프종 아형에서 더 나쁜 예후와 관련이 있고, 오비누투주맙과 유사하게 리소좀 막 투과성 및 직접적인 세포 사멸을 유도한다. 이러한 근거를 바탕으로 본 연구에서는 오비누투주맙과 TNFR1의 기전이 동시에 달성될 때 시너지 효과가 있을 것이라고 가정하고 CD20와

TNFR1에 대한 이중항체 및 융합항체를 개발하여 그 효과를 확인하였다.

오비누투주맙 결합 후 CD20와 TNFR1가 같은 위치에 있음을 확인하였으며 CD20 및 TNFR1 모두에 결합하는 항-CD20 / TNFR1 이중항체 및 Obi × TNFα 융합 항체를 개발하여 이들에 의해 유도되는 직접적인 세포 사멸 정도를 확인하였다. 이중항체는 더 낮은 직접적인 세포 사멸을 보였지만 Obi × TNF WT 및 돌연변이체 융합 항체는 오비누투주맙보다 직접적인 세포 사멸을 증가시켰다. 항체에 의한 직접적인 세포 사멸의 증가가 항체 의존성 세포독성을 향상시킬 수 있다는 이전 결과에 기초하여, Obi × TNF 융합 항체가 오비누투주맙보다 항체 의존성 세포독성을 더 향상시킬 수 있음을 확인한 결과, Obi × TNF 돌연변이는 오비누투주맙보다 거의 2배의 항체 의존성 세포독성을 유도했다. 마지막으로, 이 융합항체가 보이는 강화된 직접적인 세포사멸 및 항체 의존성 세포독성은 TNFR1 과발현된 세포에서만 관찰되어 TNFR1의존적인 현상임을 확인할 수 있었다. 또한, Obi × TNF 돌연변이는 TNFα에 의해 유도되는 NF-κB 신호전달을 유발하지 않을 뿐만 아니라 TNFα 신호를 억제하는 추가적인 효과를 보였다.

따라서, 본 연구는 CD20 및 TNFR1 이중 표적이 직접적인 세포사멸율 증가를 가져올 수 있음을 제안하고 특히, Obi × TNF 돌연변이체 융합 항체는 직접적인 세포사멸율과 항체 의존성 세포독성 증가를 보이는 바이오베터로서 B세포 비호지킨 림프종 환자에게 더 나은 치료법을 제공할 가능성을 시사한다.

핵심 되는 말: B세포 비호지킨 림프종, 오비누투주맙, 세포표면항원무리20, 종양 괴사 인자 수용체1, 종양 괴사 인자, 이중항체 및 융합 항체, 리소좀 막 투과성, 직접적인 세포 사멸, 항체 의존성 세포독성