# Development of Novel NK Engagers Exploiting Antibodies and Evaluation of its Efficacy in Cancer Therapy

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# Development of Novel NK Engagers Exploiting Antibodies and Evaluation of its Efficacy in Cancer Therapy

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# Chapter 1; General Introduction

## 1-1 Roles of NK cells in cancer-immunity

Cancer is one of the leading causes of death in developed countries[1], and it is said that one out of every two Japanese people will develop some form of cancer in their lifetime (**Figure 1.1**). The body contains immune cells called leukocytes, and the human body has an immune system that mobilizes these cells to eliminate foreign substances.

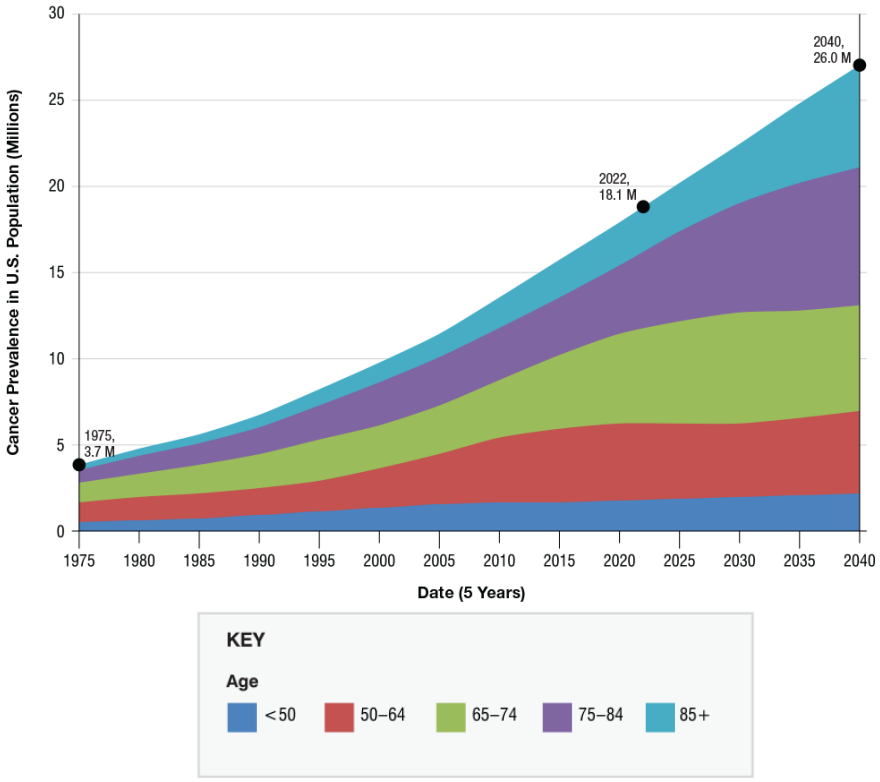


**Figure 1.1.** The number of cancer patients is increasing in developed countries
Cancer prevalence and projections in U.S. population from 1975-2040. Reprinted from ref 1.

Unlike normal cells, cancer cells continue to proliferate due to mutations caused by DNA copy errors and are "foreign substances" that should be removed from the body. Even in healthy people, cancer cells develop every day and are eliminated by immune cells. However, some of these mutated cancer cells may occur that evade the immune system, and since the immune system cannot recognize them as “foreign substances”, they continue to proliferate and accumulate into a mass to be a tumor[2] (**Figure 1.2**). One of the most important of these cells is the natural killer (NK) cell. NK cells accumulate on cancer cells via immune-related proteins called antibodies and injure cancer cells.

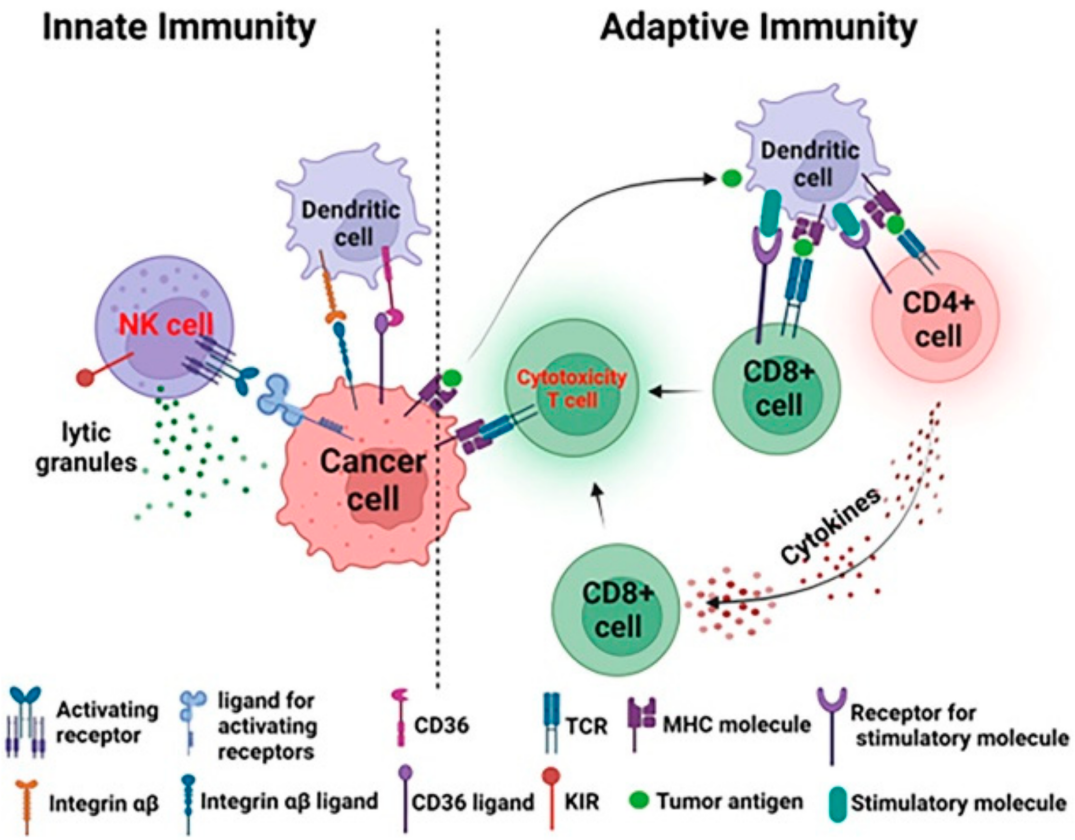


**Figure 1.2.** Two primary immunities for eliminating cancer cell
Innate immunity; NK cells and Dendritic cells recognize and attack cancer cells without prior sensitization, Adoptive immunity; Cytotoxicity T cells recognize peptide-MHC complex with prior sensitization. NK cell; Natural killer cell. MHC; major histocompatibility complex. Reprinted from ref 2.

Surgery, drug therapy, and radiotherapy have been called the three major conventional cancer treatment methods, but recently cancer immunotherapy has been attracting attention as a fourth method. Cancer immunotherapy is a cancer treatment method that utilizes immune cells that originally exist in the human body and is expected to have fewer side effects than conventional methods. NK cells are one of the immune cells that have received particular attention in recent years. One of the advantages of NK cells is that they can distinguish cancer cells and normal cells. NK cells distinguish between cancer cells and normal cells by the numerous activating or inhibitory receptors expressed on the cell membrane surface and only injure cancer cells[3] (**Figure 1.3**). In addition, cancer immunotherapy using T cells often causes severe side effects such as cytokine release syndrome, whereas NK cells are less likely to cause such immune disorders[4].

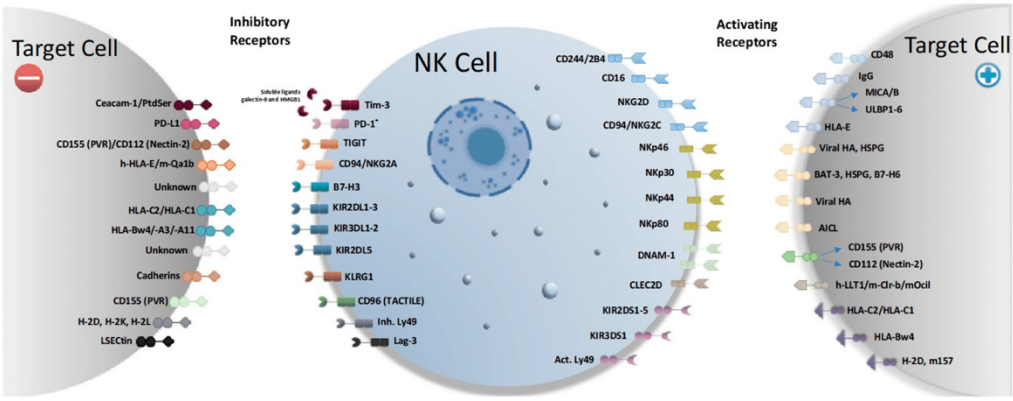


**Figure 1.3.** NK cells distinguish cancer cells and normal cells by multiple Receptors NK cell has Activating Receptors and Inhibitory Receptors and attack only the cancer cell without hurting normal cells. Reprinted from ref 3.

The FcγRIIIa differs from other receptors in that it recognizes cancer cells via antibodies and induces cellular cytotoxicity. Cancer therapeutic molecules that utilize this property are antibody drugs. Antibodies are Y-shaped proteins produced by plasma cells and consist of an Fc region with a highly conserved amino acid sequence and a Fab region with different amino acid sequences for each antibody[5] (**Figure 1.4 (A)**). In addition to the most important feature that antibodies have a very high specificity for a specific molecule derived from the Fab region, Fc regions interact with Fc receptors and some proteins of the complement system to induce immune functions called effector functions[6-8]. Among some effector effects, the one associated with NK cells is antibody-dependent cellular cytotoxicity (ADCC). NK cells bind to the Fc region of antibodies via FcγRIIIa, and signals are transmitted to NK cells through this receptor, resulting in the release of

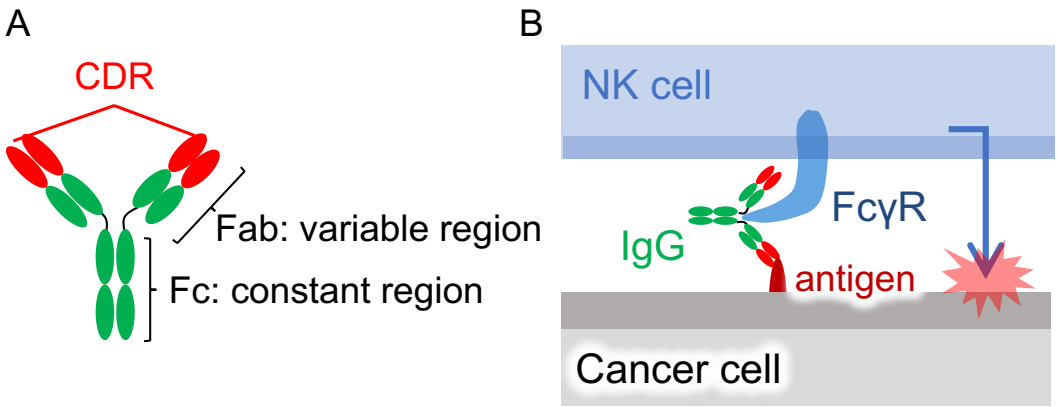


**Figure 1.4.** The structure of IgG and the mechanism of induction of ADCC

perforin, which opens pores in the membrane of cancer cells, and granzyme B, which induces apoptosis. Antibody drugs are pharmaceuticals that utilize the characteristics of antibodies (**Figure 1.4 (B)**). Therefore, T cells may attack normal cells, whereas NK cells are less likely to cause such immune disorders. Because of their superior characteristics, cancer treatment methods utilizing NK cells are being actively developed[9] (**Table 1.1**).

**Table 1.1.** NK cell-based cancer therapies
BiKES; Bi-specific antibodies termed killer-cell engager, CAR; Chimeric antigen receptor, KIR; Killer cell Ig-like receptor, TriKES; Trispecific antibodies termed killer-cell engager.

| Therapeutic approach | Development stage | Potential advantedes |
|---|---|---|
| **Activating receptors agonist** | | |
| Tumor targeting mAbs | FDA approved | Redirect NK cells against specific tumor Ag |
| mAbs + metalloproteinase inhibitor combinatin | Preclinical | Prevent ligand shedding and resulting resistance |
| mAbs + IL-2 linkage | Preclinical | Tumor-specific NK cell activity On-site IL-2-mediated activity |
| Combinations with NK cell-activating therapies | Various | FDA approced and known safety profile of IL-2, IL-5, bortezomib, lenalidomide and anti-PD-1 |
| BiKES, TriKES | Preclinical | Redirect NK cells against specific tumor Ag |
| **Brocking inhibitory signals** | | |
| mAbs to iKIRs | Clinical trial | Safe; no need for KIR genotyping |
| mAb to NKG2A-CD94 | Clinical trial | Safe; sustainable bloclade; no need for KIR genotyping |
| CTLA-4 and PD-1 checkpoint inhibitor | FDA approved/trial for combination with rituximab | Known safety profile; combination: enhanced NK activity |
| **Adoptibe therapy** | | |
| Autologous NK cells | Clinical trial | Safe |
| Allogeneic NK cells | Clinical trial | High efficacy if KIR-ligand mismatch |
| NK cell lines | Clinical trial | Easy to expand, safe to transfer postirradiation |
| CAR-NK cells | Clinical trial | Redirect NK cells against specific tumor Ag |

In cancer therapy, antibody drugs specifically bind to antigens expressed on cancer cell membranes, mobilize NK cells to cancer cells, and induce ADCC through the same mechanism as native antibodies. Antibody drugs are used clinically as very useful therapeutic agents, but their large molecular weight (150 kDa) and heterotetrameric structure make development, production, and quality control very costly[10-12]. In addition, although antibody drugs are human-derived proteins, due to their large molecular weight, anti-drug antibodies (ADA) against antibody drugs are produced by autoimmunity, and subsequent administration results in their rapid clearance from the bloodstream by the immune system, thus reducing therapeutic efficacy[13, 14] (**Figure 1.5**).

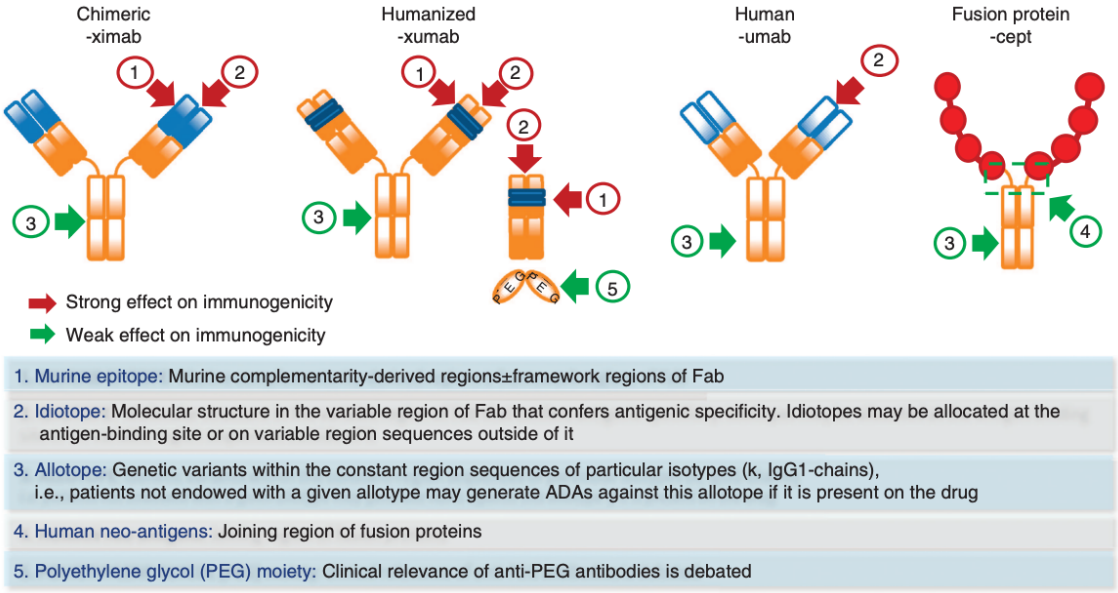


**Figure 1.5.** Immunogenic sites on anti-antibody constructs.
Antibody drugs are recognized by IgG produced by B cells and are excreted from the body in a short period. Reprinted with permission from ref 14.

## 1-2 NK cell engagers

One treatment method that can overcome the above problems is NK cell engagers (NKCEs). This method uses endogenous NK cells for cancer treatment without the need for complicated genetic modification or transplantation. However, since NK cells themselves cannot target specific cancer cells, a method to bridge cancer cells and NK cells is necessary[15]. The development of NK engagers that recruit NK cells to cancer cells has been actively underway. There are mainly two ways to recruit NK cells to cancer cells: direct cross-linking of NK cells to cancer cells and antibody-mediated recruitment of NK cells to cancer cells, which is a similar mechanism to antibody drugs.

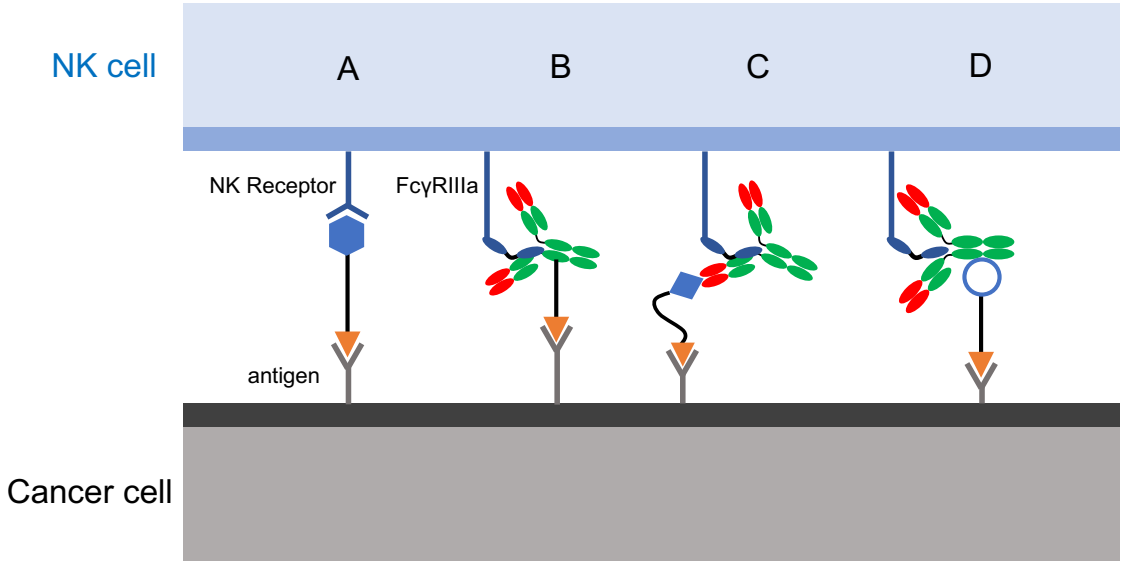


**Figure 1.6** The schematic strategies of NKCEs
Direct cross-linking of cancer cells and NK cells(A), IgG-Fc Receptor-mediated cross-linking (B-D), IgG-ligand conjugation(B), Antibody recruitment molecule (C) Fc-mediated cross-linking of antibody (D)

### 1-2-1 The method Direct crosslinking NK cell and cancer cell

Bispecific/Trispecific killer cell engagers (BiKEs/TriKES) have been reported as molecules that directly cross-link NK cells and cancer cells[16] (**Figure 1.6 (A)**). This molecule bridges FcγRIIIa and NKG2D receptors expressed on the surface of NK cells and antigens, including EpCAM, expressed on the surface of cancer cells, and its anti-tumor effect has been confirmed in ADCC. It has been reported that ADCC induction is enhanced when the affinity between the antibody and FcγRIIIa is high, and in fact, Potelligent antibodies with improved affinity to FcγRIIIa by glycosylation technology have been used to induce higher ADCC. This molecule has been used in clinical

applications. In clinical practice, Bi/TriKEs binds to NK cells in the blood and is subsequently cross-linked to cancer cells. The molecule binds to both NK cells and cancer cells with high affinity, and thus has a high ADCC induction ability. However, NK cells are relatively large and have low invasiveness into solid tumors.

### 1-2-2 antibody-mediated recruitment of NK cells to cancer cells

The antibody-mediated method is expected to have a lower ADCC induction ability than the direct NK cell-cancer cell cross-linking method, because the binding to NK cells is mediated by FcγRIIIa. There are multiple advantages to antibody-mediated modification. Proteins are normally taken up by cells through endocytosis and degraded by lysosomes. Certain proteins, such as antibodies, avoid this degradation process by binding to receptors called neonatal Fc receptor (FcRn) and migrate to the cell surface again (recycling mechanism)[17] (**Figure 1.7**). Molecules that bind to antibodies through non-covalent bonds may avoid this degradation process by binding to antibodies. In addition, compared to molecules that bind directly to NK cells, these molecules may improve tumor invasiveness.

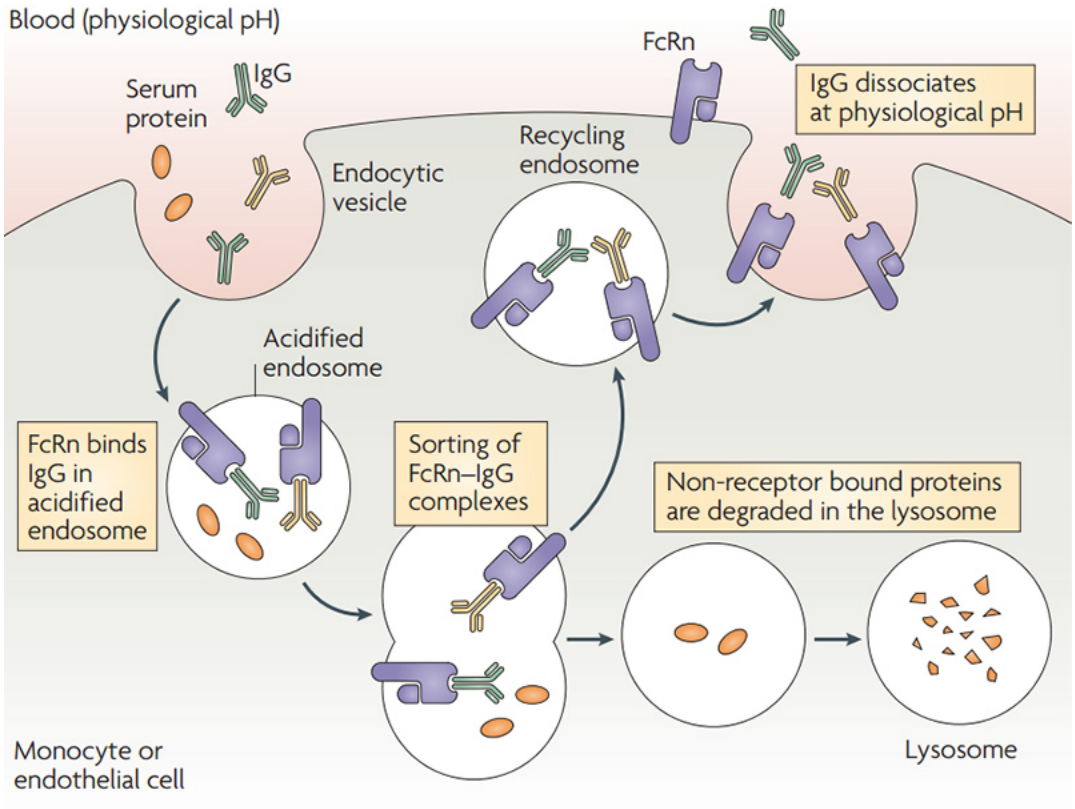


**Figure 1.7** Degradation pathways of serum proteins and antibody recycling pathways after endocytosis. Reprinted with permission from ref 17.

### 1-2-2-1 Antibody-ligand conjugation

Ligand-modified antibodies are one of the methods for antibody-mediated NK recruitment[18] (**Figure 1.6 (B)**). The purpose of modifying ligands that targets a cancer antigen to antibodies is to achieve a bispecific-like function by adding a new targeting ability by the antigen ligand in addition to the targeting ability by the Fab region of the antibody. It is reported that an IgG-ligand conjugate labeled with folate, an antigen of the folate receptor, has been developed. This IgG-FA showed induction of ADCC by folate-mediated antibody accumulation in cancer cells and inhibition of tumor growth in animal studies. However, IgG-ligands, like ordinary antibody drugs, are expected to reduce therapeutic efficacy due to the production of ADA. In addition, ligand modifications generally target the Lys or Cys of the antibody[19-22], which causes problems such as heterogeneity of therapeutic effect due to variation in the number of ligand modifications, inhibition of effector effect of the antibody due to the position of the modification, and reduction of conformational stability of the antibody[23-24].

### 1-2-2-2 Antibody recruiting molecule (ARM)

ARM is being developed to solve the above problems of antibody drugs. ARM consists of a target-binding terminus (TBT) that binds to cancer antigens and an antibody-binding terminus (ABT) that binds to endogenous antibodies[25, 26] (**Figure 1.6 (C)**). The administered ARM binds to endogenous antibodies during circulation in the blood and then accumulates on cancer cells. Antibodies accumulated on cancer cells via ARM are recognized by NK cells and induce ADCC by the same mechanism as antibody drugs. Conventional ARMs are monomeric molecules produced by organic chemistry, and their small molecular weight may solve the problem of antibody drugs in that they are not immunogenic[25]. However, they target the Fab region of endogenous antibodies for binding to antibodies, but the Fab region of endogenous antibodies is polyclonal, so antibody-ARM affinity is not constant. In addition, the amount of endogenous antibody to a specific antigen varies from person to person, resulting in heterogeneity in therapeutic efficacy[27].

### 1-2-2-3 Fc-binding ARM (Fc-ARM)

I have developed Fc-ARM as a molecule that solves the problem of conventional ARM. Fc-ARM has the property of binding to the Fc region of antibodies[28,29] (**Figure 1.6 (D)**). Since the Fc region of the antibody has a conserved amino acid sequence and structure, the affinity between Fc-ARM and the antibody is expected to be constant, resulting in uniformity of therapeutic effect. In addition, the number of endogenous

antibodies available for conventional Fab-binding ARM is only about 1%[30-34], whereas Fc-ARM can utilize more antibodies[35] and thus is expected to have a higher therapeutic effect. I have prepared Fc-ARM using folate or 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid (DUPA) as a small molecule ligand for folate receptor or Prostate Specific Membrane Antigen (PSMA), and a cyclic peptide called Fc-III or Fc-III4C as an antibody-binding molecule (**Figure 1.8**). Prepared Fc-ARMs bound the target antigen and recruited IgGs on target cancer cells. The IgG recruited via Fc-ARMs successfully induced ADCC in cell experiments and the combination of IgG and Fc-ARM2 inhibited tumor growth in animal experiments using mice[28].

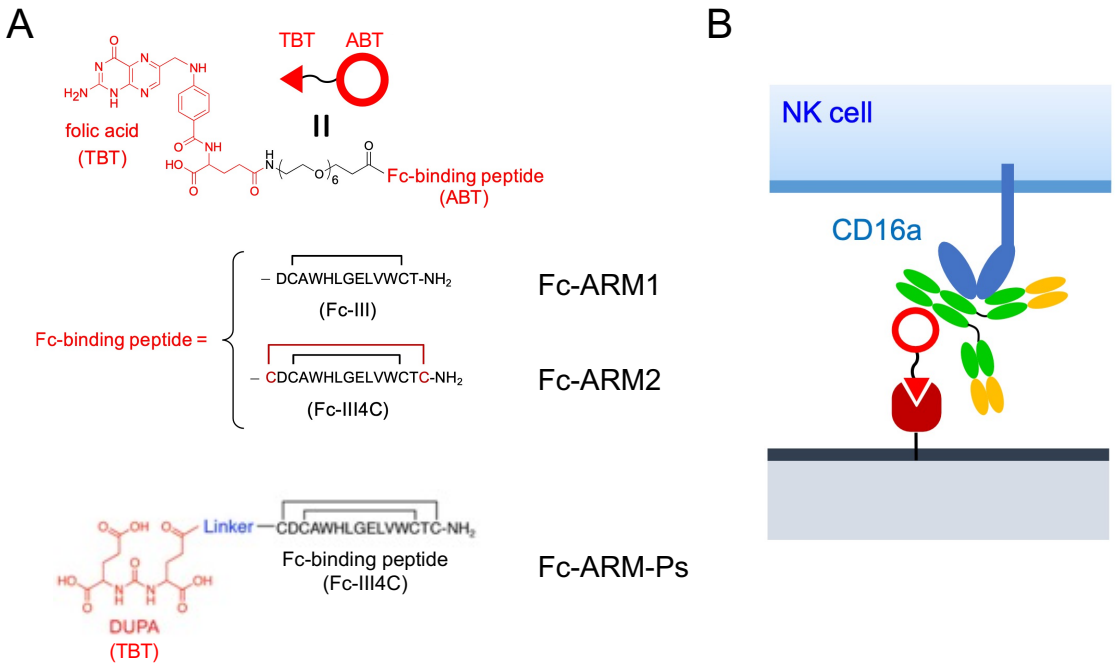


**Figure 1.8.** The structure of Fc-ARMs and the illustration of immune complex formation. (A) Molecular structure of three different Fc-ARMs with different ABT or TBT. (B) The schematic illustration of immune complex formation.

## 1-3 Overview of this thesis

In this thesis, I developed a novel NK engager that consistently focuses on the stationary region of antibodies. In Chapter 2, modification of antibodies with site-specific antigen-targeting molecules by organic chemical modification methods was evaluated in vitro. Conventional antibody modification methods target either Lys or Cys of the antibody. However, the high hydrophilicity and content of Lys make it difficult to target specific positions. The Cys-targeted modification method targets the hinge region, which overlaps with the position required for the antibody's structural stability and effector functions. I developed a molecule targeted Trp[36], a hydrophobic amino acid with low existent[37]. The position of the exposed Trp residue in the Fc region does not overlap with the recognition position required to induce effector functions and is expected to avoid inhibition of effector functions by the modification. The antibody-ligand complexes accumulated on the cells and induced ADCC, regardless of the Fab existence. It was also found that the original Fab binding ability and ADCC inducibility of the antibody was not interrupted. In Chapter 3, I prepared protein-type Fc-Arm and evaluated its functions in vitro. Previous studies have shown that the higher the binding affinity between Fc-ARM, antibody, and antigen, the more strongly ADCC is induced[28]. However, the small size of small molecule-based targeting methods limits the number of binding sites and thus limits the improvement of binding affinity. Protein-based targeting methods are expected to achieve higher affinity and thus induce higher ADCC capacity because they bind at more sites than small molecules. In Chapter 4, the author summarizes the conclusion of this thesis.

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# Chapter 2; Tryptophan-selective conjugation of mAb with folic acid preserves mAb's ADCC ability

## 2-1 Introduction

As I described in chapter 1, NK cells injured cancer cells by ADCC via Fc – FcγRIIIa interaction, but in addition to this, the Fc region also plays a crucial role by expressing effector functions via Fc - FcγR interaction[1]. Some proteins recognize the Fc region of IgG to induce these functions. Fcγ receptors (FcγRs) and complement component 1q (C1q) recognize the Fc region to exert effector functions, such as ADCC, antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC)[2,3], and Neonatal Fc receptor (FcRn) recognizes the Fc region to extend the blood half-life of IgG[4] (**Figure 2.1**).

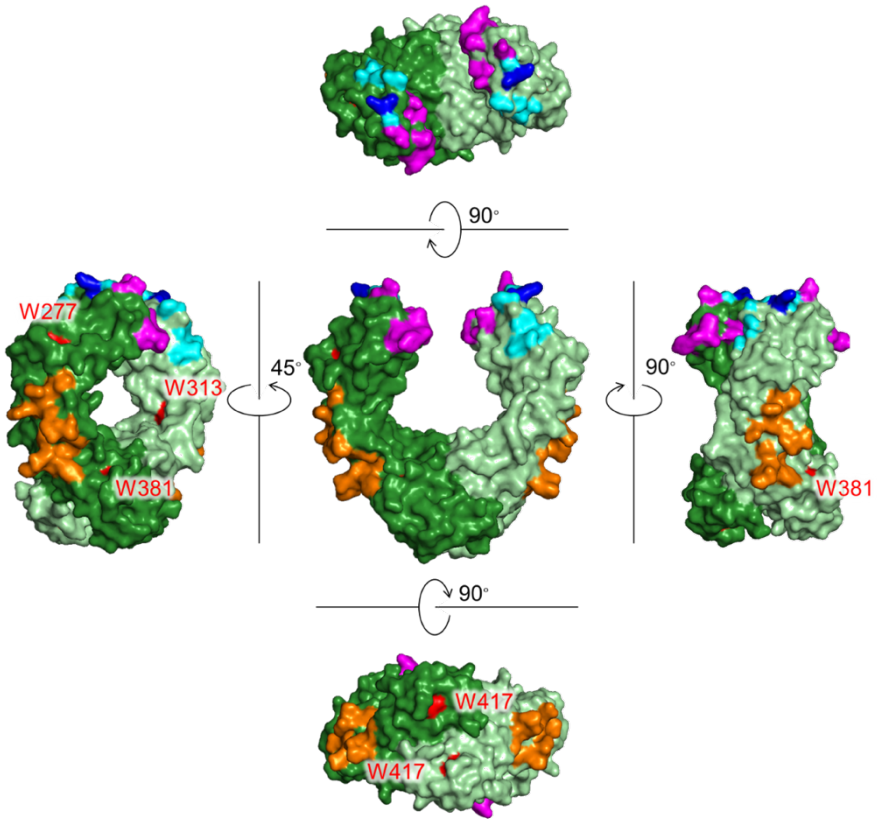



**Figure 2.1.** Crystal structures of the Fc region of IgG1 with Trp residues shown in red. Recognition residues of the three proteins are shown in different colors: magenta (FcγRIIIa, CD16a), cyan (C1q), blue (overlapped residues of FcγRIIIa and C1q), and orange (FcRn). PDB ID: 6N9T

Conjugation of mAbs to small molecules, such as drugs, fluorophores, and ligands, is widely performed to expand the functions of mAb-based therapeutics. For the effector functions to be retained in the mAb conjugates, the critical residues in the Fc regions must be preserved. For the mAb-conjugation of small molecules, lysine and cysteine residues have been used[5-8]. Since lysine residues are abundant on the surface of the Fc region, lysine-specific modification may disturb the recognition of effector proteins. Cysteine residues exist that at the hinge region are typically used for conjugation. However, cleavage of these disulfide bonds potentially lowers the structural stability of mAbs and abrogates their effector functions[9,10].

Tryptophan (Trp) is the least abundant (~1%)[11], and the least surface-exposed proteinogenic amino acid, and each Trp residue has a variety of solvent accessibilities. As shown in **Figure 2.1**, Trp residues in the Fc region do not overlap with the recognition residues of the three proteins. Thus, the controlled conjugation via Trp is not expected to disturb the recognition areas of the three effector proteins. I previously developed a tryptophan (Trp)-selective conjugation of proteins using a stable organoradical, 9-azabicylo [3.3.1] nonane N-oxyl (ABNO)[12,13].

Here I conducted a Trp-selective conjugation of a mAb to folic acid (FA), which is a well-known ligand of cancer cells (**Figure 2.2 (A)**). The FA-mAb conjugates showed ADCC against folate receptor-α (FR-α) positive cancer cells without conventional binding via the Fab region, suggesting minimized impairment of the Fc functions by Trp-conjugation (**Figure 2.2 (B)**).

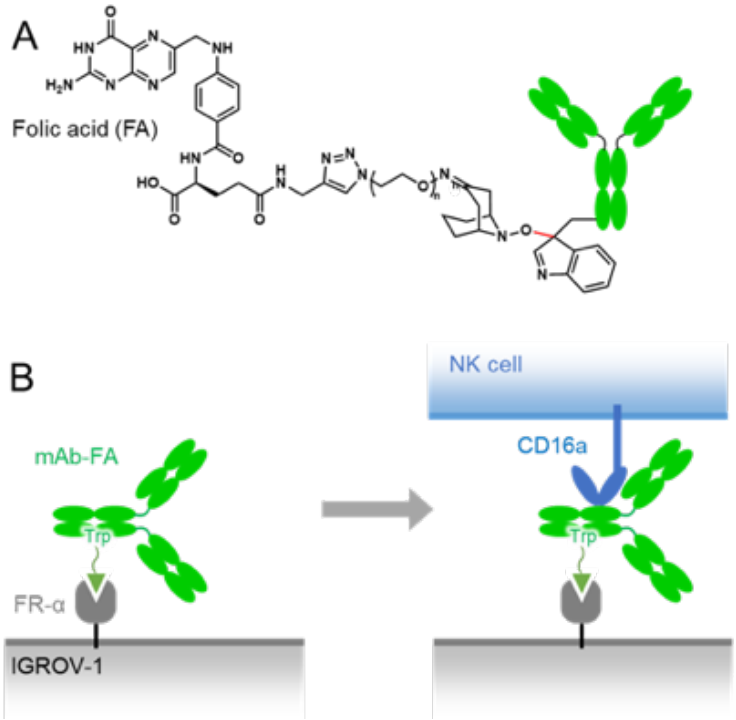


**Figure 2.2.** The structures of the mAb-FA conjugates via Trp reside (A) and schematic illustration of ADCC induction by the mAb-FA conjugates which binds to the FR-α on a cancer cell via FA (B).

## 2-2 Experimental procedures

**Cell culture**

IGROV-1 human ovarian carcinoma cells were kindly provided by Dr. T. Matsuyama (Kagoshima University), KHYG-1/CD16a-158 V cells were kindly provided by Dr. Y. Mishima (Japanese Foundation for Cancer Research). IGROV-1 cells were cultured in folate-free RPMI-1640 culture medium (Invitrogen). KHYG-1/CD16a-158 V cells were cultured in RPMI-1640 medium (Nacalai Tesque) containing 10 ng $mL^{-1}$ recombinant human IL-2 (Peprotech).

**Antibody**

The following antibodies were used in this study: anti-CD20 hIgG1 mAb (Ofatumumab, Novartis), FITC-labelled anti-hIgG-Fc Fragment Ab (BETHYL).

**Procedures for Trp-targeted conjugation of folate-PEGn-ABNO to mAb.**

Folate-PEGn-ABNO was prepared according to our previous report[13]. Anti-CD20 mAb (1.92 mg, 13.1 nmol), folate-PEG4-ABNO (112 μg, 131 nmol, 29.3 μL of 4.48 mM stock solution), NaNO2 (5.43 μg, 78.8 nmol, 5.43 μL of 1 mg/mL stock solution) and AcOH (6.56 μL) were dissolved in H2O (657 μL) and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was transferred into 20 mM PBS (pH 7.4, 200 μL) to quench the reaction. The small molecules were removed by ultrafiltration (Amicon Ultra, 30 K) (10 times, 200 μL of PBS was added in each time). UV/Vis spectrum of the sample was measured and the FA-to-antibody ratio was determined. Other conjugates with different PEGn (n = 8, 12) were prepared by the same procedures.

**Fluorescent microscopy**

IGROV-1 cells were seeded at $1 \times 10^4$ cells/well in a folate-free RPMI-1640 medium onto a 96-well glass bottom microplate (Greiner Bio-One) and incubated for 24 h. The cells were washed twice with 100 μL of PBS (-), and then incubated with mAb-FA conjugate (100 nM) or mAb (100 nM) in folate-free RPMI-1640 medium containing 1% FBS for 30 min at 4 °C. For competition experiments, folic acid (100 μM) was added simultaneously with mAb-FA conjugates. Then cells were washed twice with 100 μL of

folate-free RPMI-1640 containing 1% FBS and then incubated with folate-free RPMI-1640 containing 1% FBS and 1% FITC anti-hIgG-Fc Fragment Ab (x 100). After washing, cells were stained with Hoechst 33342 (Life Technologies) and analyzed using a BZ-8000 fluorescent microscope (Keyence).

**Design of Trp-targeted mAb-FA conjugate aiming at inducing ADCC**

FA has been widely used as a targeting ligand for drug delivery and imaging of cancer cells because some kinds of cancer cells overexpress FR-α, which has a high affinity toward FA[14,15]. Pioneering works for conjugation of mAbs with FA was reported by two groups[16,17]. One of the groups applied the conjugates to induce ADCC and succeeded in enhancing cytotoxic activity of natural killer (NK) cells toward FR-α-expressing cancer cells[16]. In these previous reports, FA was directly conjugated on the lysine residues of mAbs, which may impair the Fc functions. Moreover, the direct conjugation of FA produced mixtures of conjugates reacted via α- and γ-carboxyl groups of FA, although FA conjugated via the α-carboxyl group was reported to be irrelevant for binding to FR-α[18]. Hence, heterogeneous structures of the conjugates prepared by the direct conjugation would vary the efficacy of ADCC of each conjugate molecule. In order to prepare homogeneous conjugates, here I utilized reagents of ABNO-PEGn-FA[13] for Trp-selective conjugation of a mAb (**Figure 2.3**). These reagents enable γ-carboxyl group-selective conjugation of FA on Trp residues of a mAb via a flexible PEG linker. Even if the Trp residues in the Fc region are conjugated by the reagent, the mAb-FA conjugate is expected to induce ADCC via recognition by FcγRIIIa[19,20] because there is no Trp residue in the FcγRIIIa recognition motif of the Fc region (**Figure 2.1**).

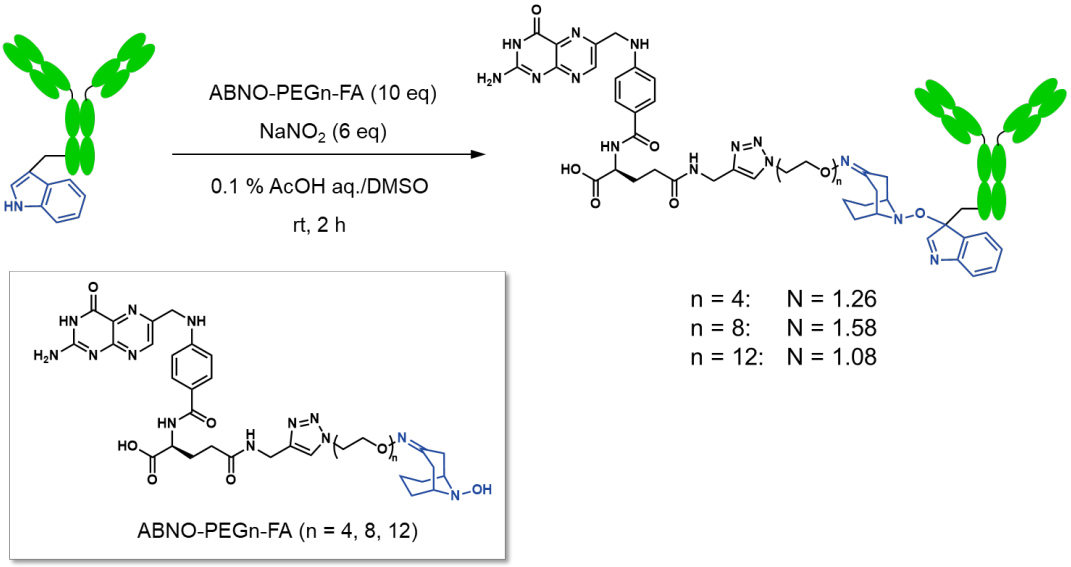



**Figure 2.3.** Preparation of the mAb-FA conjugates

**Preparation of mAb-FA**

mAb-FA conjugates were prepared based on our previous report in which I established the modification procedure of a protein with ABNO-PEGn-FA reagents (**Figure 2.3**)[13]. Here I used a mAb, namely ofatumumab (anti-CD20 IgG1), which can induce ADCC toward CD20-positive cancer cells. mAb and ABNO-PEGn-FA reagents reacted with a 10-fold molar excess of the reagents. The modification ratio of FA (N = FA/mAb) was found to be 1-2 in all the conjugates in spite of the excess molar ratio of ABNO-PEGn-FA to mAb in the reaction. This result suggests that the structures of the conjugates were homogeneous, and some highly exposed Trp residues in the mAb were modified with ABNO-PEGn-FA.

**Flow Cytometry**

IGROV-1 cells were harvested with Accutase (PAN-Biotech) and washed with PBS (-) containing 2% FBS. After cell counting, the cells were re-suspended in folate-free RPMI-1640 medium containing 2% FBS to a density of $5 \times 10^5$ cells/mL. mAb-FA conjugate (final concentration: 0.001-1000 nM) was added and the cells were further incubated for 30 min on ice. After two washes with 2% FBS/PBS (-), cells were incubated for 1 h on ice with folate-free RPMI-1640 containing 2% FBS and 1% FITC anti-hIgG-Fc Fragment Ab (x 100). After two washes with 2% FBS/PBS (-), cells were resuspended in 200 μL of 2% FBS/PBS (-) and analyzed using Cytoflex (Beckman coulter). The data was fitted using GraphPad Prism.

**ADCC assay**

IGROV-1 cells in tissue culture dishes (Greiner Bio-One) were washed in 2 mL of DPBS, detached with Accutase, and washed with folate-free RPMI-1640 medium containing 1% FBS. After cell counting, cells were re-suspended in the folate-free RPMI-1640 medium containing 1% FBS diluted to $1 \times 10^5$ cells per mL. This suspension was seeded into 96-well U-bottom plates (Greiner Bio-One) at 5000 cells per well (50 μL per well). mAb-FA conjugate (50 μL, final concentration: 10 nM) was added to the wells. Subsequently, KHYG-1/CD16a-158V was added (100 μL/well at the indicated effector/target ratio) and the plates were centrifuged (200 × g, 5 min). After incubation at 37 °C under 5% $CO_2$ for 16 h, the plates were centrifuged and 100 μL of the supernatant was transferred to a new 96-well F-bottom plate. ADCC was evaluated using the Cytotoxicity lactate dehydrogenase (LDH) Assay Kit-WST (Dojindo) according to the manufacturer's instructions. The absorbance at 490 nm was measured using the Infinite®

200 PRO M Plex (TECAN). The percentage of cytotoxicity was calculated using the following formula:

% cytotoxicity = (Abs490 sample - Abs490 effector spontaneous - Abs490 target spontaneous) ×100/(Abs490 target max - Abs490 target spontaneous)

For competition experiments, folic acid (1 μM) was added simultaneously with mAb-FA.

**Enzyme-linked immunosorbent assay**

A sample of cell supernatant (20 μL) from the ADCC assay was collected and stored at -30 °C until use. IFN-γ was quantified using the Human IFN-γ ELISA MAX Deluxe kit (BioLegend) according to the manufacturer's instructions.

**Statistical analysis**

Statistically significant differences were tested using two-tailed Student's t-test or Welch's t-test depending on the result of F-test. The symbols *, **, and *** indicate P values less than 0.05, 0.01, and 0.005, respectively; N.S., not significant.

## 2-3 Results and Discussions

**Preparation of mAb-FA conjugates**

ABNO-PEGn-FA was prepared based on our previous report. method using 9-Azabicyclo [3.3.1] nonane N-oxyl (ABNO) reported recently[11], I conducted a conjugation between mAb and folic acid (FA)-PEG-ABNO. mAb (anti-CD20 mAb; Ofatumumab, anti-HER2 mAb; Herceptin) and ABNO-PEGn -FA (10 eq.) were mixed with NaNO2 (6 eq.) in 0.1 % AcOH aq./ DMSO for 2 hours under ambient condition. Each sample's concentration and FA-to-IgG ratio was determined by UV-vis spectrometer (**Figure 2.4-8**).

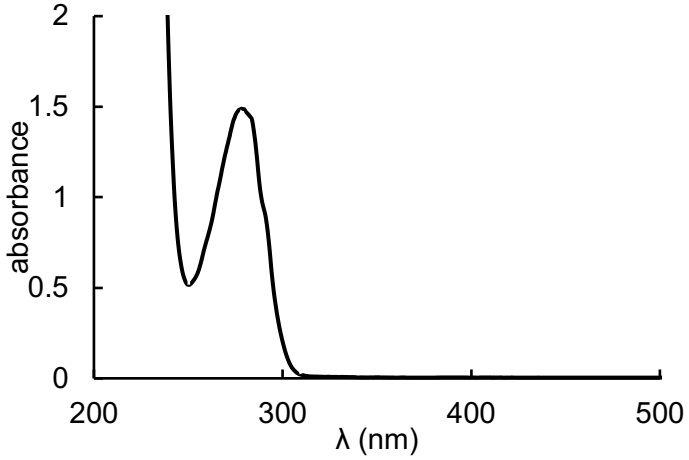



**Figure 2.4.** Absorption spectrum of anti-CD20 mAb (ca. 14 μM in $H_2O$)

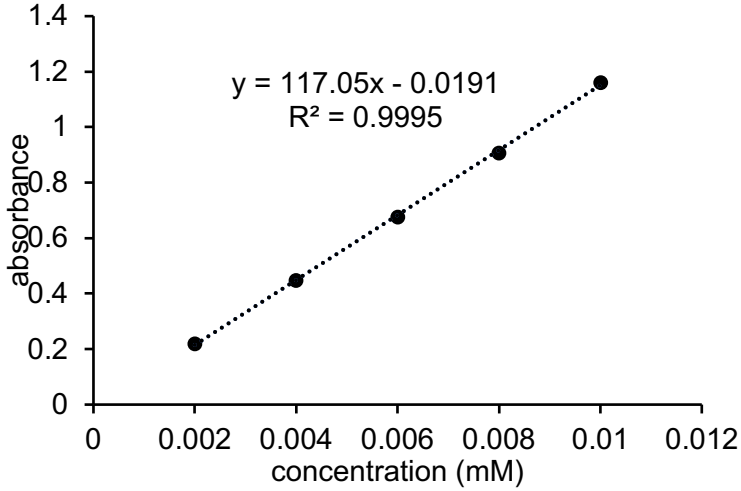



**Figure 2.5.** Calibration line (278 nm) of IgG in 20 mM PBS (pH 7.4). Molar extinction coefficients for each IgG were determined by UV-Vis spectrophotometer; ε(anti-CD20 mAb, 278 nm) = 206.6 $mM^{-1}$ $cm^{-1}$.

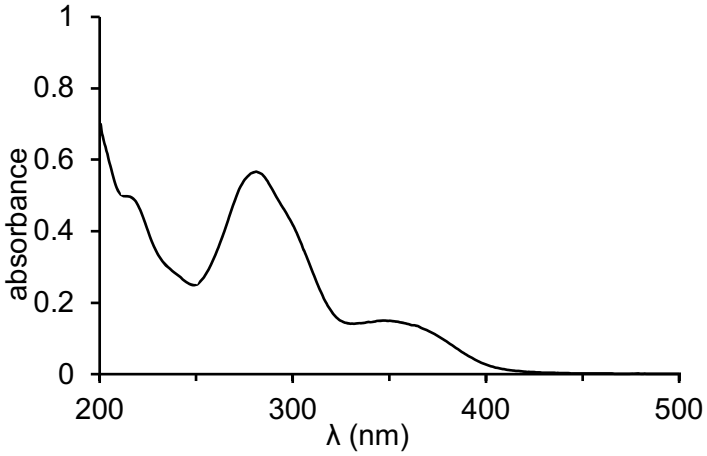



**Figure 2.6.** Absorption spectrum of folic acid (0.05 mM in 20 mM PBS, pH 7.4).

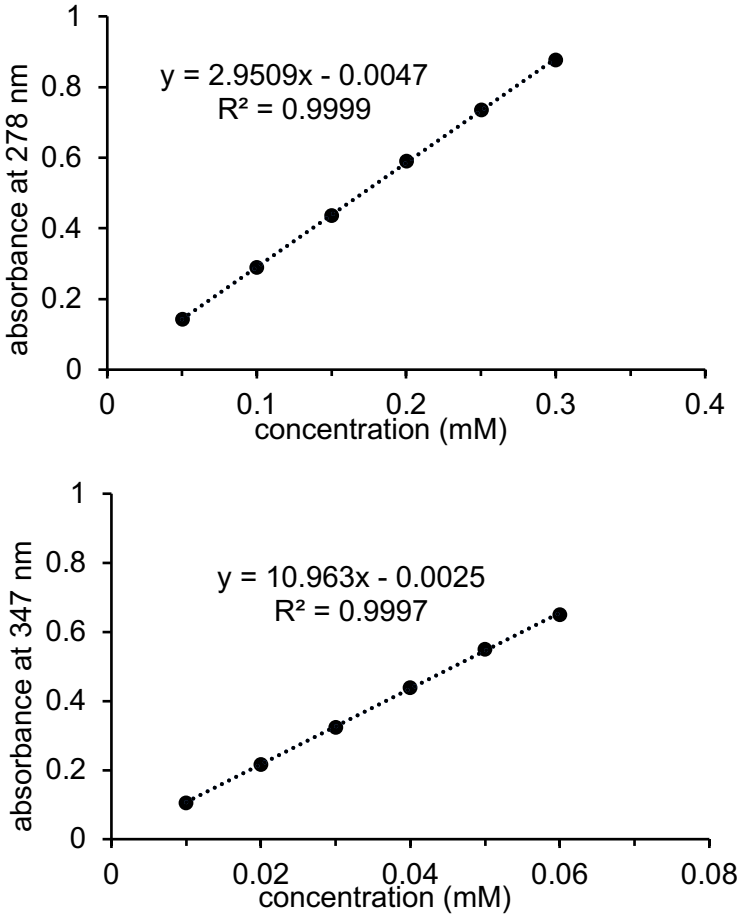
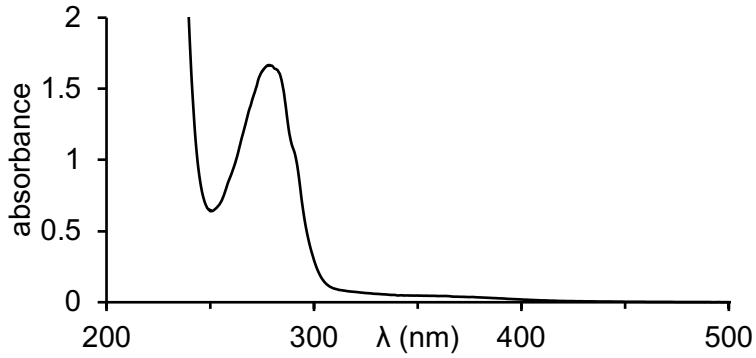
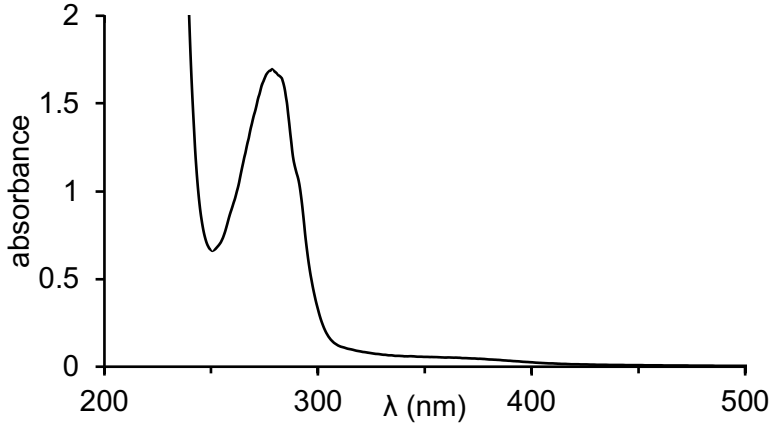



**Figure 2.7.** Calibration line (278, 347 nm) of folic acid in 20 mM PBS (pH 7.4). Molar extinction coefficient for folic acid in 278 nm and 347 nm were determined by UV-Vis spectrophotometer; ε(FA, 278 nm) = 21.926 $mM^{-1}$ $cm^{-1}$, ε(FA, 347 nm) = 5.9018 $mM^{-1}$ $cm^{-1}$.

PEG : 4




PEG : 8




PEG : 12

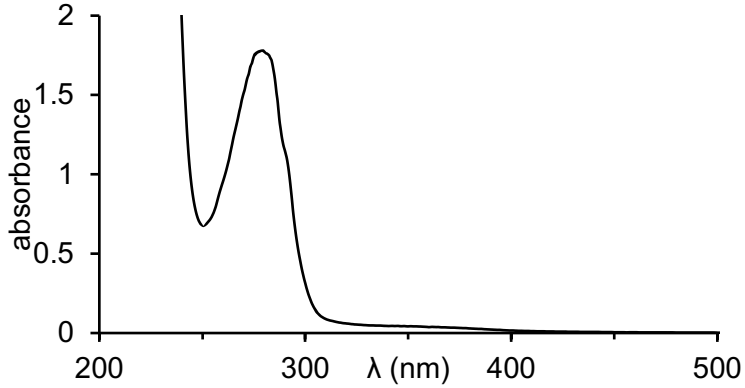
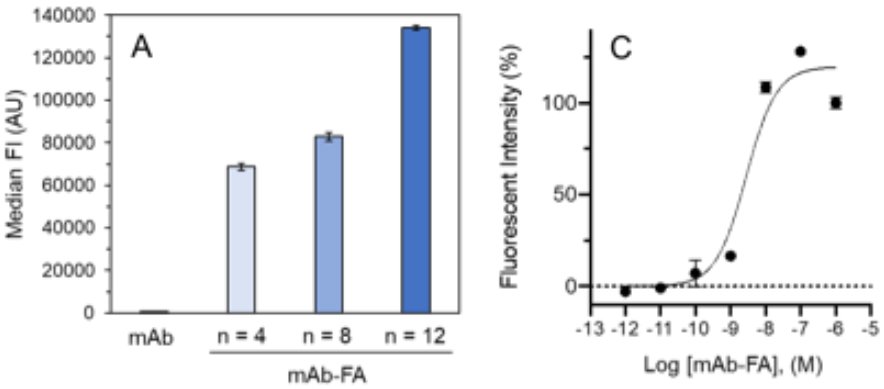



**Figure 2.8.** Absorption spectrum of mAb-FA conjugate in 20 mM PBS, pH 7.4. Concentrations and Folic acid-to-IgG modification ratio (N) were determined by UV-Vis spectroscopy; Anti-CD20 mAb-FA (PEG : 4) (Conc. = 0.0126 mM, N = 1.26), Anti-CD20 mAb-FA (PEG : 8) (Conc. = 0.0126 mM, N = 1.58), Anti-CD20 mAb-FA (PEG : 12) (Conc. = 0.0138 mM, N = 1.08).

**Binding of mAb-FA conjugates to FR-α**

I examined effect of linker lengths of the mAb-FA conjugates on binding to FR-α on cancer cells. I tested three types of mAb-FA conjugates with different linker lengths with IGROV-1 cells (FR-$\alpha^+$, $CD20^-$). The conjugates bound to the cells were detected using a FITC-labeled anti-IgG-Fc antibody and analyzed by flow cytometry (**Figure 2.9 (A)**). The result shows that the fluorescence intensity increased with the length of the PEG linker, and the conjugate with a linker of n = 12 showed the highest binding amount. Hereafter, I used the n = 12 conjugate. **Figure 2.9 (B)** shows the fluorescence microscopy image of IGROV-1 cells treated with the conjugate. Fluorescence resulting from the bound conjugate was clearly observed on the cell surface, whereas the original anti-CD20 mAb did not. Excess FA diminished the binding of the conjugate. These results demonstrate that the mAb-FA specifically binds to the FR-α on the cell surface and the PEG linker length critically affects this binding. **Figure 2.9 (C)** shows the fluorescence intensity of cells, depending on the concentration of the conjugate. A dissociation constant (Kd) calculated from the curve was 4.0 nM, which agrees with the reported values for FA and FR-α (0.1 – 1 nM)[15].



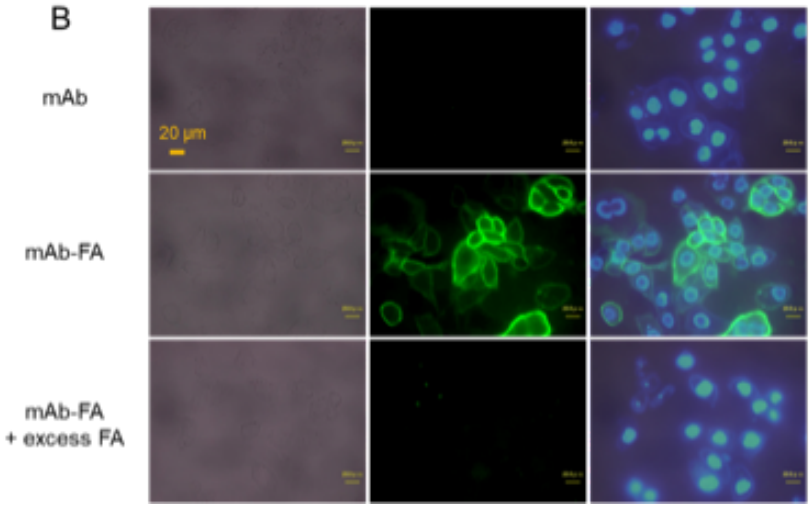



**Figure 2.9.** mAb-FA binds to the FR-α expressed on IGROV-1 cells. (A) IGROV-1 cells were incubated with anti-CD20-FA conjugates with different linker lengths (PEG, n = 4, 8, 12). After a washing step, FITC-labelled anti-IgG Fc polyclonal antibody was added to each sample, and the median of fluorescent intensity (FI) was measured using flow cytometry. (n=3, mean ± SEM.) (B) IGROV-1 cells were seeded and incubated overnight. mAb, mAb-FA, or mAb-FA + excess FA were added. After washing, mAb -FA bound to the cells was detected using anti-IgG-Fc polyclonal IgG. Cells were also stained with Hoechst 33342. (C) IGROV-1 cells were treated with increasing concentrations ofmAb-FA, and the antibodies bound to the cells were quantified. (n=3, mean ± SEM.)

**Induction of ADCC by mAb-FA conjugate**

I evaluated whether the mAb-FA conjugate bound to IGROV-1 cells can activate NK cells and induce ADCC. The conjugates and NK cells (KHYG-1/CD16a-158V) were added to IGROV-1 cells and incubated for 16 hours. Afterward, the cytotoxicity was determined by LDH assay. As shown in **Figure 2.10 (A)**, the conjugate showed significant cytotoxicity with increasing effector/target ratio, while the mAb or mAb-FA with excess FA did not induce cytotoxicity at any effector/target ratios. I directly confirmed the activation of the NK cells upon mixing with IGROV-1 cells by secretion of interferon-γ (IFN-γ). As shown in **Figure 2.10 (B)**, significant amount of IFN-γ was secreted from NK cells in the presence of the mAb-FA conjugate. Such a significant secretion of IFN-γ was not observed in the presence of the mAb or mAb-FA with excess FA. These results demonstrate that the mAb-FA conjugate which bind to cancer cells via FA/FR-α recognition induces ADCC by FcγR-mediated activation of NK cells as illustrated in **Figure 2.2 (B)**. Hence, the Trp-selective conjugation did not compromise the recognition of the Fc region of the conjugate by FcγR on NK cells.

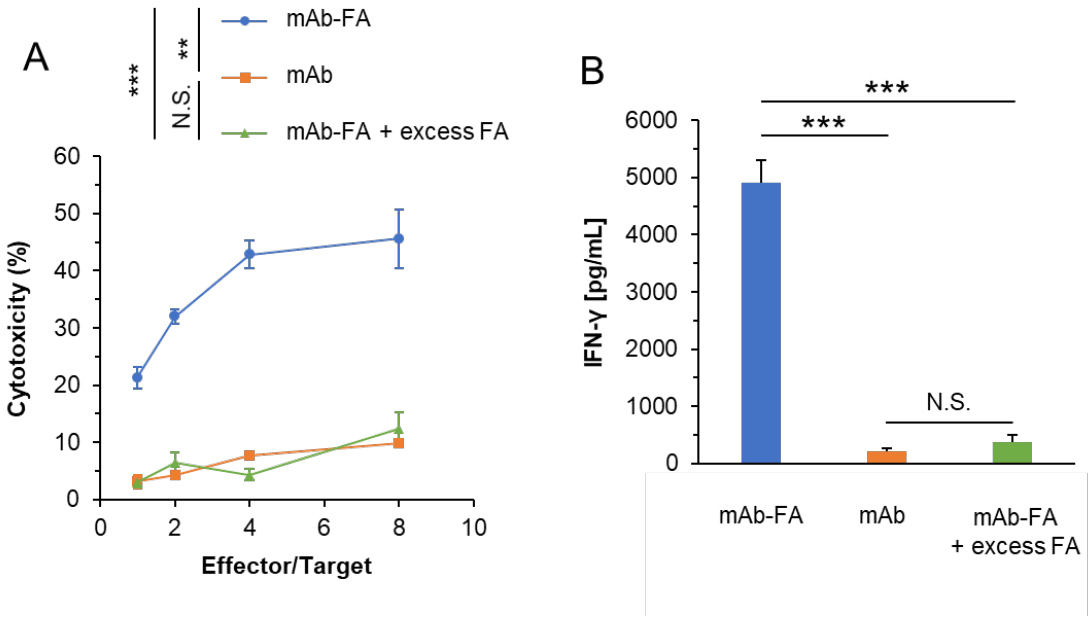



**Figure 2.10.** mAb-FA induces ADCC against FR-$\alpha^+$ IGROV-1 cells. (A) IGROV-1 cells were treated with mAb (10 nM), mAb-FA (10 nM), or mAb-FA (10 nM) + FA (1 μM) and co-cultured with KHYG-1/CD16a-158V for 16 h. Cellular cytotoxicity was quantified by an LDH assay (n=3, mean ± SEM). A statistically significant difference between "mAb-FA" and the others was observed at all Effector/Target ratios. (B) After co-culturing, culture supernatants were collected for detection of IFN-γ by ELISA (E/T = 4, n=3, mean ± SEM). Statistical analyses were carried out using two-tailed Student's t-test or Welche's t-test depending on the result of F-test. **p<0.01, ***p<0.005.

## 2-4 Conclusion

In this chapter, I applied a Trp-selective conjugation of FA to mAb to add functionality to the antibody drug without compromising effector function. The prepared anti-CD20-FA conjugate exhibited a bispecific-like effect by a modified small molecule in addition to the Fab. Anti-CD20-FA bound to FR-α on the target cells and induced ADCC via NK cells, showing that non-Fab binding to the target cells could exert intracellular signaling in NK cells to induce ADCC. The retention of the mAb's ADCC function would result from the fact that Trp residues are not included in the FcγR recognition residues in the Fc region. The Trp-selective conjugation is a promising method to add extra functions to mAbs while preserving the functions of the Fc region. The preparation of bispecific mAbs will be one of the attractive targets to apply the current method.

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# Chapter 3; The effect of cancer cellular cytotoxicity using protein-based antibody recruitment molecules with different dissociation constants

## 3-1 Introduction

In Chapter 2, the ligand for antigen cancer on mAb as a method for engaging NK cells. Previous studies have developed molecules that recruit endogenous immune cells and proteins. Among them, ARMs are being developed that could be an alternative method to antibody drugs, which use immune cells to injure cancer cells by recruiting antibodies that exist in the body to cancer cells[1-4]. To overcome the problem of the low percentage of available antibodies[5-8] and the heterogeneity in therapeutic effect caused by the inconsistent affinity between the Fab region and the ARMs, I have developed a novel molecule called Fc-ARM that targets the Fc region and has successfully induced antigen-mediated recruitment of human antibodies to cancer cells and ADCC and inhibited tumor growth[9, 10].

However, the problem with the Fc-ARM is that they require the use of existing small molecules to target cancer cells, which limits the number of antigens that can be targeted, and they do not show significant tumor growth inhibition, possibly due to affinity or dissociation time issues. Previous studies have suggested that Fc-ARMs induce higher ADCC activity by increasing the activation signal to NK cells depending on their affinity for the Fc region. In fact, it has been reported that the ADCC inducibility of IgG is higher with smaller binding dissociation constants[11]. Studies have also shown that the dissociation constant of the ligand plays a key role in the activation of immune cells[12]. Specifically, it has been shown that immune cells are activated when the dissociation constant of the ligand is smaller than a threshold value, while immune cells are inactivated when the dissociation constant is larger than the threshold value[13-15].

To solve the above problem, I considered replacing Fc-ARMs with proteins. Compared to small molecules, protein molecules are expected to interact with their target molecules at multiple points, thus increasing the affinity, especially the dissociation time, which is important for the formation of immune synapses. Unlike small molecules, it is possible to develop molecules with high specificity and affinity for any protein antigens by evolutionary molecular engineering methods such as the Phage display method.

I hypothesized that the use of a protein-based Fc-ARM molecule would result in the formation of a stable immune synapse with prolonged binding time and stronger

ADCC inducibility. Previously, other research teams have reported results that support our hypothesis, using nanobody-based cross-linked molecules to strongly induce anti-tumor immune responses in vivo by a similar mechanism[16]. I have developed a novel Fc-ARMs consisting of $Z_{HER2:342}$[17], an affibody that binds HER2, a typical tumor marker antigen, and the Z domain[18, 19], which binds to the Fc region (**Figure 3.1**). I generated a prediction of the steric structure of the complex and predicted that ADCC would be induced by the immune complex, which is the result of cross-linking of antigen and antibody by Fc-ARM **(Figure 3.1 (D)**). I also prepared monovalent and bivalent antibody-binding domain Fc-ARMs with different binding affinities and binding times and evaluated the effect of different ßlengths of time on the formation of immune synapses by assessing antibody recruitment to cancer cells and ADCC inducibility via these molecules in vitro.

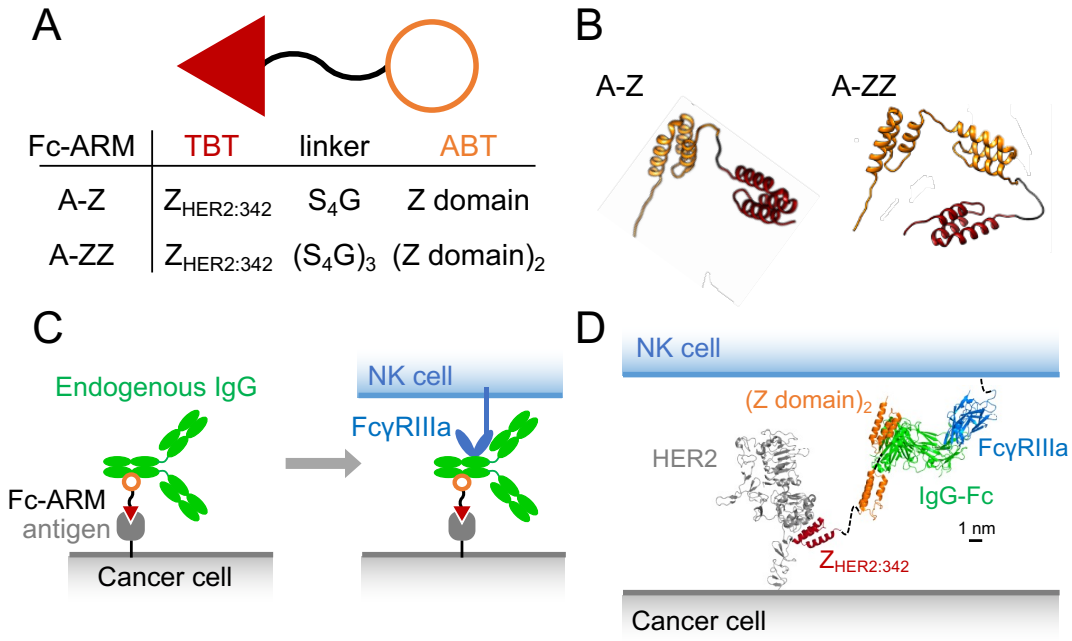



**Figure 3.1** The structure of Fc-ARM and schematic illustration of Fc-ARM strategy
(A) Two types of Fc-ARMs: $Z_{HER2:342}$ was used as target-binding terminus (TBT), and Z domain was used as antibody-binding terminus (ABT). (B) 3D structure predictions of each Fc-ARM. Orange domain; protein Z, Red domain; affibody ($Z_{HER2:342}$). (C) Schematic illustration of ADCC induction by the Fc-ARM which crosslink the FcγRIIIa and antigens. (D) Crystal structure prediction of HER2 – Fc-ARM – IgG-Fc – FcγRIIIa complex. The image was processed using PyMOL. PDBID HER2 – $Z_{HER2:342}$: 3MZW, Z domain – IgG-Fc: 6FGO, FcγRIIIa – IgG-Fc: 5VU0. Gray domain indicates HER2, red domain indicates $Z_{HER2:342}$, orange domain indicates Z domain, Green domain indicates IgG-Fc, and light blue domain indicates FcγRIIIa.

## 3-2 Experimental procedures

**Synthesis of Fc-ARM**

Genes encoding fusion proteins of each protein type Fc-ARM were designed (Sequence. 1, 2) and 15 bp of homologous sequence for in-fusion were inserted at the N- and C-termini of the genes, which were purchased from Integrated DNA Technologies (IDT). The pET22b (+) vector was used for inserting purchased genes. The purchased genes and vector were amplified by Polymerase Chain Reaction (PCR), respectively. PCR was performed using Takara PCR Thermal Cycler Dice (Takara). 0.5 μL of Dpn I (New England Biolabs) was added to the PCR product of the vector and incubated at 37 ℃ for 1 h. Then, 5 μL of 6×DNA loading buffer Dye (Thermo Scientific) was added to the vector and insert sequences. Each reaction solution was loaded onto a 1 % agarose gel containing EtBr prepared using Agarose Kanto S. Agarose electrophoresis was performed at 100 V for 30 min. The band at the target position was cut out using a gel band cutter (Nippon Genetics), and gene extraction was performed according to Qiagen's protocol. Then, the extracted DNA fragments were synthesized using In-Fusion® Snap Assembly Master Mix (Clontech) according to the manufacturer's manual. 5 μL of the prepared plasmid solution was mixed with 50 μL of E. coli JM 109 competent cell (Takara) and transformed by heat shock method. The transformants were then cultured overnight on LB agar medium (Kanto Chemical) containing ampicillin sodium (100 μg /mL). The resulting colonies were selected and incubated (37 ℃, 120 rpm, 14 h) in LB medium containing sodium ampicillin (100 μg/mL). The culture medium was centrifuged at 11,000 rpm for 1 min, and the bacteria were collected and used for plasmid extraction using the plasmid buffer (Qiagen) according to the manufacturer's protocol. Sequence analysis of the obtained plasmid DNA was performed by Eurofins Genomics (Tokyo, Japan), and the plasmid was confirmed to have the sequence of interest.

Each Fc-ARM was synthesized using an E. coli expression system. Prepared plasmids were added to BL21(DE3) competent E. coli (New England Biolabs), and transformation was performed by the heat shock method (42 °C, 30 sec). The E. coli were then cultured on LB agar medium containing sodium ampicillin (100 μg/mL) at 37 ℃ overnight. One of the resulting colonies was selected and incubated (37 ℃, 120 rpm, 6 h) in 5 mL of LB medium containing sodium ampicillin (100 μg/mL). The whole solution was added to 1 L of LB medium containing 100 μg/mL sodium ampicillin and incubated (37 ℃, 120 rpm) until the OD value reached 0.6. Isopropyl β-D-thiogalactopyranoside [IPTG] was then added at a final concentration of 0.5 mM and the cultures were incubated (17 ℃, 120 rpm, O/N). The bacteria expressing the protein of interest were then collected by centrifugation (4 ℃, 2000 ×g, 20 min). The obtained bacteria were suspended in 25

mL of PBS and ultrasonicated. Centrifugation (4 ℃, 8000 ×g, 20 min) was then performed and the supernatant containing the protein of interest was collected.

Sequence Codon sequences and amino acid sequences of the prepared molecules are below.

Sequence 1. $Z_{HER2:342}$-$S_4G$-Zdomain（A-Z）（381 bp, 127 aa, $\varepsilon_{280}$ = 9,970）

```
          10        20        30        40        50        60        70        80        90        100
  1 ATGGTTGATAATAAATTCAACAAAGAGATGCGCAACGCGTATTGGGAGATTGCTTTGCTTCCAAACCTTAACAACCAGCAGAAACGGGCCTTTATCCGTT 100
    M  V  D  N  K  F  N  K  E  M  R  N  A  Y  W  E  I  A  L  L  P  N  L  N  N  Q  Q  K  R  A  F  I  R  S

          110       120       130       140       150       160       170       180       190       200
101 CCCTGTACGATGACCCCTCACAGAGCGCCAACTTATTGGCAGAGGCGAAGAAACTGAACGACGCTCAAGCACCTAAATCGTCTTCCTCCGGAGTCGATAA 200
      L  Y  D  D  P  S  Q  S  A  N  L  L  A  E  A  K  K  L  N  D  A  Q  A  P  K  S  S  S  S  G  V  D  N

          210       220       230       240       250       260       270       280       290       300
201 TAAGTTTAACAAGGAACAACAAAACGCCTTCTATGAGATCCTTCACCTGCCGAACCTTAATGAAGAACAACGGAACGCTTTCATACAATCTCTGAAGGAC 300
      K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L  N  E  E  Q  R  N  A  F  I  Q  S  L  K  D

          310       320       330       340       350       360       370       380
301 GACCCATCGCAGAGTGCAAACTTACTTGCGGAGGCTAAAAAACTTAATGATGCTCAAGCTCCGAAGCATCATCATCACCATCAC 384
      D  P  S  Q  S  A  N  L  L  A  E  A  K  K  L  N  D  A  Q  A  P  K  H  H  H  H  H  H
```

Sequence 2. $Z_{HER2:342}$-$(S_4G)_3$-$(Zdomain)_2$（A-ZZ）（585 bp, 195 aa, $\varepsilon_{280}$ = 11,460）

```
          10        20        30        40        50        60        70        80        90        100
  1 ATGGTTGATAATAAATTCAACAAAGAGATGCGCAACGCGTATTGGGAGATTGCTTTGCTTCCAAACCTTAACAACCAGCAGAAACGGGCCTTTATCCGTT 100
    M  V  D  N  K  F  N  K  E  M  R  N  A  Y  W  E  I  A  L  L  P  N  L  N  N  Q  Q  K  R  A  F  I  R  S

          110       120       130       140       150       160       170       180       190       200
101 CCCTGTACGATGACCCCTCACAGAGCGCCAACTTATTGGCAGAGGCGAAGAAACTGAACGACGCTCAAGCACCTAAATCGTCTTCCTCCGGAAGTAGCAG 200
      L  Y  D  D  P  S  Q  S  A  N  L  L  A  E  A  K  K  L  N  D  A  Q  A  P  K  S  S  S  S  G  S  S  S

          210       220       230       240       250       260       270       280       290       300
201 TTCGGGGTCCTCCTCAAGCGGCGTCGATAATAAGTTTAACAAGGAACAACAAAACGCCTTCTATGAGATCCTTCACCTGCCGAACCTTAATGAAGAACAA 300
      S  G  S  S  S  S  G  V  D  N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L  N  E  E  Q

          310       320       330       340       350       360       370       380       390       400
301 CGGAACGCTTTCATACAATCTCTGAAGGACGACCCATCGCAGAGTGCAAACTTACTTGCGGAGGCTAAAAAACTTAATGATGCTCAAGCTCCGAAGGTCG 400
      R  N  A  F  I  Q  S  L  K  D  D  P  S  Q  S  A  N  L  L  A  E  A  K  K  L  N  D  A  Q  A  P  K  V  D

          410       420       430       440       450       460       470       480       490       500
401 ATAATAAGTTTAACAAGGAACAACAAAACGCCTTCTATGAGATCCTTCACCTGCCGAACCTTAATGAAGAACAACGGAACGCTTTCATACAATCTCTGAA 500
      N  K  F  N  K  E  Q  Q  N  A  F  Y  E  I  L  H  L  P  N  L  N  E  E  Q  R  N  A  F  I  Q  S  L  K

          510       520       530       540       550       560       570       580
501 GGACGACCCATCGCAGAGTGCAAACTTACTTGCGGAGGCTAAAAAACTTAATGATGCTCAAGCTCCGAAGCATCATCATCACCATCAC 588
      D  D  P  S  Q  S  A  N  L  L  A  E  A  K  K  L  N  D  A  Q  A  P  K  H  H  H  H  H  H
```

**Purification of Fc-ARM**

The collected supernatant was filtered through a 0.22 μm filter and purified by a chromatography system for proteins (BioLogic Duo Flow 10F QD system; BIO-RAD) using a His-Tag column (His-Trap FF crude, 5 mL; Cytiva). The purification protocol is described in the column. The purification protocol was followed according to the protocol in the user manual of the column. After chromatography, fractions with absorbance at 280 nm were collected, and the molecular weight of the proteins was confirmed by SDS-PAGE. The fractions containing the protein of interest were then concentrated by ultrafiltration, the solvent was replaced with 1 × PBS using PD-10 desalting columns packed with Sephadex G-25 resin column (Cytiva), and the concentration was determined by UV-vis spectrophotometer (Denovix Inc., USA).

**Cell culture**

SK-BR-3 human breast cancer cells and MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (ATCC). KHYG-1/CD16a-158V cells were kindly provided by Dr. Y. Mishima (Japanese Foundation for Cancer Research). SK-BR-3 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Nacalai Tesque). MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)-high (Nacalai Tesque). KHYG-1/CD16a-158V cells were cultured in RPMI-1640 containing 10 ng/mL recombinant human IL-2 (Peprotech). All the media were supplemented with 10 % of heat-inactivated fetal bovine serum (Nichirei Bioscience), 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque). Cells were cultured in a humidified atmosphere containing 5 % CO2 and 95 % air at 37 ℃. The cell lines were checked for mycoplasma contamination by the MycoAlert mycoplasma detection kit (Lonza).

**Antibodies**

The following antibodies were used: anti-CD20 (Ofatumumab, Novartis), anti-HER2 (Trastuzumab, Chugai), FITC labeled IgG from human serum (Sigma-Aldrich).

**Biolayer interferometry (BLI)**

The affinity measurement was performed against Biotinylated Human IgG1 Fc protein (IG1-H8213, ACROBiosystems), Biotinylated Mouse IgG2a Fc Protein (IGA-M8210, ACROBiosystems), Biotinylated Human Her2 / ErbB2 Protein (HE2-H82E2, ACROBiosystems), Biotinylated Human EGFR Protein (EGR-H82E3, ACROBiosystems) immobilized on Octet® SA Biosensor (18-5019, Sartorius), using

Octet RED96 System (ForteBio), as described in the manufacturer's instructions. The buffer of each sample for BLI was changed to buffer D [50 mM Hepes-KOH pH 7.5, 300 mM NaCl, 0.05 % (v/v) Tween 20, and 0.1 % (w/v) PEG6000] by using Bio-Gel P-6 Gel (1504134, Bio-Rad) before the affinity measurement. The binding assay was performed at 30˚C in the buffer D. Each step in the binding assay was as follows: equilibration for 60 s, association for 600 s, and dissociation for 600 s. Analysis was performed using 1:1 global fitting in ForteBio Data Analysis software v. 10.0.

**Fluorescent microscopy**

SK-BR-3 or MDA-MB-231 cells were seeded at $5 \times 10^4$ cells/well in a folate-free RPMI-1640 medium onto a 96-well glass bottom microplate (Greiner Bio-One) and incubated for 24 h. The cells were washed twice with 100 μL of PBS (-), and then incubated with Fc-ARM (100 nM) and FITC labeled hIgG (IgG-FITC) (1000 nM) in RPMI-1640 medium containing 1 % Super Low IgG FBS for 30 min at 4 ℃. After a wash, cells were stained with Hoechst 33342 (Life Technologies) and analyzed by BZ-8000 fluorescent microscope (Keyence, Osaka, Japan).

**Flow cytometry**

SK-BR-3 cells were harvested with Trypsin-EDTA (Gibco) and washed with PBS (-) containing 2 % super low IgG FBS. After cell counting, the cells were re-suspended in RPMI-1640 medium containing 2 % super low IgG FBS to a density of $5 \times 10^5$ cells/mL. Fc-ARMs (final concentration: 0.01-1000 nM) were added and the cells were further incubated for 30 min on ice. After two washes with 2 % Super low IgG FBS/PBS (-), cells were incubated for 1 h on ice with RPMI-1640 containing 2 % Super low IgG FBS and 1μM of FITC-IgG. After two washes with 2 % Super low IgG FBS/PBS (-), cells were resuspended in 300 μL of 2 % Super low IgG FBS/PBS (-) and analyzed using Cytoflex (Beckman coulter).

**ADCC assay**

SK-BR-3 cells in tissue culture dishes (Greiner Bio-One) were washed in 2 mL PBS (-), detached with, and washed with measurement medium; RPMI-1640 medium containing 1 % Super Low IgG FBS (Cytiva). After cell counting, SK-BR-3 cells were re-suspended in the RPMI-1640 medium containing 1 % Super Low IgG FBS diluted to $1 \times 10^5$ cells/mL. This suspension was seeded into 96-well U-bottom plates (Greiner Bio-One) at $5 \times 10^3$ cells per well (50 μL per well).

Fc-ARM and antibodies were added in the following two procedures.

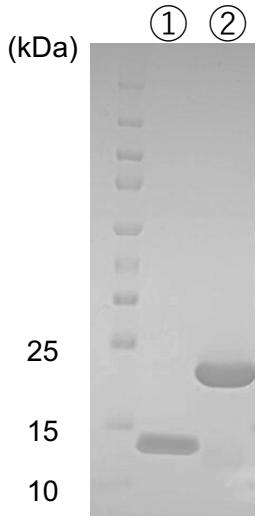
i) Sequential addition: After 25 μL/well of Fc-ARM was added, incubated for 10 min, and then 25 μL/well of measurement medium containing either anti-HER2 antibody, anti-EGFR antibody, or anti-CD20 antibody was added, and incubated for 10 min.
ii) Simultaneous addition: Fc-ARM and anti-CD20 antibody were pre-mixed in a 1.5 mL tube, and the pre-mixed solution, anti-HER2 antibody, or anti-EGFR antibody was added at 50 μL/well.
Subsequently, KHYG-1/CD16a-158V was added (100 μL/well at the indicated effector/target ratio), and the plates were centrifuged (200 ×g, 5 min).
Each sample's final concentration and control is shown below.
Sample
Fc-ARMs (A-Z, A-ZZ): 10 nM, Anti-CD20 mAb: 100 nM, Anti-HER2 mAb: 10 nM
Control
Background control: 200 μL of measurement medium, Low control: 200 μL of measurement medium containing $5 \times 10^3$ cells of target cancer cell, High control: 200 μL of measurement medium containing 1 % Triton$^{TM}$X-100 (Sigma-Aldrich) and $5 \times 10^3$ cells of target cancer cell, Effector control: 200 μL of measurement medium containing 100 U/mL of IL-2 and $5 \times 10^3$ - $4 \times 10^4$ cells of target cancer cell cells of Effector NK cell. After incubation at 37 °C under 5 % CO2 for -16 h, the plates were centrifuged and 100 μL of the supernatant was transferred to a new 96-well plate (Thermo Fisher Scientific). ADCC was evaluated using Cytotoxicity LDH Assay Kit-WST (Dojindo) according to the manufacturer's instructions. The absorbance at 490 nm was measured by Infinite® 200 PRO M Plex (TECAN).
Cytotoxicity (%) was calculated using the following formula:
Cytotoxicity (%) = ($Abs_{490}$ sample - $Abs_{490}$ effector spontaneous - $Abs_{490}$ target spontaneous) × 100 / ($Abs_{490}$ target max - $Abs_{490}$ target spontaneous)

**Dynamic Light Scattering (DLS)**

The particle size of each sample was measured by Zetasizer Ultra (Spectris Co, Ltd., Kanagawa, Japan). A-Z, A-ZZ, and anti-CD20 were prepared separately in 100 mM PBS (pH 7.4) to a final concentration of 2 μM. Then, 30 minutes before measurement, A-Z, A-ZZ, and anti-CD20 antibodies were mixed 1:1 with 100 mM PBS to a final concentration of 1 μM. Mixtures of A-Z or A-ZZ and anti-CD20 antibody were mixed 1:1 to a final concentration of 1 μM for each sample.

## 3-3 Results and Discussion

I used a protein Z or ZZ (Kd against hIgG-Fc is reportedly 70 nM and 2.5 nM, respectively)[19-21] as an ABT. Affibody $Z_{HER2:342}$ for HER2 was selected as a TBT, which has a strong affinity to each target. ABT and TBT are related to $S_4G$ or $G_4S$ linkers which are well-known peptide linkers[22], and two types of Fc-ARMs were synthesized, namely A-Z and A-ZZ. Each sample was successfully synthesized by E. coli expression system, purified by column purification systems, and identified by SDS-PAGE. Then, Fractions showing absorbance at 280 nm were recovered by His-tag column purification. Finally, the molecular weight of the protein was then confirmed by SDS-PAGE (**Figure 3.2**).




| Lane | Fc-ARM | Mw (kDa) |
|---|---|---|
| ① | A-Z | 14.6 |
| ② | A-ZZ | 22.0 |

**Figure 3.2 Sample identification**

SDS-PAGE after purification of each Fc-ARM and their calculated molecular weights.; ①A-Z, ②A-ZZ

Then, I used Biolayer interferometry (BLI) to measure affinities of Fc-ARMs against hIgG1-Fc, mIgG2a-Fc, HER2, or EGFR. I functionalized BLI sensor surface with each target and flowed Fc-ARMs over the surface. The results showed that A-Z and A-ZZ had a strong enough affinity and long dissociation time(τ) to induce ADCC for HER2 and hIgG-Fc, referring to the reported Fc-ARM (Kd and τ; 6.1 nM, 1.04 minutes for Fc and :0.19 nM for target antigen)[9] (**Table 3.1**).

**Table 3.1** Biolayer interferometry (BLI)
Dissociation constants and binding times of each Fc-ARM for each target (antigen: HER2 for A-Z and A-ZZ, FR-α for peptide Fc-ARM, human IgG; hIgG).

| | A-Z | | A-ZZ | | peptide Fc-ARM | |
|---|---|---|---|---|---|---|
| | Kd (nM) | τ (min) | Kd (nM) | τ (min) | Kd (nM) | τ (min) |
| antigen | 0.81 | 40 | 1.4 | 25 | 0.19 | - |
| hIgG-Fc | 7.8 | 6.4 | 0.28 | 98 | 6.1 | 1 |

Next, I verified if the ternary complex of target antigens Fc-ARM, and IgG could be formed. Fluorescent microscopy revealed that Fluorescence-labeled IgG was successfully bound to SK-BR-3 ($HER2^+$) cells in the presence of each Fc-ARM (**Figure 3.3**). A similar experiment was performed with cells that did not express the target antigen, but A-Z and A-ZZ did not recruit antibodies. These results showed that the ternary complex formation is driven by bispecific affinities of Fc-ARMs to target antigens and the IgG.

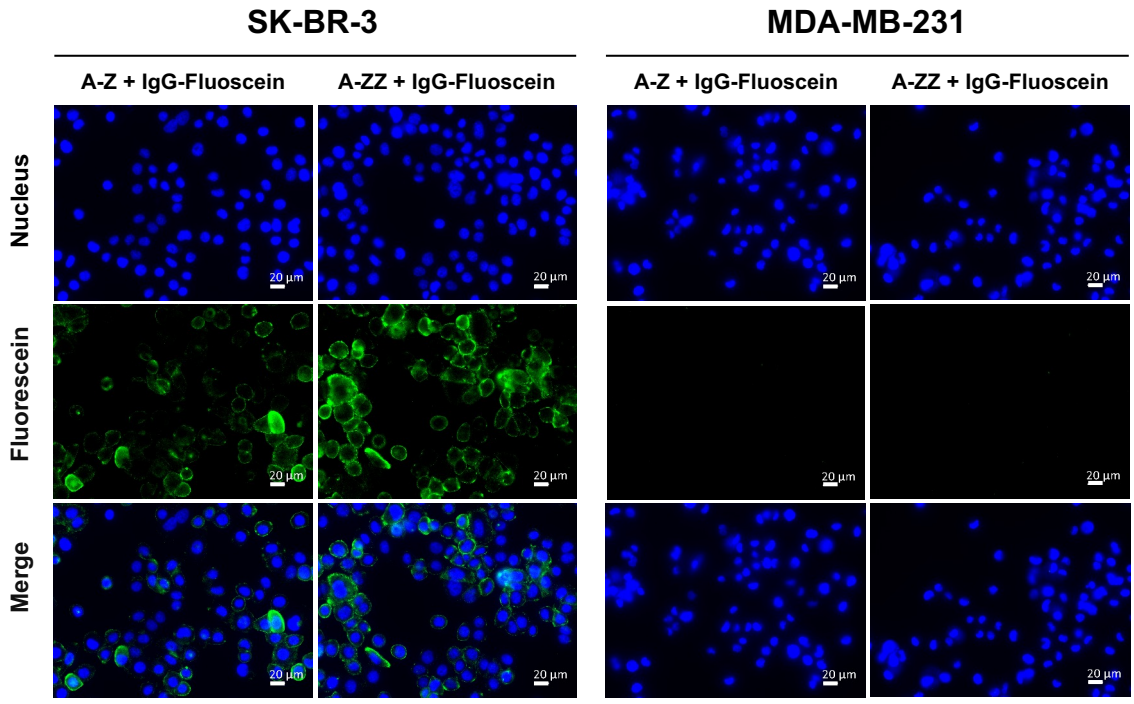



**Figure 3.3.** Fc-ARMs recruit IgG via targeting antigen
Antigen-positive cells SK-BR-3 and Antigen-negative cells MDA-MB-231 were seeded and incubated overnight. Fluorescence-labeled IgG (1000 nM) and each Fc-ARM (100 nM) were added. Nucleus were stained with Hoechst 33342 (blue). Scale bar = 20 μm.

Next, I conducted a quantitative evaluation of the ternary complex formation. Considering that 10 nM of Fc-ARM achieved the maximum fluorescent intensity (**Figure 3.4 (A) (B)**), 10 nM of Fc-ARM (A-Z, A-ZZ) and increasing concentrations of IgG-FITC were added to SK-BR-3 cells. Based on these results, future experiments were conducted with 10 nM for all of Fc-ARMs and 100 nM of IgG (**Figure 3.4 (C) (D)**).

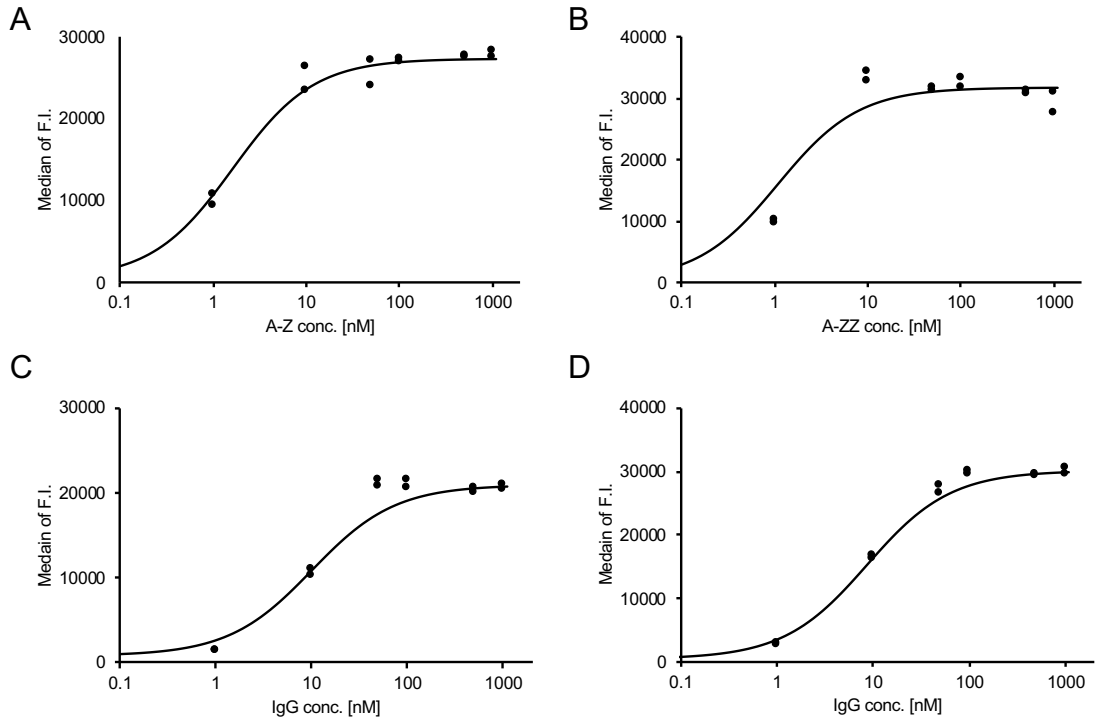



**Figure 3.4** Determination of concentration of each sample for subsequent experiments by Flowcytometry

(A), (B): SK-BR-3 cells were treated with increasing concentrations of Fc-ARM with excess amount of IgG (1 μM). (C), (D): SK-BR-3 cells were treated with increasing concentration of IgG with Fc-ARM at concentrations determined in (A) and (B) (10 nM). Y-axis indicates median of Fluorescence intensity and X-axis indicates the concentration of each sample. Two experimental repeats.

ADCC activity was evaluated using Fc-ARM and Ofatumumab (anti-CD20 human mAb). Ofatumumab has been shown to exhibit ADCC-inducing ability and does not bind to SK-BR-3 via antigen. No cytotoxicity was shown when Fc-ARM or anti-CD20 antibody was added alone. When A-Z or A-ZZ and Ofatumumab were added sequentially, the combination of A-Z and ofatumumab showed no significant increase in cytotoxicity over the other controls (**Figure 3.5 (A)**), whereas the combination of A-ZZ and ofatumumab showed higher cytotoxicity than other controls (**Figure 3.5 (B)**). When simultaneous addition, on the other hand, the combination of A-Z or A-ZZ and ofatumumab did not show significant cytotoxicity. These results suggest that the failure of A-Z to induce significant ADCC may be due to insufficient binding time to form a stable ternary complex, while they do not explain why ADCC could be induced by Fc-ARM prepared with the peptide reported previously[9]. Thus, another hypothesis is that the pertinacious Fc-ARM and antibody complex makes it difficult to be optimally spatially positioned for recognition by the FcγIIIa receptor. The difference between peptide or protein-type Fc-ARM is the number of binding sites and the size of the molecule. I hypothesized that in the case of protein, the interaction at multiple points and the size of the molecule reduced the degree of freedom of the antibody, especially in the Fc region, and that makes it difficult to change conformation to achieve a favorable spatial formation for ADCC induction. When the reason why A-ZZ could not induce ADCC in the case of simultaneous addition was examined. It became clear that the mixture of A-Z and antibody did not form aggregates, whereas the mixture of A-ZZ and antibody formed aggregates (**Figure 3.6**). This may be due to the dimerization of Z cross-links between antibodies resulted in multimerization and the formation of aggregates. Aggregation may have inhibited the formation of immune synapses and failed to transmit signals to the NK cell side, or steric hindrance prevented the FcγIIIa receptor from binding to the antibody, which may have been the cause of the failure to induce ADCC. These results suggest that both the formation of stable ternary complexes with sufficient affinity and the spatial arrangement of each molecule play a major role in inducing ADCC.

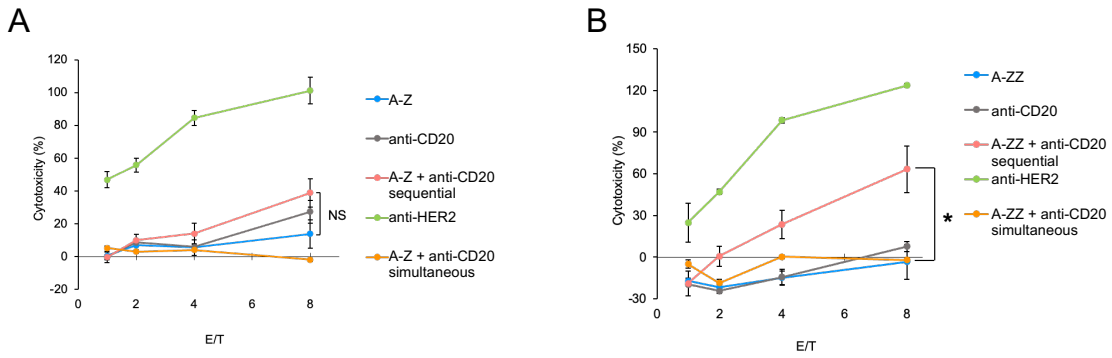



**Figure 3.5** ADCC inducibility differs depending on the method of Fc-ARM addition.

SK-BR-3 cells were treated with Fc-ARM (A) A-Z and (B) A-ZZ (10 nM), and IgG (100 nM) by two different methods. Sequential: Fc-ARM was added and incubated for 30 minutes, after which unbound molecules were washed away and IgG was added. Simultaneous: Fc-ARM and IgG were added simultaneously. The Y-axis indicates the cellular cytotoxicity ratio, and the X-axis indicates the ratio of the number of NK cells to cancer cells.

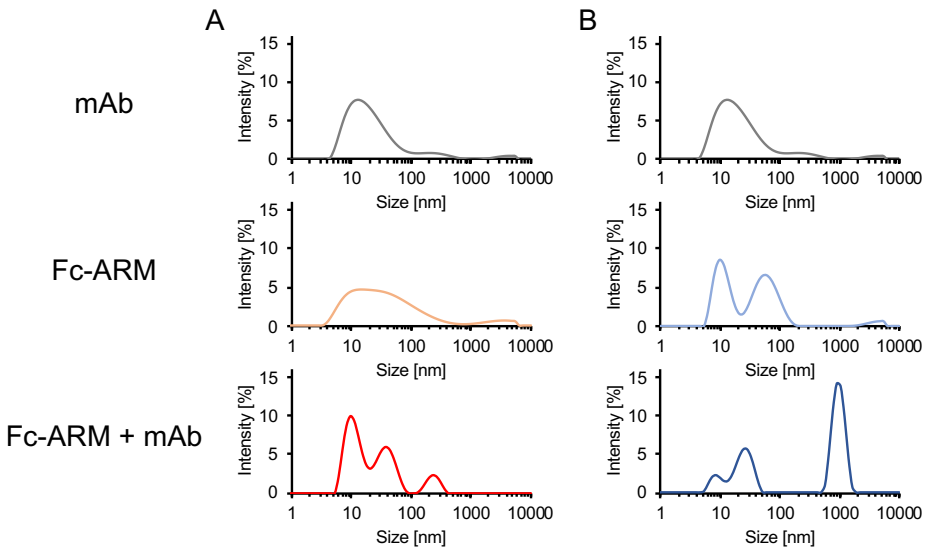



**Figure 3.6** Bivalent Fc-ARM cross-links antibodies and forms aggregation.

Particle size and aggregation properties of the prepared Fc-ARMs (A) A-Z (B) A-ZZ (1 μM), anti-CD20 mAb (1 μM), and mixtures of Fc-ARMs and antibodies were measured using dynamic light scattering (DLS). For mixtures, measurements were taken 30 minutes after mixing.

## 3-4 Conclusion

In this chapter, I developed a protein-based Fc-ARM conjugated with Affibody obtained through the phage display method. The protein-based Fc-ARMs demonstrated binding to antigens on target cells. Fc-ARM binds bivalently to the Fc induced ADCC mediated by NK cells, while monovalent Fc-ARM did not. Interestingly, even with bivalent Fc-ARM, the induction of ADCC varied depending on the method of addition. This suggests that spatial factors, such as the formation of immune synapses facilitating the transmission of activation signals, are as crucial as numerical values such as affinity and binding time. Looking ahead, the design of molecules that prevent aggregation on the cell surface will be necessary. However, protein-based targeting molecule stands out as an attractive method for recruiting immune molecules due to its ease in creating molecules that strongly bind to their targets through evolutionary engineering.

## 3-5 References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# Chpater 4; General Conclusions

Small molecule drugs such as cytotoxic anticancer drugs are passive delivery methods that do not selectively target cancer cells and can affect normal cells other than cancer cells, resulting in side effects. The combination of active targeting of antigens and NK cells that selectively attack cancer cells is expected to improve therapeutic efficacy and reduce side effects. Recently, therapies using CAR-NK and activated NK have been developed, but these cell therapies are very costly in that they require genetic modification and the patient's own NK cells must be used to avoid the Graft versus Host Disease (GvHD). To solve these problems, I focused on NK engager, which utilizes NK cells that are already present in the patient's body for cancer therapy, instead of transplanting donor NK cells.

In Chapter 2, I performed site-selective modification of small molecule ligands to antibodies and evaluated their functions in vitro. In conventional molecular modification of antibodies, the binding position and the number of modifications are random, resulting in heterogeneity in drug efficacy. Especially in the development of ADCs, the elimination of heterogeneity is an important issue, and the development of site-specific modification methods, such as AJICAP®, has been actively pursued. In addition, some binding positions may interfere with the function of antibodies in immunity, and the development of site-specific modification methods with different binding positions depending on the purpose of treatment will meet the needs of clinical use. The molecules developed in this study successfully add functions to antibodies without interfering with their affinity or ADCC inducibility. These results indicate that this method is useful as a novel modification method that solves the problems of conventional molecular modification methods and is expected to be put into practical use.

In Chapter 3, I produced a protein-based Fc-ARM and evaluated its efficacy in vitro. The purpose of this project is to improve the therapeutic efficacy of conventional Fc-ARMs. Previous reports have shown that there is a positive correlation between ADCC inducibility and the stability of immunological synapse formation. Compared to small molecule ligands, protein ligands such as affibody are expected to bind at multiple points and have longer binding times. This is expected to result in longer immunological synapse formation and improved ADCC inducibility. The highest cellular cytotoxicity was observed in molecules with longer binding times to antibodies, indicating the correctness of the strategy. There are still issues that need to be improved before Fc-ARM can be put to clinical use, such as aggregation and insufficient therapeutic effect. In the future, further function improvement of this Fc-ARM is expected by exploring molecules

with higher affinity to form more stable immune synapses.

In recent years, in addition to small molecule drugs and radiation to inhibit the growth and proliferation of cancer cells and surgical removal of tumors, the development of cancer immunotherapy, which utilizes the innate immune function of humans, has attracted attention. Cancer immunotherapy, including antibody drugs, CAR-T cells, and immune checkpoint inhibitors, still faces significant cost challenges in clinical application. The development of off-the-shelf technologies such as those in this study, which are expected to facilitate quality control, is expected to overcome these challenges. In this thesis, I focused on antibodies, NK cells, and ADCC to treat the disease of cancer. However, the essence of the idea of these strategy is the recruitment of biomolecules in situ, and it has potential applications in the treatment of other various diseases by targeting other endogenous molecules. Advances in technology have led to advances in the elucidation of disease mechanisms and the creation of ligands for specific molecules that are compatible with this approach of ours. The above technologies will contribute to the establishment of therapeutic technologies not only for cancer treatment but also for related immune diseases in the future.

# Achievements

List of publications

1. Hiroshi Tagawa, Katsuya Maruyama, Koichi Sasaki, Natsuki Konoue, Motomu Kanai, Takeshi Mori, Kounosuke Oisaki, and Yoshiki Katayama, Induction of ADCC by folic acid-mAb conjugate prepared by tryptophan-selective reaction toward folate-receptor-positive cancer cells, *RSC Advances*, **10**, 16727–16731 (2020).

2. Hiroshi Tagawa, Riku Saeki, Kenta Tanito, Shoki Munekawa, Chihaya Yamamoto, Chihiro Tanaka, Teruki Nii, Akihiro Kishimura, Hiroshi Murakami, Takeshi Mori, Yoshiki Katayama, The effect of cancer cellular cytotoxicity using protein-based antibody recruitment molecules with different dissociation constants, *RSC Advances*, **14**, 22860–22866 (2024).

List of other publications

1. K. Sasaki, M. Harada, Y. Miyashita, H. Tagawa, A. Kishimura, T. Mori,* Y. Katayama*, Fc-binding antibody-recruiting molecules exploit endogenous antibodies for anti-tumor immune responses, *Chem. Sci.*, **11**, 3208-3214 (2020).
2. T. Yoshikawa, K. Q. Phan, H. Tagawa, K. Sasaki, H. Feng, A. Kishimura, T. Mori*, Y. Katayama*, Modification of nitric oxide donors onto a monoclonal antibody boosts accumulation in solid tumors, *Int. J. Pharm.*, **583**, 119352 (2020).
3. K. Tanito, Y. Oshiro, H. Tagawa, A. Kishimura, T. Mori*, Y. Katayama*, Comparative evaluation of natural killer cell-mediated cell killing assay based on the leakage of an endogenous enzyme or a pre-loaded fluorophore, *Anal. Sci.*, **37**, 1571-1575 (2021).
4. K. Sasaki, M. Harada, T. Yoshikawa, H. Tagawa, Y. Harada, Y. Yonemitsu, T. Ryujin, A. Kishimura, T. Mori*, Y. Katayama*, Fc-binding antibody-recruiting molecules targeting prostate-specific membrane antigen: defucosylation of antibody for efficacy improvement, *ChemBioChem.*, **22**, 496-500 (2021).
5. Hiroshi Tagawa “Papers of Interest”, *Life Research Chemistry Letter*. **63** (2021).

List of presentations

1. Hiroshi Tagawa, Koichi Sasaki, Minori Harada, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama “Development of molecules that cause cytotoxicity by recruiting endogenous IgG to cancer cells”, The 24th Joint Seminar of the Kyushu Branch of the CSJ and the Busan Branch of the KCS, Kyushu Branch of the Chemical Society of Japan, Busan Branch of the Korean Chemical Society, 2019.
2. Hiroshi Tagawa, Koichi Sasaki, Minori Harada, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama ”Development of molecules that recruit endogenous IgG to cancer cells and induce cytotoxicity by NK cells”, the 56th Joint Meeting of Kyushu Branches of Chemistry Related Societies. Kyushu Branch, The Chemical Society of Japan, 2019.
3. Hiroshi Tagawa, Koichi Sasaki, Minori Harada, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama ”Development of molecules that recruit endogenous IgG to cells and induce cellular injury by NK cells” the 13th Symposium on Bio-relevent Chemistry, Sendai, Japan, September 2019 (Poster)
4. Hiroshi Tagawa, Katsuya Maruyama, Koichi Sasaki, Natsuki Konoue, Akihiro Kishimura, Motomu Kanai, Takeshi Mori, Kounosuke Oisaki, Yoshiki Katayama, Preservation of effector functions of mAb via tryptophan-selective reaction to prepare folic acid-mAb conjugate, The 69th Symposium on Macromolecules, September 2020 (Oral.)
5. Hiroshi Tagawa, Koichi Sasaki, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama, “Development of cancer immunotherapeutics in which endogenous antibodies are accumulated in cancer cells to induce cytotoxicity”, The 15th Symposium on Bio-relevent Chemistry, September 2021(Poster)
6. Hiroshi Tagawa, Koichi Sasaki, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama, Pacifichem 2021, Development of bispecific molecules which bind antigens and cancer cells to induce ADCC, December 2021 (poster)
7. Hiroshi Tagawa, Koichi Sasaki, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama, “Development of bivalent molecules that induce ADCC by cross-linking endogenous antibodies and cancer antigens”, The 38th Annual Meeting of the Japan Society of Drug Delivery System, 2021 (Oral)

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