Title:

Fc-binding antibody-recruiting molecules targeting prostate-specific membrane antigen: defucosylation of antibody for efficacy improvement

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Abstract

Synthetic small molecules that redirect endogenous antibodies to target cells are promising drug candidates because they overcome the potential shortcomings of therapeutic antibodies, such as immunogenicity. Previously, we reported a novel class of bispecific molecules targeting the antibody Fc region and folate receptor, named Fc-binding antibody-recruiting molecules (Fc-ARMs). Fc-ARMs can theoretically recruit most endogenous antibodies, inducing cancer cell elimination via antibody-dependent cell-mediated cytotoxicity (ADCC). Here, we describe new Fc-ARMs that target prostate cancer (Fc-ARM-Ps). Fc-ARM-Ps recruited antibodies to cancer cells expressing prostate membrane-specific antigen but did so with lower efficiency compared with Fc-ARMs targeting folate receptor. Upon recruitment by Fc-ARM-P, defucosylated antibodies efficiently activated natural killer cells and induced ADCC, whereas antibodies with intact N-glycans did not. The results suggest that the affinity between recruited antibodies and CD16a, a type of Fc receptor expressed on immune cells, could be a key factor controlling immune activation in the Fc-ARM strategy.

Main

Antibody-mediated clearance of malignant cells has a significant potential for treating diseases such as cancer. Once antibodies bind to the antigens on target cells, effector cells such as natural killer (NK) cells recognize the antigen-antibody complexes (immune complexes) via CD16a and lyse the target cells; this process is called antibody-dependent cell-mediated cytotoxicity (ADCC). Although therapeutic antibodies are effective against a wide variety of diseases,[1-3] their costs,[4] immunogenicity,[5, 6] and stability[7] are sometimes problematic.

To address these issues, we previously generated a novel class of antibody-recruiting small molecules (ARMs), named Fc-binding ARMs (Fc-ARMs).[8, 9] Fc-ARMs are bispecific molecules composed of an Fc-binding peptide and a target protein ligand. Unlike conventional ARMs, which utilize antigen–antibody interactions,[10-12] Fc-ARMs bind to endogenous antibodies through Fc affinity. Fc-ARM-bound endogenous antibodies are redirected to the target cells. Subsequently, effector cells recognize the antibodies on these target cells and execute cell killing, via antibody-dependent cell-mediated cytotoxicity (ADCC). Previously, we showed that Fc-ARMs designed to target folate receptor (FR) can induce ADCC against FR-positive cancer cells both *in vitro* and *in vivo*. Furthermore, we demonstrated that the Fc affinity of Fc-ARM is a critical factor regulating both the efficacy and kinetics of ADCC under these conditions. ARMs have the potential to solve the abovementioned issues associated with

therapeutic antibodies because ARMs are chemically synthesizable and possesses relatively small molecular mass compared with antibodies.[13] Fc-ARMs in particular can theoretically provide robust and sufficient opportunities for redirecting endogenous antibodies to malignant cells through exploitation of the conserved structure of the Fc region of antibodies.[9]

We sought to test the broad applicability of the Fc-ARM strategy in multiple types of target cells. For this study, we designed Fc-ARMs targeting prostate cancer (Fc-ARM-Ps) (Figure 1A). Prostate specific membrane antigen (PSMA) is overexpressed on prostate cancer cells, and its expression level correlates with the severity of malignancy.[14, 15] Thus, here we selected PSMA as a target for directing prostate cancer-specific therapy. We used an Fc-III4C peptide as an Fc binder,[16] and used 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid (DUPA) as a PSMA ligand.[17] We synthesized three Fc-ARM-Ps (P1, P2, and P3), which each possesses different lengths of linker between the Fc-III4C peptide and DUPA (Figure 1B), to investigate the potential influence of steric hindrance between the antibody and PSMA on antibody recruitment. First, we synthesized a DUPA partially protected by t-butyl groups, following a previously published protocol with minor modifications (Scheme. S1).[17] After its purification by silica gel chromatography, t-butyl-protected DUPA was identified by ^{1}H-NMR (Figure S1). Using this compound in combination with commercially available building blocks, Fc-ARM-Ps were synthesized by using solid phase peptide synthesis. Fc-ARM-Ps were characterized by reverse-phase high-performance liquid chromatography (RP-HPLC, Figure S2) and matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS, Figure S3).

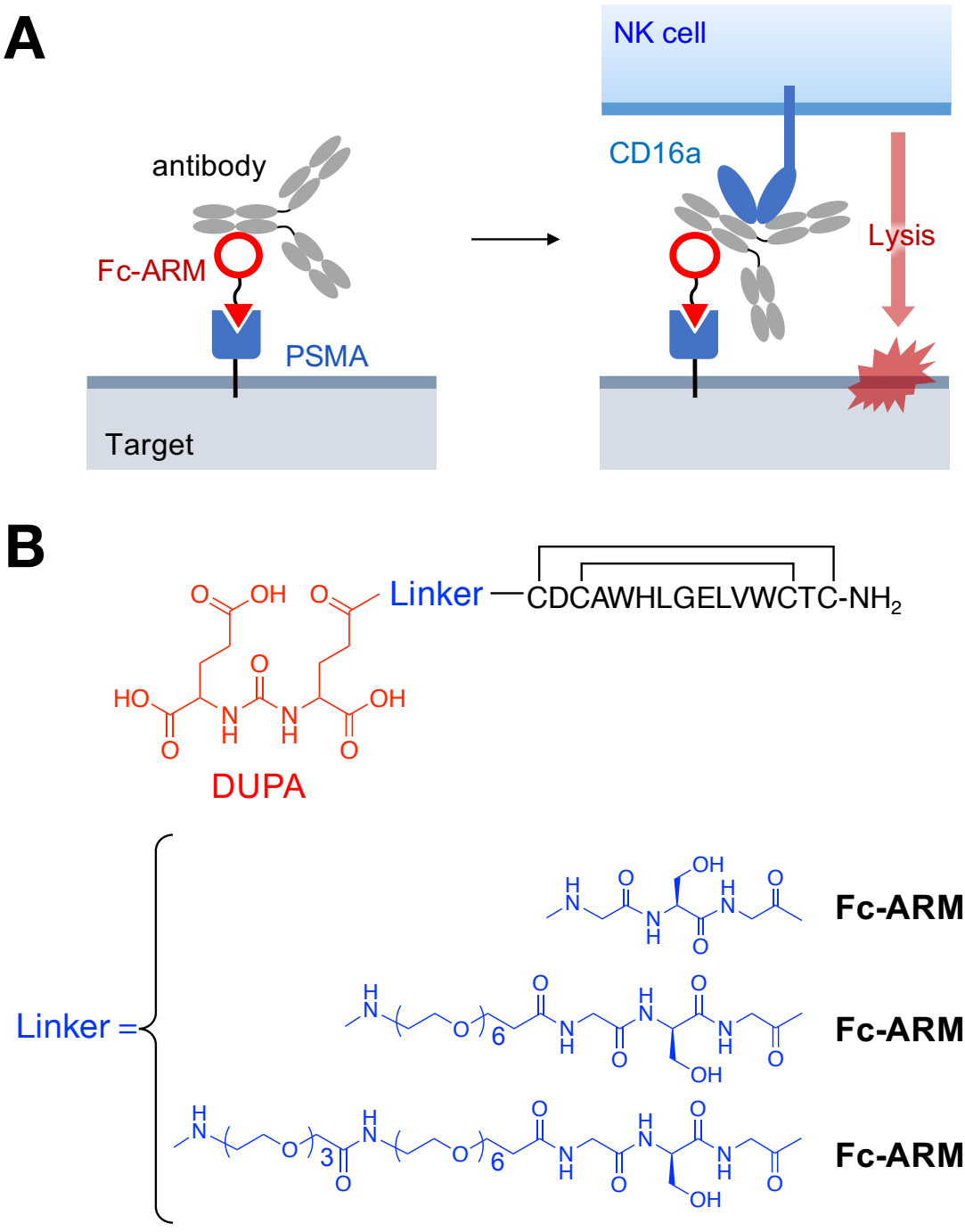


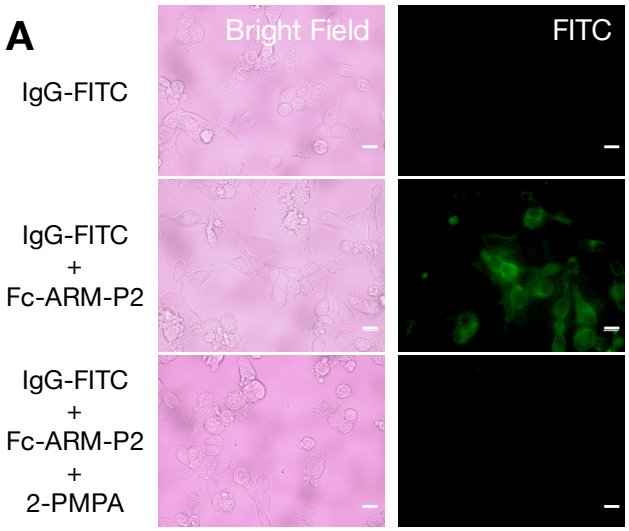
Figure 1. Fc-binding antibody-recruiting small molecules targeting prostate cancer (Fc-ARM-Ps).

(A) Schematic representation of the mechanism of action of Fc-ARM-Ps. PSMA; prostate specific membrane antigen. (B) Molecular design of Fc-ARM-Ps. DUPA; 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid.

We first used Fc-ARM-P2, which has a medium-length linker, to test if a Fc-ARM-P could recruit human IgG conjugated with fluorescein isothiocyanate (IgG-FITC) to PSMA-positive LNCaP cells. Fluorescent microscopy and flow cytometry revealed that Fc-ARM-P2 successfully recruited IgG-FITC to LNCaP cells (Figure 2A, 2B). Competition with 2-(Phosphonomethyl)pentanedioic acid (2-PMPA), a potent PSMA inhibitor,[18] diminished the fluorescence on the cells derived from recruited antibodies. Fc-ARM-P2 did not redirect IgG-FITC to PSMA-negative PC-3 cells (Figure 2C). These results demonstrate that Fc-ARMs can target not only FR, but also PSMA expressed on prostate cancer cells for antibody redirection.

Next, we evaluated the amount of Fc-ARM-P2 needed for antibody recruitment. LNCaP cells were incubated with a sufficient concentration of IgG-FITC (1,000 nM) and increasing concentrations of Fc-ARM-P2 (1–1,000 nM). After unbound molecules were removed by washing, the cells were analyzed by flow cytometry. The amount of antibody on the cells was saturated by 100 nM of Fc-ARM-P2 (Figure S4), an amount about 10-fold higher than the corresponding required amount of Fc-ARMs using folate for targeting the FR.[9] Given that folate has a stronger affinity to its receptor (K_d = 0.19 nM) [19] compared with DUPA (K_i = 8 nM),[20] and the expression level of FR in cancer cells is comparatively in the same extent with that of PSMA,[21] this difference is reasonable.

As we mentioned earlier, the length of a linker between a Fc-binding peptide and a ligand may affect the capacity of antibody recruitment. We found that Fc-ARM-P2 recruited antibodies most efficiently among three synthesized candidates (Figure 2D). Thus, we continued to use Fc-ARM-P2 for following experiments.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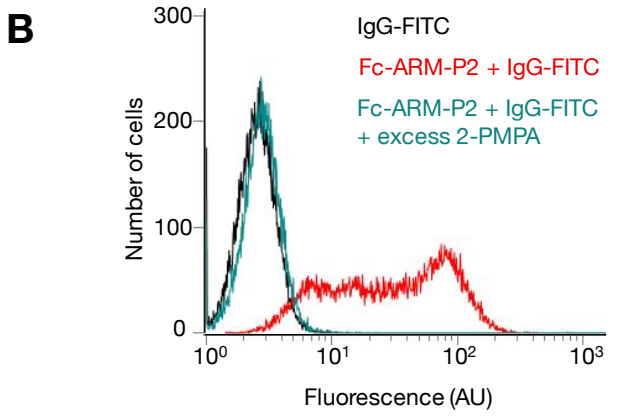
IgG-FITC
IgG-FITC
+
Fc-ARM-P2
IgG-FITC
+
Fc-ARM-P2
+
2-PMPA
Bright Field
FITC


B
IgG-FITC
Fc-ARM-P2 + IgG-FITC
Fc-ARM-P2 + IgG-FITC
+ excess 2-PMPA
Number of cells
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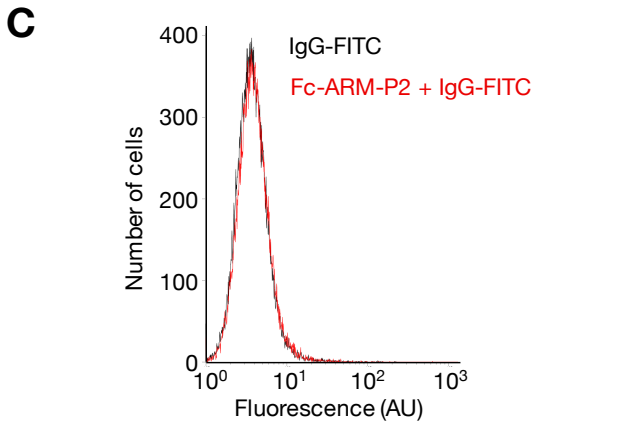
200
100
0
10^0
10^1
10^2
10^3
Fluorescence (AU)


C
IgG-FITC
Fc-ARM-P2 + IgG-FITC
Number of cells
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200
100
0
10^0
10^1
10^2
10^3
Fluorescence (AU)

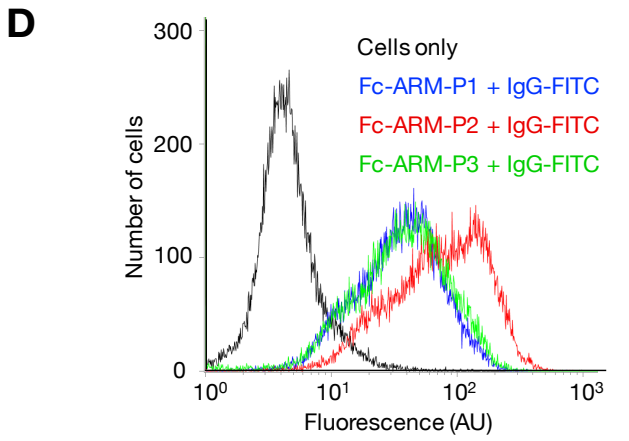
D
Cells only
Fc-ARM-P1 + IgG-FITC
Fc-ARM-P2 + IgG-FITC
Fc-ARM-P3 + IgG-FITC
Number of cells
300
200
100
0
10^0
10^1
10^2
10^3
Fluorescence (AU)

Figure 2. Fc-ARM-Ps recruit antibodies specifically to cancer cells expressing prostate membrane specific antigen (PSMA).

These experiments used IgG-FITC (500 nM), Fc-ARM-P2 (100 nM), and 2-PMPA (10 μM) unless stated otherwise. (A) LNCaP cells were seeded onto a glass-bottom plate and incubated for 24 h. After being incubated for 30 min in the presence of the indicated reagents, the cells were washed and observed by fluorescent microscopy. Scale bar = 20 μm. (B) LNCaP cells treated with the indicated reagents were incubated for 30 min. After being washed, the cells were analyzed by flow cytometry. (C) PC-3 cells were treated with IgG-FITC or Fc-ARM-Ps + IgG-FITC. (D) LNCaP cells were treated with 100 nM of Fc-ARM-Ps and 500 nM of IgG-FITC. Three (A, B) or two (C, D) experimental repeats were performed.

Finally, we evaluated whether Fc-ARM-P-mediated antibody redirection could activate NK cells and induce ADCC. Ofatumumab (anti-CD20 IgG1 monoclonal antibody [mAb]) and mogamulizumab (anti-CCR4 IgG1 mAb) were used because neither of these antibodies show significant binding to LNCaP cells on their own (Figure 3A), and they each possess different affinities to CD16a. Unlike conventional mAbs, mogamulizumab has a stronger affinity to CD16a owing to its defucosylated N-glycans in the Fc region.[22] First, we confirmed that the amount of anti-CD20 antibody recruited by Fc-ARM-P2 was similar to that of anti-CCR4 antibody (Figure 3A). We used a human NK cell line (KHYG-1/CD16a-158V) as an effector.[23] Target cells (LNCaP or PC-3 cells) were co-cultured with the NK cells in the presence of Fc-ARM-P2 and antibodies (anti-CD20 or anti-CCR4 mAb) for 16 h, after which the lactose dehydrogenase (LDH) released from lysed cells was quantified. Incubation with Fc-ARM-P2 and anti-CD20 antibody did not induce ADCC against either the LNCaP or PC-3 cells (Figures 3B and 3C). In contrast, incubation with Fc-ARM-P2 and anti-CCR4 antibody clearly induced ADCC against the LNCaP cells. The combination of Fc-ARM-P2 and anti-CCR4 antibody did not lyse PC-3 cells. NK cells released interferon (IFN)-γ upon recruitment of anti-CCR4 antibody, but not anti-CD20 antibody, to the LNCaP cells (Figures 3D and E). These results demonstrate that the Fc-ARM strategy can eliminate PSMA-positive prostate cancer cells, in agreement with findings on previously reported ARMs against prostate cancer,[24] broadening the applicability of Fc-ARMs to additional cell types. In addition, we suggest the hypothesis that the affinity between antibody and CD16a could be a key factor regulating NK cell activation in the Fc-ARM strategy. This hypothesis is in line with previous literature reporting that ADCC efficacy can be regulated by the strength of antigen–antibody interactions[25] or antibody–Fc receptor interactions.[22, 26] Because the enhanced affinity of anti-CCR4 antibody for CD16a is due to the defucosylation of the N-glycans in its Fc region,[22] recent advances in *in situ* glycoengineering[27, 28] might be applicable for enhancing the efficacy of the Fc-ARM strategy through modification of the glycans on endogenous antibodies.

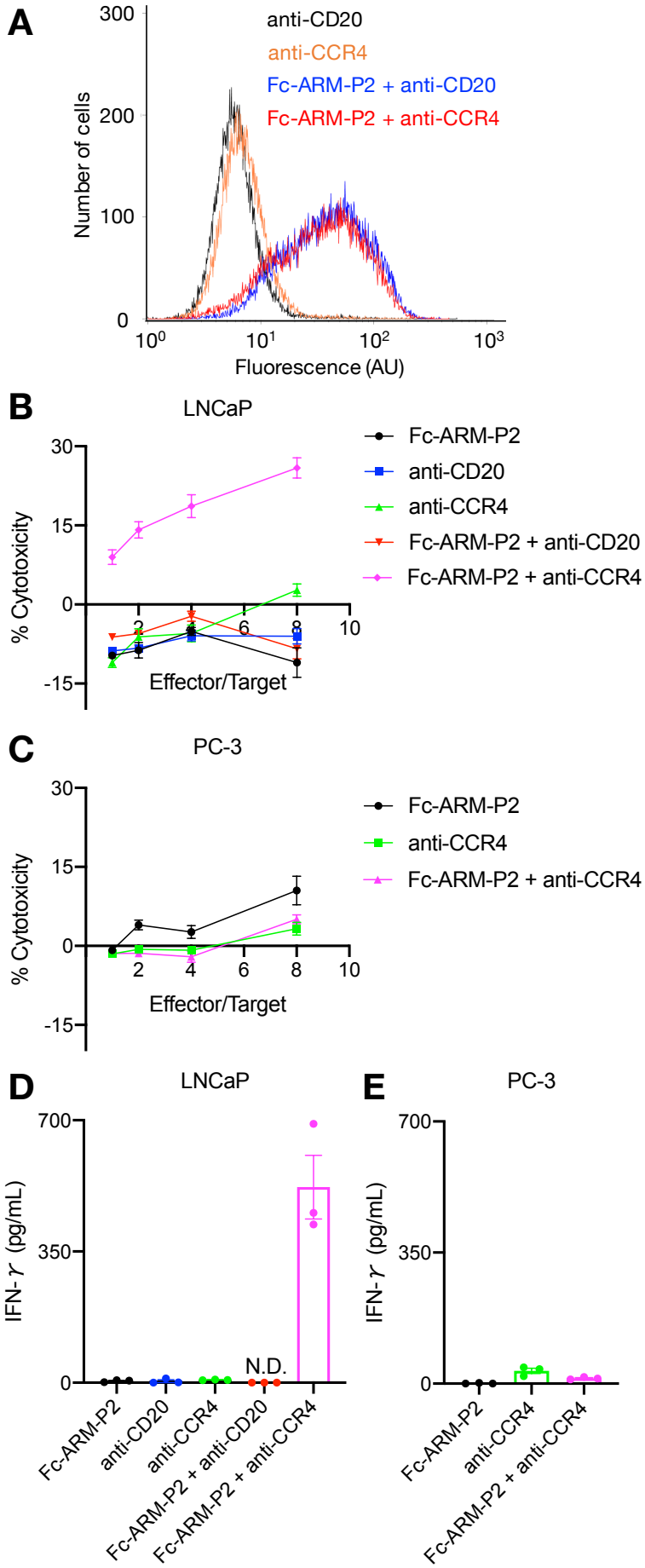
A
anti-CD20
anti-CCR4
Fc-ARM-P2 + anti-CD20
Fc-ARM-P2 + anti-CCR4
Number of cells
300
200
100
0
10^0
10^1
10^2
10^3
Fluorescence (AU)
B
LNCaP
Fc-ARM-P2
anti-CD20
anti-CCR4
Fc-ARM-P2 + anti-CD20
Fc-ARM-P2 + anti-CCR4
% Cytotoxicity
30
15
0
-15
2
6
8
10
Effector/Target
C
PC-3
Fc-ARM-P2
anti-CCR4
Fc-ARM-P2 + anti-CCR4
% Cytotoxicity
30
15
0
-15
2
4
6
8
10
Effector/Target
D
LNCaP
IFN-γ (pg/mL)
700
350
0
N.D.
Fc-ARM-P2
anti-CD20
anti-CCR4
Fc-ARM-P2 + anti-CD20
Fc-ARM-P2 + anti-CCR4
E
PC-3
IFN-γ (pg/mL)
700
350
0
Fc-ARM-P2
anti-CCR4
Fc-ARM-P2 + anti-CCR4

Figure 3. Enhancement of the Fc-ARM-P2 Fc–CD16a interaction compensates for its ADCC efficacy.

(A) LNCaP cells were treated with 100 nM of Fc-ARM-P2 and 500 nM of anti-CD20 or anti-CCR4 mAb. After being incubated for 30 min, the cells were washed, and antibodies recruited to the cells were detected using anti-human IgG secondary antibody. The cells were then analyzed by flow cytometry. (B, C) 5,000 cells/well of LNCaP cells (B) or PC-3 cells (C) were treated with 100 nM of Fc-ARM-P2 and 500 nM of an antibody (anti-CD20 or anti-CCR4). The cells were mixed with 5,000–4,0000 cells/well of KHYG-1/CD16a-158V cells and incubated for 16 h. Lactate dehydrogenase (LDH) released from the lysed cells was quantified to calculate the cytotoxicity %. (n = 3, mean ± SEM). (D, E) After the target cells and effector cells had been co-cultured, culture supernatants were collected, and human interferon (IFN)-γ was quantified by ELISA (Effector/Target = 8, mean ± SEM). Two experimental repeats were performed.

Unlike Fc-ARMs targeting the FR,[9] Fc-ARM-P2 failed to induce ADCC when used in combination with human antibody containing unmodified N-glycans. This is presumably due to the relatively weak affinity for DUPA[20] as compared with folic acid.[19] A straightforward approach to solving this problem would be developing a ligand that possesses a stronger affinity for PSMA. Furthermore, some recent advances in the field of ARMs could be informative for addressing this issue. For example, the Geest group recently reported multivalent antibody-recruiting polymers that enable efficient anchoring of endogenous antibodies onto cancer cell surfaces.[29] Multivalency may be useful for recruiting endogenous antibodies more strongly, enabling immune cell activation even when using a targeting ligand with a relatively weak affinity. The Rullo group recently reported a novel class of ARMs, named covalent immune recruiters (CIRs);[30] after forming a binary complex with an endogenous antibody, CIRs react with a lysine residue within the antibody, forming a covalent bond between the CIR and antibody. Consequently, CIRs can acquire a nearly infinite affinity to the antibody. This novel technique could be also utilized to compensate for the weak affinity of target protein ligands.

In summary, we generated Fc-ARM-Ps, a novel Fc-ARM type that targets prostate cancer cells. The Fc-ARM-Ps recruited human antibodies to PSMA-positive cancer cells. The efficiency of antibody recruitment was dependent on the linker length between the Fc-binding peptide and the targeting ligand; here, Fc-ARM-P2 showed the most efficient antibody recruitment. Once recruited by Fc-ARM-P2, anti-CCR4 antibody, which contains defucosylated N-glycans, induced ADCC against PSMA-positive LNCaP cells, whereas anti-CD20 antibody, which contains intact glycans, did not. Together with our previous report, these findings suggest that the Fc-ARM strategy is applicable to multiple target cell types and that the affinity between Fc receptors expressed on immune cells and recruited antibody could be a factor regulating ADCC efficacy. Further exploration of applicable targets and optimization of the Fc-ARM molecular structure will enhance the potential of Fc-ARM as a novel class of immunotherapeutics.

Methods

Experimental procedures are described in detail in the supporting information.

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Author contributions

K.S., M.H., T.M., and Y.K. designed the project. K.S., M.H., T. Y., and H.T. performed the experiments. K.S., M.H., T.Y., H.T., and T.M. analyzed the data. T.R. assisted with synthesis of Fc-ARM-Ps. Y.H. and Y.Y. assisted with ADCC experiments. K.S., T.M., A.K., and Y.K. wrote the manuscript. All authors read and approved the manuscript.

Notes

The authors declare no competing financial interests.

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TOC

- ✓ Fc affinity for Ab redirection
- ✓ Targeting prostate cancer
- ✓ Ab−CD16a affinity regulates ADCC