

Article

Not peer-reviewed version

Anti-CD44 Variant 10 Monoclonal Antibody Exerts Antitumor Activity in Mouse Xenograft Models of Oral Squamous Cell Carcinomas

[Kenichiro Ishikawa](#) , [Hiroyuki Suzuki](#) ^{*} , [Tomokazu Ohishi](#) , [Guanjie Li](#) , [Tomohiro Tanaka](#) , [Manabu Kawada](#) , [Akira Ohkoshi](#) , Mika K. Kaneko , Yukio Katori , [Yukinari Kato](#) ^{*}

Posted Date: 6 August 2024

doi: 10.20944/preprints202408.0238.v1

Keywords: CD44 variant 10; monoclonal antibody; ADCC; CDC; oral cancer; antitumor activity



Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Article

Anti-CD44 Variant 10 Monoclonal Antibody Exerts Antitumor Activity in Mouse Xenograft Models of Oral Squamous Cell Carcinomas

Kenichiro Ishikawa ^{1,2}, Hiroyuki Suzuki ^{1,*}, Tomokazu Ohishi ^{3,4}, Guanjie Li ¹, Tomohiro Tanaka ¹, Manabu Kawada ⁴, Akira Ohkoshi ², Mika K. Kaneko ¹, Yukio Katori ² and Yukinari Kato ^{1,*}

¹ Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai Miyagi, 980-8575, Japan; ken.ishikawa.r3@dc.tohoku.ac.jp (K.I.); clownair716@gmail.com (G.L.); tomohiro.tanaka.b5@tohoku.ac.jp (T.T.); k. mika.kaneko.d4@tohoku.ac.jp (M.K.K.)

² Department of Otolaryngology, Head and Neck Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8575, Japan; ohkoshia@hotmail.com (A.O.); yukio.katori.d1@tohoku.ac.jp (Y.Katori)

³ Institute of Microbial Chemistry (BIKAKEN), Numazu, Microbial Chemistry Research Foundation, 18-24 Miyamoto, Numazu-shi 410-0301, Japan; ohishit@bikaken.or.jp (T.O.)

⁴ Institute of Microbial Chemistry (BIKAKEN), Laboratory of Oncology, Microbial Chemistry Research Foundation, 3-14-23 Kamiosaki, Shinagawa-ku 141-0021, Tokyo, Japan; kawadam@bikaken.or.jp (M.K.)

* Correspondence: hiroyuki.suzuki.b4@tohoku.ac.jp (H.S.); yukinari.kato.e6@tohoku.ac.jp (Y.Kato); Tel.: +81-22-717-8207

Abstract: CD44 regulates cell adhesion, proliferation, survival, and stemness and has been considered a tumor therapy target. CD44 possesses the shortest CD44 standard (CD44s) and a variety of CD44 variant (CD44v) isoforms. Since the expression of CD44v is restricted in epithelial cells and carcinomas compared to CD44s, CD44v has been considered a promising target for monoclonal antibody (mAb) therapy. We previously developed an anti-CD44v10 mAb, C₄₄Mab-18 (IgM, kappa) to recognize the variant exon 10-encoded region. In the present study, a mouse IgG_{2a} version of C₄₄Mab-18 (C₄₄Mab-18-mG_{2a}) was generated to evaluate the antitumor activities against CD44-positive cells compared with previously established anti-pan CD44 mAb, C₄₄Mab-46-mG_{2a}. C₄₄Mab-18-mG_{2a} exhibited higher reactivity compared with C₄₄Mab-46-mG_{2a} to CD44v3–10-overexpressed CHO-K1 (CHO/CD44v3–10) and oral squamous cell carcinoma cell lines (HSC-2 and SAS) in flow cytometry. C₄₄Mab-18-mG_{2a} exerted a superior antibody-dependent cellular cytotoxicity (ADCC) against CD44v3–10. In contrast, C₄₄Mab-46-mG_{2a} showed a superior complement-dependent cytotoxicity (CDC) against CD44v3–10. A similar tendency was observed in ADCC and CDC against HSC-2 and SAS. Furthermore, administering C₄₄Mab-18-mG_{2a} or C₄₄Mab-46-mG_{2a} significantly suppressed CD44v3–10, HSC-2, and SAS xenograft tumor growth compared with the control mouse IgG_{2a}. These results indicate that C₄₄Mab-18-mG_{2a} could be a promising therapeutic regimen for CD44v10-positive tumors.

Keywords: CD44 variant 10; monoclonal antibody; ADCC; CDC; oral cancer; antitumor activity

1. Introduction

CD44 is involved in the tumor malignant progression through the promotion of tumor cell proliferation, migration, invasiveness, and stemness [1]. The variety of CD44 function is mediated by the alternative splicing of 20 exons [2]. CD44 standard (CD44s), the shortest isoform of CD44, is generated by the first five (1–5) and the last five (16–20) exons and expressed in a broad range of tissues. The central (6–15) exons are alternatively spliced and inserted between the first and last five

exons of CD44s. The variant exon-containing CD44 is designated as the CD44 variant (CD44v) isoform [3].

The CD44 ectodomain includes a hyaluronic acid (HA)-binding domain (HABD) [4]. The HABD is present in both CD44s and CD44v isoforms. Upon HA binding, CD44s and CD44v transduce the intracellular signaling through the cytoplasmic domain, which promotes cell migration and proliferation [5]. The variant exons-encoding regions possess a variety of functions. The v3-encoded region can be attached to a heparan sulfate side chain, which recruits heparin-binding growth factors and stimulates the signal transduction through activation of the receptors [6,7]. The v6-encoded region potentiates the MET signaling pathway through the formation of a ternary complex with its ligand, hepatocyte growth factor [8,9]. Moreover, the v8–10-encoded region regulates the intracellular reduced glutathione levels through the promotion of a cystine–glutamate transporter function [10]. These functions are essential for tumor cell proliferation, invasiveness, and survival to oxidative stress [10] and chemotherapeutic drugs [11]. Therefore, CD44 has been considered as an essential target for tumor therapy [4].

Monoclonal antibodies (mAbs) against CD44 have been evaluated in clinical trials [12,13]. RG7356, a humanized anti-pan-CD44 mAb, exhibited the antitumor effect for B cell leukemia but no cytotoxicity on normal B cells [14]. In human chronic lymphocytic leukemia-engrafted mouse model, RG7356 administration resulted in complete clearance of engrafted leukemia cells [14]. In acute myeloid leukemia [15] and advanced CD44-positive solid tumor [16], phase I clinical trials were conducted. Although RG7356 exhibited an acceptable safety profile, the studies were terminated due to the lack of dose-response relationship with RG7356 in both clinical and pharmacodynamic aspects [16].

Since CD44v expression is restricted in epithelial tissue and carcinomas, anti-CD44v mAbs were developed and evaluated in clinical studies. Humanized anti-CD44v6 mAbs (BIWA-4 and BIWA-8) labeled with ^{186}Re showed antitumor efficacy in head and neck squamous cell carcinoma (SCC) xenograft-bearing mice [17]. Moreover, the antibody-drug conjugate (ADC) of BIWA-4, bivatuzumab-mertansine, was developed and evaluated in clinical trials [13]. However, the clinical trials were terminated due to severe toxicity in the skin, probably due to the efficient accumulation of mertansine in the skin [13,18]. Therefore, anti-CD44 mAbs with more potent efficacy and lower toxicity to normal cells are desired.

The Fc region of therapeutic mAb binds to Fc γ Rs on dendritic cells, macrophages, and neutrophils, which influences the adaptive immune responses through antigen presentation and cytokine production [19]. Moreover, the Fc γ R binding results in the activation of natural killer (NK) cells and macrophages, which mediates antibody-dependent cellular cytotoxicity (ADCC) [19]. The complement-dependent cellular cytotoxicity (CDC) is also considered as an essential effector function in tumor immunotherapy [20]. The Fc region of therapeutic mAb binds to complement C1q, facilitating the assembly of active C1 complex (C1q, C1r, and C1s). The reaction of the complement cascade finally promotes the assembly of the pore-forming membrane attack complex (MAC or C5b–C9) on the tumor cell membrane, which results in the terminal cell lysis [20]. The involvement of CDC in the antitumor effect was first recognized in the treatment of B cell lymphomas by an anti-CD20 mAb, rituximab [21,22]. Furthermore, the cytolytic capacity by complements has been shown in anti-CD38 and CD52 immunotherapies for multiple myeloma and chronic lymphocytic leukemia, respectively [22–24]. Moreover, a growing body of evidence suggests that complement plays critical roles in not only tumor cell lysis but also several immunologic functions in antitumor immunity [25,26]. In the immunotherapy against solid tumors, an anti-HER2 bispecific and biparatopic antibody, zanidatamab, exerted more potent CDC against HER2-positive breast cancers compared with clinically approved anti-HER2 mAb, trastuzumab [27].

The Cell-Based Immunization and Screening method is a strategy to obtain mAbs against a membrane protein comprehensively. We immunized mice with the CD44v3–10-overexpressed cells or CD44v3–10 ectodomain and established various anti-CD44 mAbs and determined their epitopes. C₄₄Mab-5 [28] and C₄₄Mab-46 [29] are anti-pan-CD44 mAbs, which have the epitopes within the constant exon 2- and 5-encoded sequences, respectively [30–32]. We also established various CD44v-

specific mAbs. C₄₄Mab-6 recognizes variant exon 3-encoded sequences and is referred to as anti-CD44v3 mAb [33]. Furthermore, C₄₄Mab-3 (an anti-CD44v5 mAb) [34], C₄₄Mab-9 (an anti-CD44v6 mAb) [35], C₄₄Mab-34 (an anti-CD44v7/8 mAb) [36], C₄₄Mab-1 (an anti-CD44v9 mAb) [37], and C₄₄Mab-18 (an anti-CD44v10 mAb) [38] were established. C₄₄Mab-108 is an anti-CD44v4 mAb, established by peptide immunization [39]. These mAbs cover almost all variant exons and are applicable to flow cytometry, western blotting, and immunohistochemistry.

In this study, we produced an IgG_{2a}-type C₄₄Mab-18 (C₄₄Mab-18-mG_{2a}) and investigated the antitumor efficacy against CD44v3–10 and oral SCC (OSCC) xenografts by comparing anti-pan-CD44 mAb, C₄₄Mab-46-mG_{2a}.

2. Results

2.1. Flow Cytometric Analysis against CHO/CD44v3–10, HSC-2, and SAS Cells Using C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a}

We previously established an anti-CD44v10 mAb (C₄₄Mab-18, IgM, kappa) and showed the availability for flow cytometry, western blotting, and immunohistochemistry against OSCC tissues [38]. In the present study, we cloned the V_H cDNA of C₄₄Mab-18 and combined it with the C_H cDNA of mouse IgG_{2a}. We also cloned the V_L cDNA of C₄₄Mab-18 and combined it with the C_L cDNA of the mouse kappa light chain. Finally, a class-switched C₄₄Mab-18 (C₄₄Mab-18-mG_{2a}) was produced (Figure 1A). We also used mouse IgG_{2a}-type C₄₄Mab-46 (C₄₄Mab-46-mG_{2a}) as an anti-pan CD44 mAb [40]. In reduced conditions, we confirm the purity of original and recombinant mAbs by SDS-PAGE (Supplementary Figure S1). We next confirmed the reactivity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} using CHO/CD44v3–10 and CD44s cells. Both C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} detected CHO/CD44v3–10 cells in a concentration-dependent manner (Figure 1B). The reactivity of C₄₄Mab-18-mG_{2a} to CHO/CD44v3–10 was superior to that of C₄₄Mab-46-mG_{2a} (Figure 1B). In contrast, C₄₄Mab-18-mG_{2a} did not react with CHO/CD44s cells compared to C₄₄Mab-46-mG_{2a} (Figure 1C). Both C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} did not respond with CHO-K1 cells (Supplementary Figure S2). These results confirmed that C₄₄Mab-18-mG_{2a} recognizes only CHO/CD44v3–10 and that C₄₄Mab-46-mG_{2a} recognizes both CHO/CD44v3–10 and CHO/CD44s.

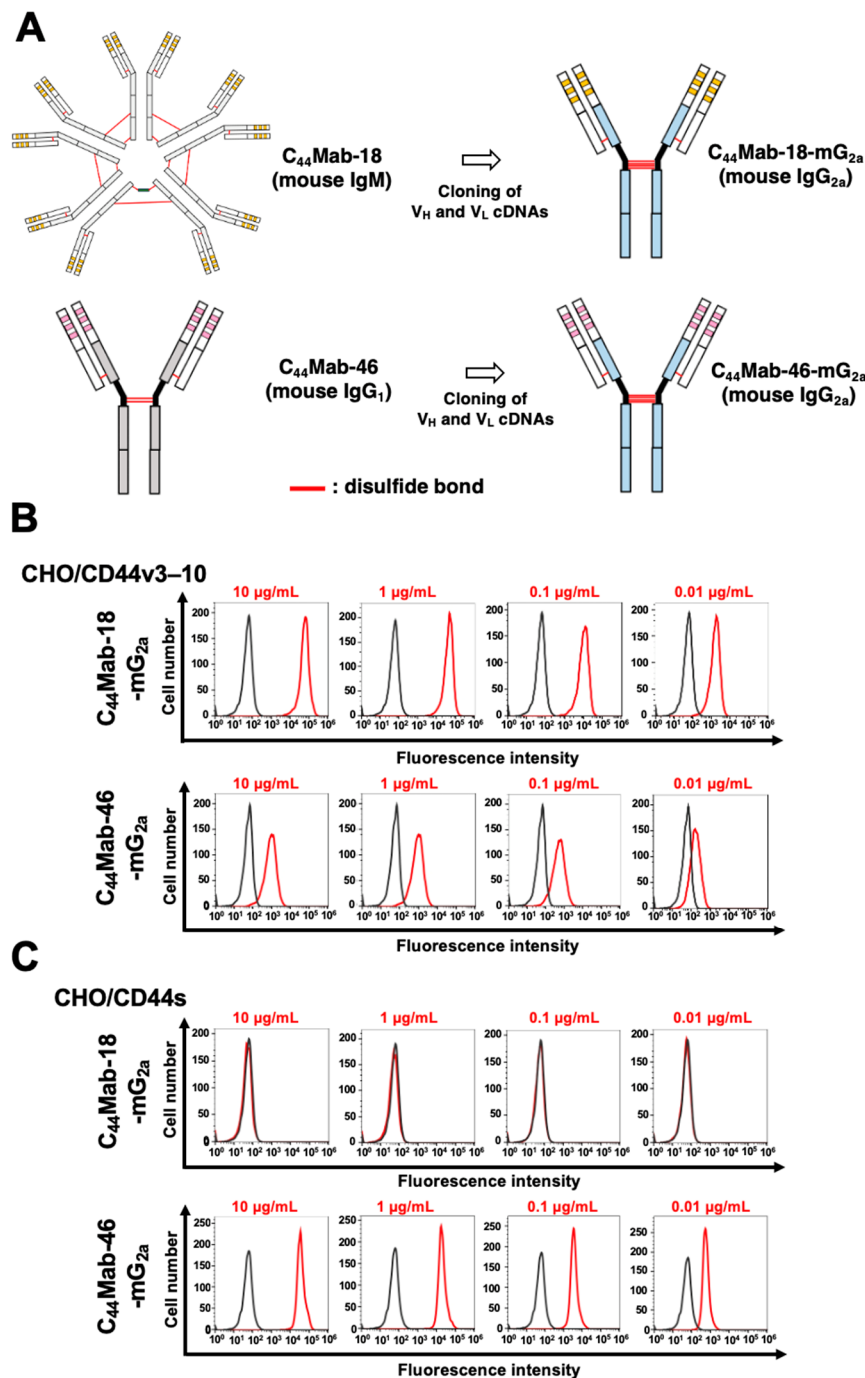


Figure 1. Flow cytometry using C44Mab-18-mG_{2a} and C44Mab-46-mG_{2a}. (A) Class-switched mouse IgG_{2a} mAbs, C44Mab-18-mG_{2a} and C44Mab-46-mG_{2a} were generated from C44Mab-18 (mouse IgM) and C44Mab-46 (mouse IgG₁), respectively. CHO/CD44v3-10 (B) and CHO/CD44s (C) cells were treated with buffer control (black) or 10-0.01 µg/mL of C44Mab-18-mG_{2a} and C44Mab-46-mG_{2a} (red). The cells were further treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were analyzed using the SA3800 Cell Analyzer.

We next investigated the reactivity of C44Mab-18-mG_{2a} and C44Mab-46-mG_{2a} against endogenous CD44-expressing OSCC cell lines, HSC-2 and SAS. Both C44Mab-18-mG_{2a} and C44Mab-46-mG_{2a} reacted with HSC-2 (Figure 2A) and SAS (Figure 2B) cells in a concentration-dependent manner. The reactivity of C44Mab-18-mG_{2a} to these cells was superior to that of C44Mab-46-mG_{2a} (Figure 2). We also used PMAb-231 as a control mouse IgG_{2a}, which did not react with CHO/CD44v3-10, HSC-2, and SAS cells (Supplementary Figure S3).

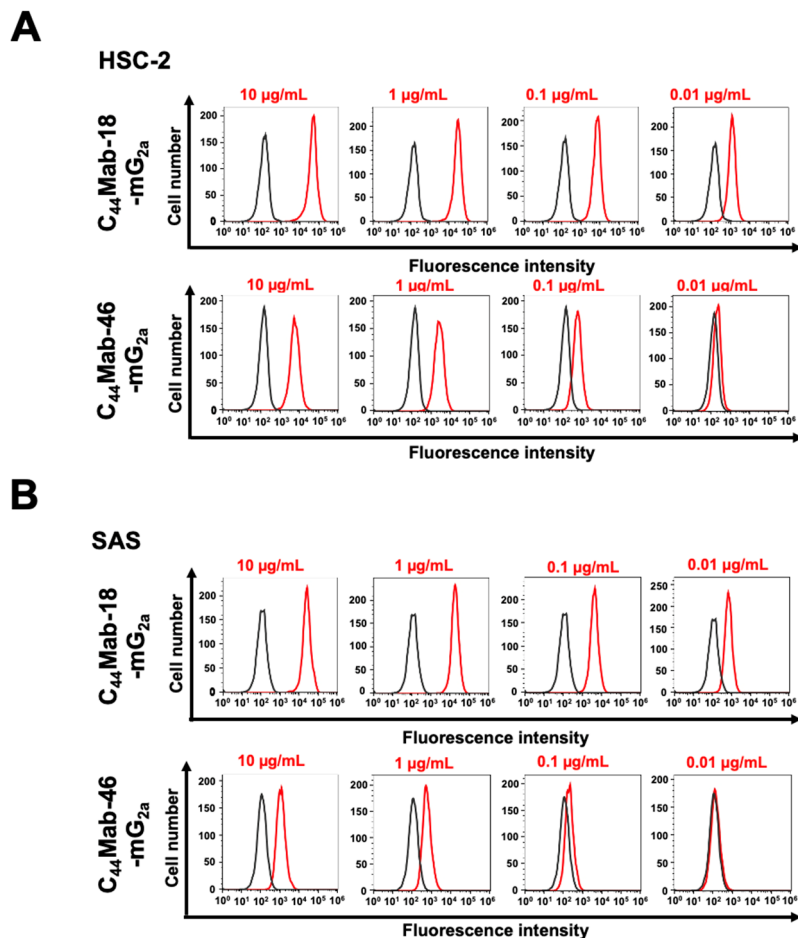


Figure 2. Flow cytometry using C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against oral squamous cell carcinoma (OSCC) cell lines. HSC-2 (A) and SAS (B) cells were treated with buffer control (black) or 10-0.01 µg/mL of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} (red). The cells were further treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were analyzed using the SA3800 Cell Analyzer.

2.2. Induction of ADCC and CDC by C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against CHO/CD44v3-10 Cells

Next, we performed *in vitro* ADCC and CDC assays to examine the cytotoxicity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against CHO/CD44v3-10 cells. As shown in Figure 3A, C₄₄Mab-18-mG_{2a} significantly exerted ADCC against CHO/CD44v3-10 cells in the presence of mouse splenocytes (30% cytotoxicity, $p < 0.01$) compared with control mouse IgG_{2a} (PMab-231, 11% cytotoxicity). Although a tendency of increased ADCC compared with the control was observed by C₄₄Mab-46-mG_{2a}, the difference was not significant.

In contrast, C₄₄Mab-46-mG_{2a} significantly exerted CDC against CHO/CD44v3-10 cells in the presence of complements (27% cytotoxicity, $p < 0.05$) compared with the control (12% cytotoxicity). We did not observe a significant difference in CDC between C₄₄Mab-18-mG_{2a} and the control (Figure 3B)

These results indicated that C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} possess different properties for exerting ADCC and CDC against CHO/CD44v3-10 cells.

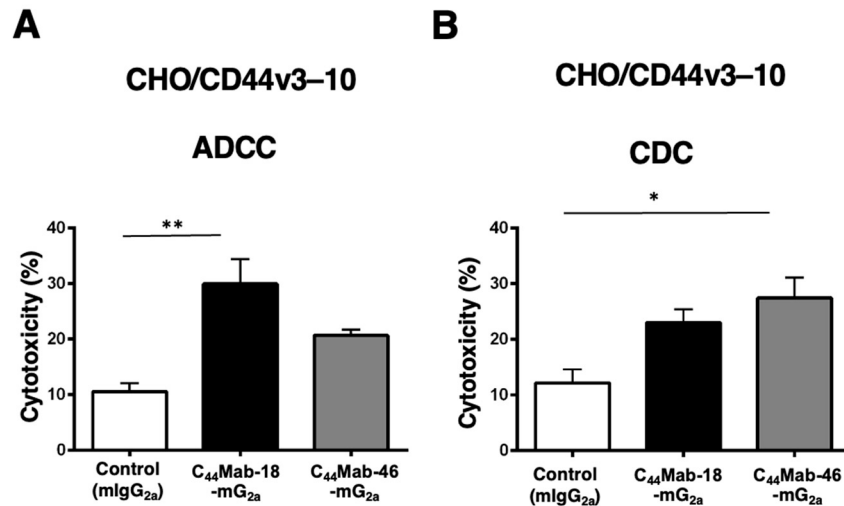


Figure 3. Evaluation of ADCC and CDC activity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against CHO/CD44v3-10 cells. The ADCC (A) and CDC (B) induced by C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mouse IgG_{2a} (mIgG_{2a}, PMab-231) against CHO/CD44v3-10 cells. Values are shown as mean $\pm$ SEM. Asterisks indicate statistical significance (** $p < 0.01$ and * $p < 0.05$; one-way ANOVA with Tukey's multiple comparisons test).

2.3. Antitumor Effects of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} in the Mouse Xenografts of CHO/CD44v3-10

We next evaluated the *in vivo* antitumor effect of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against CHO/CD44v3-10 xenograft tumors inoculated in nude mice. C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mouse IgG_{2a} (100 μ g/mouse) were intraperitoneally injected into mice on days 7, 14, and 21, following the inoculation. The tumor volume was measured on days 7, 14, 17, 21, 24, and 28. The C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} administration resulted in a significant reduction in tumor volume on days 17 ($p < 0.05$ in C₄₄Mab-18-mG_{2a}), 21 ($p < 0.01$), 24 ($p < 0.01$), and 28 ($p < 0.01$) compared with that of the control mouse IgG_{2a} (Figure 4A). The C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} administration resulted in 56% and 50% reductions in tumor volume compared with the control mouse IgG_{2a} on day 28, respectively.

The weight of CHO/CD44v3-10 tumors treated with C₄₄Mab-18-mG_{2a} was significantly lower than those treated with the control mouse IgG_{2a} (Figure 4B,C, 62% reduction; $p < 0.05$). Although a 53% reduction in tumor weight was observed by C₄₄Mab-46-mG_{2a} treatment, it is not statistically different compared to the control ($p = 0.0626$). The loss of body weight was not observed in the CHO/CD44v3-10 tumor-implanted mice during the treatments (Figure 4D,E).

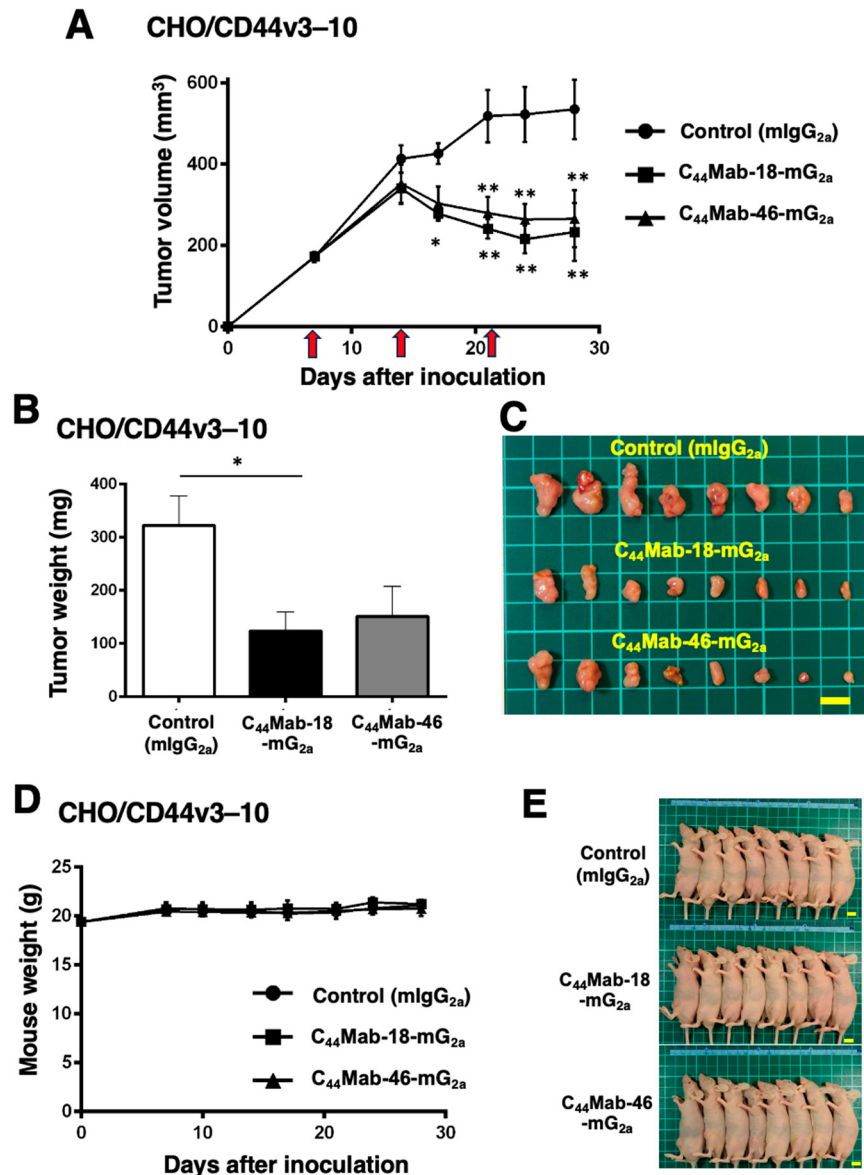


Figure 4. Antitumor activity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against CHO/CD44v3-10 xenograft. (A) Tumor volume of CHO/CD44v3-10 xenograft. CHO/CD44v3-10 cells (5×10^6 cells) were injected into mice subcutaneously. On days 7, 14, and 21, 100 μ g of C₄₄Mab-18-mG_{2a} (n=8), C₄₄Mab-46-mG_{2a} (n=8) or control mouse IgG_{2a} (mlgG_{2a}, PMab-231) (n=8) were injected into mice intraperitoneally (arrows). The tumor volume was measured on days 7, 14, 17, 21, 24, and 28 following the inoculation. Values are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ (two-way ANOVA with Tukey's multiple comparisons test). The weight (B) and appearance (C) of excised CHO/CD44v3-10 xenografts on day 28. Values are presented as the mean $\pm$ SEM. * $p < 0.05$ (one-way ANOVA with Tukey's multiple comparisons test). The body weight (D) and appearance (E) of CHO/CD44v3-10 xenograft-bearing mice treated with C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mlgG_{2a}. Scale bar, 1 cm.

2.4. Induction of ADCC and CDC by C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against HSC-2 and SAS Cells

Next, we performed *in vitro* ADCC and CDC assays to examine the cytotoxicity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against HSC-2 and SAS cells. Both C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} significantly exerted ADCC against HSC-2 cells [Figure 5A, 17% ($p < 0.01$) and 13% ($p < 0.05$) cytotoxicity, respectively] and SAS cells [Figure 5C, 19% ($p < 0.01$) and 14% ($p < 0.05$) cytotoxicity, respectively] compared with the control.

Both C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} significantly exerted CDC against HSC-2 cells [Figure 5B, 27% ($p < 0.05$) and 40% ($p < 0.01$) cytotoxicity, respectively] compared with the control. In SAS cells, C₄₄Mab-46-mG_{2a} significantly exerted CDC [Figure 5D, 26% ($p < 0.05$) cytotoxicity] compared with the control. We could not observe the significant difference of CDC against SAS cells between C₄₄Mab-18-mG_{2a} and the control (Figure 5D).

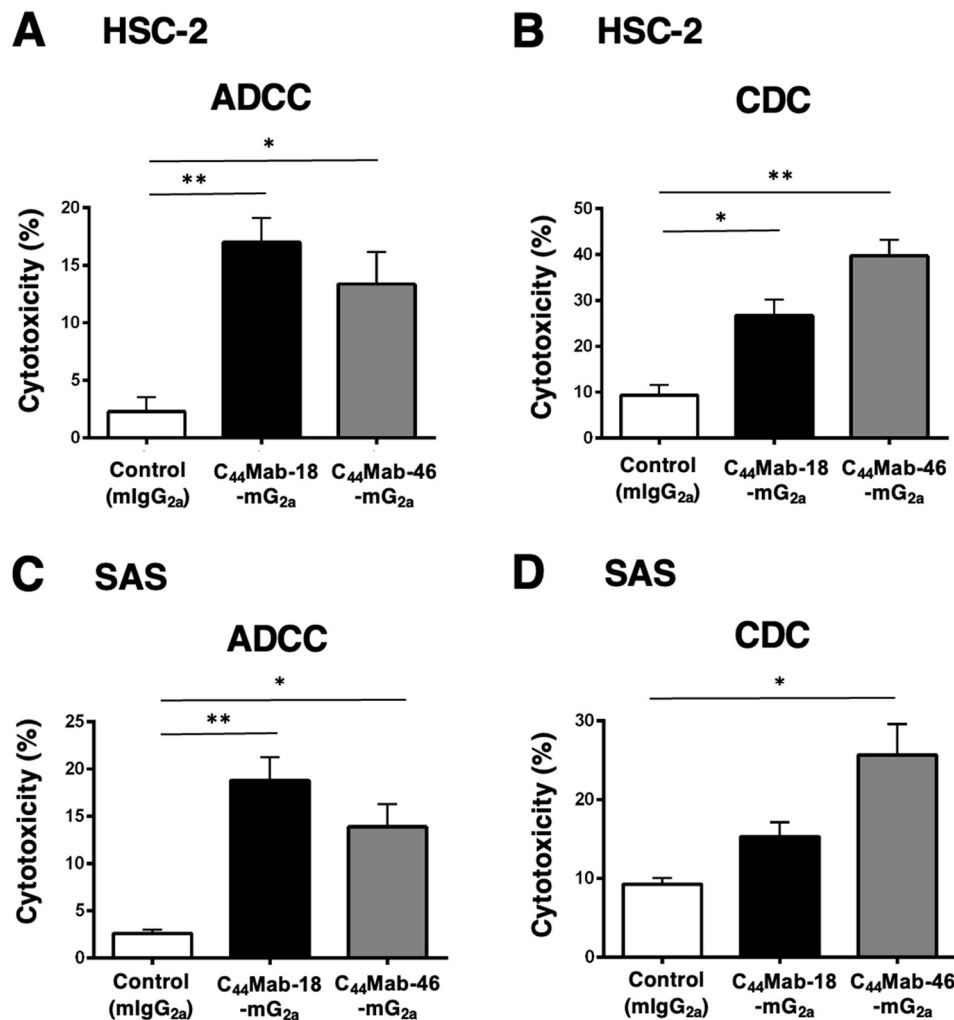


Figure 5. Evaluation of ADCC and CDC activity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against HSC-2 and SAS cells. The ADCC (A,C) and CDC (B,D) induced by C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mouse IgG_{2a} (mIgG_{2a}, PMab-231) against HSC-2 (A,B) and SAS (C,D) cells. Values are shown as mean $\pm$ SEM. Asterisks indicate statistical significance (** $p < 0.01$ and * $p < 0.05$; one-way ANOVA with Tukey's multiple comparisons test).

2.5. Antitumor Effects of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} in the Mouse Xenografts of HSC-2 and SAS

In the HSC-2 and SAS xenograft models, C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mouse IgG_{2a} (500 μ g/mouse) were intraperitoneally administrated into mice on days 7 and 14, following the inoculation. The tumor volume was measured on days 7, 14, and 17. The C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} administration resulted in a significant reduction in both tumor volume on day 14 ($p < 0.01$) and day 17 ($p < 0.01$) compared with that of the control mouse IgG_{2a} (Figure 6A,B). The C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} administration resulted in 43% and 36% reduction of HSC-2 tumor volume, respectively compared with that treated with the control mouse IgG_{2a} on day 17. The C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} administration also resulted in a 33% and 32% reduction of SAS tumor volume, respectively, compared with that treated with the control mouse IgG_{2a} on day 17.

The weight of HSC-2 tumors treated with C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} was significantly lower than that treated with the control mouse IgG_{2a} [Figure 6C, 40% ($p < 0.01$) and 30% ($p < 0.05$) reduction, respectively]. The weight of SAS tumors treated with C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} was significantly lower than that treated with the control mouse IgG_{2a} [Figure 6D, 23% ($p < 0.01$) and 19% ($p < 0.05$) reduction, respectively]. The loss of body weight was not observed in the HSC-2 and SAS tumor-implanted mice during the treatments (Figure 6E,F).

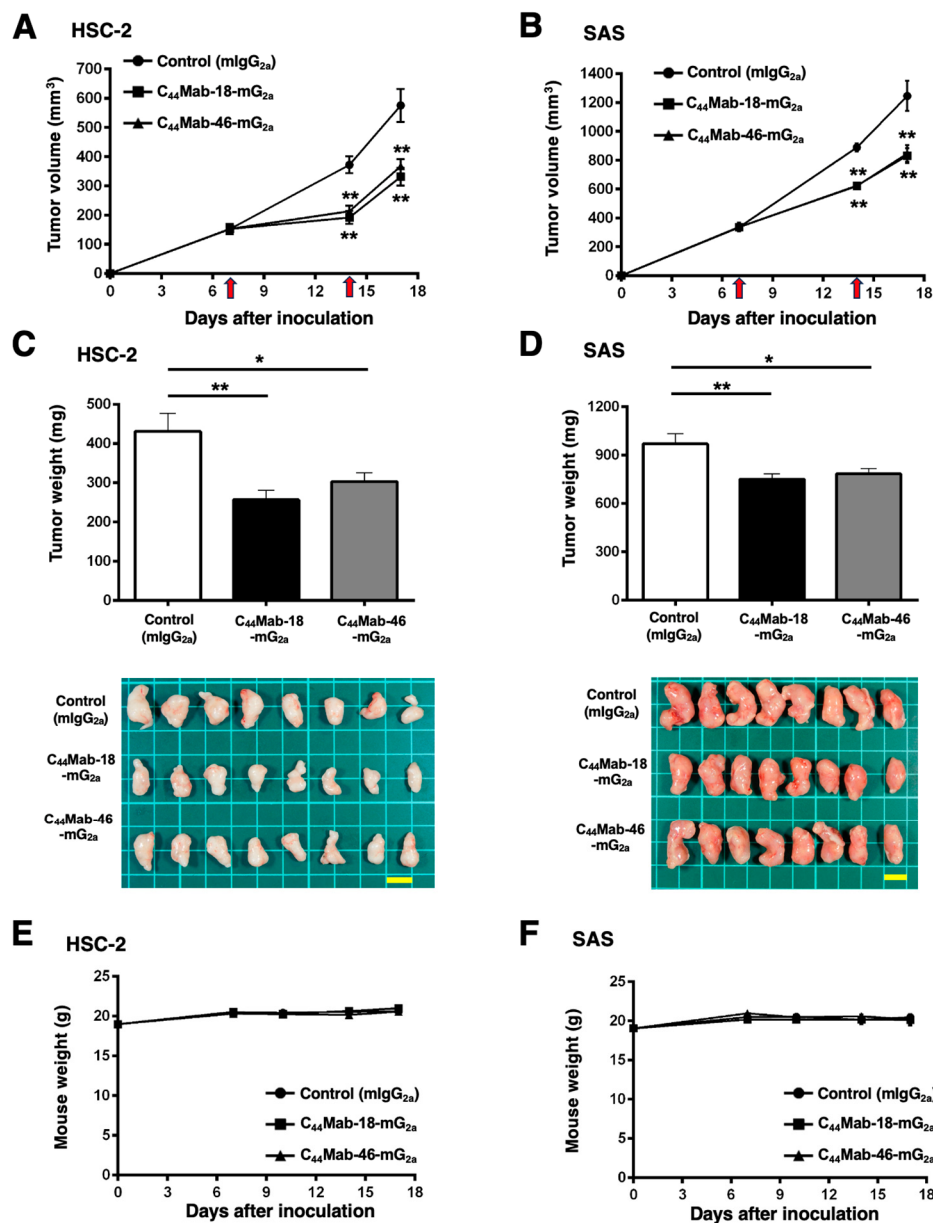


Figure 6. Antitumor activity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against HSC-2 and SAS xenograft. (A,B) Tumor volume in HSC-2 (A) and SAS (B) xenograft. HSC-2 and SAS cells (5×10^6 cells) were injected into mice subcutaneously. On days 7 and 14, 500 μ g of C₄₄Mab-18-mG_{2a} (n=8), C₄₄Mab-46-mG_{2a} (n=8), or control mouse IgG_{2a} (mIgG_{2a}, PMab-231) (n=8) were injected into mice intraperitoneally (arrows). The tumor volume was measured on days 7, 14, and 17 following the inoculation. Values are presented as the mean $\pm$ SEM. * $p < 0.01$ (two-way ANOVA with Tukey's multiple comparisons test). (C,D) The weight and appearance of the excised HSC-2 (C) and SAS (D) xenografts on day 17. Values are presented as the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ (one-way ANOVA with Tukey's multiple comparisons test). (E,F) The body weight of HSC-2 (E) and SAS (F) xenograft-bearing mice treated with C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mIgG_{2a}. Scale bar, 1 cm.

3. Discussion

Among CD44v, v10-containing isoforms include the most abundant CD44v, such as CD44v3–10, CD44v6–10, and CD44v8–10 [1,41]. Therefore, anti-CD44v10 mAbs including C₄₄Mab-18-mG_{2a} can target the broad range of CD44v-expressing tumor cells. In this study, we evaluated the antitumor activities against CD44-positive cells compared with an anti-pan-CD44 mAb, C₄₄Mab-46-mG_{2a}. C₄₄Mab-18-mG_{2a} exhibited the higher reactivity to CHO/CD44v3–10 and OSCC cells compared with C₄₄Mab-46-mG_{2a} (Figures 1 and 2). C₄₄Mab-18-mG_{2a} exhibited a superior ADCC against CD44v3–10 (Figure 3) and OSCC (Figure 5) cells. In contrast, C₄₄Mab-46-mG_{2a} showed a superior CDC against those cells. Furthermore, C₄₄Mab-18-mG_{2a} or C₄₄Mab-46-mG_{2a} similarly inhibited CD44v3–10 and OSCC xenograft growth compared with the control mouse IgG_{2a} (Figures 4 and 6). These results indicate that C₄₄Mab-18-mG_{2a} could be a promising therapeutic regimen for CD44v10-positive tumors.

In CHO/CD44v3–10 cells, C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} recognized common target, but mainly exerted ADCC and CDC activity, respectively (Figure 3). The epitope of C₄₄Mab-46 was previously determined as ¹⁷⁴TDDDV-¹⁷⁸ sequence in the constant exon 5-encoded region [30] which is relatively apart from transmembrane domain compared to variant exon 10-encoded region recognized by C₄₄Mab-18 [38]. To activate the classical pathway of complement, an ordered hexamer formation of IgG mAb is required to bind to the hexavalent complement C1q [42,43]. The structure of C₄₄Mab-46-mG_{2a}–CD44v3–10 complex may provide the appropriate space to form the hexameric structure of mAb–C1q complex and recruit the pore-forming membrane attack complex to exert CDC. In contrast, C₄₄Mab-18-mG_{2a} showed a higher reactivity to CHO/CD44v3–10 compared with C₄₄Mab-46-mG_{2a} in flow cytometry (Figure 1). The difference of the reactivity and the epitope would influence the ADCC activity. Further studies are required to reveal the relationship among the affinity of mAb, epitope, and ADCC activity.

Near-infrared photoimmunotherapy (NIR-PIT) uses a targeted mAb conjugated with a photoactivatable dye such as IRDye700DX (IR700) [44]. When the mAb binds to the antigen-expressed target cells, IR700 induces plasma membrane rupture and immunogenic cell death by NIR-light exposure. Preclinical studies of anti-pan-CD44 mAb-based NIR-PIT (IM7-IR700) were conducted. In the syngeneic mouse model of OSCC, IM7-IR700 administration and the NIR light exposure to OSCC tumors resulted in a significant reduction but failed to induce durable antitumor responses [45]. Because IM7 is a pan-CD44 mAb, IM7 might target not only tumor cells, but also CD44s-positive immune cells which involve in the antitumor immunity. The expression of CD44v is low in hematopoietic cells compared with CD44s [1]. We previously showed that C₄₄Mab-18 can distinguish tumor cells from stromal tissues in immunohistochemistry. In contrast, C₄₄Mab-46 stained both tumor and stromal tissue including fibroblasts and leukocytes [38]. Therefore, anti-CD44v10 mAb such as C₄₄Mab-18 might be a promising mAb for NIR-PIT without affecting the host immune cells in the tumor microenvironment.

Since both CD44s and CD44v are expressed in normal cells, there is a concern of adverse effects due to the recognition of normal cells by mAbs. In fact, clinical trials of the anti-CD44v6 mAb–ADC to advanced solid tumors were discontinued because of the skin toxicities [13,18]. Therefore, cancer specific mAbs (CasMabs) are desired to reduce the adverse effects. A CasMab against CD44v6 (clone 4C8) recognizes aberrantly O-glycosylated Tn (GalNAcα1-O-Ser/Thr) antigen in variant exon 6-encoded region. The 4C8 mAb was further developed to chimeric antigen receptor (CAR)-T cells, which exhibited target-specific *in vitro* cytotoxicity and significant tumor regression *in vivo* [46]. We have developed CasMabs against various tumor antigens, including HER2 (clones H₂Mab-214 [47] and H₂Mab-250 [48]) and reported the antitumor effect in mouse xenograft models using recombinant mouse IgG_{2a} or human IgG₁ mAbs [49,50]. These anti-HER2 mAbs were screened by the reactivity to cancer and normal cells in flow cytometry. H₂Mab-214 was revealed to recognize a locally misfolded structure in the Cys-rich HER2 extracellular domain 4, which usually forms a β-sheet [47]. H₂Mab-250 also shows a specific reactivity against HER2-positive tumor cells, which has been developed as CAR-T cell therapy. The phase I study has been conducted in the US (NCT06241456). We have developed CasMabs against CD44s or CD44v by comparing the reactivity against tumor and normal

cells. The anti-CD44 CasMabs could contribute to developing novel modalities such as ADCs and CAR-T cells.

4. Materials and Methods

4.1. Cell Lines and Cell Culture

CHO-K1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). OSCC cell lines, HSC-2 and SAS were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). CHO/CD44s and CHO/CD44v3-10 were previously established by transfecting pCAG-Ble/PA16-CD44s and pCAG-Ble/PA16-CD44v3-10 into CHO-K1 cells using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) [29,38].

HSC-2 and SAS was cultured in Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Inc.) containing 10% (*v/v*) heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc.), 100 U/mL of penicillin (Nacalai Tesque, Inc.), 100 µg/mL streptomycin (Nacalai Tesque, Inc.), and 0.25 µg/mL amphotericin B (Nacalai Tesque, Inc.).

CHO/CD44v3-10 was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc.) supplemented with 10% (*v/v*) FBS, antibiotics as mentioned above, and 5 mg/mL Zeocin (InvivoGen, San Diego, CA, USA). All cells were grown in a humidified incubator at 37°C with 5% CO₂.

4.2. Antibodies

An anti-pan-CD44 mAb (C₄₄Mab-46) and an anti-CD44v10 mAb (C₄₄Mab-18) were previously established [29,38]. A recombinant mouse IgG_{2a}-type mAb, C₄₄Mab-46-mG_{2a}, was generated previously [40]. To generate a recombinant mouse IgG_{2a}-type mAb from C₄₄Mab-18 (IgM, kappa), V_H cDNAs of C₄₄Mab-18 and C_H of mouse IgG_{2a} were cloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). V_L and mouse kappa light chain (C_L) cDNA of C₄₄Mab-18 was also cloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation). Using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc), the vectors were transfected into BINDS-09 cells (http://www.med-tohoku-antibody.com/topics/001_paper_cell.htm) [51] and the supernatants were collected. C₄₄Mab-18-mG_{2a} were purified using Ab-Capcher (ProteNova Co., Ltd., Kagawa, Japan). PMab-231 (a control mouse IgG_{2a}) was previously described [49].

4.3. Flow Cytometry

CHO/CD44v3-10, HSC-2, and SAS were obtained using 1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.) and 0.25% trypsin treatment. The cells were treated with C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, PMab-231, or blocking buffer [0.1% bovine serum albumin (BSA; Nacalai Tesque, Inc.) in phosphate-buffered saline (PBS)] (control) for 30 min at 4°C. Subsequently, the cells were incubated in Alexa Fluor 488-conjugated anti-mouse IgG (1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min at 4°C. Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp., Tokyo, Japan) and analyzed using SA3800 software ver. 2.05 (Sony Corp.).

4.4. ADCC

The ADCC activity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} was measured as follows. The target cells (CHO/CD44v3-10, HSC-2, and SAS) were labeled with 10 µg/mL Calcein AM (Thermo Fisher Scientific, Inc.) and plated in 96-well plates (1 × 10⁴ cells/well). The calcein-labeled target cells were mixed with the effector splenocyte (effector to target ratio, 100:1) from female BALB/c nude mice (Jackson Laboratory Japan, Inc., Kanagawa, Japan) with 100 µg/ml of C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mouse IgG_{2a} (PMab-231). After a 4.5-hour incubation at 37°C, the calcein release into the medium was measured using a microplate reader (Power Scan HT; BioTek Instruments, Inc., Winooski, VT).

The cytolyticity (% lysis) was determined: % lysis is calculated as $(E - S)/(M - S) \times 100$, where “E” indicates the fluorescence in effector and target cell cultures, “S” means the spontaneous fluorescence of only target cells, and “M” indicates the maximum fluorescence after treatment with a lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100]. Statistical analyses were performed using GraphPad PRISM 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are shown as mean $\pm$ standard error of the mean (SEM). One-way ANOVA with Tukey's multiple comparisons test was used for the statistical analyses. $p < 0.05$ was considered to indicate a statistically significant difference.

4.5. CDC

The calcein-labeled target cells were mixed with rabbit complement (final dilution 1:10, Low-Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, ON, Canada) and 100 μ g/ml of C₄₄Mab-18-mG_{2a}, C₄₄Mab-46-mG_{2a}, or control mouse IgG_{2a} (PMab-231). After a 4-hour incubation at 37°C, the calcein release into the medium was measured. Statistical analyses were performed as described above.

4.6. Antitumor Activity of C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} in Xenografts of CHO/CD44v3–10, HSC-2, and SAS

The animal study protocol was approved (approval nos. 2024-013 and 2024-038) by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Numazu, Japan). CHO/CD44v3–10, HSC-2, or SAS cells (5×10^6 cells) suspended with BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, Franklin Lakes, NJ, USA) were inoculated into the left flank of female BALB/c nude mice subcutaneously. On day 7 after the inoculation, 100 μ g of C₄₄Mab-18-mG_{2a} (n=8), C₄₄Mab-46-mG_{2a} (n=8), or control mouse IgG_{2a} (PMab-231) (n=8) in 100 μ l PBS were injected intraperitoneally. Additional antibody injections were performed on days 14 and 21. The tumor volume was measured on the indicated days.

In the HSC-2, or SAS xenograft experiment, 500 μ g of C₄₄Mab-18-mG_{2a} (n=8), C₄₄Mab-46-mG_{2a} (n=8), or control mouse IgG_{2a} (PMab-231) (n=8) in 100 μ l PBS were injected intraperitoneally on days 7 and 14 after the inoculation. The tumor volume was measured on the indicated days. The xenograft tumors were carefully removed from the sacrificed mice and weighed immediately.

All data are expressed as the mean $\pm$ standard error of the mean (SEM). In tumor weight measurement, one-way ANOVA with Tukey's multiple comparisons test was conducted. Two-way ANOVA with Tukey's multiple comparisons test was utilized for tumor volume and mice weight. GraphPad Prism 6 (GraphPad Software, Inc.) was used for all calculations. $p < 0.05$ was considered to indicate a statistically significant difference.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Confirmation of the purified mAbs.; Figure S2: Flow cytometry using C₄₄Mab-18-mG_{2a} and C₄₄Mab-46-mG_{2a} against CHO-K1 cells; Figure S3: Flow cytometry using control mouse IgG_{2a} (PMab-231) against CHO/CD44v3–10, HSC-2 and SAS cells.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, M.K., M.K.K., and Y.Kato; methodology, T.O.; formal analysis, T.T.; investigation, K.I., H.S., T.O., G.L., and T.T.; data curation, H.S. and Y.Kato; writing—original draft preparation, K.I. and H.S.; writing—review and editing, Y.Kato; supervision, A.O. and Y.Katori; project administration, Y.Kato.; funding acquisition, H.S., T.T., M.K.K., and Y.Kato. All authors have read and agreed to the published version of the manuscript.”

Funding: This research was supported in part by the Japan Agency for Medical Research and Development (AMED) under Grant Numbers 24am0521010 (to Y.Kato), JP23ama121008 (to Y.Kato), JP23am0401013 (to Y.Kato), JP23bm1123027 (to Y.Kato), and JP23ck0106730 (to Y.Kato), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) grant nos. 22K06995 (to H.S.), 21K20789 (to T.T.), 21K07168 (to M.K.K.), and 22K07224 (to Y.Kato).

Institutional Review Board Statement: The Institutional Committee for Experiments of the Institute of Microbial Chemistry approved animal experiments (approval nos. 2024-013 and 2024-038).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article and supplementary material.

Acknowledgments: The authors thank Shun-ichi Ohba and Akiko Harakawa (Institute of Microbial Chemistry [BIKAKEN], Numazu, the Microbial Chemistry Research Foundation) for technical assistance with animal experiments.

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Ponta, H.; Sherman, L.; Herrlich, P.A. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* **2003**, *4*, 33-45, doi:10.1038/nrm1004.
2. Zöller, M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer* **2011**, *11*, 254-267, doi:10.1038/nrc3023.
3. Prochazka, L.; Tesarik, R.; Turanek, J. Regulation of alternative splicing of CD44 in cancer. *Cell Signal* **2014**, *26*, 2234-2239, doi:10.1016/j.cellsig.2014.07.011.
4. Guo, Q.; Yang, C.; Gao, F. The state of CD44 activation in cancer progression and therapeutic targeting. *Febs j* **2021**, doi:10.1111/febs.16179.
5. Zöller, M. CD44, Hyaluronan, the Hematopoietic Stem Cell, and Leukemia-Initiating Cells. *Front Immunol* **2015**, *6*, 235, doi:10.3389/fimmu.2015.00235.
6. Jackson, D.G.; Bell, J.I.; Dickinson, R.; Timans, J.; Shields, J.; Whittle, N. Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. *J Cell Biol* **1995**, *128*, 673-685, doi:10.1083/jcb.128.4.673.
7. Bennett, K.L.; Jackson, D.G.; Simon, J.C.; Tanczos, E.; Peach, R.; Modrell, B.; Stamenkovic, I.; Plowman, G.; Aruffo, A. CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor. *J Cell Biol* **1995**, *128*, 687-698, doi:10.1083/jcb.128.4.687.
8. Matzke, A.; Sargsyan, V.; Holtmann, B.; Aramuni, G.; Asan, E.; Sendtner, M.; Pace, G.; Howells, N.; Zhang, W.; Ponta, H.; et al. Haploinsufficiency of c-Met in cd44^{-/-} mice identifies a collaboration of CD44 and c-Met in vivo. *Mol Cell Biol* **2007**, *27*, 8797-8806, doi:10.1128/mcb.01355-07.
9. Orian-Rousseau, V.; Chen, L.; Sleeman, J.P.; Herrlich, P.; Ponta, H. CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev* **2002**, *16*, 3074-3086, doi:10.1101/gad.242602.
10. Ishimoto, T.; Nagano, O.; Yae, T.; Tamada, M.; Motohara, T.; Oshima, H.; Oshima, M.; Ikeda, T.; Asaba, R.; Yagi, H.; et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc⁻ and thereby promotes tumor growth. *Cancer Cell* **2011**, *19*, 387-400, doi:10.1016/j.ccr.2011.01.038.
11. Wada, F.; Koga, H.; Akiba, J.; Niizeki, T.; Iwamoto, H.; Ikezono, Y.; Nakamura, T.; Abe, M.; Masuda, A.; Sakaue, T.; et al. High expression of CD44v9 and xCT in chemoresistant hepatocellular carcinoma: Potential targets by sulfasalazine. *Cancer Sci* **2018**, *109*, 2801-2810, doi:10.1111/cas.13728.
12. Birzele, F.; Voss, E.; Nopora, A.; Honold, K.; Heil, F.; Lohmann, S.; Verheul, H.; Le Tourneau, C.; Delord, J.P.; van Herpen, C.; et al. CD44 Isoform Status Predicts Response to Treatment with Anti-CD44 Antibody in Cancer Patients. *Clin Cancer Res* **2015**, *21*, 2753-2762, doi:10.1158/1078-0432.Ccr-14-2141.
13. Tjink, B.M.; Buter, J.; de Bree, R.; Giaccone, G.; Lang, M.S.; Staab, A.; Leemans, C.R.; van Dongen, G.A. A phase I dose escalation study with anti-CD44v6 bivatuzumab mertansine in patients with incurable squamous cell carcinoma of the head and neck or esophagus. *Clin Cancer Res* **2006**, *12*, 6064-6072, doi:10.1158/1078-0432.Ccr-06-0910.
14. Zhang, S.; Wu, C.C.; Fecteau, J.F.; Cui, B.; Chen, L.; Zhang, L.; Wu, R.; Rassenti, L.; Lao, F.; Weigand, S.; et al. Targeting chronic lymphocytic leukemia cells with a humanized monoclonal antibody specific for CD44. *Proc Natl Acad Sci U S A* **2013**, *110*, 6127-6132, doi:10.1073/pnas.1221841110.
15. Vey, N.; Delaunay, J.; Martinelli, G.; Fiedler, W.; Raffoux, E.; Prebet, T.; Gomez-Roca, C.; Papayannidis, C.; Kebenko, M.; Paschka, P.; et al. Phase I clinical study of RG7356, an anti-CD44 humanized antibody, in patients with acute myeloid leukemia. *Oncotarget* **2016**, *7*, 32532-32542, doi:10.18632/oncotarget.8687.
16. Menke-van der Houven van Oordt, C.W.; Gomez-Roca, C.; van Herpen, C.; Coveler, A.L.; Mahalingam, D.; Verheul, H.M.; van der Graaf, W.T.; Christen, R.; Rüttinger, D.; Weigand, S.; et al. First-in-human phase I clinical trial of RG7356, an anti-CD44 humanized antibody, in patients with advanced, CD44-expressing solid tumors. *Oncotarget* **2016**, *7*, 80046-80058, doi:10.18632/oncotarget.11098.
17. Verel, I.; Heider, K.H.; Siegmund, M.; Ostermann, E.; Patzelt, E.; Sproll, M.; Snow, G.B.; Adolf, G.R.; van Dongen, G.A. Tumor targeting properties of monoclonal antibodies with different affinity for target

- antigen CD44V6 in nude mice bearing head-and-neck cancer xenografts. *Int J Cancer* **2002**, *99*, 396-402, doi:10.1002/ijc.10369.
18. Riechelmann, H.; Sauter, A.; Golze, W.; Hanft, G.; Schroen, C.; Hoermann, K.; Erhardt, T.; Gronau, S. Phase I trial with the CD44v6-targeting immunoconjugate bivatuzumab mertansine in head and neck squamous cell carcinoma. *Oral Oncol* **2008**, *44*, 823-829, doi:10.1016/j.oraloncology.2007.10.009.
 19. Tsao, L.C.; Force, J.; Hartman, Z.C. Mechanisms of Therapeutic Antitumor Monoclonal Antibodies. *Cancer Res* **2021**, *81*, 4641-4651, doi:10.1158/0008-5472.Can-21-1109.
 20. Reis, E.S.; Mastellos, D.C.; Ricklin, D.; Mantovani, A.; Lambris, J.D. Complement in cancer: untangling an intricate relationship. *Nat Rev Immunol* **2018**, *18*, 5-18, doi:10.1038/nri.2017.97.
 21. Taylor, R.P.; Lindorfer, M.A. Cytotoxic mechanisms of immunotherapy: Harnessing complement in the action of anti-tumor monoclonal antibodies. *Semin Immunol* **2016**, *28*, 309-316, doi:10.1016/j.smim.2016.03.003.
 22. Reff, M.E.; Carner, K.; Chambers, K.S.; Chinn, P.C.; Leonard, J.E.; Raab, R.; Newman, R.A.; Hanna, N.; Anderson, D.R. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* **1994**, *83*, 435-445.
 23. de Weers, M.; Tai, Y.T.; van der Veer, M.S.; Bakker, J.M.; Vink, T.; Jacobs, D.C.; Oomen, L.A.; Peipp, M.; Valerius, T.; Slootstra, J.W.; et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol* **2011**, *186*, 1840-1848, doi:10.4049/jimmunol.1003032.
 24. Zent, C.S.; Secreto, C.R.; LaPlant, B.R.; Bone, N.D.; Call, T.G.; Shanafelt, T.D.; Jelinek, D.F.; Tschumper, R.C.; Kay, N.E. Direct and complement dependent cytotoxicity in CLL cells from patients with high-risk early-intermediate stage chronic lymphocytic leukemia (CLL) treated with alemtuzumab and rituximab. *Leuk Res* **2008**, *32*, 1849-1856, doi:10.1016/j.leukres.2008.05.014.
 25. Schmutde, I.; Laumonnier, Y.; Köhl, J. Anaphylatoxins coordinate innate and adaptive immune responses in allergic asthma. *Semin Immunol* **2013**, *25*, 2-11, doi:10.1016/j.smim.2013.04.009.
 26. Carroll, M.C.; Isenman, D.E. Regulation of humoral immunity by complement. *Immunity* **2012**, *37*, 199-207, doi:10.1016/j.immuni.2012.08.002.
 27. Weisser, N.E.; Sanches, M.; Escobar-Cabrera, E.; O'Toole, J.; Whalen, E.; Chan, P.W.Y.; Wickman, G.; Abraham, L.; Choi, K.; Harbourne, B.; et al. An anti-HER2 biparatopic antibody that induces unique HER2 clustering and complement-dependent cytotoxicity. *Nat Commun* **2023**, *14*, 1394, doi:10.1038/s41467-023-37029-3.
 28. Yamada, S.; Itai, S.; Nakamura, T.; Yanaka, M.; Kaneko, M.K.; Kato, Y. Detection of high CD44 expression in oral cancers using the novel monoclonal antibody, C(44)Mab-5. *Biochem Biophys Rep* **2018**, *14*, 64-68, doi:10.1016/j.bbrep.2018.03.007.
 29. Goto, N.; Suzuki, H.; Tanaka, T.; Asano, T.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-CD44 Monoclonal Antibody for Multiple Applications against Esophageal Squamous Cell Carcinomas. *Int J Mol Sci* **2022**, *23*, doi:10.3390/ijms23105535.
 30. Takei, J.; Asano, T.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Epitope Mapping of the Anti-CD44 Monoclonal Antibody (C44Mab-46) Using Alanine-Scanning Mutagenesis and Surface Plasmon Resonance. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 219-226, doi:10.1089/mab.2021.0028.
 31. Asano, T.; Kaneko, M.K.; Takei, J.; Tateyama, N.; Kato, Y. Epitope Mapping of the Anti-CD44 Monoclonal Antibody (C44Mab-46) Using the REMAP Method. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 156-161, doi:10.1089/mab.2021.0012.
 32. Asano, T.; Kaneko, M.K.; Kato, Y. Development of a Novel Epitope Mapping System: RIEDL Insertion for Epitope Mapping Method. *Monoclon Antib Immunodiagn Immunother* **2021**, *40*, 162-167, doi:10.1089/mab.2021.0023.
 33. Suzuki, H.; Kitamura, K.; Goto, N.; Ishikawa, K.; Ouchida, T.; Tanaka, T.; Kaneko, M.K.; Kato, Y. A Novel Anti-CD44 Variant 3 Monoclonal Antibody C(44)Mab-6 Was Established for Multiple Applications. *Int J Mol Sci* **2023**, *24*, doi:10.3390/ijms24098411.
 34. Kudo, Y.; Suzuki, H.; Tanaka, T.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-CD44 variant 5 Monoclonal Antibody C44Mab-3 for Multiple Applications against Pancreatic Carcinomas. *Antibodies* **2023**, *12*, 31, doi:10.3390/antib12020031.
 35. Ejima, R.; Suzuki, H.; Tanaka, T.; Asano, T.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-CD44 Variant 6 Monoclonal Antibody C(44)Mab-9 for Multiple Applications against Colorectal Carcinomas. *Int J Mol Sci* **2023**, *24*, doi:10.3390/ijms24044007.
 36. Suzuki, H.; Ozawa, K.; Tanaka, T.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-CD44 Variant 7/8 Monoclonal Antibody, C44Mab-34, for Multiple Applications against Oral Carcinomas. *Biomedicines* **2023**, *11*, 1099, doi:10.3390/biomedicines11041099.
 37. Tawara, M.; Suzuki, H.; Goto, N.; Tanaka, T.; Kaneko, M.K.; Kato, Y. A Novel Anti-CD44 Variant 9 Monoclonal Antibody C44Mab-1 was Developed for Immunohistochemical Analyses Against Colorectal Cancers *Curr. Issues Mol. Biol.* **2023**, *45*, 3658-3673, doi:10.3390/cimb45040238.

38. Ishikawa, K.; Suzuki, H.; Kaneko, M.K.; Kato, Y. Establishment of a Novel Anti-CD44 Variant 10 Monoclonal Antibody C(44)Mab-18 for Immunohistochemical Analysis against Oral Squamous Cell Carcinomas. *Curr Issues Mol Biol* **2023**, *45*, 5248-5262, doi:10.3390/cimb45070333.
39. Suzuki, H.; Tanaka, T.; Goto, N.; Kaneko, M.K.; Kato, Y. Development of a Novel Anti-CD44 Variant 4 Monoclonal Antibody C44Mab-108 for Immunohistochemistry. *Curr Issues Mol Biol* **2023**, *45*, 1875-1888, doi:10.3390/cimb45030121.
40. Ishikawa, K.; Suzuki, H.; Ohishi, T.; Nakamura, T.; Yanaka, M.; Li, G.; Tanaka, T.; Ohkoshi, A.; Kawada, M.; Kaneko, M.K.; et al. Antitumor activities of anti-CD44 monoclonal antibodies in mouse xenograft models of esophageal cancer. *Oncology Reports* **2024**, *in press*.
41. Zhang, H.; Brown, R.L.; Wei, Y.; Zhao, P.; Liu, S.; Liu, X.; Deng, Y.; Hu, X.; Zhang, J.; Gao, X.D.; et al. CD44 splice isoform switching determines breast cancer stem cell state. *Genes Dev* **2019**, *33*, 166-179, doi:10.1101/gad.319889.118.
42. Hiemstra, I.H.; Santegoets, K.C.M.; Janmaat, M.L.; De Goeij, B.; Ten Hagen, W.; van Dooremalen, S.; Boross, P.; van den Brakel, J.; Bosgra, S.; Andringa, G.; et al. Preclinical anti-tumour activity of HexaBody-CD38, a next-generation CD38 antibody with superior complement-dependent cytotoxic activity. *EBioMedicine* **2023**, *93*, 104663, doi:10.1016/j.ebiom.2023.104663.
43. de Jong, R.N.; Beurskens, F.J.; Verploegen, S.; Strumane, K.; van Kampen, M.D.; Voorhorst, M.; Horstman, W.; Engelberts, P.J.; Oostindie, S.C.; Wang, G.; et al. A Novel Platform for the Potentiation of Therapeutic Antibodies Based on Antigen-Dependent Formation of IgG Hexamers at the Cell Surface. *PLoS Biol* **2016**, *14*, e1002344, doi:10.1371/journal.pbio.1002344.
44. Mitsunaga, M.; Ogawa, M.; Kosaka, N.; Rosenblum, L.T.; Choyke, P.L.; Kobayashi, H. Cancer cell-selective in vivo near infrared photoimmunotherapy targeting specific membrane molecules. *Nat Med* **2011**, *17*, 1685-1691, doi:10.1038/nm.2554.
45. Nagaya, T.; Nakamura, Y.; Okuyama, S.; Ogata, F.; Maruoka, Y.; Choyke, P.L.; Allen, C.; Kobayashi, H. Syngeneic Mouse Models of Oral Cancer Are Effectively Targeted by Anti-CD44-Based NIR-PIT. *Mol Cancer Res* **2017**, *15*, 1667-1677, doi:10.1158/1541-7786.Mcr-17-0333.
46. Aasted, M.K.M.; Groen, A.C.; Keane, J.T.; Dabelsteen, S.; Tan, E.; Schnabel, J.; Liu, F.; Lewis, H.S.; Theodoropoulos, C.; Posey, A.D.; et al. Targeting Solid Cancers with a Cancer-Specific Monoclonal Antibody to Surface Expressed Aberrantly O-glycosylated Proteins. *Mol Cancer Ther* **2023**, *22*, 1204-1214, doi:10.1158/1535-7163.Mct-23-0221.
47. Arimori, T.; Mihara, E.; Suzuki, H.; Ohishi, T.; Tanaka, T.; Kaneko, M.K.; Takagi, J.; Kato, Y. Locally misfolded HER2 expressed on cancer cells is a promising target for development of cancer-specific antibodies. *Structure* **2024**, doi:10.1016/j.str.2024.02.007.
48. Kaneko, M.K.; Suzuki, H.; Kato, Y. Establishment of a Novel Cancer-specific Anti-HER2 Monoclonal Antibody H2Mab-250/H2CasMab-2 for breast cancers. *Monoclon Antib Immunodiagn Immunother* **2024**, *in press*.
49. Kaneko, M.K.; Suzuki, H.; Ohishi, T.; Nakamura, T.; Tanaka, T.; Kato, Y. A Cancer-Specific Monoclonal Antibody against HER2 Exerts Antitumor Activities in Human Breast Cancer Xenograft Models. *Int J Mol Sci* **2024**, *25*, doi:10.3390/ijms25031941.
50. Suzuki, H.; Ohishi, T.; Tanaka, T.; Kaneko, M.K.; Kato, Y. Anti-HER2 cancer-specific mAb, H2Mab-250-hG1 possesses higher complement-dependent cytotoxicity than trastuzumab. *Int. J. Mol. Sci.* **2024**, *25*, doi:10.3390/ijms25158386.
51. Li, G.; Suzuki, H.; Ohishi, T.; Asano, T.; Tanaka, T.; Yanaka, M.; Nakamura, T.; Yoshikawa, T.; Kawada, M.; Kaneko, M.K.; et al. Antitumor activities of a defucosylated anti-EpCAM monoclonal antibody in colorectal carcinoma xenograft models. *Int J Mol Med* **2023**, *51*, doi:10.3892/ijmm.2023.5221.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.