Enhancement of the Antibody-Dependent Cellular Cytotoxicity of Low-Fucose IgG1 Is Independent of FcγRIIIa Functional Polymorphism

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ABSTRACT

Purpose: **The most common polymorphic variant of Fcγ receptor type IIIa (FcγRIIIa), FcγRIIIa-158F, has been associated with inferior clinical responses to anti-CD20 chimeric IgG1 rituximab compared with FcγRIIIa-158V. As we previously found that removal of fucose residues from the oligosaccharides of human IgG1 results in enhanced antibody-dependent cellular cytotoxicity, we compared the effects of the FcγRIIIa gene *(FCGR3A)* polymorphism on normal and low-fucose versions of rituximab on antibody-dependent cellular cytotoxicity.**

Experimental Design: **The polymorphism at position 158 of FcγRIIIa was determined for the peripheral blood mononuclear cells (PBMCs) of 20 healthy donors. The PBMCs were then used as effector cells to compare the antibody-dependent cellular cytotoxicity of rituximab and a low-fucose version, KM3065. The contributions of the different cell types within the PBMC to antibody-dependent cellular cytotoxicity were examined.**

Results: **We found KM3065-mediated antibody-dependent cellular cytotoxicity was increased 10 to 100-fold compared with rituximab for each of the 20 donors. In contrast to rituximab, KM3065 antibody-dependent cellular cytotoxicity enhancement was similar for both *FCGR3A* alleles and thus independent of genotype. In addition, antibody-dependent cellular cytotoxicity of both KM3065 and rituximab requires natural killer cells but not monocytes nor polymorphonuclear cells. The antibody-dependent cellular cytotoxicity (ADCC) of each of the 20 donors correlated with the natural killer cell numbers present in the PBMCs. Importantly, using KM3065, the ADCC mediated by effector cells bearing the lower affinity variant FcγRIIIa-158F was significantly increased compared with rituximab-mediated ADCC using effector cells bearing the higher affinity FcγRIIIa-158V receptors.**

Conclusions: **The use of low-fucose antibodies might improve the therapeutic effects of anti-CD20 therapy for all patients independent of FcγRIIIa phenotype beyond that currently seen with even the most responsive patients.**

INTRODUCTION

Rituximab is a chimeric human IgG1 antibody targeting the B-cell–specific antigen CD20 and is widely used for the treatment for non-Hodgkin's lymphoma (1–4). Several *in vivo* and clinical studies indicate that antibody-dependent cellular cytotoxicity (ADCC) is an essential therapeutic mechanism of rituximab. However ADCC-mediated effects are suboptimal for the majority of patients receiving the current drug due to a polymorphic variation of antibody binding receptors (5–8).

ADCC, a lytic attack on antibody-targeted cells, is triggered after binding of lymphocyte receptors (FcγRs) to the antibody Fc region. ADCC activity is dependent on the structure of complex-type oligosaccharides linked to CH2 domain of the antibody. The content of galactose (9, 10), bisecting-GlcNAc (11, 12), and fucose (13, 14) in the antibody oligosaccharide have each been reported to effect ADCC. In a previous study, we demonstrated that fucose is the most critical antibody oligosaccharide component, and the reduction of fucose content in rituximab oligosaccharides results in >100-fold enhancement of ADCC activity (14). Moreover, we recently demonstrated that the low-fucose version of anti-CC chemokine receptor 4 chimeric IgG1 has enhanced ADCC, which results in more potent *in vivo* antitumor activity than the conventional high-fucose IgG1 (15), demonstrating reduction of the fucose content of human IgG1-type antibody, a powerful modification for the improvement of effector function.

Most therapeutic antibodies, including rituximab, are produced by Chinese hamster ovary (CHO) cells because of the ability of these cells to maintain protein productivity in cultures of high cell mass grown in well-defined media. CHO cells express high level of α1,6-fucosyltransferase, which catalyzes the fucosylation of complex oligosaccharides (14), and as a consequence, CHO-produced antibodies most often have oligosaccharides with a high fucose content. Antibodies with reduced fucose can be produced by altering the host cells from CHO cells to other cell lines with reduced fucosylation activity such as rat YB2/0 cells (14) or the CHO variant cell line, Lec13 (13).

Although the detailed mechanism by which the reduction of fucose enhances ADCC is not fully understood, Shields *et al.* (13) have demonstrated both low-fucose anti-HER2 and anti-IgE IgG1 had significantly increased binding to FcγRIIIa on natural killer (NK) cells, the predominant subtype of FcγRs

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capable of mediating ADCC. More recently, Okazaki *et al.* (16) showed, using thermodynamic and kinetic measurements, that reducing the fucose content of IgG1 enhances the binding enthalpy and association rate for IgG1-FcγRIIIa binding. However, whether other effector cell populations in addition to NK cells contribute to the enhanced ADCC of low-fucose antibody remains unknown.

FcγRIIIa gene (*FCGR3A*) allelic polymorphism generates receptors containing either a phenylalanine (F) or a valine (V) at amino acid position 158. This variation results in human IgG1 binding with higher affinity to NK cells of homozygous *FCGR3A*-158V (V/V) donors than those of homozygous *FCGR3A*-158F (F/F) donors, and this appears to activate NK cells more effectively (17, 18). Although FcγRIIIa binding affinity is presumed to be correlated with enhanced ADCC activity, there have been no quantitative investigations with effector cells from multiple donors into the relationship between *FCGR3A* polymorphism and actual ADCC value.

Importantly, several reports have recently suggested that the *FCGR3A* genotype is associated with the clinical efficacy of rituximab with the clinical response of patients bearing FcγRIIIa-158F being significantly inferior to the patients with the FcγRIIIa-158V receptors (6–8). Although rituximab has other potential effector functions, these reports underscore the importance of ADCC in clinical outcomes. However, these studies did not directly address the mechanism of the difference between the phenotypes, nor provide any potential mechanisms to overcome the resistance.

We have previously developed a low-fucose variant of rituximab designated KM3065 (14). In the present study, we measured rituximab- and KM3065-mediated ADCC using peripheral blood mononuclear cells (PBMCs) as effector cells from multiple healthy donors whose genotype at position 158 was determined to clarify the relationships between the enhanced ADCC by low-fucose IgG1 and *FCGR3A* genotype.

MATERIALS AND METHODS

Anti-CD20 Chimeric IgG1s. Rituximab, mouse-human chimeric IgG1-type anti-CD20 antibody, was purchased from Genentech, Inc. (San Francisco, CA). KM3065, low-fucose version of rituximab, was produced by rat myeloma YB2/0 cells as described previously (14).

Cell Lines. CD20-positive human B lymphoma cell line Raji and WIL2-S were purchased from the American Type Culture Collection (Manassas, VA).

Blood Donors. Blood donors were randomly selected from healthy volunteers registered in Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). All donors gave written informed consent before analyses in accordance with the process approved by the Institutional Ethical Committee.

Effector Cell Preparation. PBMCs were purified from peripheral blood using Lymphoprep (AXIS SHIELD; Dundee, United Kingdom). For effector analysis of ADCC, polymorphonuclear neutrophiles (PMNs) and mononuclear cells were purified from blood using MONO-POLY resolving medium (Dainippon Pharmaceutical, Osaka, Japan). The monocyte fraction was isolated from an aliquot of mononuclear cells by magnetic cell sorting using MACS anti-CD14 microbeads and MidiMACS separation unit (Miltenyi Biotech, Bergisch Gladbach, Germany). CD14-negative cells were then subjected to negative magnetic sorting to obtain NK cell fraction by removing CD3, CD14, CD19, CD36, and IgE-positive cells using MACS NK cell isolation kit and MidiMACS (Miltenyi Biotech). The purities of each effector cell fractions were >90%, as confirmed by flow cytometry.

ADCC Assay. Cytotoxicity was measured using a standard 4-hour ^{51}Cr-release assay as described previously (14). For monocyte-mediated ADCC, the incubation time was extended to 16 hours. In some experiments, ADCC was normalized to the number of NK cells (10^4 cells) by the following equation:

$$\text{ADCC}/10^4\ \text{NK}\ (\%) = \text{ADCC}\ (\%) \times 10^4/[\text{E/T ratio} \times \text{target cell number in an experimental well}\ (10^4) \times \text{NK percentage in PBMCs}]$$

Soluble Fc Receptors Expression. The cDNAs encoding FcγRI, FcγRIIa (131H allotype), FcγRIIb, and FcγRIIIa (158F allotype) were isolated by reverse transcription-PCR (Superscript Preamplification System, Invitrogen, Carlsbad, CA) of oligo(dT)-primed RNA from healthy donor PBMCs using specific primers that generated the fragments encoding each extracellular domain. The FcγRIIIa-158V allotype sequence was generated by PCR-based site-directed mutagenesis of the FcγRIIIa-158F cDNA. For all FcγRs, the transmembrane and intracellular domains were replaced by DNA encoding a His^6 tag. Thus, the expected proteins are composed of the extracellular domains of the FcγR at their COOH termini to His^6 at amino acid positions as follows: FcγRI, Gly^{282}; FcγRIIa, Ser^{214}; FcγRIIb, Ser^{220}; FcγRIIIa, Gly^{193} (residue numbers include the signal peptide). The cDNA were subcloned into the pKANTEX93 mammalian cell expression vector described previously (19). Plasmids were transfected into rat myeloma cell line YB2/0 by electroporation. Proteins were purified from culture supernatant by Ni-NTA chromatography (Qiagen, Valencia, CA), and their appropriate molecular weights confirmed by SDS-PAGE.

IgG1 Binding to Recombinant FcγRs. ELISA plates were coated with 5 μg/mL anti-Tetra His antibodies (Qiagen) in PBS. After blocking with PBS containing 1% BSA-PBS, purified receptors were incubated on the plates at room temperature for 2 hours. After washing with PBS containing 0.05% Tween 20 (wash buffer), serial dilutions of polymeric IgG in 1% BSA-PBS were incubated at room temperature for 2 hours. Polymeric IgG1s were prepared in 1% BSA-PBS by mixing equimolar amounts of each IgG1 and anti-F(ab′)$_2$ antibody (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 hour. After washing with the wash buffer, bound IgG1s were detected using peroxidase-labeled goat antihuman IgG (H+L) antibodies (American Qualex, San Clemente, CA), with 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) as the substrate. The reaction was stopped with 5% SDS, and absorbance at 415 nm was measured on an Emax plate reader (Molecular Devices, Menlo Park, CA). For FcγRIIIa, the study was also conducted as described above with monomeric IgG.

***FCGR3A*-158F/V Genotyping.** Genotyping of the *FCGR3A*-158V/F polymorphism was performed by using a PCR-based allele-specific restriction analysis assay with genomic DNA prepared from aliquots of peripheral blood as described previously (17).

Quantitation of Effector Cell Proportions in PBMCs. Quantitation of the NK cells and monocytes in PBMCs were determined by flow cytometric analysis as follows: PBMCs (1×10^6) were incubated on ice for 30 minutes with either FITC-labeled anti-CD3 antibody and phycoerythrin-labeled anti-CD56 antibody (Beckman Coulter, Tokyo, Japan) for NK cell quantitation, or phycoerythrin-labeled anti-CD14 antibody for monocyte quantitation in the presence of 3.8 mg/mL human IgG (Welfide, Osaka, Japan) as blocking reagent. After incubation, cells were washed twice with PBS and analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter). The $CD56^+CD3^-$ cells and $CD14^+$ cells were defined as NK cells or monocytes, respectively.

RESULTS

Effector Cell Analysis and FcγR Binding Profiles of Rituximab and Its Low-Fucose Version, KM3065. Rituximab and its low-fucose version KM3065 are composed of identical amino acid sequences and thus exhibit indistinguishable CD20 binding activity (14). They vary only the amount of N-linked oligosaccharides without fucose, which is 6% for rituximab and 91% for KM3065. This results in KM3065 exhibiting much higher ADCC than rituximab (14). To identify the effector cell populations contributing to ADCC of rituximab and its low-fucose counterpart, we used various cell fraction to measure the ADCC of each of the anti-CD20 IgG1s against $CD20^+$ B-cell lymphoma Raji cells. We compared total PBMCs, purified NK cells, PMNs, or monocytes as effector cells, all of which could potentially mediate ADCC via FcγR signaling. The effector cell populations were prepared from the same donor; however, the *FCGR3A* polymorphism at position 158 was not determined. As shown in Fig. 1*A*, KM3065 showed >100-fold higher ADCC than rituximab when total PBMCs were used as effector cells. A similar increase of ADCC was found with KM3065 only when NK cells were used as effector cells. In contrast, neither monocytes nor PMNs demonstrated measurable ADCC activity in this assay. These results demonstrate that the ADCC of both low-fucose and conventional high-fucose IgG1 is primarily mediated by NK cells in which the predominant IgG binding receptor is FcγRIIIa.

We next investigated the ELISA binding profiles of anti-CD20 IgG1s to FcγRs of the various subtypes to better understand the mechanism responsible for increased ADCC of KM3065 mediated by the NK cells. There was no significant difference in the binding of rituximab or KM3065 to FcγRI (Fig. 1*B*). KM3065 showed a slight increase in binding compared with rituximab for FcγRIIa and FcγRIIb. In contrast, the binding of KM3065 was greatly increased with equivalent binding at ~100-fold less protein to either FcγRIIIa-158F or FcγRIIIa-158V when compared with rituximab. These data are

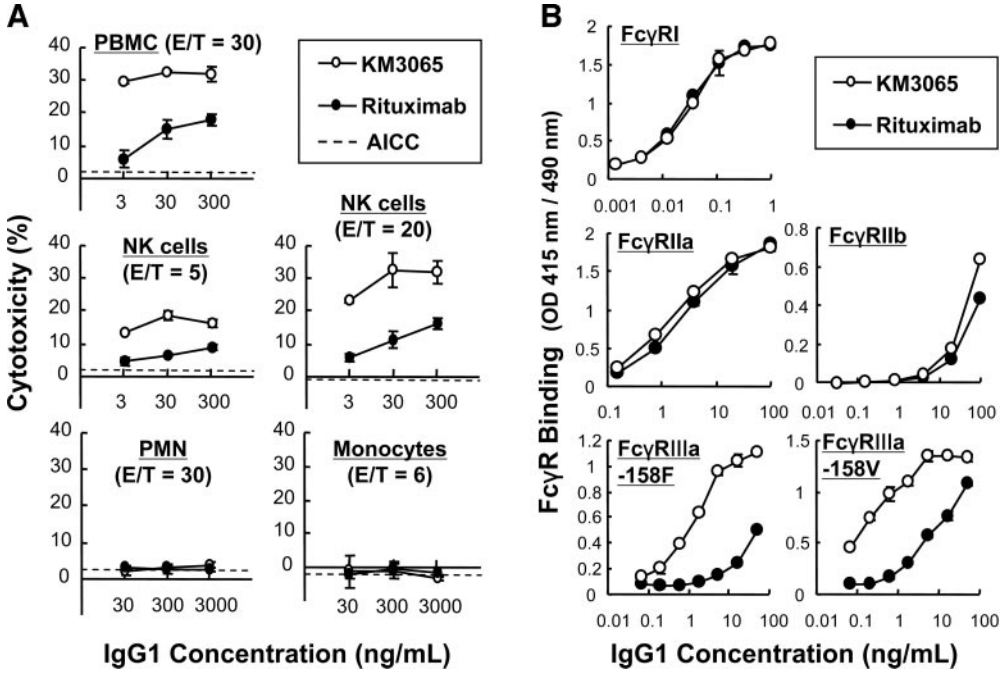

Fig. 1 Effector analysis in ADCC and FcγR binding profiles of the two anti-CD20 IgG1s. *A*, ADCC against Raji cells of the two anti-CD20 IgG1s using different cell populations from the same donor as effector cells. The kind of effector cell population (*underlined*) and E/T ratio used are indicated in each panel. Each E/T ratio is roughly proportional to the contents of each effector cell populations in peripheral blood, except for the experiment with higher number of NK cells (E/T = 20). The incubation times during the assay were 4 hours, except for 16 hours for monocyte-mediated ADCC. The mean cytotoxicities (%) ± SD of triplicates are shown. *Dashed lines* represent antibody-independent cellular cytotoxicity (AICC), the cytotoxicity in the absence of antibody. *B*, binding profiles of the two anti-CD20 IgG1s to various FcγRs. The mean values ± SD of triplicates are shown.

consistent with those previously reported for low-fucose versions of anti-HER2 IgG1 (13).

***FCGR3A*-158F/V Genotyping of 20 PBMC Donors.** We next studied the clinical implications of treating patients of different *FCGR3A* genotypes with low-fucose antibodies by determining the ADCC activity of low-fucose IgG1 using effector cells from donors for whom the *FCGR3A* genotypes had been determined. Hence, FcγRIIIa-158F/158V genotyping was conducted for 20 randomly selected healthy donors. As summarized in Table 1, the number of donors with F/F genotype was 15, and 4 donors had the V/F genotype and 1 donor had the V/V genotype. The allelic frequency calculated was 0.85 for *FCGR3A*-158F allele and 0.15 for *FCGR3A*-158V allele. The distribution observed was slightly but not significantly ($P = 0.25$, χ^2 test) biased toward the *FCGR3A*-158F allele compared with that in previous reports of the Japanese population as a whole (allelic frequency of *FCGR3A*-158F was 0.72 to 0.74; refs. 20, 21),

Comparison of Effect of *FCGR3A* Genotype on ADCC Exerted by Anti-CD20 Antibodies. We compared the ADCC of rituximab and KM3065 (1 to 100 ng/mL) using PBMCs from the 20 donors with known *FCGR3A* genotype as effector cells. Two $CD20^+$ B-cell lymphoma cell lines Raji and WIL2-S were chosen as targets. As nonspecific antibody-independent cellular cytotoxicity, primarily mediated by NK cells, can occur in these assays, the ADCC was calculated by subtracting percentage of ADCC from the percentage of cytotoxicity in the presence of antibody. The ADCC was low against Raji cells (0.448 to 13.0%) and relatively high against WIL2-S cells (13.3 to 30.3%), both of which were not significantly different between *FCGR3A* genotypes (Table 1).

The individual ADCC data separated by *FCGR3A* genotypes are shown in Fig. 2*A*. KM3065 showed more potent ADCC than rituximab for all 20 donors studied against both Raji cells and WIL2-S cells, thus ADCC enhancement by KM3065 is independent of the genotypes. Fig. 2*B* depicts the mean ADCC activity in the presence or absence of the *FCGR3A*-158V allele. The mean ADCC of KM3065 is 100-fold greater than that of rituximab for F/F donors against Raji cells, with the activity of 1 ng/mL KM3065 being comparable with that of 100 ng/mL rituximab. The improved potency of the low-fucose antibody was even greater using the WIL2-S target cells. KM3065 also demonstrated improved ADCC of V carriers (V/V or V/F); however, the difference in potency of the two IgG1s in V carriers was slightly less than for the F/F genotype.

The use of the KM3065 also reduced the difference in ADCC activity between the low-affinity and high-affinity Fc receptors. In contrast to V carriers showing a ~10-fold greater activity than F/F for rituximab, there was no significant difference for KM3065.

Importantly, the ADCC of KM3065 by the less potent F/F donor cells was significantly higher than the most responsive donor cells when rituximab was used.

Correlation between ADCC and the Percentage of NK Cells or Monocytes in PBMCs. To better understand the importance of the FcγRs and confirm the role of NK cells, we investigated the correlation between ADCC and the percentages of NK cells or monocytes, both of which express FcγRIIIa. The percentages of NK cells and monocytes in PBMCs of each donors determined by flow cytometry are summarized in Table 1. There was no significant correlation between *FCGR3A* genotype and the percentage of these two effector cells. As shown in Fig. 3, ADCC against Raji cells was shown to correlate significantly with the percentage of NK cells. In contrast, ADCC did not correlate with the percentage of monocytes. The NK cell dependence and monocyte independence of ADCC were similarly observed when WIL2-S cells were used as target cells (data not shown).

NK-Mediated ADCC Normalized for Cell Number. As both *FCGR3A* genotype and percentage of NK cells in PBMCs were shown to affect ADCC of anti-CD20 IgG1s in this study, the ADCC values obtained above were then reanalyzed by normalizing to a fixed number of NK cells (10^4 cells). We considered that the ADCC normalized for NK cell number best describes the precise relationship between *FCGR3A* genotype and ADCC. ADCC 10^4 NK cells for individuals (Fig. 4*A*) or mean value of each genotypes (Fig. 4*B*) additionally demonstrates the high potency of the low-fucose antibody and the *FCGR3A*-158V genotype, with the following rank order of potency at each IgG1 concentrations: KM3065 (V carrier) > KM3065 (F/F) > rituximab (V carrier) > rituximab (F/F) (Fig. 4*B*).

DISCUSSION

Therapeutic antibodies have been postulated to have multiple effector mechanisms *in vivo* based on *in vitro* studies. For example, with rituximab, potent ADCC, potent complement-dependent cytotoxicity, and relatively weaker direct apoptosis induction have been observed *in vitro* (3). What is important in

Table 1 Characterization of the donors

	F/F	V/F	V/V	*P*∗
No. of donors	15	4	1	
NK cells,† %, median (range)	15.1 (8.73–28.5)	13.6 (8.55–22.8)	6.27	0.404
Monocytes,‡ %, median (range)	9.85 (5.15–20.1)	10.6 (4.18–11.2)	15.9	0.874
AICC (Raji),§ %, median (range)	3.51 (1.45–13.0)	3.67 (1.96–4.82)	0.448	0.211
AICC (WIL2-S),¶ %, median (range)	16.0 (13.6–30.3)	17.3 (14.5–22.7)	13.3	0.848

∗ Determined by unpaired Student's *t* test between F/F *versus* V carriers (V/F + V/V).
† Percentage of NK cells in PBMCs.
‡ Percentage of monocytes in PBMCs.
§ Antibody-independent cellular cytotoxicity against Raji cells in 4-hour ^{51}Cr release assay.
¶ Antibody-independent cellular cytotoxicity against WIL2-S cells in 4-hour ^{51}Cr release assay.
Abbreviation: AICC, antibody-independent cellular cytotoxicity.

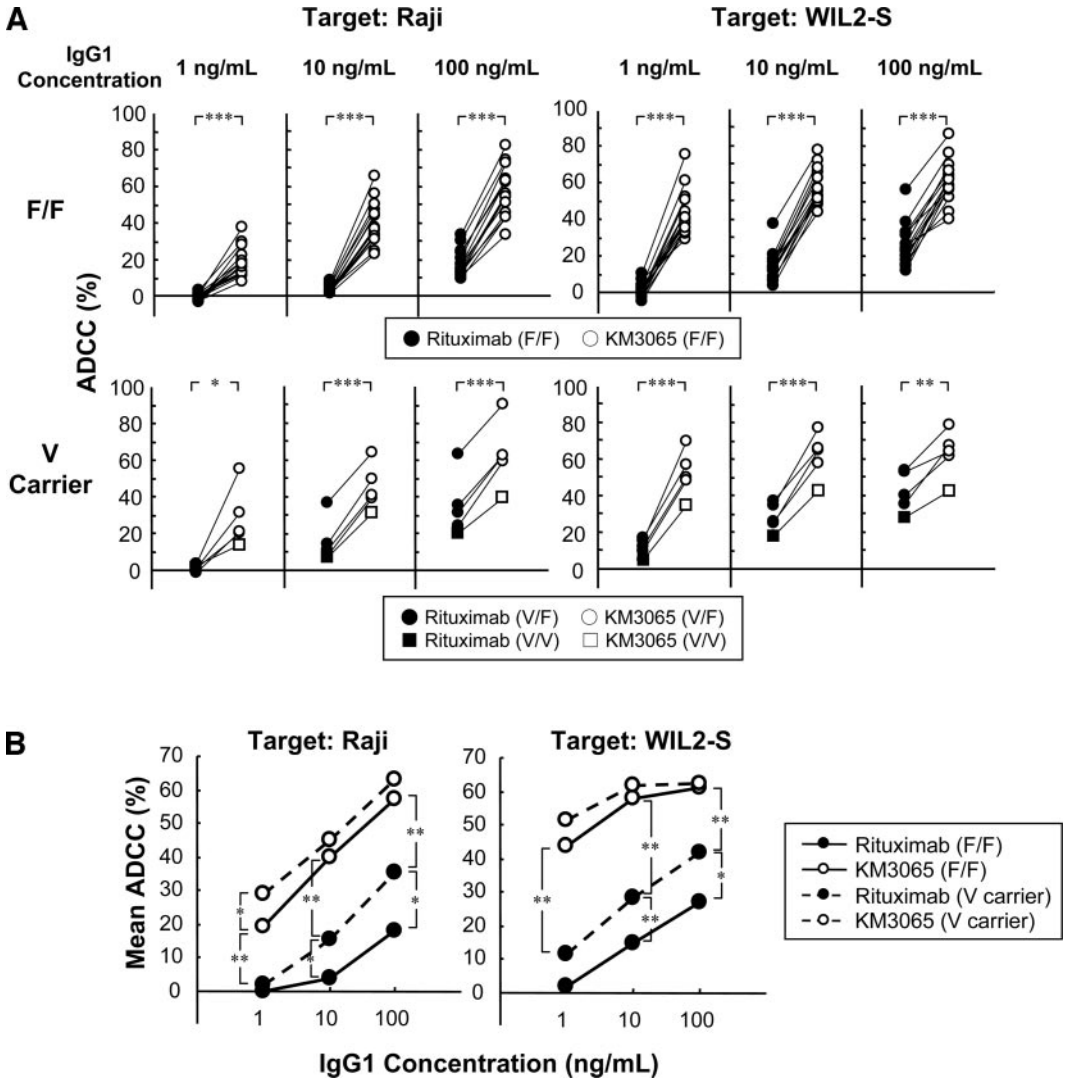

Fig. 2 Antibody-dependent cellular cytotoxicity (ADCC) of anti-CD20 IgG1s using PBMCs from 20 donors. *A*, ADCC of 20 donors according to the absence or presence of *FCGR3A*-158V allele (F/F and V carriers: *top* and *bottom panels*, respectively) in a 4-hour ^{51}Cr-release assay in the presence of the anti-CD20 IgG1s (1, 10, and 100 ng/mL) at an E/T ratio of 20/1. Raji cells or WIL2-S cells were used as target cells (*left* and *right panels*, respectively). The mean value of duplicates are shown. The plots of the same donors were connected with lines. Statistically significant differences between rituximab- and KM3065-mediated ADCC are marked by $*(P < 0.05)$, $**(P < 0.01)$, or $***(P < 0.001)$ as determined by paired *t* test. *B*, mean ADCC of each *FCGR3A* genotype groups. Statistically significant differences between different genotype groups are marked by $*(P < 0.05)$ or $**(P < 0.01)$ as determined by unpaired *t* test.

patients and, ultimately, which is critical for optimal clinical efficacy is less well understood. However, recent findings have demonstrated that ADCC plays a key role and suggests that improvements in ADCC will manifest improvements in clinical outcomes (6–8). A functional polymorphism of *FCGR3A* for patients receiving rituximab has been associated with inferior clinical responses. The polymorphic variant of FcγRIIIa of patients with inferior responses, FcγRIIIa-158F, shows a lower affinity for rituximab compared with the other variant FcγRIIIa-158V. *FCGR3A*-158F allele is more prevalent than *FCGR3A*-158V allele and the largest population, *FCGR3A*-158F/F, typically 40 to 50% in various ethnic groups (18, 20–23). Therefore, it is expected that the majority of patients would be less responsive to conventional antibody therapies that mediate ADCC. Hence, finding methods to improve the ADCC of antibodies for this major patient population is an important unmet clinical need.

As we have previously shown that low-fucose antibodies have augmented ADCC, we studied if these effects were seen in both patient populations with different Fc receptor genotypes and especially the resistant genotype. Our results clearly demonstrate that the enhanced ADCC mediated by the low-fucose anti-CD20 IgG1 is achieved independent of the *FCGR3A* genotypes; KM3065 showed greatly increased ADCC when compared with rituximab in all 20 donors. Importantly, KM3065 ADCC mediated by F/F donors with low-affinity receptors was

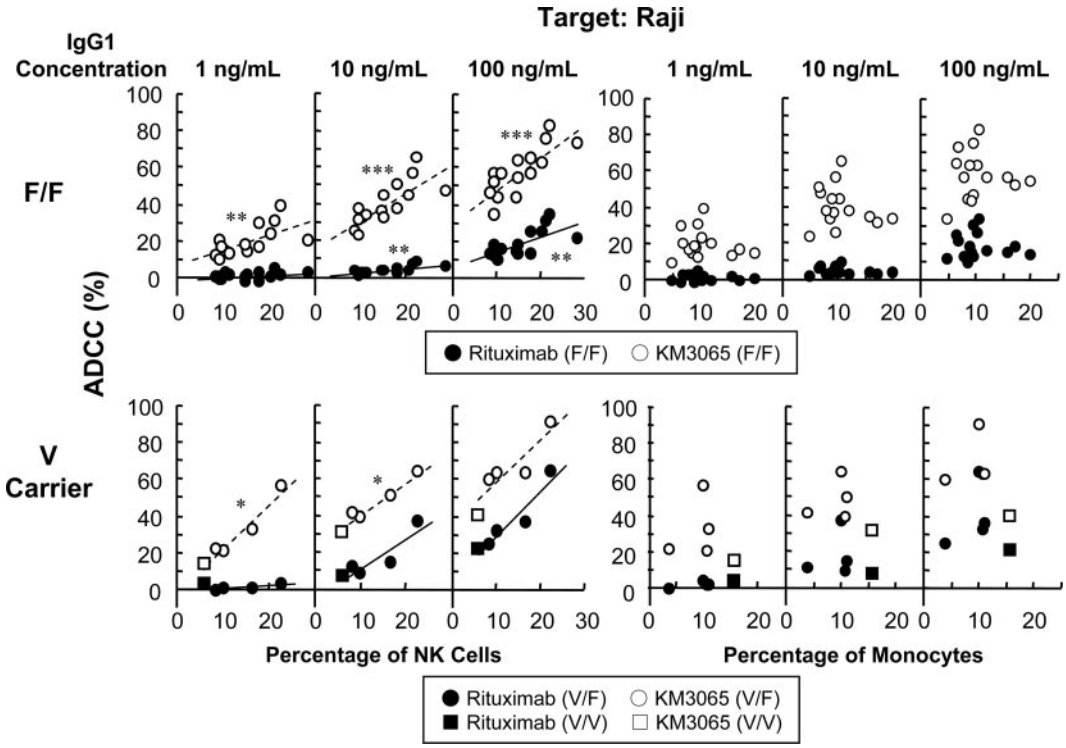

Fig. 3 Correlation between antibody-dependent cellular cytotoxicity (ADCC) and the percentage of NK cells or monocytes in PBMCs. Individual ADCC against Raji cells are plotted according to the presence or absence of *FCGR3A*-158V allele. Regression lines of rituximab- (*solid line*) and KM3065-mediated ADCC (*dashed line*) in NK cell percentage-based analyses are shown. Statistically significant correlations are marked by $*(P < 0.05)$, $**(P < 0.01)$, or $***(P < 0.001)$ as determined by parametric correlation test. No significant correlations are obtained between ADCC and monocyte percentages.

>10-fold higher than rituximab ADCC mediated by the most responsive donors with FcγRIIIa-158V receptors. This result implies that low-fucose IgG1 may overcome the reduced responsiveness to conventional IgG1 therapy of patient with low-affinity FcγRIIIa receptors and may have a improved therapeutic effect in all patients. Supporting this observation, the binding affinity of KM3065/FcγRIIIa-158F interaction was 3 to 4-fold higher than that of rituximab/FcγRIIIa-158V interaction, as we have demonstrated by calorimetric and kinetic methods very recently (16).

The study of different cell types that express Fcγ receptors demonstrated that ADCC was mediated via NK cells and not by monocytes or PMNs (Fig. 1*A*). Monocytes and PMNs are reported to effect ADCC when they are primed with cytokines (24–26), an aspect that remains to be examined. One possible reason for the observed inability of monocytes to exhibit ADCC in the current studies is the different glycosylation of FcγRIIIa receptors between leukocyte populations that result in a higher binding affinity for monomeric IgG on NK cells than on monocytes (27). PMNs express high levels of FcγRIIIb, the phosphotydylinositol-linked subtype of FcγRIII, which shares the highly homologous extracellular domain with FcγRIIIa. FcγRIIIb lacks a cytoplasmic tail and has not been demonstrated to have intrinsic signaling capacity. Although its biological role is poorly understood, it has been suggested that FcγRIIIb triggers intracellular signaling in cooperation with activation receptor FcγRIIa (28–30) or complement receptor CR3 (31). It has also been postulated that the capacity of PMNs to exert ADCC may depend on the intracellular structure of specific antigen molecules (32). The capability of low-fucose antibody to mediate ADCC by monocytes or PMNs merits additional investigated for other antigens than CD20.

In addition to *FCGR3A* polymorphism, the proportion of FcγRIII-positive cells in PBMCs has been reported to be another factor that affects ADCC (33). In the present study, NK cells were the sole effector cells in anti-CD20 IgG-mediated ADCC (Fig. 1*A*), and this result was confirmed by the significant correlation of ADCC of the 20 donor PBMC and NK cell proportions (Fig. 3). Hence, we calculated the data on ADCC for a fixed number of NK cells to provide an index for ADDC normalizing individual differences in NK cell number to more accurately measure the ADCC for each *FCGR3A* genotype. The plot of ADCC/10^4 NK cells still showed the following hierarchy: KM3065 (V carrier) > KM3065 (F/F) > rituximab (V carrier) > rituximab (F/F). This indicates the advantage of low-fucose IgG1, irrespective of the *FCGR3A* polymorphism, in the absence of heterogeneity in NK cell proportions among individuals.

There are three genotypes for *FCGR3A* polymorphism (V/V, V/F, and F/F). Although the allelic frequency determined in this study does not significantly differ from previously published data, the number of *FCGR3A*-158V carriers ($n = 5$),

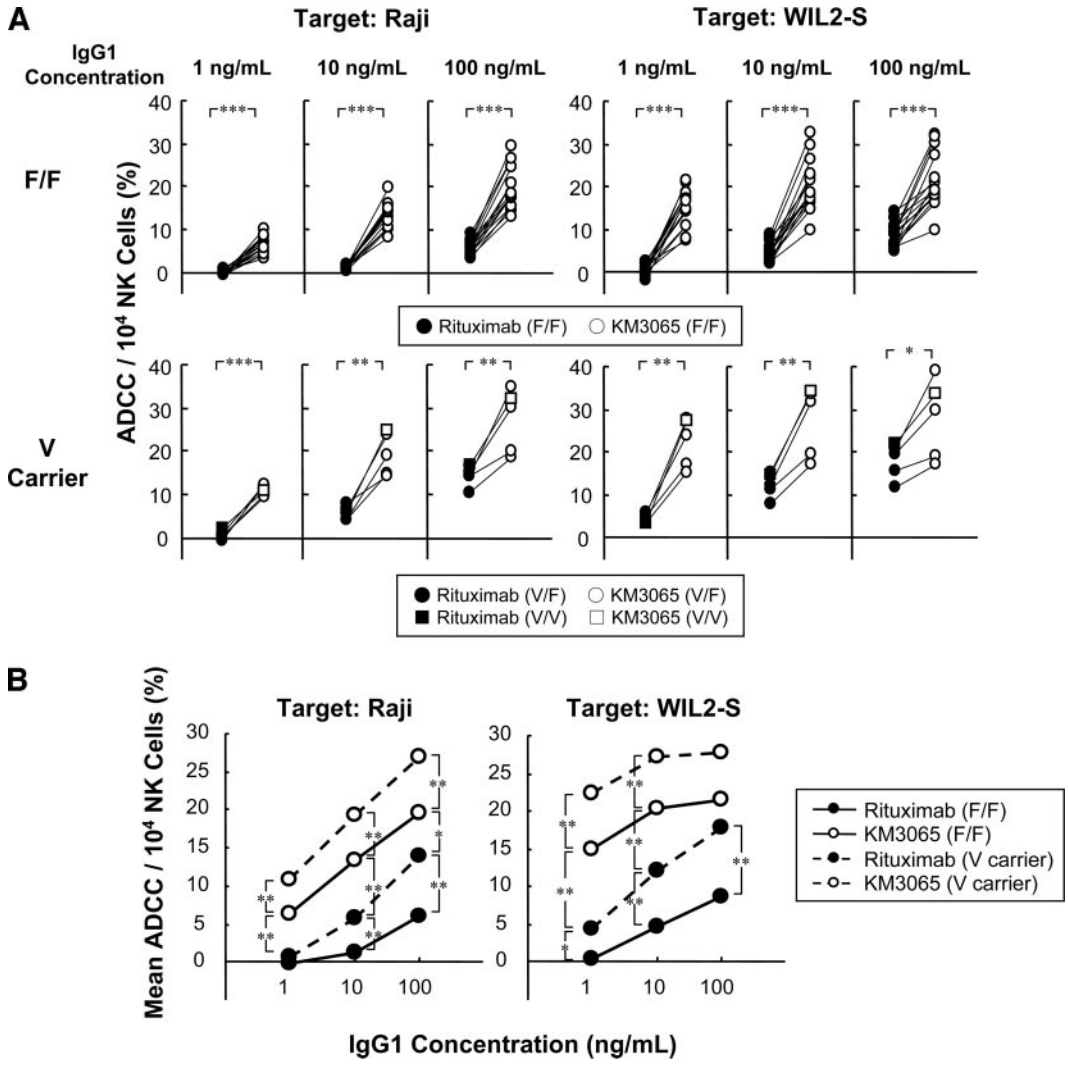

Fig. 4 Antibody-dependent cellular cytotoxicity (ADCC) per 10^4 NK cells. *A*, ADCC/10^4 NK cells of 20 donors according to the absence or presence of *FCGR3A*-158V allele (F/F and V carriers: *upper* and *lower panels*, respectively) in a 4-hour ^{51}Cr-release assay in the presence of the anti-CD20 IgG1s (1, 10, and 100 ng/mL). Raji cells or WIL2-S cells were used as target cells (*left* and *right panels*, respectively). The mean values (%) of duplicates are shown. The plots of the same donors were connected with lines. Statistically significant differences between rituximab- and KM3065-mediated ADCC/10^4 NK cells are marked by $*(P < 0.05)$, $**(P < 0.01)$, or $***(P < 0.001)$ as determined by paired *t* test. *B*, mean ADCC/10^4 NK cells of each *FCGR3A* genotype groups. Statistically significant differences between different genotype groups are marked by $*(P < 0.05)$ or $**(P < 0.01)$ as determined by unpaired *t* test.

especially V/V donors ($n = 1$), were unexpectedly low. Consequently, additional studies with more donors will be required to elucidate whether V/V and V/F NK cells inherently differ on ADCC potential. With respect to clinical data, Anolik *et al.* (7) have reported a significant difference in efficacy of B-cell depletion between *FCGR3A*-158V carriers and *FCGR3A*-158F/F in the treatment of systemic lupus erythematosus with rituximab, although they did not state the differences between V/V and V/F. In contrast, reports of B lymphoma have shown that V/V patients were more responsive for rituximab therapy than F carriers (V/F and F/F), but these studies failed to detect the differences between V/F and F/F (6, 8). Given that the V/F effector cells would be expected to have an intermediate potential, it is possible that the difference in clinical response between the three genotypes could be obscured, for example, (*a*) by some individual factors other than *FCGR3A* polymorphism that affects ADCC such as heterogeneity in NK cell proportion, or (*b*) with rituximab, some other effector functions (complement-dependent cytotoxicity, or apoptosis induction), which can also function *in vivo* (3).

In conclusion, the data presented suggests a critical need for the clinical use of low-fucose IgG1 for therapeutic antibodies that mediate ADCC as a mechanism of action. This modification might improve the clinical outcomes of all patients independent of *FCGR3A* genotypes and, in particular, overcome the shortcomings of the therapy received by the majority of patients that have the low-affinity FcγRIIIa polymorphic version. Additional study will be necessary to examine the unde-

termined question whether fucose reduction in IgG1 alters its pharmacokinetics and pharmacodynamics, including its ability to tumor penetration. Future clinical studies with low-fucose IgG1 might be required to fully address these questions.

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