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Defucosylated Chimeric Anti-CC Chemokine Receptor 4 IgG1 with Enhanced Antibody-Dependent Cellular Cytotoxicity Shows Potent Therapeutic Activity to T-Cell Leukemia and Lymphoma

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ABSTRACT

Human IgG1 antibodies with low fucose contents in their asparagine-linked oligosaccharides have been shown recently to exhibit potent antibody-dependent cellular cytotoxicity (ADCC) *in vitro*. To additionally investigate the efficacy of the human IgG1 with enhanced ADCC, we generated the defucosylated chimeric anti-CC chemokine receptor 4 (CCR4) IgG1 antibody KM2760. KM2760 exhibited much higher ADCC using human peripheral blood mononuclear cells (PBMCs) as effector cells compared with the highly fucosylated, but otherwise identical IgG1, KM3060. In addition, KM2760 also exhibited potent ADCC in the presence of lower concentrations of human PBMCs than KM3060. Because CCR4 is a selective marker of T-cell leukemia/lymphoma, the effectiveness of KM2760 for T-cell malignancy was evaluated in several mouse models. First, to compare the antitumor activity of KM2760 and KM3060, we constructed a human PBMC-engrafted mouse model to determine ADCC efficacy with human effector cells. In this model, KM2760 showed significantly higher antitumor efficacy than KM3060, indicating that KM2760 retains its high potency *in vivo*. Second, KM2760 suppressed tumor growth in both syngeneic and xenograft mouse models in which human PBMCs were not engrafted. Although murine effector cells exhibited marginal ADCC mediated by KM2760 and KM3060, KM2760 unexpectedly showed higher efficacy than KM3060 in a syngeneic mouse model, suggesting that KM2760 functions in murine effector system *in vivo* via an unknown mechanism that differs from that in human. These results indicate that defucosylated antibodies with enhanced ADCC as well as potent antitumor activity *in vivo* are promising candidates for the novel antibody-based therapy.

INTRODUCTION

Antibody-dependent cellular cytotoxicity (ADCC), a lytic attack on antibody-targeted cells, is triggered on binding of lymphocyte receptors (FcγRs) to the antibody constant region. ADCC is considered to be a major therapeutic function of antibodies, although there are others (*e.g.*, antigen binding, induction of apoptosis, and complement-dependent cellular cytotoxicity; Refs. 1, 2).

FcγRIIIa, the FcγR mainly expressed on natural killer cells and responsible for ADCC activation, has two isoforms, 158Val and 158Phe. The FcγRIIIa-158V allele shows higher binding capacity for IgG1 antibody compared with the FcγRIIIa-158F isoform (3, 4). Importantly, Cartron *et al.* (5) have reported recently that the anti-CD20 chimeric IgG1 antibody Rituxan was more effective for follicular non-Hodgkin lymphoma patients with FcγRIIIa-158Val compared with patients with FcγRIIIa-158Phe. Similar results have been reported by Anolik *et al.* (6) of Phase I/II trials of Rituxan in the treatment of systemic lupus erythematosus. These reports underscore the importance of ADCC in the clinic.

One IgG molecule contains two asparagine *N*-linked oligosaccharide sites in its Fc region (7). The general structure of IgG *N*-linked oligosaccharides is complex-type, characterized by a mannosyl-chitobiose core with or without bisecting *N*-acetylglucosamine (GlcNAc)/*L*-fucose and other chain variants including the presence or absence of galactose and sialic acid. Several groups have reported that ADCC enhancement can be achieved by manipulating human IgG1 subclass antibody oligosaccharides. ADCC requires the presence of oligosaccharides in the Fc region and is sensitive to change in the oligosaccharide structure (8–10). Among all of the sugar components in the oligosaccharide, galactose (11, 12), bisecting-GlcNAc (13, 14), and fucose (15, 16) have been reported to affect ADCC. We clarified recently the greater importance of fucose among these sugar components; defucosylation of humanized anti-interleukin 5 receptor antibody or chimeric anti-CD20 antibody enhanced their ADCC >50-fold (16). Compared with fucose, the involvement of bisecting-GlcNAc in ADCC was minimal, and galactose did not contribute to ADCC (16). The influence of nonfucosylated oligosaccharide on ADCC has been also reported by Shields *et al.* (15) using humanized anti-HER2 IgG1 and humanized anti-IgE IgG1. They showed that the improved ADCC of defucosylated IgG1 resulted from its improved binding to FcγRIIIa. Defucosylation of human IgG1-type antibody is, thus far, one of the most powerful ways to improve antibody effector function. However, the superiority of defucosylated antibodies *in vivo* has yet to be proven.

Chinese hamster ovary (CHO) cell lines are one of the most widely used host cell lines for the production of recombinant pharmaceutical proteins. Many approved or developing therapeutic antibodies are produced by CHO cells, including Rituxan and the anti-HER2 IgG1, Herceptin, both of which are increasingly used in the treatment of non-Hodgkin's lymphoma (17) and breast cancer (18), respectively. Several *in vivo* and clinical studies indicate that ADCC is one of the essential therapeutic mechanisms of Rituxan and Herceptin (2, 5, 6). However, the content of fucose in oligosaccharides of CHO-produced antibodies are relatively high, and their ADCC is much lower than that of defucosylated antibodies (15, 16).

Chemokine receptors mediate leukocyte migration through binding of soluble ligands. CC chemokine receptor 4 (CCR4) is a chemokine receptor that binds specifically to its ligands thymus and activation-regulated chemokine and macrophage-derived chemokine. CCR4 is expressed mainly on Th2-type CD4+ helper T cells in normal conditions (19–21). Th2 cells regulate humoral immunity and are also thought to play a key role in immune disorders, such as allergies and autoimmune diseases (22).

Several groups reported recently the selective expression of CCR4 on certain subsets of T-cell leukemia/lymphoma. Yoshie *et al.* (23) reported the frequent expression of CCR4 on adult T-cell leukemia (ATL) cells (22 of 24 cases were CCR4-positive by reverse transcription-PCR). ATL is the most aggressive and fatal leukemia, and there is currently no curative therapy. The very high rate of CCR4 positivity in ATL was also reported most recently by Ishida *et al.* (24). Inter-

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estingly, their data demonstrated for the first time that the CCR4 expression correlates with poor prognosis. With respect to other T-cell malignancies, anaplastic large-cell lymphoma (25, 26), mycosis fungoides in transformation (25, 27), and cutaneous T-cell lymphoma (27) have been observed for the frequent expression of CCR4.

To verify the potential of defucosylated antibody with enhanced ADCC for anticancer drugs, we established the defucosylated chimeric anti-CCR4 IgG1 antibody KM2760. KM2760 has lower fucose content than an otherwise identical CHO-produced anti-CCR4 IgG1, KM3060, and exerts much higher ADCC when human peripheral blood mononuclear cells (PBMCs) are used as effector cells. We additionally established a T-cell leukemia mouse model engrafted with human PBMCs as effector cells. KM2760 showed significantly higher antitumor efficacy in this T-cell leukemia model compared with KM3060. The effectiveness of KM2760 against T-cell malignancy was also investigated in conventional syngeneic and xenograft mouse models.

MATERIALS AND METHODS

Animals. Male C.B-17/Icr-*scid* Jcl mice and male BALB/cA Jcl-*nu* mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Male C57BL/6J Jcl mice were purchased from Taconic (Germantown, NY). The mice were maintained under specific pathogen-free conditions and used at 6–9 weeks of age. All of the *in vivo* experiments were performed in conformity with institutional guidelines in compliance with national laws and policies.

Cell Lines. CHO cell line DG44 (28) was kindly provided by Dr. Lawrence Chasin (Columbia University, New York, NY). Rat hybridoma YB2/0 [American Type Culture Collection (ATCC) CRL-1662], mouse T-cell lymphoma cell line EL4 (ATCC TIB-39), human T-lymphoblastic leukemia cell line HSB-2 (ATCC CCL-120.1), human acute T-cell leukemia cell line Jurkat (ATCC TIB-152), human acute T-lymphoblastic leukemia cell line CCRF-CEM (ATCC CCL-119), and human cutaneous T-cell lymphoma cell line Hut78 (ATCC TIB-161) were purchased from ATCC. Human acute T-lymphoblastic leukemia cell line MOLT-4 (JCRB9031), TALL-1 (JCRB0086), and PEER (JCRB0830) were purchased from Japanese Collection of Research Bioresources (Tokyo, Japan). Human T-acute lymphoblastic leukemia cell line HPB-All was kindly provided by Dr. Ryuzo Ueda (Nagoya City University Medical School, Nagoya, Japan).

Establishment of Cells Producing Chimeric Anti-CCR4 Antibodies. Hybridoma cells producing murine anti-CCR4 monoclonal antibody KM2160 was established by immunizing mice with a peptide corresponding to amino acid residues 2–29 of human CCR4 (21). KM2160 has been widely used as one of the most characterized antihuman CCR4 monoclonal antibodies (21, 23, 24, 29–31). The heavy- and light-chain variable region cDNAs prepared from hybridoma cells by PCR were cloned into the pKANTEX93 chimeric IgG1 antibody expression vector (32) to obtain the chimeric anti-CCR4 antibody expression vector pKANTEX2160. pKANTEX2160 was introduced into YB2/0 cells or DG44 cells via electroporation, and transfected cells were selected for gene amplification in methotrexate containing medium (32).

Production, Purification, and Oligosaccharide Profiling of Chimeric Anti-CCR4 IgG1s. The chimeric anti-CCR4 IgG1s were produced by transfecting pKANTEX2160 into YB2/0 cells and CHO cells, and purified by the method described previously (16). YB2/0- and CHO-produced chimeric IgG1 were designated as KM2760 and KM3060, respectively. They were then subjected to the profiling analysis of oligosaccharides as described previously (16).

Construction of an EL4 Cell Line Stably Expressing CCR4. A human CCR4 expression plasmid pcDNA3-CCR4 was kindly provided from Dr. Naofumi Mukaida (Kanazawa University, Ishikawa, Japan). A cytomegalovirus promoter was replaced with a CAG promoter in the *Bgl*II-*Hind*III site of plasmid pcDNA(CAG)-CCR3 (33) to construct CAG-pcDNA-CCR4. EL4 cells were transfected with CAG-pcDNA-CCR4 by electroporation and grown in the presence of 0.5 mg/ml G418 sulfate to obtain G418-resistant clones. The CCR4 expression level of each clone was measured by flow cytometry as described below. The highest expressing clone was selected and designated CCR4/EL4.

Flow Cytometer Analysis. Expression of cell surface CCR4 molecules was determined by flow cytometry. Leukemia T cells (2×10^6) were simultaneously stained with 10 μg/ml of biotin-labeled KM2760 and 250 μg/ml of human IgG (Welfide, Osaka, Japan) for blocking Fc receptors. Biotinylated KM2760 was prepared using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) as described by the manufacturer. Phycoerythrin-conjugated streptavidin (Becton Dickinson Japan, Tokyo, Japan) was used as the secondary reagent. The stained cells were analyzed using a EPICS XL-MCL flow cytometer (Beckman Coulter, Tokyo, Japan).

Preparation of Murine Effector Cells. Splenocytes were taken from C57BL/6 mice and stimulated in 50 ng/ml (>500 units/ml) recombinant human interleukin 2 (Peprotech EC, London, England) for 7 days. Thioglycollate-induced macrophages were taken from i.p. cavities of mice 4 days after the i.p. injection (2 ml/mouse) of 3% Brewer thioglycollate medium containing 0.3 mM thioglycollate (Difco, Detroit, MI).

ADCC Assay. ADCC was determined by 4 h ^{51}Cr release assay using PBMCs from healthy volunteers or interleukin 2-activated murine effector cells as effector cells as described previously (16). For macrophage-mediated ADCC, the incubation time was extended to 18 h.

Construction of Human PBMC-Engrafted Mice. Human PBMCs were isolated from the peripheral blood of a healthy donor using Lymphoprep (Axis Shield, Dundee, United Kingdom). PBMCs (5×10^6) were injected i.p. in a volume of 0.2 ml suspended in PBS into SCID mice under sterile conditions. The mice were pretreated with a combination of γ irradiation using a ^{137}Cesium irradiator (2.5 Gy, 3 days before PBMC injection) and 50 μl antiasialo GM1 antiserum (Wako, Richmond, VA) given i.p. 1 day before PBMC injection.

Assessment of Antitumor Activity in Hu-PBMC-SCID Mice. CCR4/EL4 cells (5×10^4) were injected i.p. into SCID mice engrafted with human PBMCs, 4 days after PBMC injection. One, 3, and 5 days later, anti-CCR4 IgG1s were injected i.p. Mice were observed daily to monitor the death due to ascites tumor development.

Experiments in a Syngeneic Disseminated Tumor Model. CCR4/EL4 cells (5×10^4) were injected i.v. into C57BL/6 mice on day 0. On days 1, 3, and 5, anti-CCR4 IgG1s were injected i.v. Twelve days after tumor injection, mice were anesthetized and sacrificed by exsanguination. Livers and kidneys were taken and their weight were measured as an indication of metastases. The organ weights were normalized by dividing by body weights. Treated *versus* control value was also used as an indication of metastasis, which was calculated as follows: treated *versus* control (%) = [(Organ weight of treated group) − (Organ weight of naïve group)]/[(Organ weight of control group) − (Organ weight of naïve group)] × 100. For *in vivo* depletion of natural killer (NK) cells, 50 μl antiasialo GM1 antisera were injected i.p. on days −3, −1, and 1, and metastases were evaluated on day 11. Almost complete elimination of NK cells in spleens of the treated mice on day 6 was confirmed using flow cytometry.

Experiments in a Human T-Cell Leukemia Xenograft Model. BALB/c-*nu* mice were injected s.c. with CCRF-CEM cells (2×10^7). Three h, 3 and 6 days later, antibodies were injected i.v. Tumor volume was calculated by the following equation:

$$\textit{Tumor volume}\ (mm^3) = 0.5 \times (\text{major diameter}) \times (\text{minor diameter})^2.$$

Statistical Analysis. Statistical significance of differential findings between experimental groups of animals was determined by two-tailed unpaired *t* test.

RESULTS

Generation and Characterization of Chimeric Anti-CCR4 IgG1. We have demonstrated previously that human IgG1 antibodies produced by rat myeloma YB2/0 cells have lower fucose contents in their *N*-linked oligosaccharides compared with those produced by CHO cells (16). In this study, we generated chimeric anti-CCR4 IgG1 produced by YB2/0 cells, designated KM2760, and that produced by CHO/DG44 cells, designated KM3060, to compare the antitumor activity of defucosylated IgG1 to highly fucosylated IgG1. These two IgG1s have the same amino acid sequence; only their *N*-linked oligosaccharide structures vary. Oligosaccharide profile analyses showed that the content of nonfucosylated oligosaccharides in KM2760 was 93%, whereas that in KM3060 was only 9% (Fig. 1;

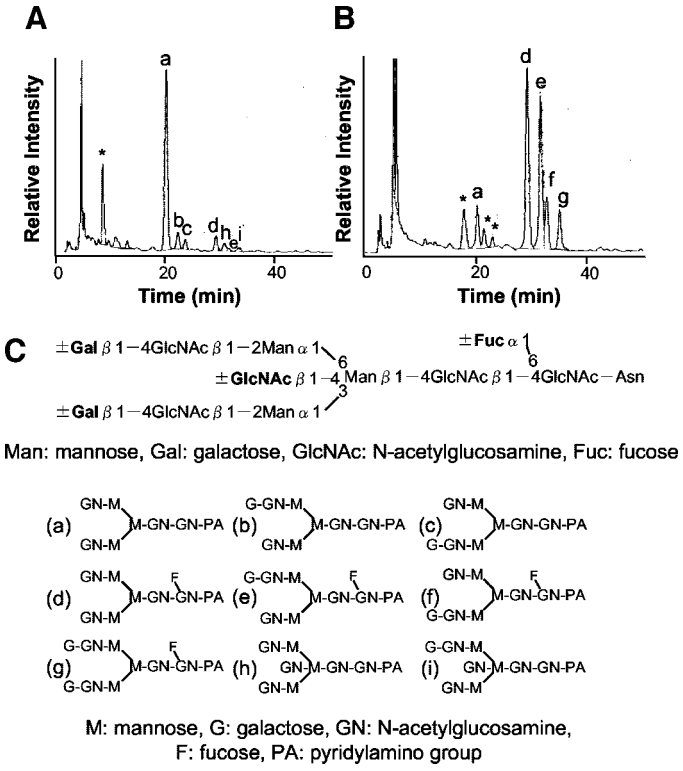

Fig. 1. Oligosaccharide profiles of chimeric anti-CC chemokine receptor 4 IgG1. Oligosaccharides derived from YB2/0-produced KM2760 (*A*) and Chinese hamster ovary-produced KM3060 (*B*) were pyridylaminated and applied to reverse-phase high-performance liquid chromatography as described in "Materials and Methods." The alphabetical peak codes correspond to those of oligosaccharide structure in *C*. A conserved oligosaccharide core, linked to the Asn, is composed of three mannose (*Man*) and two *N*-acetylglucosamine (*GlcNAc*) monosaccharide residues. Additional GlcNAcs are normally β1,2-linked to the α6 Man and α3 Man (α6 and α3 arms, respectively), whereas the monosaccharide residues in *boldface type*, galactose (*Gal*), fucose (*Fuc*), and the bisecting GlcNAc, can be present or absent. Peaks indicated with *asterisks* are artifacts of pyridylamino derivatives (38). The percentage of nonfucosylated oligosaccharides was calculated by the following equation: (Total peak area of a, b, c, h, and i)/(Total peak area)×100.

Table 1). Although the difference in the contents of fucose was most prominent, there were also some changes in other sugar components, such as galactose or GlcNAc, their differences were not considered sufficient to contribute to the difference in ADCC (16). The CCR4-binding activities of KM2760 and KM3060 were measured by ELISA, and the two antibodies showed identical binding to CCR4 (data not shown).

KM2760 Exerts Much More Potent Human PBMC-Mediated ADCC Than KM3060. Next we compared ADCC of various concentrations of KM2760 and KM3060 using CCR4/EL4 cells as target cells and a constant number of PBMCs as effector cells (E:T ratio = 25) from three different donors (donor A, B, and C; Fig. 2, *A–C*, respectively). The CCR4/EL4 cells were murine thymoma EL4 cells expressing high levels of exogenous human CCR4, established by transfecting the EL4 cells with an expression vector encoding CCR4. KM2760 exhibited much higher ADCC than KM3060 in all three of the experiments. The difference in potency was prominent especially for donor A (Fig. 2*A*, the maximum cytotoxicity of KM2760 and KM3060 were 51% and 8%, respectively) and donor B (Fig. 2*B*, 100% and 4%, respectively). KM2760 also showed higher potency for donor C; however, the maximum cytotoxicity of KM3060 was relatively high in this case (Fig. 2*C*, 100% and 71%, respectively). These results suggest that defucosylated IgG1 antibodies exhibit much more potent ADCC than highly fucosylated CHO-produced IgG1s. This is true despite the considerable variation in ADCC of CHO-produced IgG1s among individuals.

ADCC was also measured in the presence of varying numbers of PBMCs from donor A at a constant antibody concentration (3 μg/ml). Notably, KM2760 needed much fewer effector cells to achieve the same cytotoxicity as shown by KM3060 (Fig. 2*D*). A similar advantage of defucosylated IgG1 in E:T ratio-based observation has also been reported for humanized anti-HER2 IgG1 (15).

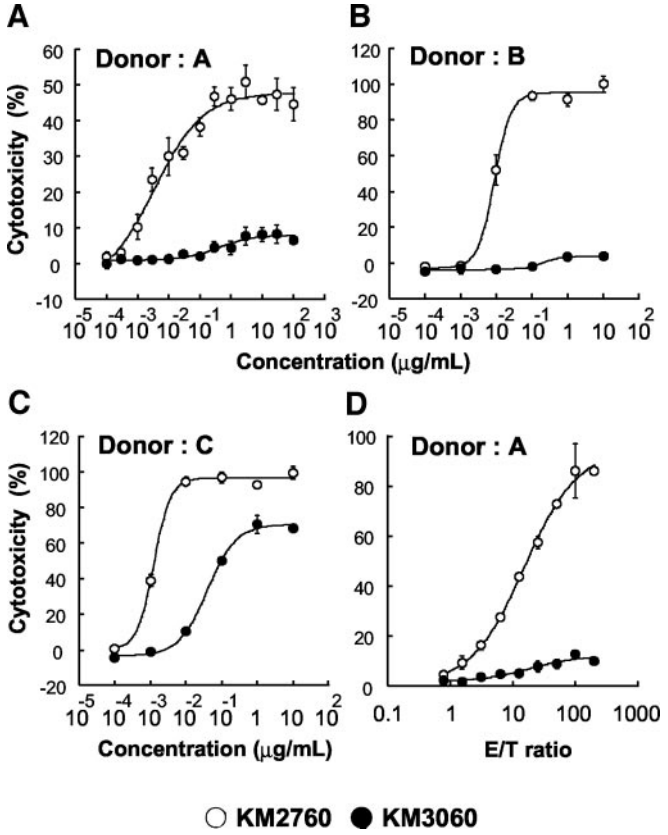

Fig. 2. Antibody-dependent cellular cytotoxicity (ADCC) of chimeric anti-CC chemokine receptor 4 IgG1 against CCR4/EL4 cells. Cytotoxicity was measured by 4 h-^{51}Cr release assay, in the presence of KM2760 or KM3060 and human peripheral blood mononuclear cells as effector cells. Cytotoxicity (%) is indicated on the *Y* axis as mean (n = 3); *bars*, ±SD. *A–C*, ADCC of KM2760 and KM3060 at the concentrations indicated on the *X* axis with PBMCs from different three donors. E:T ratio was held constant at 25. *D*, ADCC of KM2760 and KM3060 in the presence of various numbers of PBMCs from donor A. Antibody concentrations were held constant at 3 μg/ml. The *X* axis represents E/T value.

Table 1 *Oligosaccharide composition of anti-CCR4*[a] *IgG1s*

Pyridylaminated oligosaccharides were analyzed for their compositions by reverse-phase HPLC as described under "Materials and Methods." The percentages of the total oligosaccharide are given on a molar basis. The structures of each oligosaccharide are represented in Fig. 1. Fuc (−) is total percentage of non-fucosylated oligosaccharides. Bis (+) indicates total percentage of bisecting GlcNAc-binding oligosaccharides. G0, G1, and G2 indicate total percentage of non-galactosylated, mono-galactosylated, and di-galactosylated oligosaccharides.

IgG1	a	b	c	d	e	f	g	h	i	Total	Fuc(−)	Bis(+)	G0	G1	G2
KM2760	76	7	4	6	1	ND	ND	4	2	100	93	6	86	14	ND (%)
KM3060	9	ND	ND	37	34	11	9	ND	ND	100	9	ND	46	45	9 (%)

[a] CCR4, CC chemokine receptor 4; HPLC, high-performance liquid chromatography; ND, not detected.

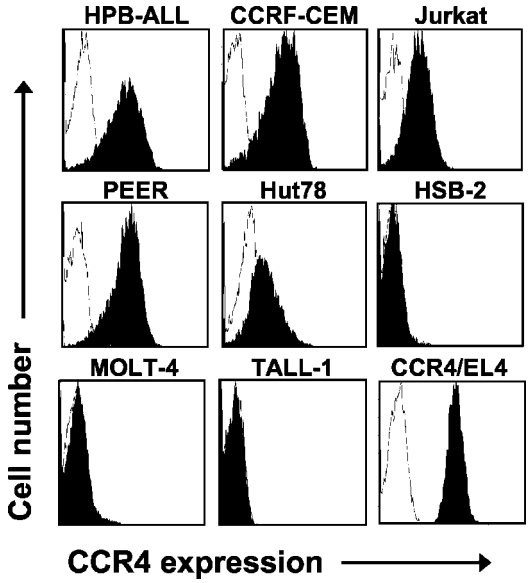

Fig. 3. Expression of CC chemokine receptor 4 (*CCR4*) on human neoplastic T-cell lines. Cell lines indicated above each panel were stained with biotinylated KM2760 (*filled histograms*) or buffer alone (*blank histograms*). Phycoerythrin-conjugated streptavidin was used as the secondary reagent. The stained cells were analyzed by flow cytometry.

We also confirmed by flow cytometry that KM2760 or KM3060 did not bind to parental EL4 cells not expressing human CCR4. Likewise, KM2760 and KM3060 did not exert any ADCC on the parental EL4 cells (data not shown), indicating that the potent cytotoxicity of KM2760 depends on the presence of CCR4.

T-Cell Lymphoma and Leukemia Cell Lines Frequently Expressing CCR4 Are Lysed by KM2760-Mediated ADCC. To confirm the reactivity of KM2760 to neoplastic T cells, we used biotinylated KM2760 to stain eight randomly selected human T-cell leukemia/lymphoma cell lines. CCR4/EL4 cells were stained as the positive control. As shown in Fig. 3, five of eight cell lines were significantly KM2760-positive (Fig. 3, *A–E*), indicating that T-cell leukemia/lymphoma frequently express CCR4. This finding coincides well with recent reports (23, 25–27).

Using fresh human PBMCs (donor D) as effector cells, we measured ADCC of KM2760 against five of the eight cell lines selected above. As shown in Fig. 4, all of the target cell lines were lysed in the presence of KM2760, although the extent of antibody-dependent and independent cytotoxicities were varied among cell lines.

KM2760 Shows Potent *in Vivo* Antitumor Activity in a T-Cell Leukemia Mouse Model Engrafted with Human PBMCs. To evaluate the *in vivo* antitumor activity of chimeric anti-CCR4 antibodies in a mouse model in which ADCC was effected by human effector cells, we constructed a human PBMC-engrafted SCID mouse model. According to the method reported by Shpitz *et al.* (34), SCID mice were pretreated with irradiation and anti-asialo GM1 antisera, followed by i.p. injection of human PBMCs. The engrafted PBMCs were derived from donor A, whose PBMC-mediated ADCC was shown in Fig. 2, *A* and *D*. Our preliminary study showed that the engrafted PBMCs survive for at least 2 weeks in the peritoneal cavity, and human PBMCs obtained from peritoneal cavities of engrafted mice *ex vivo* induced potent ADCC by KM2760 and weak ADCC by KM3060 (data not shown), in the same manner as when fresh PBMCs were used as effector cells.

The antitumor activity of antibodies in human PBMC-engrafted mice is shown in Fig. 5*A*. When CCR4/EL4 cells were injected i.p. 4 days after the engraftment of human PBMCs, without additional treatment the mice survived only for an additional 15.2 days on average because of the ascites tumor development. Significant survival prolongation was observed in mice that received either KM2760 or KM3060. However, both 1 μg and 10 μg of KM2760 were significantly more potent (the mean survival time, 22.7 days and 23.2 days, respectively) than the same and larger doses (10 μg and 100 μg) KM3060 (17.7 days and 18.0 days, respectively). In contrast, 10 μg KM2760 showed no significant survival prolongation in mice without human PBMCs (Fig. 5*B*), indicating that the antitumor efficacy shown in Fig. 5*A* entirely depended on human PBMCs.

***In Vivo* KM2760 Antitumor Activity in a Syngeneic Disseminated T-Cell Leukemia Model.** Human IgG1 exerts antitumor activity *in vivo* via murine Fc receptor signaling (2). To additionally verify whether advantage of defucosylated IgG1 is evaluable in the conventional mouse model without introducing human effector cells, we measured KM2760- and KM3060-mediated ADCC with effector cells from C57BL/6 mice (interleukin 2-stimulated splenocytes and thioglycollate-induced peritoneal macrophages; Fig. 6*A*). When splenocytes were used as effector cells, they showed increased natural cytotoxicity in the absence of IgG1 primarily induced by lymphokine-activated killer cells. However, regarding IgG1-dependent cytotoxicity, the activated splenocytes or macrophages could only show marginal activities, and no significant differences were found between the two IgG1s. Taken together, these data indicate that murine effector cells do not reproduce the enhanced ADCC of KM2760; consequently, murine effector system might differ from that of human regarding the mechanism of action of defucosylated IgG1.

Next we investigated the *in vivo* antitumor efficacy of KM2760 and KM3060 in a syngeneic tumor model that does not bear human effector cells. When CCR4/EL4 cells were injected i.v. in syngeneic

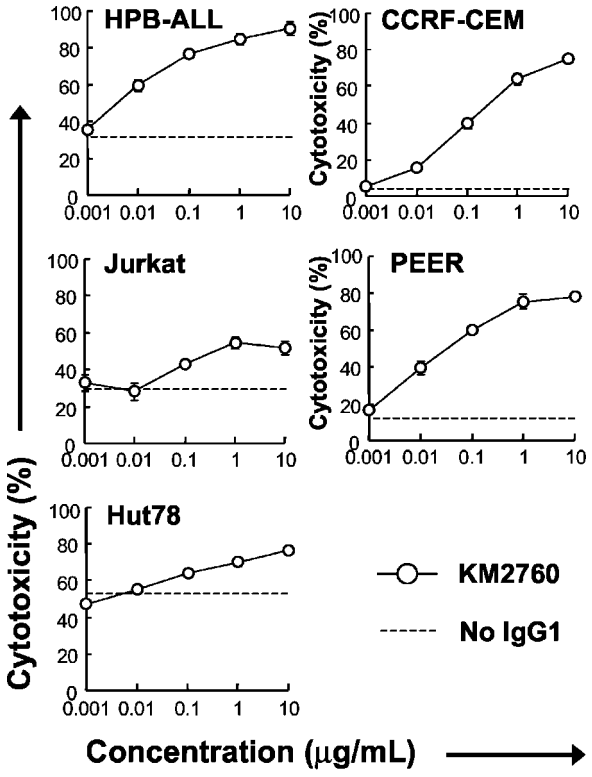

Fig. 4. Antibody-dependent cellular cytotoxicity of KM2760 against CC chemokine receptor 4-positive T-cell leukemia cells. Cytotoxicity was measured by 4 h-^{51}Cr release assay in the presence of KM2760 at the concentrations on the *X* axis and human peripheral blood mononuclear cells (from donor D) as effector cells. E:T ratio was held constant at 25. Five T-cell leukemia cell lines were used as target cells as indicated in each panel. Cytotoxicity (%) is indicated on the *Y* axis as mean (n = 3); *bars*, ±SD. *Dashed lines* indicate the cytotoxicity in the absence of antibody.

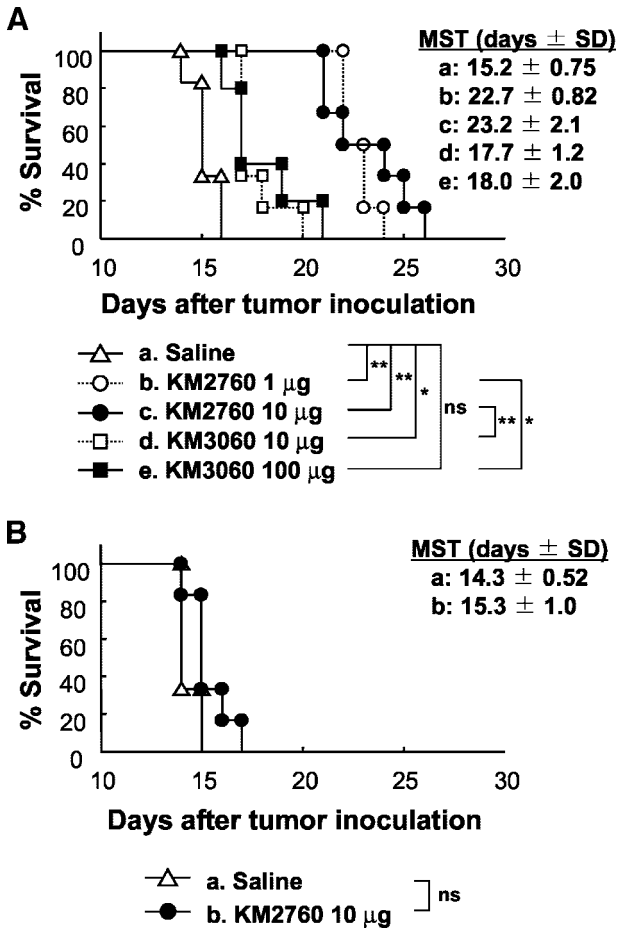

Fig. 5. Kaplan-Meier survival plot of SCID mice inoculated with CCR4/EL4 cells. Indicated treatments were given i.p. on 1, 3, and 5 days after tumor inoculation. *A*, mice were engrafted with human peripheral blood mononuclear cells 4 days before tumor inoculation. *B*, mice were not engrafted with human peripheral blood mononuclear cells for the control experiment. Each group consisted of five or six mice. The mean survival time (*MST*) of each group were shown in the *inset; bars*, ±SD. Similar results were found in two repeat experiments. Significant differences between experimental groups are indicated by ∗ ($P < 0.05$), ∗∗ ($P < 0.01$), or ns (not significant).

C57BL/6 mice, metastases caused swelling and measurable weight gain of livers and kidneys by day 12. All of these metastases were confirmed by flow cytometry or immunohistochemical analysis (data not shown). In this model, KM2760 significantly suppressed the experimental metastases (Fig. 6*B*). Unexpectedly, KM2760 showed more potent efficacy than KM3060. The difference was more profound for kidney metastases, *i.e.*, the weight of treated organs were almost the same as that of naïve animals, whereas the difference for liver metastases were found to be modest. Depletion of NK cells by administration of anti-asialo GM1 antisera did not significantly affect the treated *versus* control value of KM2760- and KM3060-treated organs (Fig. 6*C*). These results suggest that there is an unidentified mechanism of the superiority of KM2760 in murine effector system that differs from human effector mechanism in which enhanced ADCC of defucosylated IgG1 is due to the strong binding to FcγRIIIa on NK cells (15). We also performed the same experiment with FcRγ chain-deficient mice of C57BL/6 background. In contrast with wild-type mice, the antitumor efficacy of both KM2760 and KM3060 were completely abrogated in FcRγ-deficient mice (data not shown), indicating that the antitumor efficacy observed in the syngeneic tumor model using wild-type mice depended on the activating signals via murine FcγRs.

***In Vivo* KM2760 Antitumor Activity of KM2760 in Mice Bearing Human T-Cell Leukemia.** *In vivo* antitumor activity of KM2760 against human neoplastic T cells was also investigated in a xenograft model bearing murine effector system. We chose human CCRF-CEM cells for this study, because CCRF-CEM was found to be the only human cell line among all five of the selected CCR4-positive cell lines shown in Fig. 3, *A–E*, which reproducibly formed a s.c. solid tumor in nude mice (data not shown). As shown in Fig. 7, KM2760 showed a significant tumor formation delay (3–4 weeks later than the control group).

DISCUSSION

In the present study, we generated the defucosylated chimeric anti-CCR4 IgG1 antibody KM2760, and demonstrated its extremely potent ADCC and antitumor activity to T-cell leukemia/lymphoma in various mouse models.

The high frequency of CCR4 expression on neoplastic T cells has been reported (29, 31–33) and is especially high in ATL (80–90%). Our results also showed that CCR4 is frequently expressed on neoplastic T-cell lines (5 of 8) and that all of these CCR4-expressing cell lines were lysed by KM2760-mediated ADCC. KM2760 is also expected to recognize and lyse ATL cells in patients, because Ishida *et al.* (24) have shown that neoplastic cells from ~90% of ATL patients can be stained by KM2160, the original murine antibody of chimeric KM2760 sharing the same variable region with KM2760. Because CCR4 expression is mostly localized to Th2 cells (19–21), we expect low toxicity of CCR4-targeted therapy. There is currently no curative therapy for ATL, and the 4-year survival rate is only 5–6% for acute or lymphoma type patients (35). CCR4-targeted antibody therapy with potent effector function is, therefore, a promising candidate to overcome this difficulty.

KM2760 with low fucose-containing oligosaccharides (93% defucosylated) exerted potent ADCC with human PBMCs as effector cells. In this study, we observed that KM2760 ADCC with PBMCs from three donors was much higher than that of KM3060 (9% defucosylated oligosaccharides). Interestingly, KM3060 ADCC was relatively high in the experiment with donor C (Fig. 2*C*). We did not investigate the FcγRIIIa genotypes of PBMC donors in this study; however, it may be that one of three donors used in this study carries the FcγRIIIa-158Val genotype, which corresponds to an isoform capable of stronger binding to IgG1. In addition, KM2760 can exert potent ADCC in the presence of fewer effector cells (Fig. 2*D*). This feature could be therapeutically beneficial, because the number of effector cells able to penetrate into a large tumor mass is much less than available for circulating tumors.

In this report, the therapeutic efficacies of KM2760 and KM3060 were compared in a CCR4-positive T-cell leukemia model with human PBMC-engrafted mice. Human PBMCs can be engrafted into the peritoneal cavity of SCID mice for a limited duration (34). We adapted this mouse model to reflect therapeutic efficacy of ADCC by human effector cells. In this mouse model, KM2760 was shown to be significantly more effective in survival prolongation than larger amounts of KM3060. This result suggested that the high potency of KM2760 was retained *in vivo*. In support of the clinical relevance of the model, the antitumor activity of KM2760 entirely depended on the presence of engrafted PBMCs. This is the first report to demonstrate the superiority of defucosylated IgG1 as a therapeutic agent *in vivo*. The potent *in vivo* efficacy of defucosylated IgG1 achieved with a very low dose could ameliorate the problem of cost, which is one of the major defects of antibody drugs.

Additional evidence of the effectiveness against T-cell malignancy was shown in a syngeneic or a xenograft mouse model that does not

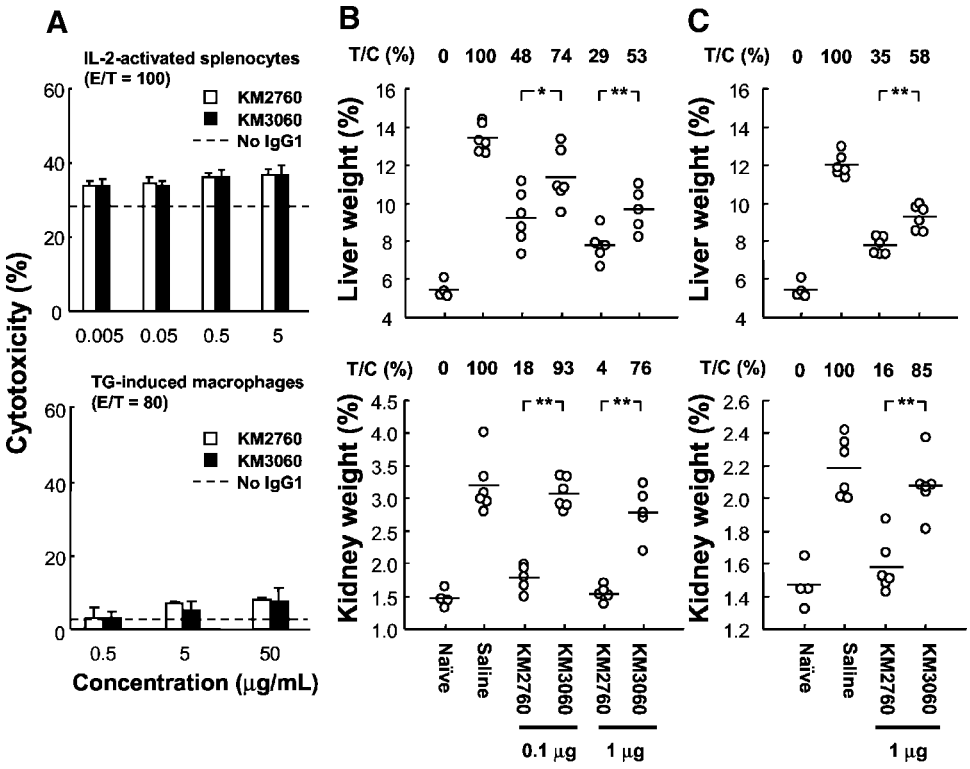

Fig. 6. Antitumor activity of KM2760 and KM3060 in murine effector system. *A*, antibody-dependent cellular cytotoxicity of KM2760 and KM3060 with murine effector cells. *In vitro* cytotoxicities of the two IgG1s were determined in ^{51}Cr release assay using interleukin 2-activated splenocytes (*upper*) or thioglycollate-induced macrophages (*lower*) as effector cells. Cytotoxicity (%) is indicated on the *Y* axis as mean ($n = 3$); *bars*, ± SD. *Dashed lines* indicate the natural cytotoxicity in the absence of antibody (29% for splenocyte cytotoxicity and 2.6% for macrophage cytotoxicity). E:T ratios are shown *above each panel*. In splenocyte cytotoxicity assay, similar data were also obtained with E:T = 30, with 15% natural cytotoxicity and very weak antibody-dependent cytotoxicities for the two IgG1s, which were not significantly different from each other (data not shown). *B*, effect of KM2760 and KM3060 on syngeneic disseminated tumor. Experimental metastases were induced by i.v. inoculation of CCR4/EL4 cells into C57BL/6 mice. Of the two IgG1s or control saline, 0.1 or 1 μg was injected on 1, 3, and 5 days after inoculation i.v. Each group consisted of five or six mice. On day 12, livers (*upper*) and kidneys (*lower*) were taken from all mice including healthy ones that did not receive tumor inoculation (*naïve*) and measured their relative weight as a percentage to the total body weight to evaluate the metastasis. *Horizontal lines* represent mean value in each group. Treated *versus* control (%) of each animal group were shown *above each panel*. Significant differences between the two IgG1s are indicated by * ($P < 0.05$) or ** ($P < 0.01$). Similar results were found in two repeat experiments. *C*, effect of natural killer cell-depletion on the antitumor activities of KM2760 and KM3060. To deplete natural killer cells, all mice were additionally given anti-asialo GM1 antisera (50 μg, i.p.) on day −3, −1, and 1 to the same experimental protocol as described in *B*.

bear human effector cells. Using FcRγ chain-deficient mice, Clynes *et al.* (2) have shown that the mechanisms of action in clinically effective Rituxan and Herceptin in such mouse models were FcγR mediated, suggesting that antibodies with a human IgG1 constant region could exert antitumor activity *in vivo* via murine FcγR on murine leukocytes. However, unlike human PBMC-mediated ADCC, the ADCC of the two anti-CCR4 IgG1s on CCR4/EL4 cells mediated by mouse effector cells were very weak and indistinguishable from each other in this study. On the other hand, we unexpectedly found that KM2760 was more efficacious than KM3060 in the syngeneic tumor model, especially for kidney metastases in a FcγR-dependent way. Taken together, our results suggested that KM2760 functions in mice more efficiently than KM3060 via an unidentified mechanism, which differs from that in humans, and which may vary with the tumor microenvironment. A similar phenomenon has been reported for the case of anti-gp75 antibody, in which the antibody was capable of suppressing syngeneic melanoma in mice in a FcγR-dependent manner, whereas the ADCC by murine effector cells could not be detected *in vitro* (36, 37), although it was not a comparative study of fucose variants. This inconsistency between *in vivo* and *in vitro* activity profiles observed in the present study and in the case of anti-gp75 antibody lead us to postulate the two possible mechanisms: (*a*) murine ADCC effective *in vivo* sometimes might be too weak to be detected *in vitro*; or (*b*) the antibodies can attack tumor not in a direct cytolytic way *in vivo*. Because KM2760 and KM3060 does not exhibit complement-dependent cellular cytotoxicity nor direct cytotoxicity *in vitro* (data not shown), these effector functions are not likely to be important for anti-CCR4 IgG1s in mouse model. In addition, we also found the incapability of murine NK cells in *in vivo* efficacy of KM2760 and KM3060, implying the involvement of FcγR-expressing effector cells other than NK cells, such as macrophages. The impor-

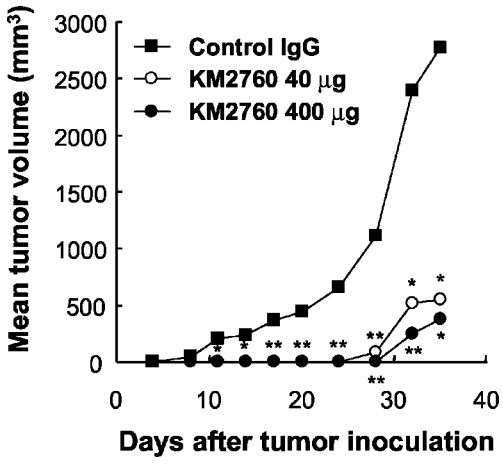

Fig. 7. Effect of KM2760 on tumor growth of human T-cell leukemia cells in nude mice. CCRF-CEM (2×10^7) cells were injected s.c. into nude mice. Three h, and 3 and 5 days later, mice were treated i.v. with indicated dose of KM2760 or control human IgG. Each group consisted of five mice. Significant differences *versus* control group are indicated by * ($P < 0.05$) or *** ($P < 0.01$) Similar results were found in two repeat experiments.

tance of macrophages in FcγR-mediated IgG1 therapy in the mouse model has also been pointed out by Clynes *et al.* (2), as they found that the therapeutic efficacies of Rituxan and Herceptin were potentiated in mice that lack inhibitory receptor FcγRIIb, which are expressed on macrophages but not on NK cells. In contrast to NK cells, which selectively express FcγRIII, macrophages express all of the three subtypes of FcγRs both in human and mouse systems. One possible explanation is that the *in vivo* superiority of KM2760 in mouse system may be due to the differential binding of the two fucose variant IgG1s to at least one of the FcγRs on macrophages. At this point, we have no additional clues to how KM2760 functions in murine effector system, and it requires additional investigation. Considering the clinical relevance of the model, our results suggest that the improved ADCC of defucosylated human IgG1 could be evaluated only in a human effector system, such as human PBMC-engrafted mice.

The antitumor efficacy of KM2760 was also confirmed in the xenograft model bearing human leukemia CCRF-CEM cells. The efficacy in a xenograft model seemed to be somewhat modest, because even 400 μg of administration did not lead to complete suppression. A possible explanation for this is that the two mouse models differ in the accessibility of effector cells to tumor cells, *i.e.*, tumor cells disseminated by blood flow in the syngeneic model can be easily accessed by mouse effector cells, and, therefore, more sensitive to antibody therapy compared with s.c. tumor model. In addition, a little lower CCR4 expression level in CCRF-CEM cells compared with CCR4/EL4 cells (Fig. 3) may also contribute to the lower responsiveness of the xenograft model to the KM2760 therapy.

Many therapeutic antibodies currently approved or under clinical development are produced by CHO cells. Compared with YB2/0 cells, CHO cells express higher levels of α1,6-fucosyltransferase, which catalyzes the fucosylation of complex-type oligosaccharides (16), and in consequence, CHO-produced antibodies are likely to have highly fucosylated oligosaccharides. In support of this, we recently analyzed the oligosaccharide profile of Rituxan and demonstrated that the content of nonfucosylated oligosaccharide was only 6% and the ADCC of Rituxan was much lower than that of YB2/0-produced defucosylated anti-CD20 antibody (16). Defucosylated antibody with enhanced ADCC and potent antitumor activity gives the additional value to the next generation of therapeutic antibodies.

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