Cancer Therapy: Preclinical

Clinical Cancer Research

Defucosylated Humanized Anti-CCR4 Monoclonal Antibody KW-0761 as a Novel Immunotherapeutic Agent for Adult T-cell Leukemia/Lymphoma

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Abstract

Purpose: Adult T-cell leukemia/lymphoma (ATLL) has a very poor prognosis. We have developed the humanized defucosylated anti–CC chemokine receptor 4 (CCR4) monoclonal antibody KW-0761 as a next generation immunotherapeutic agent. The first aim of the present study was to evaluate whether the antitumor activity of KW-0761 would likely be sufficient for therapeutic clinical application against ATLL. The second aim was to fully elucidate the mechanism of antibody-dependent cellular cytotoxicity (ADCC) mediated by this defucosylated monoclonal antibody.

Experimental Design: The antitumor activity of KW-0761 against ATLL cell lines was evaluated *in vitro* using human cells and in mice *in vivo*. Primary ATLL cells from 23 patients were evaluated for susceptibility to autologous ADCC with KW-0761 by two independent methods.

Results: KW-0761 showed potent antitumor activity against ATLL cell lines both *in vitro* and in the ATLL mouse model *in vivo*. In addition, KW-0761 showed potent antitumor activity mediated by highly enhanced ADCC against primary ATLL cells both *in vitro* and *ex vivo* in an autologous setting. The degree of KW-0761 ADCC against primary ATLL cells in an autologous setting was mainly determined by the amount of effector natural killer cells present, but not the amount of the target molecule CCR4 on the ATLL cell surface.

Conclusion: KW-0761 should be sufficiently active for therapeutic clinical application for ATLL. In addition, combination treatment strategies that augment natural killer cell activity should be promising for amplifying the effect of KW-0761. In the near future, the actual efficacy of KW-0761 will be established in pivotal clinical trials. *Clin Cancer Res; 16(5); 1520–31. ©2010 AACR.*

The use of therapeutic monoclonal antibodies (mAb) for the treatment of cancer has evolved into a promising approach over the last several years. In the clinical field of hematologic malignancies, development of the chimeric anti-CD20 mAb rituximab has changed the standard therapy in patients with B-cell lymphomas and has markedly improved their prognoses (1, 2). In contrast, T-cell lymphomas still have very poor prognoses, and no standard treatment strategies for these diseases have been developed (3). Among these malignancies, adult T-cell leukemia/lymphoma (ATLL), caused by human T-cell leukemia virus type 1 (HTLV-1), has an extremely unfavorable prognosis (3, 4). Because we had previously found that CC chemokine receptor 4 (CCR4) is expressed on tumor cells from most ATLL patients (5), as well as on tumor cells from a subgroup of peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS), which also has an unfavorable prognosis (6), we postulated that this molecule might represent a novel molecular target for immunotherapy against refractory T-cell leukemia/lymphoma. Accordingly, we have developed a chimeric anti-CCR4 mAb, KM2760 (7), the Fc region of which is defucosylated, resulting in highly enhanced antibody-dependent cellular cytotoxicity (ADCC) due to increased binding affinity to the Fcγ receptor (FcγR) on effector cells (8, 9). As a result, this mAb KM2760 indeed showed potent antitumor activity mediated by highly enhanced ADCC against CCR4-expressing tumors both *in vitro* and in mice *in vivo* (7, 10–15). These promising preclinical data prompted

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Translational Relevance

We have developed a humanized therapeutic defucosylated anti–CC chemokine receptor 4 monoclonal antibody (mAb), KW-0761. We are currently conducting a phase II clinical trial of KW-0761 for relapsed/refractory adult T-cell leukemia/lymphoma (ATLL). To the best of our knowledge, KW-0761 is the first defucosylated therapeutic mAb to be used clinically in cancer patients. Defucosylation of mAb should be a promising next-generation technology with improved antibody-dependent cellular cytotoxicity (ADCC), and could be applied to many other mAbs. Therefore, a better understanding of ADCC mediated by KW-0761 will allow the development of novel, more effective treatment strategies using not only KW-0761 but also other defucosylated mAbs. Thus, the present data not only directly provide the basis for a clinical application of KW-0761 in ATLL, but also for the clinical development of many other defucosylated mAbs in other cancers. In addition, the present observation that the degree of autologous KW-0761 ADCC was mainly determined by the amount of effector natural killer cells present will help in the design of subsequent clinical protocols.

us to proceed with the clinical development of this defucosylated anti-CCR4 mAb.

Because it is generally accepted that replacement of mouse immunoglobulin constant regions with the human equivalents effects the largest reduction in immunogenicity, and humanization of variable domains effects a further decrease (16), we have now generated a defucosylated humanized anti-CCR4 mAb, KW-0761, for clinical application. The present study had two major aims. The first was to evaluate the antitumor activity of KW-0761 both *in vitro* and in mice *in vivo* to determine whether it would be sufficient for therapeutic clinical application against ATLL. The second aim was to fully elucidate the mechanism of ADCC mediated by this defucosylated mAb. In particular, we focused on effector or target cell–associated factors that determine susceptibility to KW-0761 ADCC. KW-0761 is the first defucosylated therapeutic mAb used clinically in cancer patients. Here, we report preclinical studies of KW-0761 for the first time. We believe that the defucosylation of therapeutic mAb should be a promising next-generation technology with improved ADCC, and therefore that it will be applied to many other therapeutic mAbs in the near future (9, 17). Thus, a better understanding of ADCC mediated by KW-0761 will facilitate the development of novel, more effective treatment strategies using not only KW-0761 but also other defucosylated next-generation therapeutic mAbs. To achieve the two aims set out above, we used cells from 23 primary ATLL patients, and evaluated the autologous ADCC of KW-0761 by two independent methods.

Materials and Methods

Anti-CCR4 mAbs. The mouse antihuman CCR4 IgG1 mAb KM2160 was previously described (5). The defucosylated human/mouse chimeric anti-CCR4 IgG1 mAb KM2760 was also previously described (7). KW-0761 is a defucosylated humanized IgG1 mAb generated from a mouse anti-CCR4 mAb by Kyowa Hakko Kirin Co., Ltd. (17, 18). KM2760 can induce only ADCC activity, but does not mediate complement-dependent cytotoxicity or direct antitumor activities (10, 15). Similarly, KW-0761 induces only ADCC activity, not complement-dependent cytotoxicity or direct effects (data not shown).

Biosensor analysis. A peptide corresponding to positions 12 to 29 of the NH_2-terminus residues of the CCR4 protein (H-DESIYSNYYLYESIPKPC-OH), CCR4-1-9′, was synthesized by the Fmoc solid phase method using an automated peptide synthesizer, PSSM-8 (Shimadzu), followed by biotinylation of the COOH-terminus using EZ-Link PEO-maleimide-activated biotin (Thermo Fisher Scientific Inc.). The biotinylated CCR4-1-9′ peptide was dissolved in HBS-EP buffer (0.01 M HEPES pH 7.4, 150mM NaCl, 3mM EDTA, 0.005% surfactant P20) containing 16.7% (v/v) of N, *N*-dimethylformamide (Wako Pure Chemical Industry) at a concentration of 1.0 mg/mL and further diluted in HBS-EP buffer to 0.05 μg/mL. The diluted CCR4-1-9′ peptide was injected over a streptavidin biosensor chip SA (BIACORE) at a flow rate of 5 μL/min for 1 min. KM2760 or KW-0761 diluted to 20, 10, 5, 2.5, 1.25, or 0.625 μg/mL in HBS-EP buffer was injected over the CCR4-1-9′-captured sensor surface at a flow rate of 5 μL/min for 4 min to monitor the surface plasmon resonance (SPR) of the on-rate kinetics. Following the completion of the on-rate kinetics phase, the SPR of the off-rate kinetics phase was monitored with HBS-EP buffer running over the sensor surface at 5 μL/min for 4 min. At the end of the off-rate kinetics phase, residual bound antibody was removed by flushing with 10 mmol/L Glycine-HCl (pH 1.5; BIACORE) at a flow rate of 5 μL/min for 1 min. Intensity of SPR was expressed as resonance units (RU). Data were analyzed by BIAevaluation version 3.0 kinetic evaluation program (BIACORE).

Cells. Blood samples were obtained from ATLL patients, and peripheral blood mononuclear cells (PBMC) were isolated with Ficoll-Paque (Pharmacia). Diagnosis and classification of clinical subtypes of ATLL was according to the criteria proposed by the Japan Lymphoma Study Group (19). ATLL patients with the acute and lymphoma subtypes have an aggressive clinical course, whereas ATLL patients with the chronic and smoldering subtypes have longer survival (19). Thus, here we designated the acute subtype of ATLL as the aggressive variant, and the chronic and smoldering subtypes of ATLL as indolent variants. The expression of surface molecules such as CD4, CD16, CD25, and CD56 was also analyzed in ATLL patient blood mononuclear cells, and HTLV-1 proviral DNA load in PBMC (copies/1,000 PBMC), as part of the routine clinical

blood examination at SRL, Inc. (Tokyo, Japan). PBMCs were also isolated in the same way from healthy individuals for use as effector cells in ADCC assays. In autologous ADCC assays, CD3-positive cells were isolated from fresh PBMCs obtained from ATLL patients with anti-human CD3 microbeads (Miltenyi Biotec) and were used as target cells. Cells from the remaining CD3-negative subset were used as effector cells. A CD3-negative subset was also isolated from a single healthy individual and the cells were used as standard control effector cells throughout all *in vitro* autologous KW-0761 ADCC assays. All donors provided informed written consent before sampling according to the Declaration of Helsinki, and the present study using human samples was approved by the institutional review boards of Nagoya City University Graduate School of Medical Sciences and Imamura Bun-in Hospital.

ATLL cell lines. ATN-1, ATL102, and MT-2 (10), and S-YU (20) were previously described. TL-Om1 was kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University.

***ADCC assay* in vitro**. Standard 4-h ^{51}Cr release assays were carried out as described previously (10). All experiments were done in triplicate and the percent cell lysis is presented as a mean value ± SD. The effector:target (E:T) ratio was fixed at 50:1.

Antibodies and flow cytometry analysis. FITC-conjugated anti-CD16 (clone 3G8), PerCP-conjugated anti-CD4 (SK3), and phycoerythrin (PE)-conjugated anti-CD25 (M-A251), and the appropriate isotype control antibodies, were purchased from BD Biosciences. Cells were analyzed by FACScalibur (BD Biosciences) with the aid of CXP cytometer software (Beckman Coulter).

Animals. Male severe combined immunodeficient (SCID) mice were purchased from CLEA Japan, Inc., and used at 6 to 8 weeks of age. All *in vivo* experiments were done in conformity with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia research (2nd edition).

ATLL tumor-bearing mouse model. S-YU cells (1×10^7) suspended in 0.2 mL RPMI-1640 medium were s.c. inoculated into SCID mice. KW-0761 or control (saline) injections into tumor-bearing SCID mice were started 7 d after tumor inoculations, when the tumor volume had reached 112 to 316 mm^3. Ten mice were divided into two groups for KW-0761 or control injections, such that the average tumor volumes were the same in each group. Tumor volume was calculated by the following formula: tumor volume (mm^3) = 0.5 × (major diameter) × (minor diameter)^2. KW-0761 at a dose of 20 mg/kg or control was injected into the tail vein of SCID mice weekly for 4 wk.

S-YU cells (1×10^7) suspended in 0.2 mL RPMI-1640 medium were i.p. inoculated into SCID mice. KW-0761 or control injections were started 7 d after tumor inoculations, when the plasma sIL-2Rα levels had reached 2,963 to 7,973 pg/mL. Ten mice were divided into two groups for KW-0761 or control injections, such that the average plasma sIL-2Rα levels were the same in each group. KW-0761 at a dose of 20 mg/kg or control was injected into the tail vein of SCID mice weekly for 4 wk. Plasma sIL-2Rα concentrations were measured by ELISA using Human sIL-2Rα Immunoassay Kits (R&D Systems, Inc.) according to the manufacturer's instructions.

Ex vivo *ATLL cell depletion assay.* Heparinized peripheral blood samples from ATLL patients, diluted with an equal volume of RPMI-1640 medium, were incubated at 37°C, 5% CO_2 for 24 h, with or without 10 μg/mL KW-0761. Thereafter, the sample was stained with PerCP-conjugated anti-CD4, FITC-conjugated anti-CD25 (M-A251), and PE-conjugated anti-CCR4 (clone 1G1), or their appropriate isotype controls (all antibodies from BD Biosciences). Stained cells were treated with FACSlysing solution (BD Biosciences), and analyzed by FACScalibur with the aid of CXP cytometer software. The ATLL cell-depleting activity of KW-0761 was determined by the following gating strategy. The lymphocyte population was gated on forward and side scatter, the CD4-positive cells were further gated, and then CCR4 and CD25 expression was plotted. The percentage of ATLL cells in the lymphocyte population was calculated according to the following formula: ATLL % = A × B/100 %, where A is the percentage of CD4-positive cells among the lymphocyte population, and B the percentage of ATLL cells determined by CCR4 and CD25 expression levels within the CD4-positive lymphocytes. The anti-CCR4 antibody used here, clone 1G1, is specific for an epitope different from that bound by KW-0761; the binding of 1G1 to CCR4 was not affected by the presence of KW-0761 (data not shown).

Statistical analysis. Correlations between two variables obtained from primary ATLL samples were assessed using the Spearman rank correlation coefficient (Rs). Differences of the variables between two groups were examined with the Mann-Whitney *U*-test. Mouse survival analyses were done by the Kaplan-Meier method, and survival curves were compared using the log-rank test. All statistical analyses were done using the SAS software program (Release 9.1.3, SAS Institute Inc.). In our study, $P < 0.05$ was considered significant.

Results

Biosensor analysis of KW-0761 and KM2760. Association rate constants (Ka) of KM2760 and KW-0761 were calculated as 2.18 × 105 mol/L^{-1}s^{-1} and 1.18 × 105 mol/L^{-1}s^{-1}, respectively. Dissociation rate constants (Kd) could not be calculated for either antibody due to their very slow dissociation ($<10^{-5} \sim 10^{-6}$; Fig. 1A).

KW-0761 and KM2760 ADCC against ATLL cell lines. Flow cytometry analysis showed that CCR4 was expressed on TL-Om1 (data not shown). We compared KW-0761 ADCC with KM2760 ADCC against the CCR4-positive ATLL cell lines ATN-1, ATL102, MT-2, and TL-Om1 in the presence of PBMC from three healthy donors. As shown in Fig. 1B, KW-0761 induced robust ADCC that was comparable with that induced by KM2760 in the presence of any donor PBMC.

KW-0761 induces potent antitumor activity in ATLL tumor-bearing mice. In the s.c. inoculation model, injection of KW-0761 showed therapeutic efficacy as seen by its effect on tumor volume. Two of five mice achieved complete remission in the KW-0761 group. The tumor volume of KW-0761 and control groups at day 27 after tumor inoculation was 1,456 ± 1,365 and 3,839 ± 814 mm^3, respectively. This difference was statistically significant ($P = 0.0212$; Fig. 2A). In the i.p. inoculation model, the KW-0761 group had a significant prolongation of survival compared with controls ($P = 0.0342$; Fig. 2B, left). The concentration of human sIL2-Rα in the plasma of mice in the KW-0761–treated and control groups at day 14 after tumor inoculation was 168,347 ± 100,091 and 310,990 ± 92,101 pg/mL, respectively ($P = 0.0367$; Fig. 2B, right). No toxicity attributable to

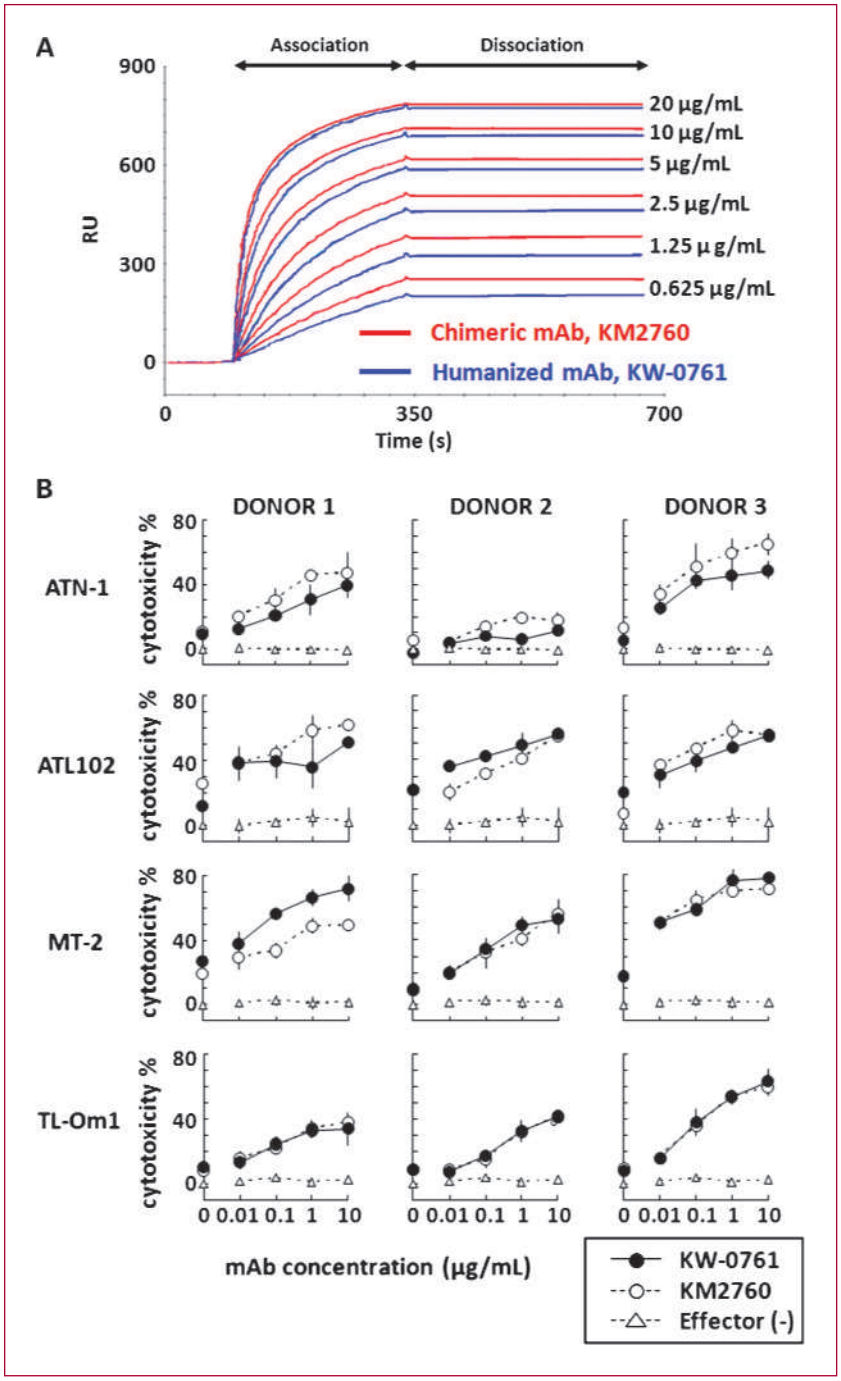

Fig. 1. Comparison of binding activity and ADCC activity of KW-0761 and KM2760. A, biosensor analysis of KW-0761 and KM2760. Y-axis, amount of bound antibodies, in resonance units (RU); X-axis, time in seconds. B, ADCC of KW-0761 and KM2760 against ATLL cell lines measured by standard 4-h ^{51}Cr release assays using PBMCs from three healthy donors as effector cells. X-axis, concentrations of mAbs. The E:T ratio was fixed at 50:1. All experiments were done in triplicate, and the percent cell lysis is presented as mean ± SD.

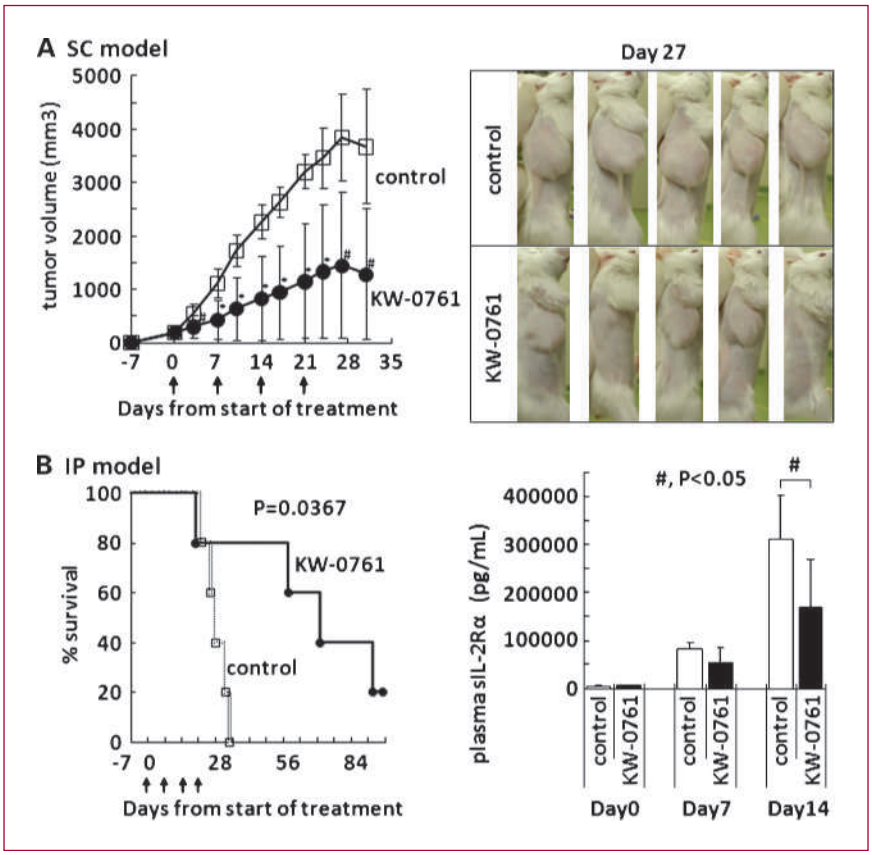

Fig. 2. KW-0761–induced potent antitumor activity in ATLL tumor-bearing mice. A, antitumor activity of KW-0761 against pre-established s.c. S-YU tumor. Injections of KW-0761 were started when S-YU tumor size reached ca. 190 mm^3 (7 d after S-YU inoculation). Tumor volume was measured twice weekly, and mean ± SD are presented. Arrows, KW-0761 or control injections. Each group consisted of five mice. □, control (saline); ●, KW-0761. Left, significant differences between KW-0761 and vehicle group (*, $P < 0.01$; #, $P < 0.05$). Right. photographs of each mouse 27 d after starting treatment. B, antitumor activity of KW-0761 against pre-established peritoneal S-YU tumor. Injections of KW-0761 were started when the plasma sIL-2Rα levels reached ca. 5,000 pg/mL (7 d after S-YU inoculation). Kaplan-Meier survival plot of SCID mice is presented. Arrows, KW-0761 or control injections. Each group consisted of five mice. □, control; ●, KW-0761. Left, significant difference between the control and treatment groups ($P < 0.05$). On days 0, 7, and 14 from the start of treatment, concentrations of plasma sIL-2Rα were measured. The data represent the mean + SD of each group. Right, #, significant differences versus the control group ($P < 0.05$).

KW-0761 injections was observed in any of the mice in this setting.

KW-0761 mediates potent* in vitro *ADCC activity against primary ATLL cells in an autologous setting. We tested KW-0761 ADCC in 22 cases with primary ATLL cells in an autologous setting. A representative result obtained with patient ATLL6 is shown in Fig. 3. In this case, the CD3-positive target cell population contained 51.0% CD4-, CD25- and CCR4–triple positive ATLL cells, expressing CCR4 at a mean fluorescence intensity (MFI) ratio of 12.1 (8.5/0.7; Fig. 3A). The CD3-negative effector cell population contained 37.5% CD16-positive cells, and 20.5% CD16-expressing natural killer (NK) cells and 2.7% monocytes as determined by side scatter height (SSC-H) (Fig. 3B). The maximum cell lysis by KW-0761 ADCC mediated by autologous effector cells was 60.4% at an antibody concentration of 10.0 μg/mL, whereas cytotoxicity mediated by allogeneic effector cells was 78.6% at the same antibody concentration (Fig. 3C). In all 22 cases tested, *in vitro* KW-0761 ADCC mediated by autologous effector cells tended to be lower than that mediated by control effector cells from a healthy individual. The results with all ATLL cases which were tested for *in vitro* autologous KW-0761 ADCC are summarized in Table 1.

Correlations between the percentage of CD16-positive cells among effector cells and the CCR4 MFI ratio of ATLL cells, and* in vitro *autologous KW-0761 ADCC. The level of autologous KW-0761 ADCC showed no significant correlations with allogeneic control KW-0761 ADCC (Rs = 0.310, $P = 0.1603$) or the CCR4 MFI ratio of target ATLL cells (Rs = 0.073, $P = 0.7473$; Fig. 3D, top left). The CCR4 MFI ratio also failed to correlate with the degree of allogeneic control KW-0761 ADCC (Rs = −0.002, $P = 0.9940$). However, significant correlations were found between the percentages of CD16-positive cells and CD16-expressing NK cells within the effector population, and the degree of autologous KW-0761 ADCC [Rs = 0.531, $P = 0.0109$ (Fig. 3D, top right), and Rs = 0.813, $P < 0.0001$ (Fig. 3D, bottom left), respectively], but not between the percentage of CD16-expressing monocytes and autologous KW-0761 ADCC (Rs = 0.369, $P = 0.0906$; Fig. 3D, bottom right).

Ex vivo *ADCC activity of KW-0761*. We further investigated the *ex vivo* ADCC activity of KW-0761 using diluted whole blood from 20 cases with primary ATLL. This model is certainly more relevant for evaluation of the intravascular environment of ATLL patients *in vivo*, because no manipulations are made which could potentially cause artifacts, such as purifying effector cells from individuals, mixing large numbers of the purified effector cells and low numbers of target cells, or incubating the mixture in nonhuman serum without any human plasma components. In addition, this model

represents a purely autologous setting without any exception. A representative result, again from patient ATLL6, is shown in Fig. 4A. CD4-positive cells constituted 44.3% of the lymphocyte population, of which 78.1% were tumor cells as determined by CCR4 and CD25 expression. Therefore, the percentage of ATLL cells within the lymphocyte population, as determined by CD4, CD25, and CCR4 staining, was 34.6% (44.3 × 78.1/100).

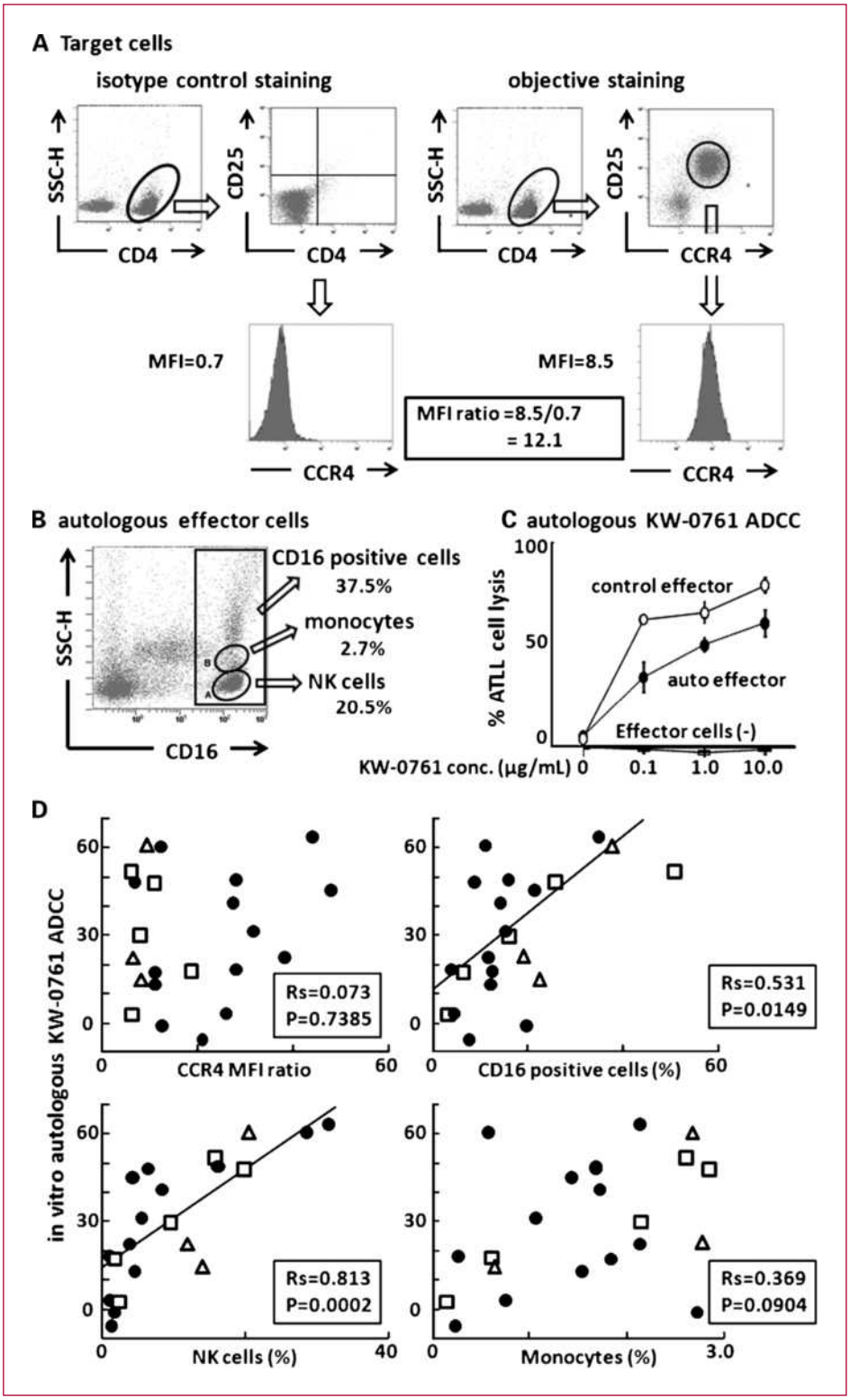

Fig. 3. *In vitro* autologous KW-0761 ADCC against primary ATLL cells. A, target tumor cells (the CD3-positive subset) were obtained from patient ATLL6 as a representative case. CCR4 expression level on target tumor cells is presented as the MFI ratio, i.e., the MFI with the anti-CCR4 mAb, KM2160 (right) divided by isotype control (left). B, autologous effector cells (the CD3-negative subset of PBMC) obtained from ATLL6. CD16-expressing NK cells and monocytes are determined by their side scatter height (SSC-H) levels. C, KW-0761 ADCC against the CD3-positive subset measured by a standard 4-h ^{51}Cr release assay in the presence of the CD3-negative subset at the E:T ratio of 50:1. As control effector cells, the CD3-negative subset from the same healthy individual was included in every assay. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD. D, correlations between the CCR4 MFI ratio of target ATLL cells (top left), the percentage of CD16-positive cells (top right), CD16-expressing NK cells (bottom left), or monocytes (bottom right) within the effector cell population and the degree of autologous KW-0761 ADCC. Each dot plot in each panel, one ATLL case; ●, ATLL acute type; □, chronic type; Δ, smoldering type. The correlation coefficients (Rs) and *P* values in each panel were assessed by Spearman rank correlation coefficient testing.

Table 1. ATLL cases tested for KW-0761 ADCC

Sample name	Subtype	*In vitro* autologous KW-0761 ADCC						*Ex vivo* KW-0761 ADCC			
		Autologous cytotoxicity (%)	Allogeneic cytotoxicity (%)	CCR4 MFI ratio	Total CD16 (%)	CD16 NK (%)	CD16 monocyte (%)	Cytotoxicity (%)	CD16/56 in lymph (%)	CD4/25 in lymph (%)	HTLV-1 load (copies/ 1,000 PBMC)
ATLL1	Chronic	30.0	45.0	7.8	15.8	9.4	2.1	N.T.	2.5	83.8	833.0
ATLL2	Acute	49.2	64.9	27.9	15.8	16.1	1.7	N.T.	0.9	90.9	758.9
ATLL3	Smoldering	22.1	83.1	6.3	19.0	12.0	2.8	10.2	13.9	10.9	232.6
ATLL4	Acute	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	4.2	1.9	85.4	969.9
ATLL5	Smoldering	14.4	47.8	8.2	22.4	14.1	0.6	62.5	39.9	38.4	214.3
ATLL6	Smoldering	60.4	78.6	12.1	37.5	20.5	2.7	51.5	13.4	47.8	82.5
ATLL7	Chronic	51.7	93.5	5.8	50.7	15.7	2.6	28.7	6.0	72.1	541.8
ATLL8	Chronic	48.0	84.6	10.6	25.5	19.8	2.8	21.5	9.5	40.7	334.8
ATLL9	Chronic	17.4	58.6	18.4	12.5	1.5	0.6	−0.3	4.1	84.9	867.0
ATLL10	Acute	31.1	93.2	31.6	15.2	5.5	1.1	11.9	6.7	28.1	110.3
ATLL11	Acute	−6.1	62.8	20.8	7.7	1.3	0.2	−0.8	0.9	80.5	445.9
ATLL12	Acute	63.6	82.2	43.8	34.7	31.5	2.1	0.1	N.T.	N.T.	504.3
ATLL13	Acute	45.3	105.1	47.9	21.3	4.2	1.4	32.0	N.T.	N.T.	99.4
ATLL14	Acute	48.1	107.1	6.7	8.8	6.5	1.7	−0.7	0.5	18.3	554.5
ATLL15	Acute	3.1	47.6	25.9	4.5	1.1	0.8	−1.0	1.3	88.7	234.0
ATLL16	Acute	17.9	91.4	27.9	4.0	1.1	0.3	3.6	4.6	76.6	332.6
ATLL17	Acute	22.0	86.9	38.3	11.5	3.8	2.1	−3.4	4.0	68.4	482.3
ATLL18	Acute	12.7	81.3	10.9	12.1	4.5	1.5	N.T.	2.2	76.9	314.1
ATLL19	Acute	17.2	80.6	11.0	6.5	1.8	1.8	14.8	3.4	66.1	478.5
ATLL20	Chronic	2.6	119.6	6.2	3.1	2.4	0.1	−18.0	1.2	87.4	845.0
ATLL21	Acute	−1.2	31.9	12.4	19.5	1.6	2.7	4.6	7.1	83.8	1270.0
ATLL22	Acute	60.6	74.2	9.2	11.1	28.4	0.6	50.9	18.9	26.4	167.9
ATLL23	Acute	40.9	98.5	27.3	14.3	8.2	1.7	6.6	6.8	51.6	375.1

Abbreviation: N.T., not tested.

This was decreased in the presence of 10 μg/mL KW-0761 to 16.8%, calculated in the same manner. In this example, the ATLL cell-depleting activity of KW-0761 (*ex vivo* KW-0761 ADCC) was calculated as follows: (34.6-16.8)/ 34.6 × 100 = 51.5% (Fig. 4A). The results with all ATLL cases tested for *ex vivo* KW-0761 ADCC are also summarized in Table 1.

Correlations among* in vitro *autologous KW-0761 ADCC, percentage of NK cells, percentage of ATLL cells, and* ex vivo *KW-0761 ADCC. The same blood samples used for *ex vivo* KW-0761 ADCC were assessed for their content of NK cells as determined by both CD16 and CD56 expression, as well as their content of ATLL cells determined by both CD4- and CD25-positivity (SRL, Inc.). The HTLV-1 proviral DNA load in PBMC was also determined by SRL, Inc. The degree of *ex vivo* KW-0761 ADCC correlated with the presence of NK cells among blood lymphocytes (Rs = 0.802, $P = 0.0001$; Fig. 4B, top left). *Ex vivo* KW-0761 ADCC was inversely correlated with the numbers of ATLL cells in the blood (Rs = −0.581, $P = 0.0115$; Fig. 4B, top right), as well as with the HTLV-1 proviral DNA load in PBMC (Rs = −0.582, $P = 0.0071$; Fig. 4B, bottom left). There were also significant correlations between the *ex vivo* KW-0761 ADCC and *in vitro* autologous KW-0761 ADCC determined by ^{51}Cr release assay (Rs = 0.456, $P = 0.0497$; Fig. 4B, bottom right). In addition, the presence of NK cells was inversely correlated with the number of ATLL cells (Rs = −0.600, $P = 0.0041$) and HTLV-1 proviral DNA load in PBMC (Rs = −0.523, $P = 0.0150$). Finally, the proportion of ATLL cells in blood lymphocytes was found to correlate with the HTLV-1 proviral DNA load as determined in PBMC (Rs = 0.617, $P = 0.0029$).

Influence of factors such as aggressive (acute) and indolent (chronic and smoldering) ATLL clinical variants on* in vitro *autologous KW-0761 ADCC. There were no significant differences between aggressive and indolent ATLL variants in the degree of *in vitro* autologous KW-0761 ADCC (Fig. 5, top left) or allogeneic KW-0761 ADCC (Fig. 5, top right). The CCR4 MFI ratio of the aggressive variant was significantly higher than that of indolent variant ($P = 0.0037$, Fig. 5, bottom left).

Comparison of* ex vivo *KW-0761 ADCC-associated factors according to ATLL clinical variants. There were no significant differences between the two ATLL clinical variants in the degree of *ex vivo* KW-0761 ADCC ($P = 0.2346$; Fig. 5, bottom right), the proportion of $CD16^+CD56^+$ NK cells ($P = 0.1031$) or $CD4^+CD25^+$ ATLL cells in blood lymphocytes ($P = 0.6638$), or the HTLV-1 proviral DNA load in PBMC ($P = 0.9229$).

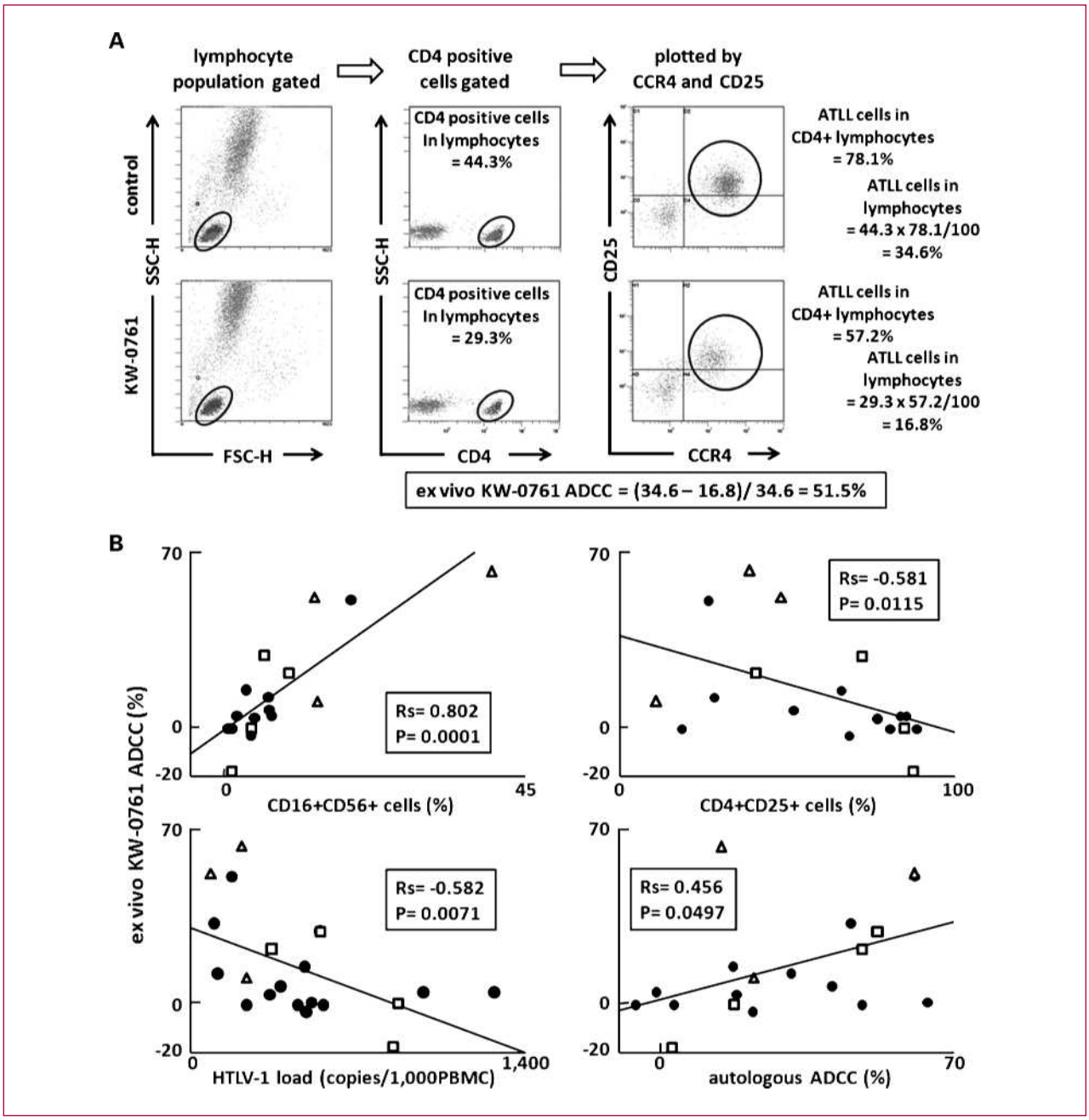

Fig. 4. *Ex vivo* ADCC activity of KW-0761 in ATLL. A, the ATLL cell-depletion activity of KW-0761 was determined by a flow cytometric method. Analysis of ATLL6 is again shown as a representative case. The percentage of ATLL cells determined by CD4, CD25, and CCR4 expression within the lymphocyte population in the control culture was 34.6% (top three panels). The percentage of ATLL cells remaining in the presence of 10 μg/mL KW-0761 was 16.8% calculated in the same manner (bottom three panels). In this case, ATLL cell-depleting activity by KW-0761 (designated *ex vivo* KW-0761 ADCC) was calculated as follows: (34.6-16.8)/34.6 × 100 = 51.5%. B, correlations between the numbers of NK cells determined by both CD16 and CD56 positivity (top left), and ATLL cells determined by both CD4 and CD25 positivity (top right), HTLV-1 proviral DNA load in PBMC (bottom left), and *in vitro* autologous KW-0761 ADCC against primary ATLL cells (bottom right) and the degree of *ex vivo* KW-0761 ADCC. Each dot plot in each panel, one ATLL case; ●, ATLL acute type; □, chronic type; Δ, smoldering type. The correlation coefficients (Rs) and *P* values in each panel were assessed by Spearman rank correlation coefficient testing.

Discussion

In the present study, we set out to evaluate whether the antitumor activity of KW-0761 would be sufficient for therapeutic clinical application against ATLL and to elucidate the mechanism of ADCC mediated by this defucosylated mAb. We found that, first, KW-0761 indeed showed potent antitumor activity against ATLL cell lines both *in vitro* and in the ATLL mouse model *in vivo*. In addition, KW-0761 showed potent antitumor activity mediated by highly enhanced ADCC against primary ATLL cells both *in vitro* and *ex vivo* in an autologous setting. We believe that collectively these preclinical data show the suitability of KW-0761 for therapeutic clinical application in ATLL. Second, we found that the degree of KW-0761 ADCC against primary ATLL cells in an autologous setting was mainly determined by the amount of effector NK cells present, but not the amount of the target molecule CCR4 on the ATLL cell surface.

Although the generation of humanized antibodies with a high binding affinity for antigen generally requires overcoming some difficulties such as additional transfer of one or more framework-region residues from the parent mouse antibody (21, 22), the present biosensor analysis indicated that our humanized antibody KW-0761 showed potent binding activity that is almost identical to that of chimeric antibody KM2760. The present study also showed that KW-0761 induced a robust ADCC activity comparable with that induced by KM2760 in the presence of PBMC from healthy individuals. In addition, KW-0761 exhibited potent and significant antitumor activities both in the s.c. and i.p. inoculation ATLL mouse models. These findings, together with our previous report that KM2760 exhibited potent antitumor activity mediated by ADCC *in vitro* and *in vivo* mouse models (7, 10–15), indicate that application of KW-0761 should be an ideal and promising treatment strategy for patients with ATLL.

The ultimate goal of this type of immunotherapy is to obtain sufficient antitumor activity by simply administering mAb to patients *in vivo*; however, the therapeutic effect may be hampered by the immunocompromised state common in patients with ATLL (23–25). Therefore, the efficacy of the autologous system must be shown in ADCC evaluations. Here, we adopted two different methods to examine KW-0761 ADCC activity mediated by autologous effector cells against primary ATLL cells. One was the standard 4-hour ^{51}Cr release assay, the other an *ex vivo* ATLL cell depletion assay. The former employed a fixed E:T ratio, and effector cells from the same healthy person were included as a standardized control in every assay. This assay is appropriate for separately evaluating effector or target cell–associated factors determining susceptibility to KW-0761 ADCC. In the latter assay, KW-0761 or control was simply added to whole blood samples from individual ATLL patients, and reduction of the proportion of ATLL cells was determined after incubation. This model is certainly more likely to reflect the natural intravascular environment of ATLL patients *in vivo*, such as the E:T ratio, and the presence of endogenous plasma IgG which inhibits ADCC mediated by the therapeutic mAb (26, 27).

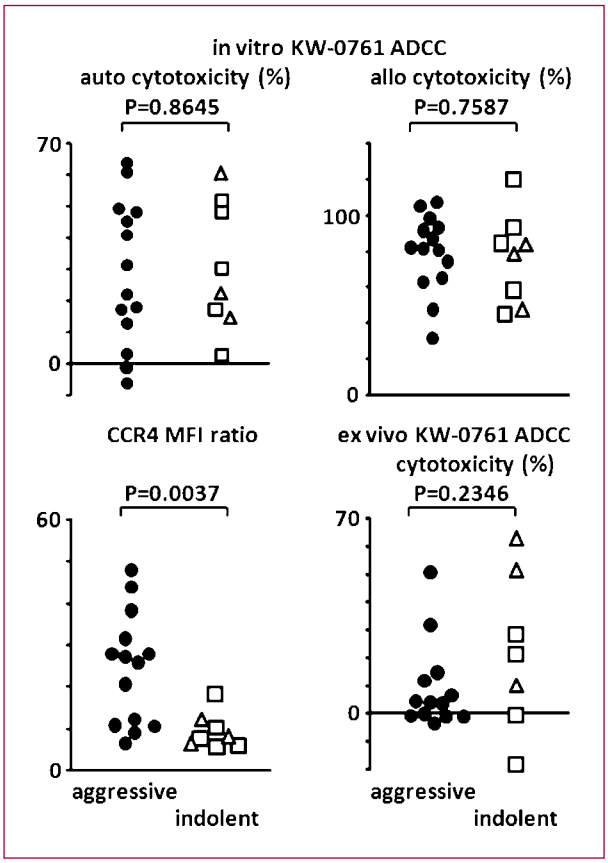

Fig. 5. Comparison of *in vitro* autologous KW-0761 ADCC and *ex vivo* KW-0761 ADCC in different ATLL clinical variants. Differences in the *in vitro* autologous (top left) or allogeneic (top right) KW-0761 ADCC against primary ATLL cells, CCR4 MFI ratio of target ATLL cells (bottom left), and *ex vivo* KW-0761 ADCC (bottom right) between aggressive and indolent clinical variants assessed using the Mann-Whitney *U*-test. Each dot plot in each panel, one ATLL case, ●, ATLL acute type; □, chronic type; Δ, smoldering type. The *P* values are indicated in each panel.

In the former assay, the level of *in vitro* autologous KW-0761 ADCC observed did not correlate with the CCR4 MFI ratio of the target ATLL cells. This result is a novel significant finding, because it has been generally accepted that the expression level of the target molecule is one of the most important factors that determine susceptibility to conventional types of therapeutic mAb ADCC (28–30). We have previously reported that defucosylation of therapeutic mAb resulted in the same degree of ADCC induction at lower target antigen levels than mediated by unmodified antibody; these previous experiments, however, were conducted using established cell lines as targets and allogeneic cells from healthy donors as effectors (31). The present study is the first to show that defucosylated mAb KW-0761 could overcome one of the main obstacles preventing efficient ADCC caused by reduced

amounts of target antigen, using primary tumor cells and autologous effector cells.

Lymphoma patients homozygous for FcγRIIIa-158VV, which has the highest affinity for the Fc region of IgG1, have the best clinical responses to rituximab (32, 33). The clear role of FcγR-bearing effectors in mediating the response to rituximab in clinical settings further shows the importance of the effector cells for ADCC. In addition, within ADCC effector cells, NK cells are thought to be particularly important (11, 15, 28, 34–36). These observations are consistent with the present findings of correlations between *in vitro* autologous KW-0761 ADCC and the amount of CD16-expressing cells, in particular NK cells. On the other hand, we must take into account the present finding that KW-0761 ADCC mediated by autologous effector cells tended to be lower than that mediated by control effector cells in all 22 cases. This does suggest some impairment of ATLL patients' ADCC-mediating NK cell activity.

The present *ex vivo* ATLL cell depletion assays also showed the importance of the amount of effector NK cells for KW-0761 ADCC. In addition, *ex vivo* KW-0761 ADCC also inversely correlated with the proportion of ATLL cells in the blood and with the HTLV-1 proviral DNA load in PBMC. These findings are consistent with observations that the population of NK cells in blood lymphocytes also inversely correlated with variables reflecting the number of ATLL cells in patients' blood, such as the proportions of $CD4^+CD25^+$ cells and the HTLV-1 proviral DNA load. Significant correlations of ADCC activity assessed by different methods, i.e., *ex vivo* KW-0761 ADCC and *in vitro* autologous KW-0761 ADCC, seemed to be due to the amount of NK cells present in the ATLL blood sample.

Treatment strategies for ATLL patients should be selected according to clinical subtypes (37, 38). Therefore, we compared the efficacy of KW-0761 ADCC in the different ATLL clinical variants. We found no significant differences in the degree of *in vitro* autologous or allogeneic KW-0761 ADCC, or *ex vivo* KW-0761 ADCC, between the ATLL clinical variants. This indicates that the susceptibility of ATLL tumor cells to KW-0761 ADCC was not influenced by the clinical variant from which they were isolated. This was despite the finding that there were significant differences in the CCR4 MFI ratios between ATLL clinical variants, a novel finding, although this did not affect susceptibility to lysis. There are at least two possible explanations for different CCR4 ratios: first, that CCR4 expression by ATLL cells increased with disease progression from smoldering, chronic to acute subtypes; second, ATLL clones that highly express CCR4 selectively survive in the face of host immune system, and proliferate during disease progression from smoldering, chronic to acute subtypes. The latter would be consistent with the fact that CCR4-positive ATLL cells from a subset of patients actually function as regulatory T (Treg) cells (24).

Most importantly, based on the promising results of the preclinical work presented here, we have conducted a phase I clinical trial of the humanized defucosylated anti-CCR4 mAb KW-0761 in a single-agent, dose-escalation, multicenter study for patients with relapsed or refractory CCR4-positive PTCL, including ATLL, in Japan (ClinicalTrials.gov Identifier: NCT00355472). In this phase I study, we confirmed that KW-0761 infusion was well tolerated in humans, and determined the recommended phase II dose. In addition, one acute-type ATLL patient treated at 0.01 mg/kg has achieved a hematologic complete remission. This exciting clinical effect seen at a dose of 0.01 mg/kg of KW-0761 is consistent with the concept of employing the defucosylation of therapeutic mAb to highly enhance ADCC (39). We have completed a phase I, and are currently conducting a phase II, clinical trial (ClinicalTrials.gov Identifier: NCT00920790). In addition, in the United States, a phase I/II clinical trial of KW-0761 for PTCL started in 2009, and is currently ongoing (ClinicalTrials.gov Identifier: NCT00888927). The present observation that the degree of autologous KW-0761 ADCC was mainly determined by the amount of effector NK cells provides us with evidence for planning more effective combination strategies with KW-0761 in the future. Immunomodulatory agents such as lenalidomide (40, 41) or interferons-α and γ (13) that augment NK cell function, may be promising candidates for combination therapy together with KW-0761. In addition, quantification of NK cells in the blood may be a useful biomarker to predict the clinical effect of KW-0761 treatment. Furthermore, because CCR4 is expressed on Treg cells (9, 42–46), we believe that KW-0761 could also be used as a novel strategy for treatment of many other types of cancer to overcome their suppressive effect on the host's immune response to tumor cells. In addition, other investigators reported that Treg cells directly inhibited not only other T cells but also NK cell effector function (47–49). With these important observations taken together, it is proposed that KW-0761 itself would enhance NK cell mediation of ADCC by killing CCR4-expressing Treg cells, which would otherwise inhibit NK cell effector function, in humans *in vivo*. In the near future, the efficacy of KW-0761 will be established in pivotal clinical trials.

Disclosure of Potential Conflicts of Interest

Ishii T, Akinaga S, and Shitara K are employed by Kyowa Hakko Kirin Co Ltd. The other authors have no potential conflicts of interest to disclose.

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References

1. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 2002;346:235–42.
2. Pfreundschuh M, Trümper L, Osterborg A, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol 2006;7:379–91.
3. Armitage J, Vose J, Weisenburger D. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. J Clin Oncol 2008;26:4124–30.
4. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood 1977;50:481–92.
5. Ishida T, Utsunomiya A, Iida S, et al. Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma (ATLL): its close association with skin involvement and unfavorable outcome. Clin Cancer Res 2003;9:3625–34.
6. Ishida T, Inagaki H, Utsunomiya A, et al. CXC chemokine receptor 3 and CC chemokine receptor 4 expression in T-cell and NK-cell lymphomas with special reference to clinicopathological significance for peripheral T-cell lymphoma, unspecified. Clin Cancer Res 2004;10:5494–500.
7. Niwa R, Shoji-Hosaka E, Sakurada M, et al. Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T cell leukemia and lymphoma. Cancer Res 2004;64:2127–33.
8. Shinkawa T, Nakamura K, Yamane N, et al. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 2003;278:3466–73.
9. Ishida T, Ueda R. CCR4 as a novel molecular target for immunotherapy of cancer. Cancer Sci 2006;97:1139–46.
10. Ishida T, Iida S, Akatsuka Y, et al. The CC chemokine receptor 4 as a novel-specific molecular target for immunotherapy in adult T-cell leukemia/lymphoma. Clin Cancer Res 2004;10:7529–39.
11. Ito A, Ishida T, Yano H, et al. Defucosylated anti-CCR4 monoclonal antibody exercises potent ADCC-mediated antitumor effect in the novel tumor-bearing humanized NOD/Shi-scid, IL-2Rgamma(null) mouse model. Cancer Immunol Immunother 2009;58:1195–206.
12. Yano H, Ishida T, Imada K, et al. Augmentation of antitumour activity of defucosylated chimeric anti-CCR4 monoclonal antibody in SCID mouse model of adult T-cell leukaemia/lymphoma using G-CSF. Br J Haematol 2008;140:586–9.
13. Yano H, Ishida T, Inagaki A, et al. Defucosylated anti CC chemokine receptor 4 monoclonal antibody combined with immunomodulatory cytokines: a novel immunotherapy for aggressive/refractory Mycosis fungoides and Sezary syndrome. Clin Cancer Res 2007;13:6494–500.
14. Ishida T, Ishii T, Inagaki A, et al. The CCR4 as a novel-specific molecular target for immunotherapy in Hodgkin lymphoma. Leukemia 2006;20:2162–8.
15. Ito A, Ishida T, Utsunomiya A, et al. Defucosylated anti-CCR4 monoclonal antibody exerts potent ADCC against primary ATLL cells mediated by autologous human immune cells in NOD/Shi-scid, IL-2Rγ^{null} mice *in vivo*. J Immunol 2009;183:4782–91.
16. Hwang WY, Foote J. Immunogenicity of engineered antibodies. Methods 2005;36:3–10.
17. Satoh M, Iida S, Shitara K. Non-fucosylated therapeutic antibodies as next-generation therapeutic antibodies. Expert Opin Biol Ther 2006;6:1161–73.
18. Kanda Y, Yamane-Ohnuki N, Sakai N, et al. Comparison of cell lines for stable production of fucose-negative antibodies with enhanced ADCC. Biotechnol Bioeng 2006;94:680–8.
19. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87). Br J Haematol 1991;79:428–37.
20. Imada K, Takaori-Kondo A, Sawada H, et al. Serial transplantation of adult T cell leukemia cells into severe combined immunodeficient mice. Jpn J Cancer Res 1996;87:887–92.
21. Gonzales NR, De Pascalis R, Schlom J, Kashmiri SV. Minimizing the immunogenicity of antibodies for clinical application. Tumour Biol 2005;26:31–43.
22. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 1986;321:522–5.
23. Taylor GP, Matsuoka M. Natural history of adult T-cell leukemia/lymphoma and approaches to therapy. Oncogene 2005;24:6047–57.
24. Yano H, Ishida T, Inagaki A, et al. Regulatory T-cell function of adult T-cell leukemia/lymphoma cells. Int J Cancer 2007;120:2052–7.
25. Inagaki A, Ishida T, Ishii T, et al. Clinical significance of serum Th1-, Th2- and regulatory T cells-associated cytokines in adult T-cell leukemia/lymphoma: high interleukin-5 and -10 levels are significant unfavorable prognostic factors. Int J Cancer 2006;118:3054–61.
26. Iida S, Misaka H, Inoue M, et al. Nonfucosylated therapeutic IgG1 antibody can evade the inhibitory effect of serum immunoglobulin G on antibody-dependent cellular cytotoxicity through its high binding to FcγRIIIa. Clin Cancer Res 2006;12:2879–87.
27. Preithner S, Elm S, Lippold S, et al. High concentrations of therapeutic IgG1 antibodies are needed to compensate for inhibition of antibody-dependent cellular cytotoxicity by excess endogenous immunoglobulin G. Mol Immunol 2006;43:1183–93.
28. Inagaki A, Ishida T, Yano H, et al. Expression of the ULBP ligands for NKG2D by B-NHL cells plays an important role in determining their susceptibility to rituximab-induced ADCC. Int J Cancer 2009;125:212–21.
29. Lewis GD, Figari I, Fendly B, et al. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol Immunother 1993;37:255–63.
30. Sarup JC, Johnson RM, King KL, et al. Characterization of an anti-p185HER2 monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. Growth Regulation 1991;1:72–82.
31. Niwa R, Sakurada M, Kobayashi Y, et al. Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. Clin Cancer Res 2005;11:2327–36.
32. Cartron G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. Blood 2002;99:754–8.
33. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 2003;21:3940–7.
34. Niwa R, Hatanaka S, Shoji-Hosaka E, et al. Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 Is independent of FcγRIIIa functional polymorphism. Clin Cancer Res 2004;10:6248–55.
35. Fischer L, Penack O, Gentilini C, et al. The anti-lymphoma effect of antibody-mediated immunotherapy is based on an increased degranulation of peripheral blood natural killer (NK) cells. Exp Hematol 2006;34:753–9.
36. Bhat R, Watzl C. Serial killing of tumor cells by human natural killer cells-enhancement by therapeutic antibodies. PLoS ONE 2007;2:e326.
37. Tsukasaki K, Utsunomiya A, Fukuda H, et al. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. J Clin Oncol 2007;25:5458–64.
38. Tsukasaki K, Hermine O, Bazarbachi A, et al. Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. J Clin Oncol 2009;27:453–9.
39. Yamamoto K, Utsunomiya A, Tobinai K, et al. Phase I study of KW-0761, a defucosylated humanized anti-CCR4 antibody, in relapsed patients with adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma. J Clin Oncol, In press. DOI: 10.1200/JCO.2009.25.3575.
40. Tai YT, Li XF, Catley LR, et al. Immunomodulatory drug lenalidomide (CC-5013, IMiD3) augments anti-CD40 SGN-40-induced cytotoxicity

in human multiple myeloma: clinical implications. Cancer Res 2005; 65:11712–20.
41. Reddy N, Hernandez-Ilizaliturri FJ, Deeb G, et al. Immunomodulatory drugs stimulate natural killer-cell function, alter cytokine production by dendritic cells, and inhibit angiogenesis enhancing the anti-tumour activity of rituximab *in vivo*. Br J Haematol 2008;140: 36–45.
42. Iellem A, Mariani M, Lang R, et al. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 andCCR8 by CD4(+)CD25(+) regulatory T cells. J Exp Med 2001;194:847–53.
43. Hirahara K, Liu L, Clark RA, Yamanaka K, Fuhlbrigge RC, Kupper TS. The majority of human peripheral blood CD4 + CD25highFoxp3 + regulatory T cells bear functional skin-homing receptors. J Immunol 2006;177:4488–94.
44. Baatar D, Olkhanud P, Sumitomo K, Taub D, Gress R, Biragyn A. Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4 + Tregs and unprimed CCR4+ Tregs, regulate effector T cells using FasL. J Immunol 2007;178:4891–900.
45. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10:942–9.
46. Ishida T, Ishii T, Inagaki A, et al. Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. Cancer Res 2006;66:5716–22.
47. Ralainirina N, Poli A, Michel T, et al. Control of NK cell functions by CD4+CD25+ regulatory T cells. J Leukoc Biol 2007;81:144–53.
48. Smyth MJ, Teng MWL, Swann J, Kyparissoudis K, Godfrey DI, Hayakawa Y. CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. J Immunol 2006;176:1582–7.
49. Ghiringhelli F, Ménard C, Terme M, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-β-dependent manner. J Exp Med 2005;202:1075–85.