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.
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.

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 NH2-termninus residues of the CCR4 protein (H-DESYNYYLYESIPKPC-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 BiEvaluation 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 evaluation program (BIACORE).
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 FACS calibur (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 ($1 \times 10^7$) 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$^3$) = $0.5 \times$ (major diameter) $\times$ (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. Plasma sIL-2R$\alpha$ concentrations were measured by ELISA using Human sIL-2R$\alpha$ 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 respective 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 $2.18 \times 10^5$ mol/L$^{-1}$s$^{-1}$ and $1.18 \times 10^5$ mol/L$^{-1}$s$^{-1}$, respectively. Dissociation rate constants (Kd) could not be calculated for either antibody due to their very slow dissociation ($<10^{-3} \sim 10^{-2}$; 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 mm³, 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
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), 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 51Cr 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; Δ, chronic type; ▽, smoldering type. The correlation coefficients (Rs) and P values in each panel were assessed by Spearman rank correlation coefficient testing.
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: \((34.6 - 16.8)/34.6 \times 100 = 51.5\%\) (Fig. 4A). The results with all ATLL cases tested for ex vivo KW-0761 ADCC are also summarized in Table 1.

### Table 1. ATLL cases tested for KW-0761 ADCC

<table>
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<th>Sample name</th>
<th>Subtype</th>
<th>Autologous cytotoxicity (%)</th>
<th>Allogeneic cytotoxicity (%)</th>
<th>CCR4 MFI ratio</th>
<th>Total CD16 (%)</th>
<th>CD16 NK (%)</th>
<th>CD16 monocyte (%)</th>
<th>Cytotoxicity (%) in lymph</th>
<th>CD16/56 (%) in lymph</th>
<th>CD4/25 (%) in lymph</th>
<th>HTLV-1 load (copies/1,000 PBMC)</th>
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<td>19.8</td>
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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: \((34.6-16.8)/34.6 \times 100 = 51.5\%\) (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 $\mu$g/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: $(34.6 - 16.8)/34.6 \times 100 = 51.5\%$.

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; △, 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 51Cr 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).

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 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

**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; △, smoldering type. The P values are indicated in each panel.
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amounts of target antigen, using primary tumor cells and autologous effector cells.

Lymphoma patients homozygous for FcyRIIa-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 FcyR-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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