**Featured Article**

The CC Chemokine Receptor 4 as a Novel Specific Molecular Target for Immunotherapy in Adult T-Cell Leukemia/Lymphoma

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**ABSTRACT**

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell neoplasm with dismal prognosis, and no optimal therapy has been developed. We tested the defucosylated chimeric anti-CC chemokine receptor 4 (CCR4) monoclonal antibody, KM2760, to develop a novel immunotherapy for this refractory tumor. In the presence of peripheral blood mononuclear cells (PBMCs) from healthy adult donors, KM2760 induced CCR4-specific antibody-dependent cellular cytotoxicity (ADCC) against CCR4-positive ATLL cell lines and primary tumor cells obtained from ATLL patients. We next examined the KM2760-induced ADCC against primary ATLL cells in an autologous setting. Antibody-dependent cellular cytotoxicity mediated by autologous effector cells was generally lower than that mediated by allogeneic control effector cells. However, a robust ADCC activity was induced in some cases, which was comparable with that mediated by allogeneic effector cells. It suggests that the ATLL patients’ PBMCs retain substantial ADCC-effector function, although the optimal conditions for maximal effect have not yet been determined. In addition, we also found a high expression of FoxP3 mRNA and protein, a hallmark of regulatory T cells, in ATLL cells, indicating the possibility that ATLL cells originated from regulatory T cells. KM2760 reduced FoxP3 mRNA expression in normal PBMCs along with CCR4 mRNA by lysis of CCR4+ T cells in vitro. Our data suggest not only that the CCR4 molecule could be a suitable target for the novel antibody-based therapy for patients with ATLL but also that KM2760 may induce effective tumor immunity by reducing the number of regulatory T cells.

**INTRODUCTION**

Adult T-cell leukemia/lymphoma (ATLL) is a peripheral T-cell neoplasm that is characterized by highly pleomorphic lymphoid cells and is caused by human T lymphotrophic virus type 1 (HTLV-1; ref. 1). It has a very poor prognosis (1–3) because patients are usually highly immunocompromised and suffer from frequent severe infections and because tumor cells are usually resistant to conventional chemotherapeutic agents (4). Allogeneic stem cell transplantation may improve survival of ATLL patients only when an appropriate degree of graft versus host disease develops (5). However, only a small fraction of patients may benefit from allogeneic stem cell transplantation because ATLL has a long latency and occurs in elderly individuals with a median age of 55 years. Therefore, alternative treatment strategies for ATLL patients are needed to improve their prognosis.

The use of therapeutic monoclonal antibody for the treatment of cancer has become a promising approach over the last few years, as exemplified by the success of the anti-CD20 chimeric monoclonal antibody rituximab used for the treatment of B-cell non-Hodgkin’s lymphoma (6–9). Other promising monoclonal antibodies are also emerging, such as Campath 1H (anti-CD52) for the treatment of B-cell chronic lymphocytic leukemia (10), anti-CD33 for acute myelocytic leukemia (11), anti-p185HER2/neu for breast cancer (12), and anti-vascular endothelial growth factor for colorectal cancer (13). In regard to ATLL, an anti-CD25 monoclonal antibody therapy has shown substantial effects, but the benefit over conventional chemotherapy has to be determined (14). Thus, development of promising monoclonal antibodies against dismal T-cell neoplasms, including ATLL, is an urgent issue.

We have recently developed a new chimeric monoclonal antibody, KM2760, that binds specifically to CC chemokine
receptor 4 (CCR4) and whose Fc region is artificially defucosylated to enhance ADCC activity by increasing its binding affinity to FcγR on effector cells (15, 16). We have shown that KM2760 exhibits potent ADCC against non-ATLL CCR4-positive T-cell leukemia/lymphoma lines with human peripheral blood mononuclear cells (PBMCs) as effector cells both in vitro and in vivo mouse models (16). We have also shown that tumor cells obtained from a large majority of patients with ATLL express CCR4 and that the extent of CCR4 expression is significantly associated with skin involvement and poor prognosis (17). On the basis of these observations, we describe here the potent KM2760-induced ADCC against both ATLL cell lines and primary tumor cells obtained from patients with ATLL. In addition, we describe a correlation between CCR4 and FoxP3 gene expression, the latter of which is a hallmark of immunoregulatory T cells, suggesting that the KM2760 may provoke effective tumor immunity by reducing the number of immunoregulatory T cells.

MATERIALS AND METHODS

Chimeric Anti-CCR4 Monoclonal Antibody KM2760. We generated a chimeric anti-CCR4 IgG1 monoclonal antibody, KM2760, whose Fc region was defucosylated to enhance FcγR-mediated binding affinity with effector cells of ADCC (16).

Cell Lines and Patient Cells. HUT102, ATL102, and ATN-1 are human T-cell lines established from patients with ATLL (17, 18–20) with clonal integration of HTLV-1. MT-2 is a human T-cell line transformed by infection of HTLV-I (17, 19–21). CCRF-CEM (16), PEER (16), TALL-1 (16), Jurkat (16), MOLT-4F (22), MOLT-3 (23), HPB-ALL (24), and NCU-LBL-1 are non-ATLL T-cell lines. NCU-LBL-1 was a human T lymphoblastic lymphoma cell line established in our laboratory. All cell lines were interleukin 2 independent and were maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum. PBMCs containing CD4+CD25+CCR4+ ATLL cells were isolated from ATLL patients’ peripheral blood with Ficoll-Paque (Pharmacia, Uppsala, Sweden) and used as targets in ADCC assays. PBMCs from all healthy volunteer donors used throughout the study were prepared as above and used as effector cells. In the autologous setting, a CD3-positive subset was isolated from fresh PBMCs obtained from ATLL patients with anti-human CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions, and used as target cells. The remaining CD3-negative subset was used as effector cells. A CD3-negative subset was also isolated from one healthy adult volunteer (PBMC 1) and used as control effector cells. Patients and volunteers gave informed written consent before the sampling procedure and informed consent was provided according to the Declaration of Helsinki.

Flow Cytometry. Expression of CCR4 antigen on cell lines was examined by flow cytometry. One million cells were incubated at 4°C with fluorescein isothiocyanate (FITC)-conjugated mouse anti-CCR4 monoclonal antibody KM2160 at the final concentration of 10 μg/mL for 30 minutes. After washing twice, cells were analyzed by FACScan with the aid of CellQuest software (Becton Dickinson, San Jose, CA). In addition, the following antibodies were used: FITC-conjugated anti-CD55 (clone IA10), FITC-conjugated anti-CD59 (clone p282), FITC-conjugated anti-CD16 (clone 3G8), peridinin chlorophyll protein-conjugated anti-CD4 (clone SK3), and phycoerythrin-conjugated anti-CD25 (clone M-A251) with appropriate control monoclonal antibodies. All monoclonal antibodies, except for KM2160, were purchased from BD PharMingen (San Jose, CA).

 Establishment of a CCR4-Expressing Stable Transfected. A full open reading frame of CCR4 cDNA was cloned into the retroviral vector pLPBC [ref. 25; the backbone plasmid, LZRSpBMN-Z was kindly provided by Dr. Garry Nolan, Stanford University (Stanford, CA)] with HindIII and NotI sites. CCR4-negative HUT102 cells (17) were transduced with retroviruses carrying either CCR4 or green fluorescent protein cDNA (26) and selected in the presence of puromycin (0.7 μg/mL) as described previously (25). The expression of CCR4 on the transduced HUT102 cells was verified by flow cytometry as described above and was 100% positive (data not shown).

ADCC Assay. ADCC was determined by a standard 4-hour chromium 51 release assay. Target cells (1 × 10⁶ cells) were labeled with 1.5 kBq of Na₂¹⁵⁰CrO₄ (¹⁵⁰Cr) for 2 hours and kept for 15 minutes on ice and washed twice. Aliquots of the labeled tumor cells (2.5 × 10⁵ cells/50 μL) were mixed with effector PBMCs (50 μL) and serial dilutions of monoclonal antibodies (100 μL) in 96-well U-bottomed plates and incubated at 37°C, 5% CO₂ for 4 hours. Effector PBMCs obtained from four healthy adult volunteers (numbered from 1 to 4) and ATLL patient samples were used at the fixed E:T ratio of 50:1. Then, supernatants were removed and counted in a gamma counter. The percentage of specific lysis was calculated according to the following formula: percentage of specific lysis = (E − S)/(M − S) × 100, where E is the experimental release, S is the spontaneous release, and M is the maximum release by 1.5% Triton X-100. All expressed values were averages of triplicate experiments.

Complement-Dependent Cytotoxicity (CDC) Assay. The target cells were labeled in the same way as in the ADCC assay. Aliquots of the labeled cells were distributed into 96-well U-bottomed plates (5 × 10³ cells/50 μL) and incubated with monoclonal antibodies (50 μL) serially diluted in RPMI 1640 supplemented with 40% of either heat-inactivated or intact pooled human serum (100 μL) obtained from 10 healthy adult volunteers. Pooled human serum, not heat-inactivated, was used as the source of complement. After 1.5 hours of incubation at 37°C, 5% CO₂, supernatants were removed and counted in a gamma counter, and the percentage of specific lysis was calculated in the same way as used in ADCC assays. In some experiments, blocking antibodies against CD55 (clone 1C6) and CD59 (clone 1F5) were used in the CDC assay to block the function of the CD55/59 (27, 28) at the final concentration of 10 μg/mL.

Cell Proliferation Assay. Cell proliferation was analyzed by the CellTiter 96 AQueous One solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI) according to the manufacturer’s instructions. Aliquots of the ATLL cell lines or fresh ATLL cells were distributed into 96-well flat-bottomed microtiter plates and incubated with serial dilutions of KM2760 (0.1, 1.0, and 10.0 μg/mL) in RPMI 1640 supplemented with 10% heat-inactivated pooled human serum. Combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-

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2-(4-sulfonyl)-2H-tetrazolium/phenazine methosulfate solution (provided with the kit) was added at 0, 24, 48, 72, and 96 hours to each well, and 2 hours (ATLL cell lines) or 4 hours (fresh ATLL cells) after incubation at 37°C, 5% CO₂, the absorbance at 490 and 650 nm were recorded with an ELISA plate reader. Subtraction of the 650-nm reference absorbance from the 490-nm absorbance, which is directly proportional to the number of living cells, provides elimination of background. Subtraction of the average absorbance of the three “no cell” control wells from all other experimental absorbance values is considered to yield corrected absorbance. All expressed values were averages of triplicate experiments. The influence of cytokines upon cell proliferation in the presence of KM2760 was also evaluated by adding recombinant human IFN-α-2b or recombinant human IFN-γ at the final concentration of 100 units/mL.

**FCGR3A Genotyping.** Genotyping of the FCGR3A polymorphism was done by a reverse transcription-PCR followed by direct sequencing. Total RNA was prepared from the effector PBMCs obtained from four healthy adult volunteers and was reverse transcribed as a first-strand cDNA solution, and each aliquot was used to amplify FcyRIIa mRNA. Nested PCR was used to produce a 928-hp fragment of the FcyRIIa cDNA spanning the polymorphic site; primer pairs used were as follows: sense, 5’-CAGACTGAGAATCAGATGA-3’, and antisense, 5’-GGTCTATGTTCCTGCTGCT-3’; and nested sense, 5’-GGTCTATGTTCCTGCTGCT-3’, and nested antisense, 5’-TGGAGATGAGTTGGTTGCAAA-3’. This former primer set was designed not to amplify the FcyRIIb cDNA. The nested PCR products were directly sequenced using the following internal primers: sense, 5’-AACACTGCTCTCGATAAGGT-3’, and antisense, 5’-ATATAAGCCTGTTGCTGGA-3’, with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

**Real-Time PCR for CCR4 and FoxP3.** Total RNA was prepared from fresh PBMCs of 8 patients with acute type ATLL, 1 HTLV-1 carrier, 11 healthy adult volunteers, and 6 ATLL cell lines (ATN-1, MT-2, ATL102, HUT102, CCR4-transduced HUT102, and green fluorescent protein-transduced HUT102) and 8 non-ATLL T-cell lines. Both CD4+CD25+ and CD4+CD25− subsets were isolated with a human CD4+CD25+ regulatory T-cell isolation kit (Miltenyi Biotec) from fresh PBMCs of one healthy adult volunteer (PBMC 1), according to the manufacturer’s instructions. CD4+CCR4+ and CD4+CCR4− subsets were isolated in a similar way with e2b-in-conjugated anti-CCR4 monoclonal antibody (KM2160) and anti-biotin microbeads (Miltenyi Biotec). cDNA aliquots prepared from the purified CD4+CD25+, CD4+CD25−, CD4+CCR4+, and CD4+CCR4− subpopulations were subjected to conventional RT-PCR for CD4, CD25, CCR4, and β-actin.

Fresh PBMCs from healthy adult volunteers were incubated with or without 10 μg/mL KM2760 in RPMI 1640 supplemented with 10% of heat-inactivated pooled human serum. After 6 hours of incubation at 37°C, 5% CO₂, total RNA was purified and incubated with DNase I and then reverse transcribed to first-strand cDNA. These aliquots were used to quantitate CCR4, FoxP3, or β-actin mRNA. CCR4 was PCR amplified with a primer set purchased from Roche Molecular Biochemicals (Mannheim, Germany), according to the manufacturer’s instructions. FoxP3 was amplified with the following exon-spanning primers: sense, 5’-GAGGACTTCCTCAAG-CACT-3’, and antisense, 5’-TGCACTGCACTGCTCT-3’. PCR was carried out with FastStart DNA master SYBR Green I (Roche Molecular Biochemicals) with the aid of a LightCycler Quick System 330 (Roche Molecular Biochemicals). β-Actin was used as an internal control (primer set was purchased from Roche Molecular Biochemicals). The standard curve for each gene was generated by amplifying serially diluted plasmids incorporating cDNA of the individual gene. The quantitative assessment of the mRNA of interest was done by dividing its expression level by that of β-actin and expressed as a copy-number ratio. All assays were conducted in triplicate, and the mean value was used as the mRNA level. Consequently, the CCR4 copy number ratio 1 was defined as 4.48 × 10² copies of CCR4 mRNA per β-actin mRNA. The FoxP3 copy number ratio 1 was defined as 5.65 × 10⁻⁵ copies of FoxP3 mRNA per β-actin mRNA.

**Western Blot Analysis.** As for FoxP3 protein expression, cell lysates extracted from PBMCs derived from the ATLL patients and healthy adult volunteers in addition to various cell lines, including ATLL and non-ATLL T cell lines, were used for Western blot analysis. Goat antihuman FoxP3 (Abcam, Cambridge, United Kingdom) and goat antiguinea Igs (Santa Cruz Biotechnology, Santa Cruz, CA) was used as an internal control.

**Statistical Analysis.** The differences in the CCR4/FoxP3 copy number ratio between the two groups was examined with the Mann-Whitney U test. Data were analyzed with the aid of StatView software, version 5.0 (SAS Institute, Cary, NC). In this study, P < 0.05 was considered as significant.

**RESULTS**

**KM2760-Induced ADCC against ATLL Cell Lines.** The dependence on CCR4 expression of KM2760-induced ADCC was verified with the CCR4-negative HUT102 cell line transduced with CCR4 cDNA or control green fluorescent protein cDNA by using a standard 4-hour ⁵¹Cr-release assay. Only the CCR4-transduced HUT102 cell line was lysed effectively in the presence of PBMCs from four healthy adult donors (20 to 50% lysis in the presence of 1.0 μg/mL KM2760; data not shown). We next examined the KM2760-induced ADCC against four cell lines, including HUT102 as a negative control. Expression of CCR4 on these ATLL cell lines is shown in Fig. 1A. KM2760 induced a robust ADCC activity against CCR4-positive ATLL cell lines, but not HUT102, in a dose-dependent manner (Fig. 1B). Even in the absence of KM2760, 5 to 35% lysis by PBMCs, presumably due to natural killer (NK) cell activity, was observed; however, significant enhancement of lytic activity by KM2760 was obtained at a concentration as low as 0.1 μg/mL.

Susceptibility to lysis varied among cell lines and was influenced by the individual PBMCs used. Interestingly, the cellular composition of PBMCs from these healthy individuals thus might be associated with the ADCC activity. The percentages of CD16+ cells in the PBMC 1, 2, 3 and 4 were 15.3, 10.7, 4.2, and 12.5%, respectively, and PBMC 3 showed a tendency
to reproducibly induce lower ADCC activity throughout the study. Genotyping results for the polymorphic status of amino acid position 158 in the \textit{FCGR3A} encoding FcRIIIa revealed that PBMC 1, 2 and 3 were \textit{FCG3A-158 F/F} homozygous, and PBMC 4 was \textit{FCG3A-158V/F} heterozygous.

**KM2760-Induced ADCC against Freshly Isolated ATLL.** Unlike established cell lines, tumor cells present in patients with ATLL may be heterogeneous and behave differently in KM2760-induced ADCC. To examine whether ATLL cells freshly isolated from patients also are susceptible to KM2760-induced lysis, we tested PBMC samples from three patients with refractory acute ATLL. KM2760 induced ADCC against all fresh ATLL cells in a dose-dependent manner in the presence of PBMCs from four healthy adult donors (Fig. 2). The lysis ranged from 25 to 75% of CD4$^+$ CD25$^+$ CCR4$^+$ fresh ATLL cells was observed in the presence of 1.0 $\mu$g/mL KM2760, although the lytic activity varied among BMCD donors as observed in the case of ATLL cell lines.

**Fig. 2** KM2760-induced ADCC against ATLL cells obtained from patients. A, freshly isolated PBMCs from three patients with refractory acute-type ATLL were analyzed by flow cytometry with peridinin chlorophyll protein-conjugated anti-CD4, phycoerythrin-conjugated anti-CD25 and FITC-conjugated KM2160 monoclonal antibodies. ADCC against the patient PBMCs containing ATLL cells was measured by a standard 4-hour $^{51}$Cr release assay in the presence of KM2760 and effector PBMC obtained from four normal volunteers at the E:T ratio of 50:1. B, All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD.

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**Fig. 1** CCR4 expression in ATLL cell lines and KM2760-induced ADCC. A, Four ATLL cell lines were stained with FITC-conjugated anti-CCR4 monoclonal antibody (KM2160) at the concentration of 10 $\mu$g/mL (blank histograms) or isotype control monoclonal antibody (filled histograms). B, ADCC against ATLL cell lines was measured by standard 4-hour $^{51}$Cr release assay in the presence of effector PBMCs obtained from four normal volunteers and KM2760 at the concentrations indicated on the X axis. The percentages of CD16$^+$ cells in PBMC 1, 2, 3, and 4 were 15.3, 10.7, 4.2, and 12.5%, respectively. The E:T ratio was fixed at 50:1. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD. Each result represents three independent experiments.
The ultimate goal of immunotherapy is to obtain sufficient tumoricidal activity by simply administrating monoclonal antibodies in vivo; however, the therapeutic effect may be hampered by the immunocompromised situation that is common in patients with ATLL (please see the latter section). We thus next examined KM2760-induced ADCC in several patients with ATLL in an autologous setting. In contrast to the lysis induced by allogeneic PBMCs as effector cells, which is presumably due to NK cell activity, no lysis of fresh ATLL cells was induced by autologous PBMCs in the absence of KM2760. As shown in Fig. 3B, the extent of lysis of the fresh CD4^+CD25^+CCR4^+ ATLL mediated by autologous effector cells varied among the cases. In two acute-type ATLL patients (case 1 and case 3), a robust ADCC mediated by autologous cells was observed, which was comparable with that mediated by allogeneic cells. The latter patient, whose percentage of CD16^+ cells in the CD3-negative subset of PBMCs (12.8%, Fig. 3C) was about half of the CD16^+ cell percentage in the CD3-negative subset of allogeneic PBMC 1 (26.0%; Fig. 3D), was hematologically in partial remission after systemic chemotherapy. In the other two patients with acute- and chronic-type ATLL, a less efficient ADCC mediated by autologous cells was observed compared with that mediated by allogeneic ones. The percent CD16^+ cells in the CD3-negative subset of PBMCs in these two cases was ~23%, which was comparable with that in allogeneic PBMC 1 (26.0%; Fig. 3D). These data suggest that the ADCC-effector function of the PBMCs obtained from these two patients was suppressed to a greater extent.

**Autologous KM2760-Induced ADCC against Freshly Isolated Peripheral T-Cell Lymphoma Cells.** We have recently shown that nearly 40% of cases with peripheral T-cell lymphoma (PTCL) unspecified are positive for CCR4 (29). To test whether CCR4-positive PTCL, unspecified cells are susceptible to KM2760, we sought and found one patient diagnosed...
percent cell lysis is presented as the average was fixed at 50:1. All experiments were done in triplicate, and the involvement or PBMCs from a normal donor as a control. The E:T ratio

in the presence of the autologous PBMCs that were free from tumor cell

amounted to 44.4 ± 2.3% of CD4+ CD25+ (CCR4+) immunoregulatory T cells. Three-color fluorescence-activated cell sorting analysis for PBMCs obtained from four healthy volunteers revealed that 44.4 ± 2.3% of CD4+ CD25+ cells were positive for CCR4, and 70.0 ± 7.6% of CD4+ CCR4+ cells were positive for CD25 (data not shown). These data indicate that the majority of CD4+ CCR4+ T cells do express CD25 in the normal condition. As expected, FoxP3 was expressed in the CD4+ CCR4+ T cells at a level nearly nine times higher than that in CD4+ CCR4- T cells obtained from healthy volunteers (FoxP3 copy number ratios were 92.2 and 10.1, respectively; Fig. 5B, left panel). In addition, there was a significant difference in the FoxP3 copy number ratio of PBMCs obtained from eight ATLL patients and 11 healthy volunteers (82.0 ± 81.4 versus 9.0 ± 4.8; P = 0.0003; Fig. 5B, right panel), implying the presence of a profound immunosuppressive state in the patients with ATLL. In addition, expression of FoxP3 protein assessed by Western blot analysis was almost proportional to that of FoxP3 mRNA level in each case (Fig. 5C). We also analyzed the FoxP3 copy number ratio of six ATLL cell lines and eight non-ATLL T-cell lines. In contrast, all of them, irrespective of HTLV-1 involvement and CD4/CD25/CCR4 positivity, showed extremely low levels of the FoxP3 copy number ratio, which ranged from 0 to 5.0 (data not shown). Corresponding to this result, Western blot analysis of FoxP3 protein in these cell lines detected no band or extremely faint bands compared with those of normal PBMCs. In any case, expressed level of FoxP3 protein correlated well with that of FoxP3 mRNA level in each cell line (data not shown).

Finally, we examined whether KM2760 treatment would affect the mRNA expression of CCR4 and FoxP3 in fresh PBMCs from four healthy adult volunteers. A 6-hour KM2760 treatment reduced the FoxP3 mRNA expression level in parallel with CCR4 mRNA (Fig. 6). These results indicate that KM2760 induced ADCC against CCR4-positive T cells and that the majority of the lysed CCR4-positive T cells simultaneously expressed FoxP3.

KM2760-Induced CDC against ATLL Cell Lines and Fresh ATLL Cells. We next examined whether KM2760 possesses CDC activity; however, no activity was observed in either ATLL cell lines (Fig. 7A), or in fresh ATLL cells obtained from several acute type patients (data not shown). To investigate the underlying mechanism accounting for the lack of CDC activity, we analyzed the expression levels of complement inhibitors (CD55 and CD59) present on the surface of these ATLL cell lines (35). High levels of CD55 and CD59 expression were observed in ATLL cell lines (Fig. 7B). To confirm that CD55 and/or CD59 expression on the cell surface of the ATLL cell lines can inhibit CDC activity, we carried out blocking experiments in various combinations of complement, KM2760, and anti-CD55 and/or CD59 antibodies. As shown in Fig. 7C, the antibody treatments had no significant effect on ATN-1 and MT-2. On the other hand, the anti-CD55 but not anti-CD59

with CCR4-positive PTCL unspecified, who was seronegative for HTLV-1. Mononuclear cells containing tumor cells were obtained from one of the affected lymph nodes (LN) of a patient with PTCL unspecified were analyzed as above. The LN contained 19.4% of CD4+ CCR4+ PTCL cells. KM2760-induced ADCC against the isolated mononuclear cells containing PTCL cells was measured by a 4-hour 51Cr release assay in the presence of the autologous PBMCs that were free from tumor cell involvement or PBMCs from a normal donor as a control. The E:T ratio was fixed at 50:1. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD.

Correlation between CCR4 and FoxP3 Expression in ATLL. Using quantitative reverse transcription-PCR, we analyzed CCR4 mRNA level in PBMCs obtained from six healthy volunteers and eight acute-type ATLL patients. As shown in Fig. 5A, the CCR4 copy number ratio of PBMCs obtained from six healthy volunteers, 5.0 ± 2.5 (average ± SD), were significantly lower than that of PBMCs obtained from eight ATLL patients, 101.8 ± 60.8 (P = 0.0045). It is generally known that the surface phenotype of ATLL cells is represented by CD4 and CD25, and we have recently reported that most ATLL cells also express CCR4 (17). Immunoregulatory T cells, which function by actively suppressing self-reactive T cells, exist in the CD4+CD25+ T-cell population. Immunoregulatory T cells engaged in the maintenance of immunologic self-tolerance (30–33) have been shown to be controlled by the transcription factor FoxP3 (34). One of the clinical characteristics of ATLL is a highly immunocompromised state correlated with its poor prognosis; thus, we hypothesized that ATLL cells might originate from CD4+CD25+ (CCR4+) immunoregulatory T cells. Three-color fluorescence-activated cell sorting analysis for PBMCs obtained from four healthy volunteers revealed that 44.4 ± 2.3% of CD4+CD25+ cells were positive for CCR4, and 70.0 ± 7.6% of CD4+CCR4+ cells were positive for CD25 (data not shown). These data indicate that the majority of CD4+CCR4+ T cells do express CD25 in the normal condition. As expected, FoxP3 was expressed in the CD4+CCR4+ T cells at a level nearly nine times higher than that in CD4+CCR4- T cells obtained from healthy volunteers (FoxP3 copy number ratios were 92.2 and 10.1, respectively; Fig. 5B, left panel). In addition, there was a significant difference in the FoxP3 copy number ratio of PBMCs obtained from eight ATLL patients and 11 healthy volunteers (82.0 ± 81.4 versus 9.0 ± 4.8; P = 0.0003; Fig. 5B, right panel), implying the presence of a profound immunosuppressive state in the patients with ATLL. In addition, expression of FoxP3 protein assessed by Western blot analysis was almost proportional to that of FoxP3 mRNA level in each case (Fig. 5C). We also analyzed the FoxP3 copy number ratio of six ATLL cell lines and eight non-ATLL T-cell lines. In contrast, all of them, irrespective of HTLV-1 involvement and CD4/CD25/CCR4 positivity, showed extremely low levels of the FoxP3 copy number ratio, which ranged from 0 to 5.0 (data not shown). Corresponding to this result, Western blot analysis of FoxP3 protein in these cell lines detected no band or extremely faint bands compared with those of normal PBMCs. In any case, expressed level of FoxP3 protein correlated well with that of FoxP3 mRNA level in each cell line (data not shown).

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antibody increased the CDC lysis of ATL102 irrespective of the presence of KM2760, indicating that ATL102 can be lysed with complement alone if CD55 is blocked functionally. In addition, KM2760 did not induce CDC activity against any of eight non-ATLL T-cell lines irrespective of the presence of CD55 and/or CD59 (data not shown).

Effect of KM2760 on Proliferation of ATLL Cell Lines and Fresh ATLL Cells. We investigated whether KM2760 could inhibit proliferation of both ATLL cell lines and fresh ATLL cells obtained from several acute type patients. No inhibitory effect induced by KM2760 on proliferation of ATLL cell lines or fresh ATLL cells was observed (data not shown). Addition of recombinant human IFN-α or recombinant human IFN-γ did not affect the proliferation in any of them, whereas IFN-α alone induced growth inhibition of MT-2 and all fresh ATLL cells (data not shown).

DISCUSSION

In the present study, we extended our previous observations of potent KM2760-induced ADCC activity against CCR4-positive non-ATLL T-cell lines (16) to ask whether tumor cells from patients with ATLL and PTCL also could be good targets in KM2760-based immunotherapy. Our data clearly showed the potent KM2760-induced ADCC against both ATLL cell lines and fresh ATLL cells obtained from patients. This KM2760-induced ADCC was completely dependent on the cell surface expression of CCR4 on the target cells. However, the observed ADCC activity differed in individual PBMCs used for the assay and in the cell lines tested. There are several potential explanations to account for these observations: CCR4 expression level, percentage of CD16+ cells, and genotype of the FCGR3A polymorphism. Firstly, the cell surface expression level of CCR4 seemed to be an important factor for better ADCC activity as MT-2 cell line, which was stained the best with CCR4 antibody, showed the highest ADCC activity. Secondly, the percentage of CD16+ cells among PBMCs is most likely critical because it was almost reproducibly correlated with ADCC activity, as reported in myeloma study with a plasma cell-specific antibody (36). However, it is of note that one of the normal donors (PBMC 3) who had only 4.2% of CD16+ cells in PBMCs still showed sufficient ADCC activity against CCR4-positive ATLL cells, supporting our previous report that defucosylated chimeric anti-CCR4 monoclonal antibody needed much fewer effector cells to achieve the same cytotoxicity as in KM2760-based immunotherapy. Our data clearly showed the potent KM2760-induced ADCC against both ATLL cell lines and fresh ATLL cells obtained from patients. This KM2760-induced ADCC was completely dependent on the cell surface expression of CCR4 on the target cells. However, the observed ADCC activity differed in individual PBMCs used for the assay and in the cell lines tested. There are several potential explanations to account for these observations: CCR4 expression level, percentage of CD16+ cells, and genotype of the FCGR3A polymorphism. Firstly, the cell surface expression level of CCR4 seemed to be an important factor for better ADCC activity as MT-2 cell line, which was stained the best with CCR4 antibody, showed the highest ADCC activity. Secondly, the percentage of CD16+ cells among PBMCs is most likely critical because it was almost reproducibly correlated with ADCC activity, as reported in myeloma study with a plasma cell-specific antibody (36). However, it is of note that one of the normal donors (PBMC 3) who had only 4.2% of CD16+ cells in PBMCs still showed sufficient ADCC activity against CCR4-positive ATLL cells, supporting our previous report that defucosylated chimeric anti-CCR4 monoclonal antibody needed much fewer effector cells to achieve the same cytotoxicity as
that shown by nondefucosylated antibody (16). This feature also should be therapeutically beneficial because the number of effector cells capable of penetrating into tumor masses could be much less than that of tumor cells in the clinical settings. Finally, it has been shown that human IgG1 binds more strongly to FcγRIIIa on NK cells homozygous for FCGR3A-158V allele than to those homozygous or heterozygous for 158F alleles (37, 38). FcγRIIIa is expressed on both NK cells and monocytes, which are the most important natural cytotoxic effectors. Homozygosity for the 158V allele is associated with better clinical and molecular responses to the chimeric anti-CD20 IgG1 monoclonal antibody rituximab in follicular lymphoma (39) but not in chronic lymphocytic leukemia (40). The FCGR3A genotype of the effector PBMCs used throughout the current study was examined. Unfortunately, PBMCs from donors homozygous for the 158V allele were not found among our donors; nevertheless, a robust ADCC activity was observed irrespective of the absence of donors homozygous for the 158V allele. We have recently shown that defucosylated chimeric IgG1 monoclonal

![Fig. 6](image-url) Reduction of CCR4 and FoxP3 mRNA levels in PBMCs treated with KM2760. Fresh PBMCs from four normal volunteers were incubated in the presence (+) or absence (−) of 10 μg/mL KM2760 in RPMI 1640 supplemented with 10% of heat-inactivated pooled human serum. After 6 hours of incubation at 37°C, 5% CO2, cDNA was prepared from harvested cells and assessed for the expression of CCR4 and FoxP3 by quantitative reverse transcription-PCR, and the copy number ratio for each was calculated. ○, PBMC 1; ●, PBMC 2; △, PBMC 3; and ▲, PBMC 4.

![Fig. 7](image-url) CDC activity of KM2760 against ATLL cell lines and effect of CD55 and CD59 blocking. A. CDC activity against ATLL cells was measured by 1.5 hours of 51Cr release assay. Cells were incubated with the indicated concentrations of KM2760 in RPMI 1640 supplemented with intact pooled human serum (PHS) at the final concentration of 20%. All experiments were done in triplicate, and the percent lysis is presented as the average ± SD. B. ATLL cell lines were stained with FITC-conjugated anti-CD55 or anti-CD59 monoclonal antibodies (blank histograms). Staining with isotype control monoclonal antibody is shown as filled histograms. The percentage of positive cells is indicated in each panel. C. Blocking antibodies against CD55 (1C6) and CD59 (1F5) were added to the CDC assay to block the function of the CD55 and CD59, individually or in combination. The concentration of KM2760, anti-CD55, and anti-CD59 monoclonal antibodies were fixed at 10 μg/mL. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD. A and C each represents three independent experiments.
antibodies, anti-CD20 and anti-CCR4, can induce much stronger ADCC than nondefucosylated ones (15, 16). These findings can probably explain why the PBMCs, even from donors homozygous or heterozygous for the 158F allele, induced remarkable antibody-dependent cellular cytotoxicity activity in the presence of defucosylated chimeric anti-CCR4 monoclonal antibody KM2760. This feature should be therapeutically beneficial because a comparable ADCC activity of KM2760 can be expected irrespective of FcGR3a genotype.

From the clinical point of view, KM2760 induced a potent ADCC activity at concentrations that are considered to be clinically attainable (1 to 10 μg/mL) against freshly isolated tumor cells in the presence of allogeneic PBMCs as effector cells. Antibody-dependent cellular cytotoxicity against freshly isolated ATLL cells mediated by autologous effector cells was generally lower than that mediated by allogeneic control ones. However, a robust activity, which was comparable with that mediated by allogeneic effector cells, was induced in two of the four cases. It has been shown that HTLV-1 can infect many human cell types other than CD4-positive T-lymphocytes such as NK cells but not hematopoietic progenitor cells (41–47). Previous reports have revealed that the NK cell activity was significantly decreased in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis, although the underlying mechanism remains unknown (48, 49). If HTLV-1 integration into effector cells is the direct reason for their impaired effector function, newly generated cells from the hematopoietic progenitor cells would be free from the virus and expected to show normal function until they become infected. Because close cell-to-cell interaction is required to transmit viruses from HTLV-1-bearing cells to noninfected cells (50), and this process should be time-consuming, there must be an open-window period in which to repopulate normal effector cells. Alternatively, effector cells differentiated or remaining in an immunocompromised environment caused by the copresence of ATLL cells might have impaired cytolytic function (51). In any case, our observation that one patient in partial remission after chemotherapy showed much better ADCC activity despite the lower percentage of autologous CD16+ effector cells in the CD3-negative subset of PBMCs, indicates that chemotherapy before KM2760 administration could be an optimal choice for patients with large tumor burdens in peripheral blood. In addition, treatment with KM2760 for patients with lymphoma type ATLL or PTCL, the latter of which was shown in this study, could be clinically effective because these patients are free from peripheral blood involvement of the tumor cells.

In this report, we showed not only that FoxP3 mRNA was expressed in the CD4+CCR4+ T-cell subset at a much higher level than that in the CD4+CCR4− T-cell subset obtained from normal volunteers but also that FoxP3 was highly expressed in freshly isolated ATLL cells. It is known that the surface phenotype of ATLL cells is represented by positivity for CD4, CD25, and CCR4. Our novel findings suggest that ATLL cells might originate from CD4+CD25+ (CCR4+) immunoregulatory T cells. Thus, it can be envisaged that FoxP3-expressing ATLL cells would give rise to a profound immunosuppressive environment around themselves so that they can escape from the host’s immunosurveillance. Interestingly, all established ATLL cell lines expressed extremely low or no detectable FoxP3 when assessed by quantitative PCR and Western blot analysis. We surmise that they do not need to express FoxP3 because there exists no effector T cells that attack the ATLL cell lines in culture. In addition, the suppression of the host’s normal effector T cells by these ATLL cells can result in a severe immunocompromised state, which is one of the clinical characteristics of patients with ATLL. A similar situation has been shown in Hodgkin’s lymphoma (52). Moreover, it has been shown that tumor cells in Hodgkin’s lymphoma express thymus and activation-regulated chemokine, which is one of the specific ligands for CCR4, and that reactive lymphocytes surrounding the tumor cells do express CCR4 (53). These studies support our hypothesis of the close relationship between FoxP3 and CCR4 expressions in ATLL cells. Because KM2760 reduced the FoxP3 mRNA expression level, presumably by specifically killing FoxP3-coexpressing CCR4+ T cells in PBMCs obtained from normal volunteers, KM2760 may be beneficial in reducing the immunosuppressive effect of not only FoxP3-expressing ATLL cells but also FoxP3-expressing normal immunoregulatory T cells and subsequently provoke effective tumor immunity or restore the host’s profound immunosuppressive state. Collectively, our findings strongly suggest that KM2760 also can be used as a potential immunomodifier.

KM2760 showed no CDC activity against CCR4-positive target cells. ATLL cell lines showed high expression of complement inhibitors such as CD55 and CD59, whereas addition of blocking antibodies against CD55 or CD59 did not induce CDC activity by KM2760. Besides CDC, we did not detect any direct inhibitory effect on proliferation of the ATLL cell lines or fresh ATLL cells, even when combined with IFN-α or IFN-γ, which have been used for the treatment of several hematologic malignancies, including ATLL. Collectively, we conclude that the major antitumor activity of KM2760 is mediated by ADCC.

In conclusion, the present study shows a promising ADCC activity of the defucosylated chimeric anti-CCR4 monoclonal antibody, KM2760, against tumor cells of ATLL and PTCL, although the optimal conditions for obtaining maximal effector function of autologous effector cells from patients still need to be explored. Moreover, the ability of KM2760 to act as an immunomodifier and break tolerance to tumor cells is also encouraging. As rituximab, a chimeric anti-CD20 monoclonal antibody, has changed the standard therapy in elderly patients with diffuse large B-cell lymphoma (54), now KM2760 could be an ideal treatment modality against patients with ATLL and CCR4-positive PTCL.

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CCR4 as a Novel Molecular Target for Immunotherapy


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The CC Chemokine Receptor 4 as a Novel Specific Molecular Target for Immunotherapy in Adult T-Cell Leukemia/Lymphoma

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