Because CC chemokine receptor 4 (CCR4) is expressed on tumor cells from most patients with ATL,[8,9] we postulated that it might represent a novel molecular target for immunotherapy. Accordingly, KW-0761, a next-generation humanized anti-CCR4 immunoglobulin G1 (IgG1) monoclonal antibody (mAb) with a defucosylated Fc region, which markedly enhances antibody-dependent cellular cytotoxicity (ADCC), was developed.[10,11] We demonstrated that robust ADCC by the defucosylated anti-CCR4 mAb against primary tumor cells from patients with ATL mediated by autologous effector cells was triggered both in vitro and in a humanized mouse model in vivo.[11-13] These promising preclinical results prompted us to conduct a phase I clinical trial of KW-0761 for patients with relapsed CCR4-positive peripheral T-cell lymphoma (PTCL), including ATL. This study demonstrated good tolerability, predictable pharmacokinetics, and preliminary evidence of potent antitumor activity and resulted in a recommended dose of 1.0 mg/kg for subsequent clinical trials.[14] Herein, we report the results of a multicenter phase II study designed to assess the efficacy, pharmacokinetic profile, and safety of KW-0761 monotherapy in patients with relapsed CCR4-positive aggressive ATL.

PATIENTS AND METHODS

Patients

Patients 20 years of age or older with CCR4-positive aggressive ATL (acute, lymphoma, or unfavorable chronic type)[1,4] who had relapsed after at least one prior chemotherapy regimen were eligible. The unfavorable chronic type of ATL was defined by the presence of at least one of the following three factors: low serum albumin, high lactate dehydrogenase, or high blood urea nitrogen concentration.[5] CCR4 expression was determined by immunohistochemistry or flow cytometry using a mouse anti-CCR4 mAb (KM2160)[8,14] and confirmed by a central review committee. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2. Eligibility criteria also included the following laboratory values: absolute neutrophil count $\geq$ 1500/μL, platelet count $\geq$ 50,000/μL, hemoglobin $\geq$ 8.0 g/dL, AST $\leq$ 2.5 $\times$ the upper limit of the normal range (UNL), ALT [lteuq] 2.5 $\times$ UNL, total bilirubin $\leq$ 1.5 $\times$ UNL, serum creatinine $\leq$ 1.5 $\times$ UNL, corrected serum calcium $\leq$ 11.0 mg/dL, and arterial partial oxygen pressure $\geq$ 65 mmHg or arterial blood oxygen saturation $\geq$ 93%. Patients were excluded if they had an active infection, a history of organ transplantation, active concurrent cancers, CNS involvement, a bulky mass requiring emergent radiotherapy, or seropositivity for hepatitis B virus antigen, hepatitis C virus antibody, or HIV antibody.

Study Design

This study was a multicenter, single-arm, phase II trial. Objectives of the study were to evaluate the efficacy, pharmacokinetic profile, and safety of KW-0761 monotherapy. Patients received intravenous infusions of KW-0761 once per week for 8 weeks at a dose of 1.0 mg/kg.[14] Oral antihistamine and acetaminophen were administered before each KW-0761 infusion to prevent infusion reactions. The primary end point was overall response rate (ORR), and secondary end points included the best response by disease site, PFS, and OS. Objective responses were assessed after the fourth and eighth infusions of KW-0761 by an independent efficacy assessment committee according to the modified response criteria for ATL.[4] It was estimated that 25 patients would be required to detect a lower limit of the 95% CI exceeding the 5% threshold of ORR based on the assumptions that the minimum required ORR for a new drug for relapsed, aggressive ATL is 5%,[15] with an expected ORR for KW-0761 of 30%[14] with 90% power. Adverse events (AEs) were graded according to the National Cancer Institute Common Terminology Criteria for AEs, version 3.0. The presence of human anti-KW-0761 antibodies in the patients' plasma was examined using enzyme-linked immunosorbent assay. Blood samples collected at times strictly in accordance with the protocol were employed for the pharmacokinetic analysis. Samples were obtained from patients who had received at least one dose of KW-0761 up to all eight doses. When any event resulted in an alteration in the infusion protocol, only those samples taken before the alteration were used for the analysis. The following parameters were calculated for plasma KW-0761: maximum drug concentration and trough drug concentration of each KW-0761 administration, area under the blood concentration time curve from 0 to 7 days after the first and eighth doses, and half-life period ($t_{1/2}$) after the eighth dose. As an additional research parameter, we investigated blood T-cell subset distribution during and after KW-0761 treatment and compared these values with those of 10 healthy donors as controls (five men, five women; median age, 45 years; range, 41 to 57 years).

Statistical Analysis

Survival estimates were calculated using the Kaplan-Meier method. PFS was defined as the time from the first dose of KW-0761 to progression, relapse, or death resulting from any cause, whichever occurred first. OS was measured from the day of the first dose to death resulting from any cause. Regarding T-cell subset analysis, differences between the patients' values before KW-0761 treatment and those of the controls were examined using the Mann-Whitney U-test. Differences between KW-0761 pretreatment values and those at each time point after KW-0761 treatment were examined using the Wilcoxon signed-rank test. All analyses were performed with SPSS Statistics 17.0 (SPSS, Chicago, IL). In this study, $P < .05$ was considered significant.

Study Oversight

The study was sponsored by Kyowa Hakko Kirin Company (Tokyo, Japan). The academic investigators and the sponsor were jointly responsible for the study design. The protocol was approved by the institutional review board at each participating site, and all patients and controls provided written informed consent before enrollment according to the Declaration of Helsinki.

RESULTS

Patients

Of the 28 patients enrolled onto the study, 27 (12 men, 15 women) received at least one infusion of KW-0761. One patient was withdrawn for aggravation of the general condition before the administration of KW-0761. Demographics and clinical characteristics of the 27 patients are summarized in Table 1. Median age was 64 years (range, 49 to 83). The disease subtypes included 14 acute, six lymphoma, and seven unfavorable chronic type ATL. Of these 27 patients, 14 (52%) completed the schedule of eight planned infusions. Of the remaining 13 patients, 11 (41%) discontinued treatment because of disease progression, one (4%) because of skin rash, and another (4%) because of concurrent colon cancer, for which this patient was excluded from the efficacy evaluation.

Efficacy of KW-0761

Of 26 patients evaluable for efficacy, objective responses were noted in 13 patients (ORR, 50%; 95% CI, 30% to 70%), including eight complete responses (CRs). Responses according to disease site were 100% (13 of 13; all CRs) for blood, 63% (five of eight) for skin, and 25% (three of 12) for nodal and extranodal lesions. Responses according to disease subtype were 43% (six of 14) for acute, 33% (two of six) for lymphoma, and 83% (five of six) for unfavorable chronic type ATL. Responses according to number of prior chemotherapy regimens were 48% (10 of 21) in those who had one prior regimen and 60% (three of five) for those who had two or three prior regimens. Median PFS and OS were 5.2 and 13.7 months, respectively (Figs 1A, 1B).

Table 1. Patient Demographics and Clinical Characteristics (n = 27)*

Characteristic	No.	%
Age, years		
Median	64	
Range	49-83	
≥ 65	13	48
Sex		
Male	12	44
Female	15	56
ECOG performance status†		
0	15	56
1	7	26
2	5	19
Disease subtype		
Acute	14	52
Lymphoma	6	22
Chronic	7	26
Prior chemotherapy regimens, No.		
1	22	82
2	3	11
3	2	7

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
*Of 28 patients enrolled, 27 received at least one infusion of KW-0761.
†ECOG performance status scores range from 0 (normal activity) to 5 (death), with higher scores indicating more severe disability.

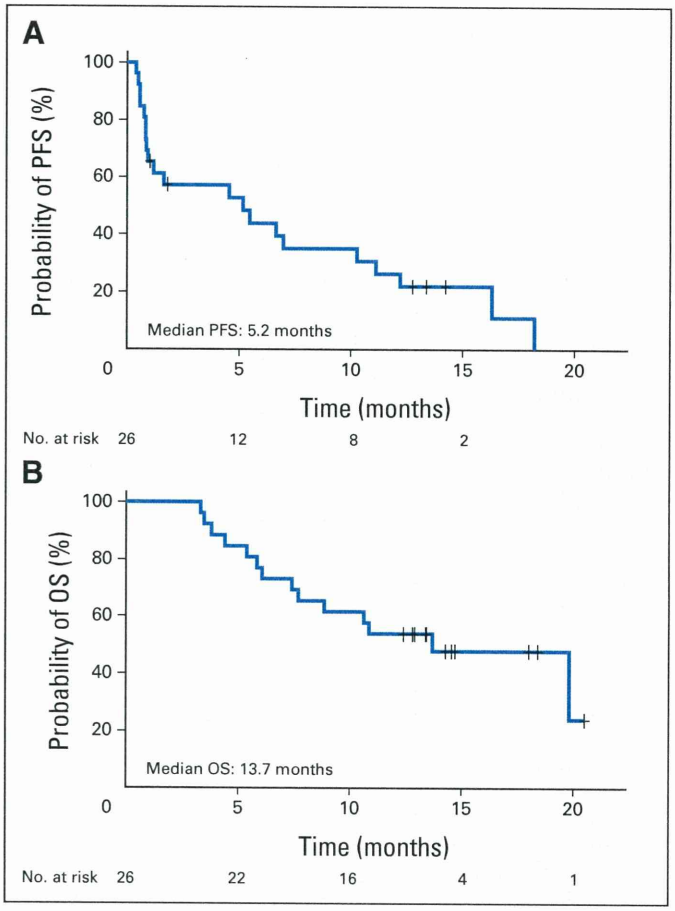

Fig 1. Kaplan-Meier curves of estimated (A) progression-free survival (PFS; median, 5.2 months) and (B) overall survival (OS; median, 13.7 months).

Pharmacokinetics

KW-0761 plasma concentrations over eight infusions, once per week, at 1.0 mg/kg are shown in Figure 2. Mean maximum drug concentration and trough drug concentration (± standard deviation) of the eighth infusion were 42.9 ± 14.2 μg/mL and 33.6 ± 10.6 μg/mL, respectively. Mean area under the blood concentration time curve from 0 to 7 days after the eighth infusion was 6,297 ± 1,812 μg × hours/mL. The mean $t_{1/2}$ after the eighth infusion was 422 ± 147 hours.

AEs

Table 2 lists AEs that occurred in at least 15% of patients or at grades 3 to 4, which were determined as possibly, probably, or definitely KW-0761 related. The most common nonhematologic AE was an infusion reaction (89%). In addition, 80% or more of the following recorded AEs occurred along with an infusion reaction: fever, chills, tachycardia, hypertension, nausea, and hypoxemia (Table 2). These events occurred primarily at the first infusion, becoming less frequent with subsequent treatments. The infusion reactions and component events were transient, and all patients recovered, although some needed systemic steroids. Skin rashes were observed as another frequent nonhematologic AE (63%), mostly occurring after the fourth or subsequent infusions. Of the 14 patients who developed grade 2 or higher skin rashes, objective responses were noted in 13 patients (93%), including eight CRs. On the other hand, of the 12 patients who developed no or grade 1 skin rashes, no objective responses were observed. A typical clinical course of the rash is depicted in Appendix Figures A1A and A1B (online only). The skin rash observed in this patient appeared after the seventh infusion, and the corresponding skin biopsy revealed mild perivascular CD8-positive cells dominating an inflammatory reaction, with an absence of ATL cells. The skin rash recovered on application of topical steroid. Of the 17 patients who developed skin rashes, one developed Stevens-Johnson syndrome, which was determined as possibly KW-0761 related, although that patient also received trimethoprim/sulfamethoxazole, fluconazole, and acyclovir for prevention of infection according to the protocol. This patient stopped those preventive agents and was treated with

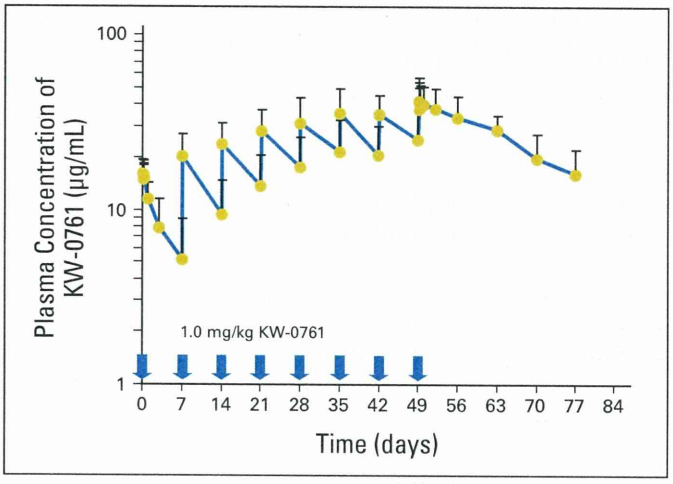

Fig 2. Pharmacokinetics of KW-0761. Mean KW-0761 plasma concentrations during and after 1.0 mg/kg KW-0761 infusions once per week for 8 weeks. Bar indicates upper limit of standard deviation.

Table 2. Adverse Events (n = 27)*

Adverse Event	Grade (No. of patients) 1	2	3	4	All Grades No. of Patients	%	Infusion Reaction Related (No. of patients) All Grades	≥ Grade 2
Nonhematologic								
Infusion reaction	1	22	1	0	24	89		
Fever	20	2	0	0	22	82	18	2
Rash	3	9	5	0	17	63	1	0
Chills	14	2	0	0	16	59	16	2
ALT	5	4	2	0	11	41		
AST	3	5	2	0	10	37		
Tachycardia	9	0	0	0	9	33	9	0
Hypertension	6	2	0	0	8	30	8	1
Albuminemia	7	1	0	0	8	30		
ALP	4	2	0	0	6	22		
Weight gain	5	0	0	0	5	19		
Nausea	4	1	0	0	5	19	5	1
Hyponatremia	5	0	0	0	5	19		
Hypoxemia	0	2	3	0	5	19	4	4
Hypotension	2	2	0	0	4	15	3	1
Pruritus	0	3	1	0	4	15		
γ-GTP	0	1	3	0	4	15		
Hypophosphatemia	0	4	0	0	4	15		
Hyperuricemia	4	0	0	0	4	15		
Hypercalcemia	1	1	0	1	3	11		
Hypokalemia	1	0	2	0	3	11		
Erythema multiforme†	0	0	1	0	1	4		
Hyperglycemia	0	0	1	0	1	4		
Tumor lysis syndrome	0	0	1	0	1	4		
Metabolic/laboratory, other‡	4	7	3	0	14	52		
Hematologic								
Lymphopenia§	0	6	9	11	26	96		
Leukocytopenia	3	7	8	0	18	67		
Thrombocytopenia	7	2	3	2	14	52		
Neutropenia	5	4	5	0	14	52		
Hemoglobin	4	3	1	0	8	30		

Abbreviations: ALP, alkaline phosphatase; BUN, blood urea nitrogen; CRP, C-reactive protein; GTP, glutamyl transpeptidase.

*Of 28 patients enrolled, 27 received at least one infusion of KW-0761. Listed are adverse events determined as possibly, probably, or definitely KW-0761 related that occurred in at least 15% of patients or were of grade 3 to 4 severity.

†One patient diagnosed as having Stevens-Johnson syndrome.

‡Other metabolic and laboratory test abnormalities included hypoproteinemia, BUN elevation, CRP, glycosuria, hypochloremia, and hyperammoniemia.

§Lymphopenia included decrease of abnormal lymphocytes.

systemic steroids, but improvement required the passage of 4 months. Lymphopenia, including a decrease in the number of ATL cells, occurred in 26 (96%) of the 27 patients. Grades 3 to 4 thrombocytopenia was observed in five patients (19%) but was not associated with bleeding, and grade 3 neutropenia also occurred in five patients but did not lead to a febrile episode. The latter two hematologic AEs improved in all patients. None of the patients developed detectable anti–KW-0761 antibody.

T-Cell Subset Analysis

The numbers of circulating blood CD4+ CCR4+, CD4+ CD25+ FOXP3+, CD4+ CCR4−, and CD4− CD8+ cells from KW-0761–treated patients and those from the 10 controls are presented as box and whisker plots in each graph (Appendix Figs A2A to A2D, online only). The numbers of CD4+ CCR4+ and CD4+ CD25+ FOXP3+ cells in patients with ATL before treatment were significantly higher than those in the controls but were significantly reduced after the first KW-0761 infusion. The reduction lasted for at least 4 months after the eighth infusion (Appendix Figs A2A, A2B; online only). The numbers of CD4+ CCR4−, and CD4− CD8+ cells in patients with untreated ATL were significantly lower than those in the controls. KW-0761 treatment led to a transient further reduction of those cells; however, recovery took place by the fifth infusion (Appendix Figs A2C, A2D; online only).

DISCUSSION

In the present multicenter phase II study, KW-0761 monotherapy demonstrated significant responses in patients with relapsed ATL with an acceptable toxicity profile. An ORR of 50% and median PFS and OS values of 5.2 and 13.7 months, respectively, were observed. Because the lower limit for an ORR with a 95% CI was 30%, this study met the primary end point. These results suggest an improvement over what has been achieved with other agents in relapsed ATL.[15] Cladribine was associated with an ORR of 7% (one of 15 patients),[16] and irinotecan hydrochloride treatment had an ORR of 38% (five of 13 patients) with a median duration of response of 31 days.[17] Antiviral therapy consisting of a combination of zidovudine and interferon, which has been proposed as a standard first-line therapy in leukemic subtypes of ATL,[18] was initially reported as having a median OS of 3.0 months in 19 patients with acute or lymphoma type ATL.[19] In addition, White et al[20] reported three objective responses lasting longer than 1 month with zidovudine plus interferon in 18 patients with ATL, of whom 15 had received prior therapy. Those observations collectively suggest that KW-0761 may offer an advantage over or provide an additional therapeutic option to the currently available therapy for relapsed ATL, although there were no direct comparisons.

On examining the results of ATL treatment according to disease site, disease in blood seemed to be more sensitive to KW-0761 than at other disease sites. Currently, we are unable to fully explain this difference; however, factors such as the KW-0761 delivery or the amount of ADCC effector cells such as natural killer (NK) cells and monocytes/macrophages in each disease site may be important.

Pharmacokinetic analyses demonstrated that the $t_{1/2}$ after the eighth administration of KW-0761 was nearly the same as that of circulating endogenous human IgG1, indicating good stability of this antibody in vivo. In addition, no anti-KW-0761 antibody was detected, suggesting that the antigenicity of this novel defucosylated mAb is not likely to be a problem clinically, consistent with findings in our preceding phase I study.[14]

The infusion reactions observed in the present study may also provide novel insights into problems associated with antibody therapy. It is generally recognized that complement plays a major role in infusion reactions,[21] but this mechanism cannot apply to KW-0761, because the agent is unable to mediate complement-dependent cytotoxicity.[11] Therefore, the infusion reactions observed here may have a different mechanism compared with those of other antibody therapies, such as rituximab. KW-0761 has a defucosylated Fc region, which markedly enhances ADCC because of increased binding affinity to the

Fcγ receptor on effector cells. Defucosylated IgG1 is a more potent activator of NK cells than nondefucosylated IgG1 during ADCC.[22] We surmise that the infusion reactions to KW-0761 were mainly induced by cytokines and related cytotoxic molecules released from highly activated NK cells.

The present study demonstrated that compared with the levels in the controls, KW-0761 led to a significant and lasting decrease in the number of CD4+ CCR4+ but not CD4+ CCR4− or CD4− CD8+ cells in patients with ATL. Consistent with the fact that CCR4 is expressed not only on T-helper type 2 cells but also on regulatory T (Treg) cells,[23-26] KW-0761 treatment also resulted in a significant and lasting decrease in CD4+ CD25+ FOXP3+ cells, including both ATL cells and endogenous non-ATL Treg cells.[27-29] Reduction or suppression of Treg cells is expected to be a potentially promising strategy for boosting antitumor immunity in patients with cancer, as observed in studies with ipilimumab,[30-33] although ipilimumab and KW-0761 have different targets; the former suppresses Treg cell function, and the latter decreases their number. Hence, KW-0761 could also lead to activation of antitumor immunity, which might also contribute to its potent anti-ATL response. Because ipilimumab causes immune-related AEs such as diarrhea and colitis, we were especially vigilant in monitoring for this type of AE. Because CCR4 contributes to lymphocyte skin-specific homing,[34] it was not surprising that skin rashes, which could be an immune-related AE, were frequently observed in the present KW-0761 study. Skin rashes, including the most severe case of Stevens-Johnson syndrome, the causal association of which with concomitant medications other than KW-0761 could not be excluded, proved to be manageable, and patients improved in all cases, although some needed systemic or topical steroid treatment. The observed better responses to KW-0761 in patients with grade 2 or higher skin rashes were highly impressive. However, the underlying mechanisms for this finding are not clear; thus, further detailed investigation is warranted. All of the 14 patients who developed grade 2 or higher skin rashes received five or more KW-0761 infusions according to the protocol, whereas only three of the 12 patients who developed no or grade 1 skin rashes received five or more KW-0761 infusions. This suggests the possibility that skin rashes were associated with the number of KW-0761 infusions. The Cochran-Mantel-Haenszel test stratified by the number of KW-0761 infusions ($\leq$ four v $\geq$ five) indicated a significant association between clinical response and skin rashes (no or grade 1 v grades 2 to 4; $P = .009$). However, the sample size is insufficient to draw such a conclusion.

Following on a phase III study (JCOG9801 [Japan Clinical Oncology Group 9801]) for untreated aggressive ATL,[5] the present promising results for KW-0761 monotherapy prompted us to conduct a subsequent randomized trial of VCAP-AMP-VECP chemotherapy with or without KW-0761 for previously untreated ATL (Clinicaltrials.gov: NCT01173887). CCR4 is also expressed on tumor cells from a subgroup of PTCL other than ATL, which also has an unfavorable prognosis.[2,35,36] Thus, we are currently conducting a phase II study of KW-0761 monotherapy for relapsed CCR4-positive PTCL (Clinicaltrials.gov:NCT01192984). In addition, Duvic et al[37] recently reported a phase I/II study of KW-0761 for refractory cutaneous T-cell lymphoma. They found that KW-0761 was well tolerated at doses of 0.1 to 1.0 mg/kg, and a promising ORR of 39% (15 of 38 patients) was achieved, although expression of CCR4 on lymphoma cells was not included as one of the eligibility criteria (Clinicaltrials.gov: NCT00888927). Furthermore, clinical trials of KW-0761 for patients with Hodgkin's lymphoma may be worth trying, because it has been reported that Hodgkin's lymphoma tumor cells produce CCR4 ligand molecules, and migratory CCR4-expressing Treg cells prevent a host immune attack on tumor cells, thereby creating an immunologically favorable environment for the tumor cells.[38]

Although this phase II study offers a novel promising treatment option (KW-0761) for patients with relapsed ATL, some limitations should be discussed. First, the present phase II study was relatively small, with consequent limitations on drawing definitive conclusions about the efficacy and safety profile of KW-0761. Second, patients received different prior systemic chemotherapy regimens, which could affect the results of the present study. Finally, the enrolled patients all had aggressive ATL, but three clinical subtypes (acute, lymphoma, and unfavorable chronic type) were included. Although there may be no significant differences in susceptibility to conventional chemotherapies between these subtypes, the heterogeneity of the enrolled patients might have affected the results.

In conclusion, this multicenter phase II study demonstrated that KW-0761 monotherapy showed clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Further investigation of KW-0761 for ATL and other T-cell neoplasms is warranted on the basis of the present results.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: Shiro Akinaga, Kyowa Hakko Kirin (C) **Consultant or Advisory Role:** Michinori Ogura, Kyowa Hakko Kirin (C) **Stock Ownership:** None **Honoraria:** Takashi Ishida, Kyowa Hakko Kirin **Research Funding:** Takashi Ishida, Kyowa Hakko Kirin **Expert Testimony:** None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Takashi Ishida, Naokuni Uike, Kazuhito Yamamoto, Atae Utsunomiya, Kunihiro Tsukasaki, Shiro Akinaga, Masao Tomonaga, Kensei Tobinai, Ryuzo Ueda
Financial support: Shiro Akinaga
Provision of study materials or patients: Takashi Ishida, Tatsuro Joh, Naokuni Uike, Kazuhito Yamamoto, Atae Utsunomiya, Shinichiro Yoshida, Yoshio Saburi, Toshihiro Miyamoto, Shigeki Takemoto, Hitoshi Suzushima, Kunihiro Tsukasaki, Kisato Nosaka, Hiroshi Fujiwara
Collection and assembly of data: Takashi Ishida, Tatsuro Joh, Naokuni Uike, Kazuhito Yamamoto, Atae Utsunomiya, Shinichiro Yoshida, Yoshio Saburi, Toshishiro Miyamoto, Shigeki Takemoto, Hitoshi Suzushima, Kunihiro Tsukasaki, Kisato Nosaka, Hiroshi Fujiwara, Kensei Tobinai
Data analysis and interpretation: Kenji Ishitsuka, Hiroshi Inagaki, Michinori Ogura, Kensei Tobinai
Manuscript writing: All authors
Final approval of manuscript: All authors

REFERENCES

1. 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 79:428-437, 1991
2. Vose J, Armitage J, Weisenburger D: International peripheral T-cell and natural killer/T-cell lymphoma study: Pathology findings and clinical outcomes. J Clin Oncol 26:4124-4130, 2008
3. Uchiyama T, Yodoi J, Sagawa K, et al: Adult T-cell leukemia: Clinical and hematologic features of 16 cases. Blood 50:481-492, 1977
4. 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 27:453-459, 2009
5. 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 25:5458-5464, 2007
6. Utsunomiya A, Miyazaki Y, Takatsuka Y, et al: Improved outcome of adult T cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant 27:15-20, 2001
7. Hishizawa M, Kanda J, Utsunomiya A, et al: Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: A nationwide retrospective study. Blood 116:1369-1376, 2010
8. Ishida T, Utsunomiya A, Iida S, et al: Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: Its close association with skin involvement and unfavorable outcome. Clin Cancer Res 9:3625-3634, 2003
9. Yoshie O, Fujisawa R, Nakayama T, et al: Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. Blood 99:1505-1511, 2002
10. 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 278:3466-3473, 2003
11. Ishii T, Ishida T, Utsunomiya A, et al: Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. Clin Cancer Res 16:1520-1531, 2010
12. 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 10:7529-7539, 2004
13. 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-2R gamma(null) mice in vivo. J Immunol 183: 4782-4791, 2009
14. 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 28:1591-1598, 2010
15. Tobinai K: Current management of adult T-cell leukemia/lymphoma. Oncology (Williston Park) 14: 1250-1256, 2009
16. Tobinai K, Uike N, Saburi Y, et al: Phase II study of cladribine (2-chlorodeoxyadenosine) in relapsed or refractory adult T-cell leukemia-lymphoma. Int J Hematol 77:512-517, 2003
17. Tsuda H, Takatsuki K, Ohno R, et al: Treatment of adult T-cell leukaemia-lymphoma with irinotecan hydrochloride (CPT-11): CPT-11 Study Group on Hematological Malignancy. Br J Cancer 70:771-774, 1994
18. Bazarbachi A, Plumelle Y, Carlos Ramos J, et al: Meta-analysis on the use of zidovudine and interferon-alfa in adult T-cell leukemia/lymphoma showing improved survival in the leukemic subtypes. J Clin Oncol 28:4177-4183, 2010
19. Gill PS, Harrington W Jr, Kaplan MH, et al: Treatment of adult T-cell leukemia-lymphoma with a combination of interferon alfa and zidovudine. N Engl J Med 332:1744-1748, 1995
20. White JD, Wharfe G, Stewart DM, et al: The combination of zidovudine and interferon alpha-2B in the treatment of adult T-cell leukemia/lymphoma. Leuk Lymphoma 40:287-294, 2001
21. van der Kolk LE, Grillo-López AJ, Baars JW, et al: Complement activation plays a key role in the side-effects of rituximab treatment. Br J Haematol 115:807-811, 2001
22. 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 11:2327-2336, 2005
23. Imai T, Nagira M, Takagi S, et al: Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 11: 81-88, 1999
24. Iellem A, Mariani M, Lang R, et al: Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. J Exp Med 194: 847-853, 2001
25. Yagi H, Nomura T, Nakamura K, et al: Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 16:1643-1656, 2004
26. Ishida T, Ueda R: CCR4 as a novel molecular target for immunotherapy of cancer. Cancer Sci 97:1139-1146, 2006
27. Ishida T, Ueda R: Immunopathogenesis of lymphoma: Focus on CCR4. Cancer Sci 102:44-50, 2011
28. Karube K, Ohshima K, Tsuchiya T, et al: Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. Br J Hematol 126:81-84, 2004
29. Yano H, Ishida T, Inagaki A, et al: Regulatory T-cell function of adult T-cell leukemia/lymphoma cells. Int J Cancer 120:2052-2057, 2007
30. Hodi FS, O'Day SJ, McDermott DF, et al: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711-723, 2010
31. Takahashi T, Tagami T, Yamazaki S, et al: Immunologic self-tolerance maintained by $CD25^{+}CD4^{+}$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 192:303-310, 2000
32. Zou W: Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 6:295-307, 2006
33. O'Day SJ, Hamid O, Urba WJ: Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4): A novel strategy for the treatment of melanoma and other malignancies. Cancer 110:2614-2627, 2007
34. Campbell JJ, Haraldsen G, Pan J, et al: The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature 400:776-780, 1999
35. Ishida T, Inagaki H, Utsunomiya A, et al: CXCR3 and CCR4 expression in T-cell and NK-cell lymphomas with special reference to clinicopathological significance for peripheral T-cell lymphoma, unspecified. Clin Cancer Res 10:5494-5500, 2004
36. Weisenburger DD, Savage KJ, Harris NL, et al: Peripheral T-cell lymphoma, not otherwise specified: A report of 340 cases from the International Peripheral T-cell Lymphoma Project. Blood 117: 3402-3408, 2011
37. Duvic M, Pinter-Brown L, Foss FM, et al: Results of a phase 1/2 study for KW-0761, a monoclonal antibody directed against CC chemokine receptor type 4 (CCR4), in CTCL patients. Blood 116, 2010 (abstr 962)
38. 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 66:5716-5722, 2006

Acknowledgment

We thank all nurses and clinical research coordinators who were involved in this study for their patient care and schedule management. We are also grateful to Takeshi Takahashi, PhD, Masatoshi Sugiura, PhD, and Nobuhito Matsunaga, PhD (Kyowa Hakko Kirin) for their help in preparing this article.

Appendix

List of the investigators, review committees, and medical experts who participated in this trial:

Ilseung Choi and Emi Honda: Department of Hematology, National Kyushu Cancer Center; Yasuhiko Miyazaki: Department of Hematology, Oita Prefectural Hospital; Yuji Moriwaki: Department of Hematology, Nagasaki Medical Center; Hiroshi Onoda and Dai Chihara: Department of Hematology and Cell Therapy, Aichi Cancer Center; Atsushi Inagaki: Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences; Jun Yamanouchi: Department of Bioregulatory Medicine,

Graduate School of Medicine, Ehime University; Koji Kato: Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences; Yoshitaka Imaizumi: Department of Hematology and Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Science; Yuko Watanabe: Department of Hematology and Immunology, NTT West Kyushu Hospital; Shogo Takeuchi and Masahito Tokunaga: Department of Hematology, Imamura Bun-in Hospital; Kazuo Tamura: Internal Medicine/Division of Medical Oncology, Hematology and Infectious Disease, School of Medicine; Shigeo Nakamura: Department of Clinical Pathophysiology, Nagoya University Graduate School of Medicine; Koichi Ohshima: Department of Pathology, School of Medicine, Kurume University; Junichi Tsukada: Cancer Chemotherapy Center and Hematology, University of Occupational and Environmental Health; Kazunari Yamaguchi: Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University; Yasuaki Yamada: Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences; Shuichi Hanada: Department of Internal Medicine, National Hospital Organization Kagoshima Medical Center.

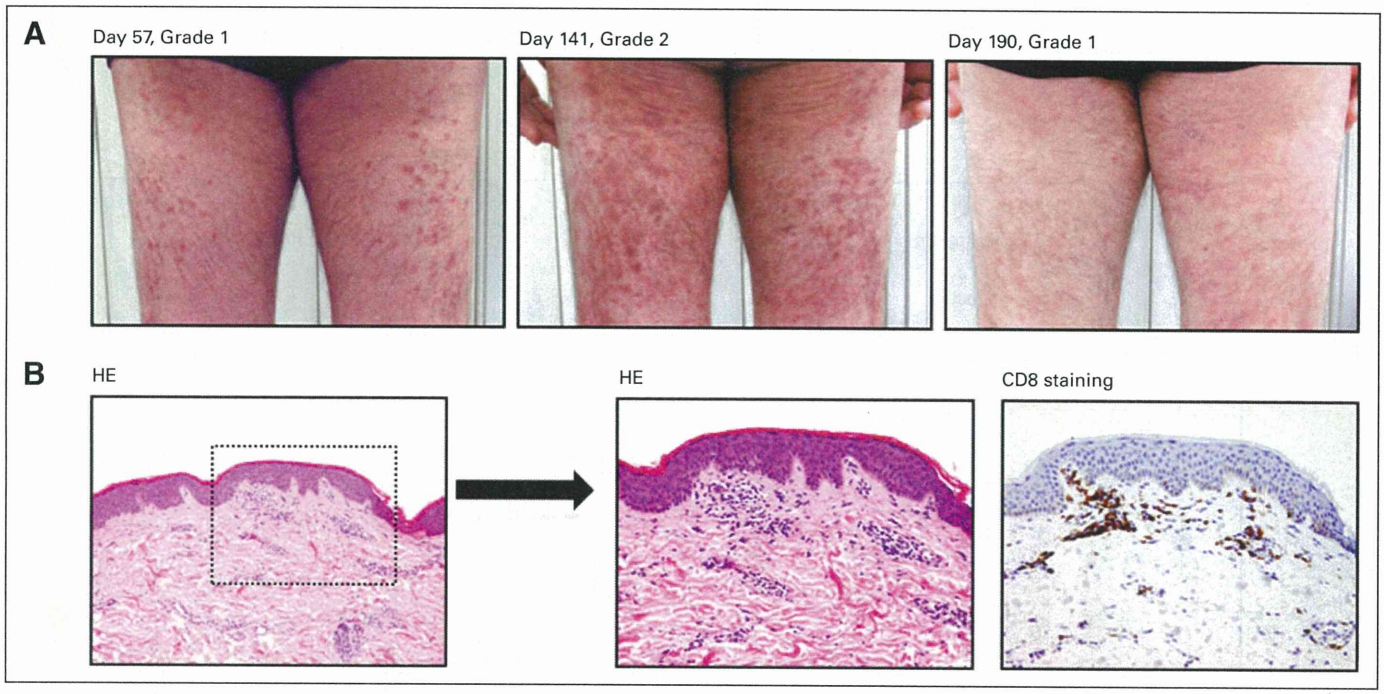

Fig A1. Example of typical clinical course of skin rash after KW-0761 treatment. (A) Macroscopic observations of patient whose skin eruptions appeared after the seventh infusion. (B) Corresponding skin biopsy at day 57 showing mild spongiosis and mild perivascular inflammation with dominant CD8-positive cells but absence of adult T-cell leukemia-lymphoma cells. The skin rash recovered on treatment with topical steroid. HE, hematoxylin and eosin.

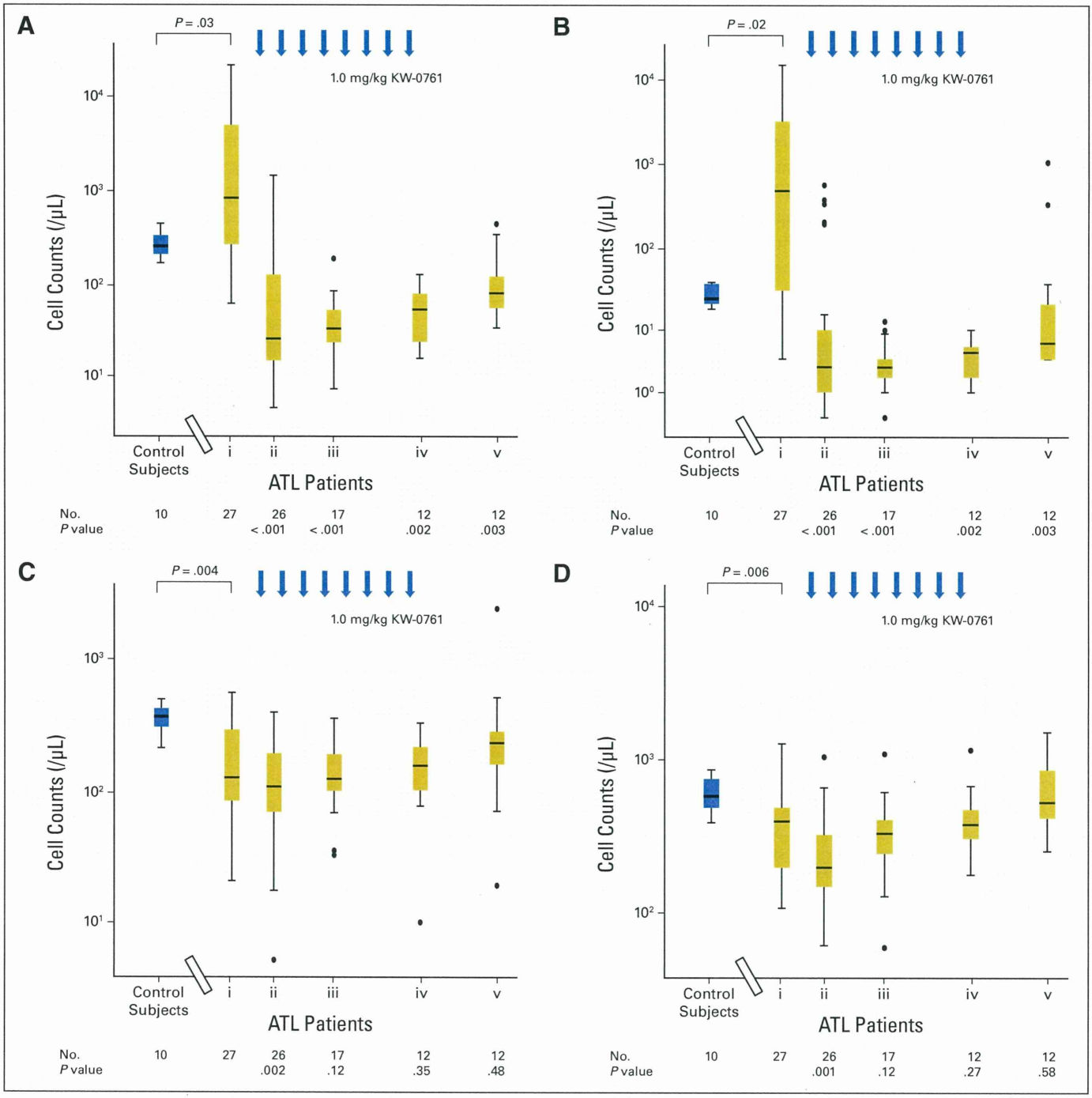

Fig A2. T-cell subset analysis. Blood samples collected at times in accordance with the protocol were employed. When any event resulted in a discontinuation of the infusion protocol, only those samples taken before this time were used for the T-cell subset analysis. (A) Numbers of CD4+ CCR4+ cells from KW-0761–treated patients with adult T-cell leukemia-lymphoma (ATL) in blood samples taken (i) just before the first KW-0761 infusion, (ii) just before the second infusion, (iii) just before the fifth infusion, (iv) 1 week after the eighth infusion, and (v) 4 months after the eighth infusion and those from 10 controls are shown as box and whisker plots indicating minimum, lower quartile, median, upper quartile, and maximum values. Differences between the patients' values before KW-0761 treatment and those of the controls are indicated as a *P* value (Mann-Whitney U-test) in the graph. The number of samples used for analysis at each point is indicated below the graph. The differences between before and each point after KW-0761 treatment [(i) *v* (ii), (iii), (iv), or (v)] are indicated as a *P* value (Wilcoxon signed-rank test) below the graph. CCR4 was detected by a monoclonal antibody (clone 1G1), where its binding to CCR4 was not affected by the presence of KW-0761. Numbers of (B) CD4+ CD25+ FOXP3+; (C) CD4+ CCR4−; and (D) CD4− CD8+ cells are presented in the same manner.

Scientific category: Immunobiology

Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/lymphoma

Running title: Novel immunotherapy targets of ATLL

Hiroyoshi Nishikawa,[1] Yuka Maeda,[1] Takashi Ishida,[2] Sacha Gnjatic,[3] Eiichi Sato,[4] Fumiko Mori,[2] Daisuke Sugiyama,[1] Asahi Ito,[2] Yasuo Fukumori,[5] Atae Utsunomiya,[6] Hiroshi Inagaki,[7] Lloyd J. Old,[3] Ryuzo Ueda,[2] and Shimon Sakaguchi[1,8]

[1]Experimental Immunology, Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan; [2]Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan; [3]Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York, NY 10065; [4]Department of Anatomic Pathology, Tokyo Medical University, Tokyo 160-8402, Japan; [5]Osaka Blood Center, Osaka 536-8505, Japan; [6]Department of Hematology, Imamura Bun-in Hospital, Kagoshima 890-0064, Japan; [7]Department of Clinical Pathology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan; [8]Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

Key words: Tumor immunity, Adult T-cell leukemia/lymphoma, Cancer/testis antigens, $CD8^+$ T cells, Antibody responses.

Correspondence should be addressed to:
Shimon Sakaguchi, M.D., Ph.D.
Experimental Immunology, Immunology Frontier Research Center,
Osaka University
3-1 Yamadaoka, Suita, Osaka 565-0871, Japan.
Tel: +81-6-6879-4461 / Fax: +81-6-6879-4464
E-mail: shimon@ifrec.osaka-u.ac.jp

Ryuzo Ueda, M.D., Ph.D.
Department of Medical Oncology and Immunology,
Nagoya City University Graduate School of Medical Sciences,
1Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi, 467-8601, Japan.
Tel: +81-52-851-5511 / Fax: +81-52-852-0849
E-mail: ueda@med.nagoya-cu.ac.jp

Footnote: H.N., Y.M. and T.I. contributed equally to this study.
This article is dedicated to the memory of Lloyd J. Old, M.D.

Abbreviations: APC, antigen presenting cell; ATLL, Adult T-cell leukemia/lymphoma; CCR4, CC chemokine receptor 4; CT antigen, Cancer/testis antigen; FoxP3, Forkhead

Box P3; HBZ, HTLV-1 bZIP factor; HTLV-1, Human T-lymphotropic virus type 1; mAb, monoclonal antibody; PBMC, Peripheral blood mononuclear cell; PTCL-NOS, peripheral T-cell lymphomas, not otherwise specified; RT-PCR, Reverse transcription-polymerase chain reaction; Tregs, regulatory T cells.

Abstract

Adult T-cell leukemia/lymphoma (ATLL) is an intractable hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1), which infects approximately 20 million people worldwide. Here, we have explored the possible expression of cancer/testis (CT) antigens by ATLL cells, as CT antigens are widely recognized as ideal targets of cancer immunotherapy against solid tumors. A high percentage (87.7%) of ATLL cases (n=57) expressed CT antigens at the mRNA level; NY-ESO-1 (61.4%), MAGE-A3 (31.6%), and MAGE-A4 (61.4%). CT antigen expression was confirmed by immunohistochemistry. This contrasts with other types of lymphoma or leukemia, which scarcely express these CT antigens. Humoral immune responses, particularly against NY-ESO-1, were detected in 11.6% (5/43) and NY-ESO-1-specific $CD8^+$ T cell responses were observed in 55.6% (5/9) of ATLL patients. NY-ESO-1-specific $CD8^+$ T cells recognized autologous ATLL cells and produced effector cytokines. Thus, ATLL cells characteristically express CT antigens and therefore vaccination with CT antigens can be an effective immunotherapy of ATLL.

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a distinct hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1).[1,2] HTLV-1 is endemic in southwestern Japan, Africa, South America and the Caribbean Islands, and approximately 20 million people worldwide are infected.[3] Five percent of the infected individuals develop ATLL after a long latency period.[2] ATLL cells are CD4 positive and the majority, if not all, of them express the transcription factor FoxP3 (Forkhead Box P3), CD25, CTLA-4, and CCR4 (CC chemokine receptor 4), and are functionally immunosuppressive, thus phenotypically and functionally resembling naturally occurring regulatory T cells (Tregs).[3-9] Because of its immunosuppressive property and resistance to conventional chemotherapy, aggressive ATLL has a poor prognosis with a mean survival time of less than 1 year.[2,8] A recent phase III trial of a dose-intensified multidrug chemotherapy for untreated ATLL patients (acute, lymphoma and unfavorable chronic type) showed a median progression-free and overall survival of only 7.0 and 12.7 months, respectively.[10] This and other reports indicate that chemotherapy alone is of limited success for ATLL, and mostly fails to cure the disease.[10,11] Allogeneic hematopoietic stem cell transplantation has been introduced over the last decade as a potential therapy for ATLL with a long-term remission in only a small fraction of patients who are young, well controlled in disease progression, and have an appropriate stem cell source.[12] More effective strategies to treat ATLL are therefore required.

Several HTLV-1 components have been explored as targets for immunotherapy of ATLL. HTLV-1 Tax, which is crucial for ATLL oncogenesis, has generally been considered to be a main target of the host's cellular immune responses. Yet, the

frequency of Tax expression in HTLV-1 infected cells reduces in the course of disease progression, and *Tax* transcripts are detected only in ~40% of the established ATLL cases,[13] thus limiting Tax-targeted immunotherapy to a subset of patients. HBZ (HTLV-1 bZIP factor), another HTLV-1 component, which contains an N-terminal transcriptional activation domain and a leucine zipper motif at its C-terminal, also plays an important role in the proliferation of ATLL cells and is detectable in almost all ATLL cases.[2] However, $CD8^+$ T cells specific for HBZ could only recognize peptide-pulsed target cells, but not ATLL cells themselves.[14] Furthermore, HTLV-1 is transmitted mainly from mothers to infants through breast milk, and such vertical infection in early life may induce tolerance to the virus and result in insufficient HTLV-1-specific T-cell responses.[15,16] For these reasons, targeting the HTLV-1 components alone may be insufficient for successful immunotherapy of ATLL, necessitating identification of novel tumor-associated target antigens for the immunotherapy.

The expression of cancer/testis (CT) antigens, of which more than 100 have been identified so far, is normally limited to human germ line cells in the testis and in various types of human cancers.[17,18] This restricted expression pattern in normal tissues makes them ideal cancer antigens for tumor immunotherapy. NY-ESO-1 and MAGE family antigens, a subset of CT antigens, are indeed able to elicit spontaneous humoral and cellular immune responses in cancer patients.[17-19] Clinical trials of CT antigen vaccination are currently under way with several types of vaccine formulation including peptide, protein and DNA, and some of the treated patients have experienced clinical benefits from vaccination.[19,20] This promising result of CT antigens as a target of tumor immunotherapy has prompted intensive studies of their expression in a wide range of human cancers. However, detailed analysis of CT antigen expression in hematologic disorders has been limited.[21,22] In the present study, we

have investigated possible expression of CT antigens by ATLL cells and possible humoral and cellular immune responses against CT antigens in ATLL patients.

Methods

Primary ATLL cells

Blood or lymph node samples were obtained from ATLL patients, and mononuclear cells were isolated with Ficoll-Paque (Pharmacia, NJ). Diagnosis and classification of clinical subtypes of ATLL were according to the criteria proposed by the Japan Lymphoma Study Group.[23] All donors provided informed written consent before sampling according to the Declaration of Helsinki, and the present study was approved by the institutional ethics committees of Nagoya City University Graduate School of Medical Sciences, Osaka University, and Imamura Bun-in Hospital.

ATLL cell lines

ATN-1, ATL102, HUT102, MT-2, and MT-1 were previously described.[24,25] TL-Om1 and TL-Su were kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). MT-4 was purchased from the Health Science Research Resources Bank (Osaka, Japan). MJ was purchased from ATCC (Manassas, VA). TCL-Kan was kindly provided from Professor M. Kannagi (Tokyo Medical and Dental University, Tokyo, Japan).

Reagents

Phycoerythrin-conjugated anti-IFN-γ (4S.B3) monoclonal antibody (mAb) and Phycoerythrin-Cyanine7 conjugated anti-TNF-α (MAb11) mAb were purchased from eBioscience (San Diego, CA). Fluorescein isothiocyanate-conjugated anti-IL-2 (MQ1-17H12) mAb and Allophycocyanin-Cyanine7-conjugated anti-CD8 (SK1) mAb were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-NY-ESO-1 mAb (E978,

mouse IgG1)[26] and pan-MAGE mAb (57B, mouse IgG1)[27] were purified from hybridoma supernatant by protein G affinity chromatography. Synthetic peptides of NY-ESO-1_{1-20} (MQAEGRGTGGSTGDADGPGG), NY-ESO-1_{11-30} (STGDADGPGGPGIPDGPGGN), NY-ESO-1_{21-40} (PGIPDGPGGNAGGPGEAGAT), NY-ESO-1_{31-50} (AGGPGEAGATGGRGPRGAGA), NY-ESO-1_{41-60} (GGRGPRGAGAARASGPGGGA), NY-ESO-1_{51-70} (ARASGPGGGAPRGPHGGAAS), NY-ESO-1_{61-80} (PRGPHGGAASGLNGCCRCGA), NY-ESO-1_{71-90} (GLNGCCRCGARGPESRLLEF), NY-ESO-1_{81-100} (RGPESRLLEFYLAMPFATPM), NY-ESO-1_{91-110} (YLAMPFATPMEAELARRSLA), NY-ESO-$1_{101-120}$ (EAELARRSLAQDAPPLPVPG), NY-ESO-$1_{111-130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-$1_{119-143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-$1_{131-150}$ (NILTIRLTAADHRQLQLSIS), NY-ESO-$1_{139-160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-$1_{151-170}$ (SCLQQLSLLMWITQCFLPVF), NY-ESO-$1_{161-180}$ (WITQCFLPVFLAQPPSGQRR) were obtained from Invitrogen.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with RNeasy Mini Kit (Qiagen). cDNA was synthesized from 0.1 μg of total RNA using SuperScript III reverse transcriptase kit (Invitrogen) and the Oligodt primer in a total volume of 20 μl. cDNA was amplified in a final volume of 20 μl containing 10 μM of each CT antigen primer as reported[21,28] except NY-ESO-1 (sense, 5'-AGT TCT ACC TCG CCA TGC CT-3'; antisense, 5'- TCC TCC TCC AGC GAC AAA CAA-3') and 0.2 μl of Ex-Taq polymerase (Takara Bio, Shiga, Japan) in according to the instruction provided by the manufacturer.

ELISA

Patients' sera were analyzed by ELISA for seroreactivity to bacterially-produced recombinant proteins NY-ESO-1/CTAG1B, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT7/MAGE-C1, CT10/MAGE-C2, SSX1, SSX2, and SSX4.[29] Sera were diluted serially from 1/100 to 1/100,000 and added to low-volume 96-well plates (Corning, NY) coated overnight at 4°C with 1 µg/ml antigen in 25 µl volume and blocked for 2 hours at room temperature with phosphate buffered saline containing 5% non-fat milk. After overnight incubation, plates were extensively washed and rinsed with phosphate buffered saline. Antigen-specific IgG was detected with specific mAb conjugated with alkaline-phosphatase (Southern Biotech, Birmingham, AL). Following addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured using a fluorescence reader Cytofluor Series 4000 (PerSeptive Biosystems, Framingham, MA). A reciprocal titer was calculated for each sample as the maximal dilution still significantly reacting to a specific antigen. This value was extrapolated by determining the intersection of a linear trend regression with a cutoff value. The cutoff was defined as 10x the average of OD values from the first 4 dilutions of a negative control pool made of 5 healthy donor sera. In each assay, sera of patients with known presence or absence of specific reactivity were used as controls.

Immunohistochemistry

All tissue specimens were fixed with formalin and embedded in paraffin. Tissue sections of 3 µm thickness on charged glass slides were deparaffinized and rehydrated. Antigen retrieval was performed by autoclave (105°C for 20 minutes) using Tris-EDTA buffer (pH9.0) as heating solution. Primary mAb for NY-ESO-1 (E978) and pan-MAGE protein (57B) were used at the concentration of 5 µg/ml and 2 µg/ml respectively. Endogeneous peroxidase activity was blocked with 3% hydrogen

peroxidase and 1% sodium azide. Simple stain max-PO (Multi) (Nichirei, Tokyo, Japan) was used for secondary detection. 3,3'-diamino-benzidine was used as chromogen. Human testicular tissue was served as positive control.

In vitro sensitization

NY-ESO-1-specific $CD8^+$ and $CD4^+$ T cells were pre-sensitized as described previously.[30,31] Briefly, $CD8^+$ T cells and $CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) using a CD8 Microbeads and a $CD4^+$ T cell Isolation Kit, respectively (Miltenyi Biotec). The purity of isolated populations was confirmed to be >90%. The non-$CD8^+$/$CD4^+$ cell population was pulsed with 10 μM of pooled peptides overnight and was used as antigen presenting cells (APCs). After irradiation, 5-10 x 10^5 APCs were added to round-bottom 96-well plates (Corning) containing 1-5 x 10^5 $CD8^+$ or $CD4^+$ T cells and were fed with IL-2 (10 U/ml; Roche Diagnostics) and IL-7 (20 ng/ml; R&D Systems). Subsequently, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/ml) and IL-7 (40 ng/ml) twice per week.

Intracellular cytokine staining

$CD8^+$ T cells from PBMCs from ATLL patients were pre-sensitized for 10-18 days. These pre-sensitized $CD8^+$ T cells were re-stimulated for 6 hours with peptide-pulsed T-APCs[31] and GolgiStop reagent (BD Biosciences) was added 1 hour later. Cells were stained for cell surface markers and for intracellular cytokines such as IFN-γ and TNF-α after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Tetramer assay

Tetramer staining was performed as previously described.[31] Briefly, pre-sensitized $CD8^+$ T cells were stained with Phycoerythrin-labeled tetramers (prepared at the Ludwig Institute Core Facility by Drs. P. Guillaume and I. Luescher, Lausanne, Switzerland) for 15 minutes at 37°C before additional staining with Allophycocyanin-Cyanine7-conjugated anti-CD8 mAb for 15 minutes at 4°C. After washing, results were analyzed by FACSCanto and FlowJo software.

Statistical analysis

The significance of the difference in each CT antigen expression between two groups was assessed by Fisher's exact test. *P* values <0.05 were considered significant.

Results

A subset of CT antigens is highly expressed in ATLL cell lines.

To examine possible expression of 11 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, SSX-4, and SCP-1) by ATLL cells, we assessed mRNA expression by RT-PCR in 10 ATLL cell lines (MT-1, MT-2, MT-4, MJ, ATL102, ATN-1, TL-Om-1, TL-Su, TCL-kan, and HUT102). In sharp contrast to a previous report showing that the majority of T-cell lymphomas did not express CT antigens except SCP-1,[21] a high percentage of ATLL cell lines expressed NY-ESO-1, MAGE-A3, and MAGE-A4 (90%, 50% and 70%, respectively) in the present study (Supplemental Table 1). SCP-1, another CT antigen, was also detected in 40% of ATLL cell lines, as in other T cell lymphomas (Supplemental Table 1).

NY-ESO-1, MAGE-A3, and MAGE-A4 are widely expressed in primary ATLL cells.

Given the high percentage of CT antigen mRNA expression in ATLL cell lines, we next examined the expression of 11 CT antigens in primary tumor cells from 57 individual ATLL patients. As shown in Figure 1A, Table 1, and Supplemental Table 2, NY-ESO-1, MAGE-A3 and MAGE-A4 mRNA expression was detected in 61.4% (35/57), 31.6% (18/57), and 61.4% (35/57), respectively, of primary ATLL patients. SCP-1 expression was also detected in 23.2% (13/56). Among four subtypes (acute, lymphoma, chronic, and smoldering) of ATLL, the acute and lymphoma types show aggressive clinical courses (aggressive types), whereas the chronic and smoldering types progress more indolently (indolent types).[2,23] There were no significant differences between the ATLL patients with aggressive and indolent types in the expressions of NY-ESO-1, MAGE-A3, and MAGE-A4; yet there was a trend for more frequent expression of CT antigens

in aggressive ATLL types (Table 1). Immunohistochemical analysis confirmed the expression of NY-ESO-1 and pan-MAGE at the protein level in samples available for pathological analysis (Figure 1B). By contrast, expression of other CT antigens was limited: MAGE-A1 8.8%(3/34), MAGE-A10 0%(0/33), CT-7 0%(0/33), CT-10 0%(0/33), SSX-1 0%(0/33), SSX-2 0%(0/33) and SSX-4 0%(0/33) by RT-PCR.

Taken together, CT antigens such as NY-ESO-1, MAGE-A3 and MAGE-A4 are expressed in a significant fraction of primary ATLL cases and 87.7% (50/57) of ATLL patients expressed at least one of these three CT antigens.

Humoral immune responses against NY-ESO-1 are detected in a subset of ATLL patients.

We next asked whether ATLL patients spontaneously developed humoral and cellular immune responses specific for CT antigens. Serum samples from 43 primary ATLL patients were assessed by ELISA for the reactivity to 10 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4). Significant antibody titers were detected against NY-ESO-1 in five out of 43 (11.6%) patients and against MAGE-A1, MAGE-A3 and MAGE-A4 in 0% (0/43), 2.3% (1/43) and 0% (0/43), respectively (Figure 2). Humoral immune responses against CT antigens whose expression was not detected by RT-PCR (MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4) were limited (Figure 2). These data collectively indicate that NY-ESO-1 expressed in primary ATLL cells elicits spontaneous antibody responses in ATLL patients as in patients with NY-ESO-1 expressing solid tumors.[19]

Cellular immune responses against NY-ESO-1 in ATLL patients