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ORIGINAL REPORT

Defucosylated Anti-CCR4 Monoclonal Antibody (KW-0761) for Relapsed Adult T-Cell Leukemia-Lymphoma: A Multicenter Phase II Study

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A B S T R A C T

Purpose
Adult T-cell leukemia-lymphoma (ATL) is usually resistant to conventional chemotherapies, and there are few other treatment options. Because CC chemokine receptor 4 (CCR4) is expressed on tumor cells from most patients with ATL, KW-0761, a humanized anti-CCR4 monoclonal antibody, which markedly enhances antibody-dependent cellular cytotoxicity, was evaluated in the treatment of patients with relapsed ATL.

Patients and Methods
A multicenter phase II study of KW-0761 for patients with relapsed, aggressive CCR4-positive ATL was conducted to evaluate efficacy, pharmacokinetic profile, and safety. The primary end point was overall response rate, and secondary end points included progression-free and overall survival from the first dose of KW-0761. Patients received intravenous infusions of KW-0761 once per week for 8 weeks at a dose of 1.0 mg/kg.

Results
Of 28 patients enrolled onto the study, 27 received at least one infusion of KW-0761. Objective responses were noted in 13 of 26 evaluable patients, including eight complete responses, with an overall response rate of 50% (95% CI, 30% to 70%). Median progression-free and overall survival were 5.2 and 13.7 months, respectively. The mean half-life period after the eighth infusion was 422 ± 147 hours (± standard deviation). The most common adverse events were infusion reactions (89%) and skin rashes (63%), which were manageable and reversible in all cases.

Conclusion
KW-0761 demonstrated clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Further investigation of KW-0761 for treatment of ATL and other T-cell neoplasms is warranted.

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INTRODUCTION

Adult T-cell leukemia-lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus type I. The disease is resistant to conventional chemotherapeutic agents, and there currently exist limited treatment options; thus, it has a poor prognosis.[1-4] A recent phase III trial for previously untreated patients with aggressive ATL (acute, lymphoma, or unfavorable chronic type) age 33 to 69 years demonstrated that a dose-intensified multidrug regimen, VCAP-AMP-VECP (vincristine, cyclophosphamide, doxorubicin, and prednisone; doxorubicin, ranimustine, and prednisone; and vindesine, etoposide, carboplatin, and prednisone), resulted in median progression-free (PFS) and overall survival (OS) of 7.0 and 12.7 months, respectively.[5] This remains unsatisfactory compared with responses in other hematologic malignancies. Allogeneic hematopoietic stem-cell transplantation has evolved into a potential approach to treating patients with ATL over the last decade. However, only a small fraction of patients with ATL have the opportunity to benefit from transplantation, such as those who are younger, have achieved sufficient disease control, and have an appropriate stem-cell source.[6,7] Therefore, the development of alternative treatment strategies for patients with ATL is an urgent issue.

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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).

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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
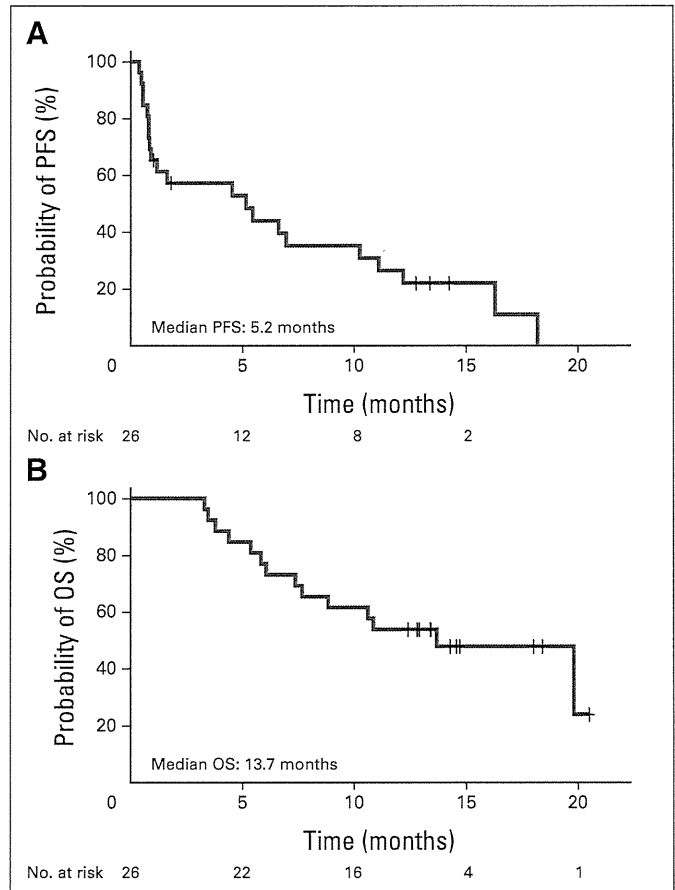
developed skin rashes, one developed Stevens-Johnson syndrome, which was determined as possibly KW-0761 related, although that patient also received trimethoprim/sulfamethoxazole, fluconazole, and acyclovir for prevention of infection according to the protocol. This patient stopped those preventive agents and was treated with
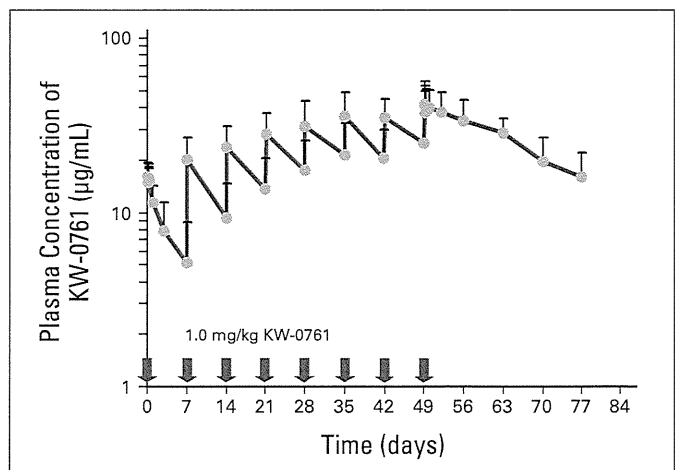

Fig 2. Pharmacokinetics of KW-0761. Mean KW-0761 plasma concentrations during and after 1.0 mg/kg KW-0761 infusions once per week for 8 weeks. Bar indicates upper limit of standard deviation.

Adverse Event	Grade (No. of patients)				All Grades		Infusion Reaction Related (No. of patients)	
	1	2	3	4	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		

Table 2. Adverse Events (n = 27)*

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

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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.

AUTHOR CONTRIBUTIONS

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ORIGINAL ARTICLE

Heterogeneity in clonal nature in the smoldering subtype of adult T-cell leukemia: continuity from carrier status to smoldering ATL

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Abstract To better understand indeterminate HTLV-1 carriers and smoldering (SM) subtype of adult T-cell leukemia (ATL), HTLV-1 proviral integrated status, proviral load (PVL) and ATL-related biomarkers were examined in 57 smoldering cases, including unusual carriers with a percentage of ATL-like cells. We found that according to Southern blot hybridization analytic features, 28 patients with SM ATL could be divided into 3 groups consisting of 16 (57.4%) patients with a monoclonal band, 6 (21.4%) with oligoclonal bands and the remaining 6 with smears. Although no clinical differences were observed among the 3 SM subtypes, HTLV-1-infected CD4 T-cell counts increased in order of poly-, oligo- and monoclonal subtypes. This trend began in the carrier stage and also was observed in PVL, CD25 and CCR4, indicating that a clone consisting of leukemic phenotypic cells was continuously growing. Moreover, the antigen modulation rates of CD26 and CD7 and the increasing rate of CD25 and CCR4 cells were closely correlated to growing clonal size, indicating

that these markers had the possibility to predict a monoclonal band. In particular, CD26 or the ratio of CD26/CD25 had a validity differential for leukemic nature and predictive detection of clonal band. Conclusively, the present study shows that smoldering ATL is heterogeneous in the leukemogenic process, and the behavior of CD26 plays a central role in the evolution from early occult to overt smoldering ATL.

Keywords ATL · HTLV-1 · Provirus · Southern blot · Leukemogenesis

Abbreviations

HTLV-1	Human T-cell leukemia virus type-1
ATL	Adult T-cell leukemia
SBH	Southern blotting hybridization
PMNC	Peripheral blood mononuclear cell
LDH	Lactate dehydrogenase
sIL-2R	Soluble-interleukin-2 receptor

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) was found to be a causative retrovirus of a T-cell malignancy, designated as adult T-cell leukemia (ATL) [1, 2]. All ATL cells, without exception, carry the HTLV-1 provirus in the same genomic site in each case, indicating that provirus insertion is an excellent biomarker for the cellular clonality of ATL and the integrated provirus can be used as the proviral load (PVL) [3] to estimate viral and cellular burden. Proof of clonality is essential for a diagnosis of malignant neoplasm, but it should be noted that HTLV-1-infected cells also are clonally expanded to maintain

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persistent infection [2, 4]. Thus, changes in the abundance of HTLV-1-infected cell clones play an important role in persistent infection and ATL leukemogenesis. The clonality of provirus-carrying cells is usually demonstrated by Southern blot hybridization (SBH). However, since the detection sensitivity of this assay is limited (about 5%) [5, 6], it is unavailable for samples including small clones with 5% or fewer monoclonal cell populations.

Recently, we have had many opportunities to see patients with smoldering ATL and unusual carriers with high HTLV-1 PVL or with a proportion of ATL-like cells. We sometimes struggle to distinguish such borderline cases between carriers and smoldering ATL. ATL cells phenotypically resemble Treg cells expressing CD4, CCR4 and CD25. On the other hand, ATL cells aberrantly express 100 or more cell surface receptors and ligands [7, 8]. Such aberrantly expressed receptors consist mainly of natural, adaptive and ectopic types, some of which are considered to be involved in leukemogenesis [9]. In particular, downregulation of CD3, CD7 and CD26 are observed during the early phase of leukemogenesis [9]. However, little is known about the behavior of cells concurrently expressing CD4, CD25, CCR4 and CD26 in the carrier to SM stages using in vivo practical samples. Accordingly, to better understand indeterminate carriers and smoldering ATL, the present study was focused on the implication between the SBH features reflecting clone size and cellular changes in phenotype and number. In particular, CD26 is noted to be one of the prodromal cellular changes, because the downregulation of CD26 begins in the carrier stage and persists continuously till the completion of ATL.

Materials and methods

White blood cell counts, morphological data, serum lactate dehydrogenase (LDH) activity and soluble interleukin-2 receptor (sIL-2R) were used from routine laboratory data. Peripheral blood samples were collected from our ATL and HTLV-1 carrier clinic, consisting of 28 patients with smoldering ATL carrying 5% or more ATL-like cells in blood, 12 unusual carriers with around 5% ATL-like cells, and 17 common (healthy) carriers. Thirty-four samples from patients with leukemic chronic or acute ATLs were used as a positive control. Morphological evaluation was microscopically conducted by hematological specialists.

High-molecular-weight DNA was extracted from peripheral blood mononuclear cells (PMNC) using a QIAmp DNA Blood Mini Kit (Qiagen GmbH, Hilden Germany). PVL was quantified by LightCycler Technology (Roche Diagnostic K.K., Tokyo, Japan) using hydro-probes and previously described primers [10–12]. Normalization was done using the β-globin gene and the PVL was expressed as copy number per 10^4 cells or percent for PMNC. This study was done under the approval of our institutional board.

Clone assay of SBH

SBH analysis was performed by a method described previously with modification, using 7 mixtures of probes covering the total region of the digoxigenined provirus and the restriction enzymes of EcoR-1 and Pst-1 [13, 14]. Pst-1 cuts 4 sites of the provirus, but Eco-R1 cannot cut within the provirus. Therefore, to determine clonally related sharp band or polyclonally related smear bands, Eco-R1 digestive genomic fragments were used. To assay clonality accurately, we monitored 1.5, 3 and 5% clonal cell controls every time. Band patterns were estimated using a densitometer (Fujifilm Life Science, Science Lab 2005, and Tokyo, Japan).

Flow cytometry analysis for cell surface antigens

The positive rate for CD3, CD4, CD7, CD25, CD26 and CCR4 was measured by a routine method using whole blood according to the manufacturer's instructions (BD FACSCato-II, Nixon BD. Inc, Tokyo, Japan). The association between CD7 and CD26 antigen modulation and the positivity of CD4, CD25 and CCR4 cells was assessed by using the four-color staining method according to the manufacturer's instructions. Co-expression with CD4, CD25, CCR4 and CD26 was assessed by a four-color flow cytometric method using CD26-FITC, CD4-PerCP, CD25-APC and CCR4-PE. The rate (%) of $CD4^{(+)}CD25^{(+)}CCR4^{(+)}$ cells relative to all CD4 cells and the co-expression rate of CD26 with $CD4^{(+)}CD25^{(+)}CCR4^{(+)}$ cells was measured according to the BD FACSCato-II protocol (BD, Inc., Tokyo, Japan).

Statistical analysis

Data are expressed mainly as the median and analyzed using the Mann–Whitney test. P value of below 0.05 was considered to be statistically significant. Analyses were performed with Stat Flex version 6.5 software packages (Artech Inc., Osaka, Japan).

Results

Classification features of band patterns using a densitometer

The band patterns in SBH analysis using Eco-R1-digestive genomic fragments were mainly estimated by a densitometer graph. The densitometer graphs equivalent to band

status are classified into five patterns (Fig. 1a): patterns-1 and -2 are light and dense smear bands with no difference in staining density; pattern-3 is a hill ridge, irregular shape with vertical stripes or a low and broad projection with smear bands; pattern-4 is a low/sharp peak type; and pattern-5 is a sharp high peak. In vivo clonal status of the five patterns based on SBH features, as shown in Fig. 1b, is considered to correspond to few small clones in pattern (P)-1, many small clones with few growing clones in P-2, oligoclones of a detection limit in P-3, and clearly detectable monoclone(s) with various background clonal status in P-4 and 5.

SBH analysis allocated 57 cases to 17 of P-1, 11 of P-2, 12 of P-3, 6 of P-4 and 11 of P-5. The relations between intra- or inter-disease status and the band patterns are summarized in Table 1. Consequently, smoldering ATL was the most heterogeneous for SBH patterns; 16 (57.1%) of 28 smoldering ATLs were P-4 and -5 (large clone consisting of at least 5% monoclonal cells), while the remaining 12 (42.9%) were P-1, -2 and -3 (equivalent to polyclonal or oligoclonal band). Actually, Fig. 2 is an interesting example of an SM subtype showing dense

smear bands and abnormal cells with an aberrant phenotype of 73% CD4, 77% CD25 and 21% CD26.

Cyto-oncological characteristics of the three SM subtypes

To characterize ATL-related biomarkers in the three subtypes of SM, the findings were comprehensively compared with those of healthy carriers, unusual carriers and patients with chronic ATL (Table 2). First, clonal expansion-associated biomarkers, such as PVL, HTLV-1-infected CD4 T-cell counts in 1 μL peripheral blood and the serum level of sIL-2R increased regularly in the order of poly-, oligo- and monoclonal SM subtypes. Figure 3 shows the line graphs on increasing fold (rate) of PVL and total lymphocyte, all CD4 T-cell and HTLV-1-infected CD4 T-cell counts converted from Table 2. The graph shows two distinctive patterns (solid lines of PVL and infected CD4 T-cell vs. broken lines of total lymphocyte and all CD4 T-cell). In contrast to the horizontal part of the broken lines, the solid lines are gradually elevated, meaning that the provirus-carrying CD4 T-cells gradually increase

(A) Pattern Classification of SBH features
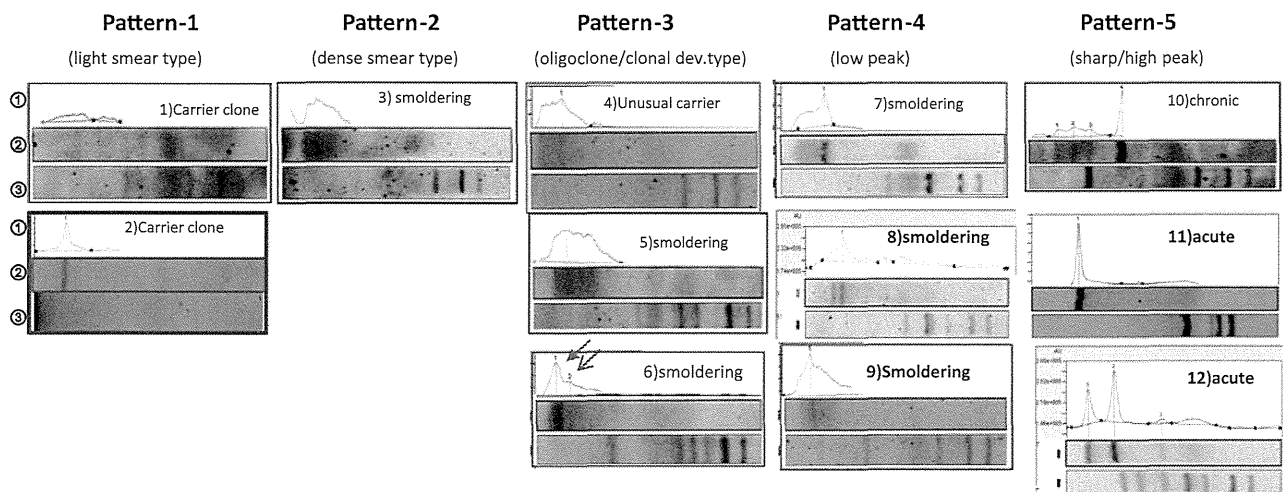
(B) The images of in vivo clonal status in each Pattern

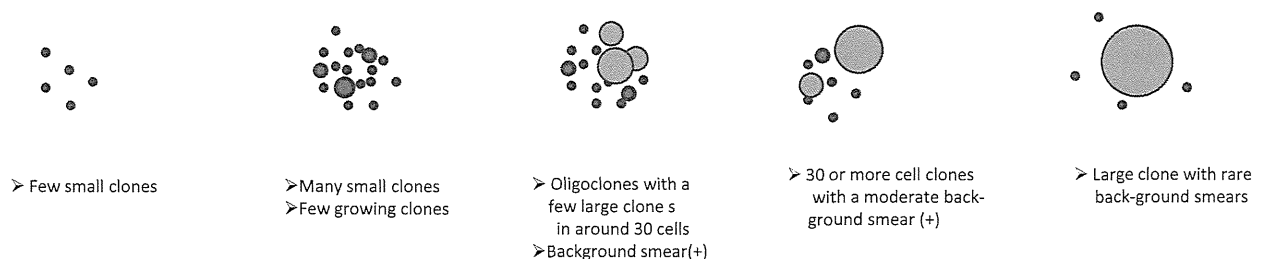**Fig. 1** **a** The densitometry patterns for HTLV-1 proviral integration status according to SBH band features using a restrictive enzyme of Eco-R1 and representative cases. Subjects were mainly classified into five patterns according to densitometer images. ① Densitometry graph. ② SBH analysis for Eco-R1-digestive genomic fragments. ③ SBH analysis for Pst-1-digestive genomic fragments. **b** The image of in vivo clonal status in each pattern

Table 1 The pattern distribution of SBH features in intra- and inter-diseases

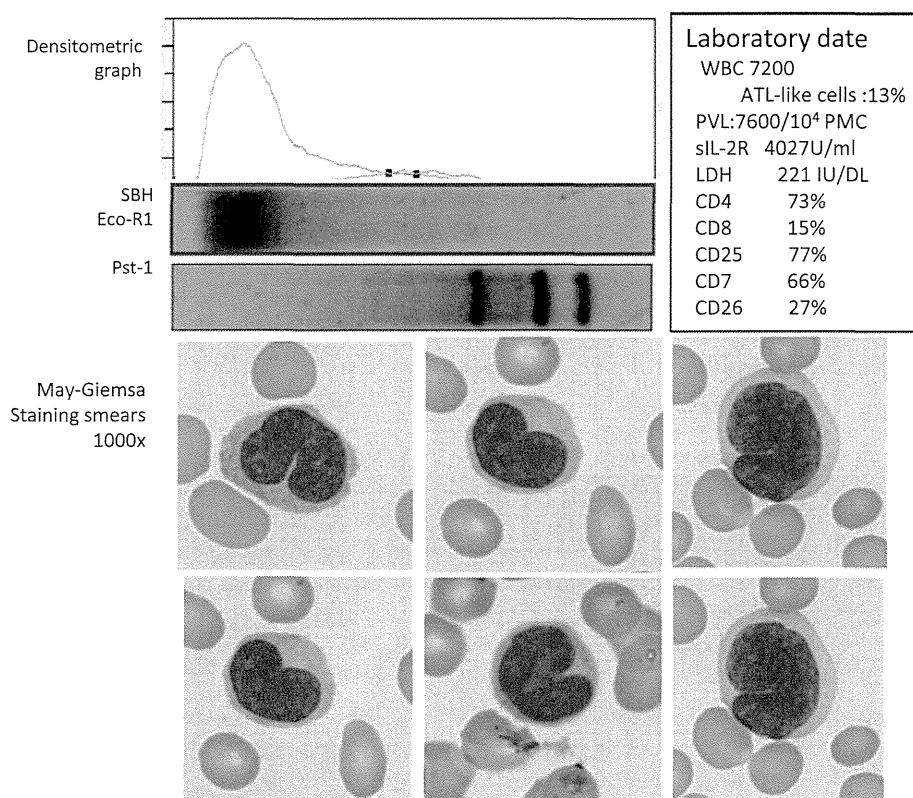|	Smear band		Oligoclonal bands	Monoclonal bands		Total
	P-1	P-2	P-3	P-4	P-5	
Common carriers	11	5	0	0	(1)[a]	17
Unusual carriers	5	1	6	0	0	12
Smoldering ATL	1	5	6	6	10	28

The criteria of the classification is explained in the text. P-1, P-2, P-3, and P-4 and P-5 generally correspond to light smears, dense smears, oligoclonal bands and monoclonal bands, respectively. Of leukemic type ATL, including the chronic and acute type, SBH features in smoldering ATL were the most heterogeneous

Common carriers HTLV-1-seropositive individuals without any HTLV-1-associated disorders

Unusual carriers those who have clinico-cytological findings similar to that of the smoldering subtype of ATL

[a] Carrier clone

Fig. 2 A representative case presentation of the polyclonal smoldering subtype (SM) showing a polyclonal dense smear in SBH analysis and the smoldering subtype with full hematological criteria

Densitometric graph

SBH
Eco-R1

Pst-1

May-Giemsa
Staining smears
1000x

Laboratory date
WBC 7200
    ATL-like cells :13%
PVL:7600/10⁴ PMC
sIL-2R 4027U/ml
LDH   221 IU/DL
CD4   73%
CD8   15%
CD25   77%
CD7   66%
CD26   27%

regardless of the almost stable counts of non-infected CD4 T-cells during the entire period of smoldering ATL.

The positive values of CD4, CD25, CCR4, CD7 and CD26 subsets (%) were observed to change continuously and concurrently in the order of common carriers, unusual carriers, polyclonal SM, oligoclonal SM and monoclonal SM. In order to interpret these data in detail, a line graph was used (Fig. 4). CCR4 and CD25 cells increased concurrently and sharply from the common carrier stage to the oligoclonal stage. The down-regulation of CD26 was initiated in the unusual carrier stage and kept falling continuously by chronic stage. The fluctuations of CD4 and

CD26 showed an opposing trend, and the interval between CD4 and CD26 (solid triangle and gray circle) gradually enlarged with the increasing cell number of 32, 54, 115 and 163 cells. Such behavior of CD26 was expected to play a central role in budding of ATL in the early stage of multistep leukemogenesis.

Clinico-oncological usefulness of CD26

From the results described above, CD26 appears to be closely associated with the evolution of SM. In contrast to characteristic phenotypes in overt ATL cells, those of

Table 2 Comparison of the measurement value (mean) of ATL-related biomarkers among the polyclonal, oligoclonal and monoclonal SM subtypes

	Carrier stage		Smoldering stage			Chronic
	Common	Unusual	Polyclonal	Oligoclonal	Monoclonal	
PVL (%)	5.9	17.9	22.6*	28.0	39.3*	78.3
Total Ly counts	1750	1950	2308	1732	2306	7659
All CD4 T-cell	786	882	1570	1513	1377	7220
Infected CD4	102	330	480*	628	744*	8346
LDH (IU/mL)	199	200	222	186	179	257
sIL-2R (U/mL)	868	765	1425	1877	1887	6106
CD4 (%)	43	48	52	55	56	79
CD25 (%)	14	22	32	45	44	75
CCR4 (%)	14	25	38*	50	58**	76
CD7 (%)	63	65	70	47	50	11
CD26 (%)	43	41	41*	28	22**	7

Statistically significant ($P < 0.05$) between * and ** in PVL, infected CD4 cell number, CCR4 (%) and CD26 (%)

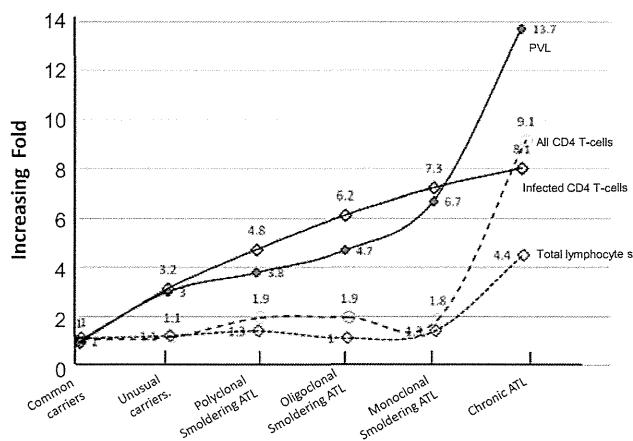HTLV-1-infected CD4 T-cell number/1 μL p-blood = total Ly counts × CD4%/100 × PVL (%/100)

Fig. 3 The difference in the line-graph patterns between HTLV-1-infected and uninfected cells. PVL and infected CD4 T-cell counts gradually increased from the early carrier stage to the last stage of smoldering, while the HTLV-1-uninfected cell population was stable, indicating that the discrepancy was explained by the infected leukemic clonal expansion alone

occult ATL (SM) cells are now controversial. One of the reasons for this is thought to be the difficulty in identifying SM cells. Therefore, to overcome these problematic issues, a dot-plot graph for CD26 versus CD25 and a four-color staining method were applied. The dots of CD26 and CD25 were mainly clustered into two areas (Fig. 5): 11 of monoclonal SM were clustered into a solid line circle, while oligoclonal and polyclonal SM were widely distributed. On the other hand, carriers were compactly clustered within the broken line circle. This indicates that also the 3 SM subtypes are not always homogeneous in biological character. Since the CD25 versus CD26 dot graph only
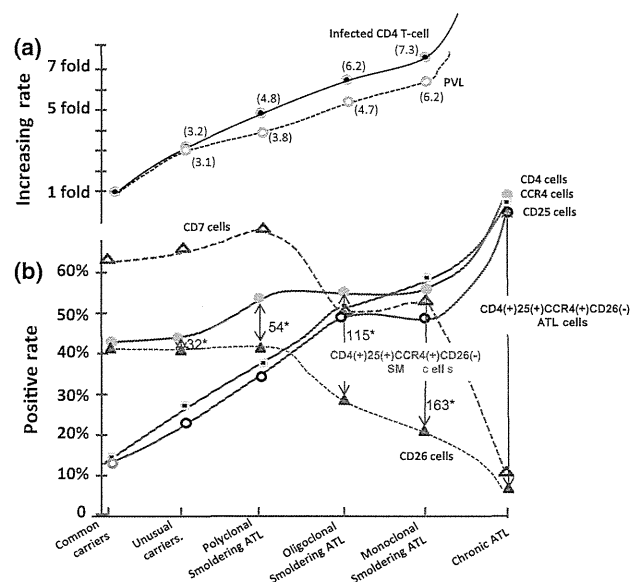

Fig. 4 The rate of change in each ATL-related biomarker. There were two major types of curves; a concurrent increasing type with tumor burden and a decreasing type with aberrant down-regulation. **a** The increasing rate (fold) relative to 102 HTLV-1-infected CD4 T-cells in 1 μL of blood. **b** Comparison of the positive rates in each disease state equivalent to P-1 (common carriers), P-2 (dense smears), P-3 (oligoclonal), and P-4 and P-5 corresponding to the monoclonal phase. *Asterisks* represent predicted CD4$^{(+)}$CD26$^{(-)}$ cell number equivalent to a major clonal expansion representing the absolute increased tumor burden

hinted at the heterogeneity of SM, we examined the clinico-oncological role of CD26 using 3 parameters of HTLV-1-infected cell counts, the CD26/CD25 ratio and SBH features. As shown in Fig. 6, the number of HTLV-1-infected CD4 T-cells was closely correlated to the ratio of
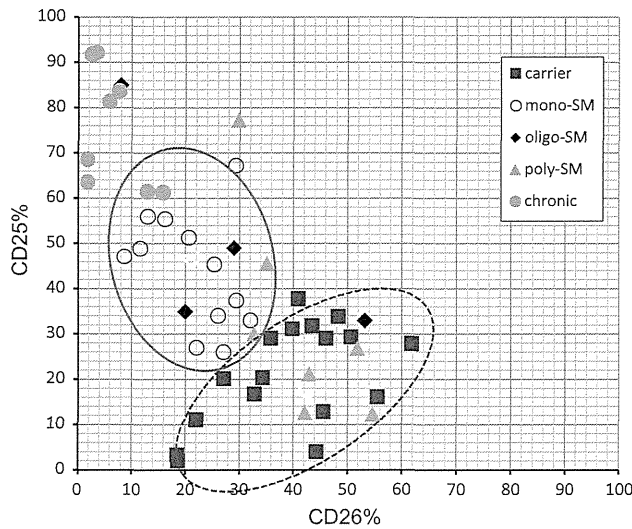
Fig. 5 CD26 versus CD25 twin dot graph, showing that there were two hot areas. The cells clustering in the same area have uniform bio-characteristics. Monoclonal SM was concentrated in the same area, but polyclonal SM and carriers were distributed sparsely and widely. *Red squares within dotted line circle* 3SM subtypes were scattered into both circles

CD26/CD25 ($R^2 = 0.6586$), and the clustering patterns were characteristic.

Samples with monoclonal band were mainly clustered in a high area within 0 to <1 of the X-axis. Most other samples were widely distributed in a area of around 1.00–11.00 of the Y-axis. Thus, the CD26/CD25 ratio represents the degree of advance in the leukemic process, comparable to the growing level of an ATL cell clone. Actually, this was demonstrated to be an indicator of a monoclonal band using a distribution graph and a receiver operating curve (ROC). That is, as shown in Fig. 7a, the ratios of CD26/CD25 were clearly separated by the presence or absence of a monoclonal band. The ROC analysis gave a high area under the curve (AUC) of 0.90, and sensitivity, specificity, and positive and negative predictive values were 87.0, 83.0, 80.0 and 89.0%, respectively (Fig. 7b), when the ratio of cutoff value (COV) was 1.04. The detective test performances of CD26 and CD7 alone for a monoclonal band were 0.82 and 0.81 AUC, respectively. This simple predictive method as an alternative to the SBH test, which is time- and labor-consuming, may be practically useful.
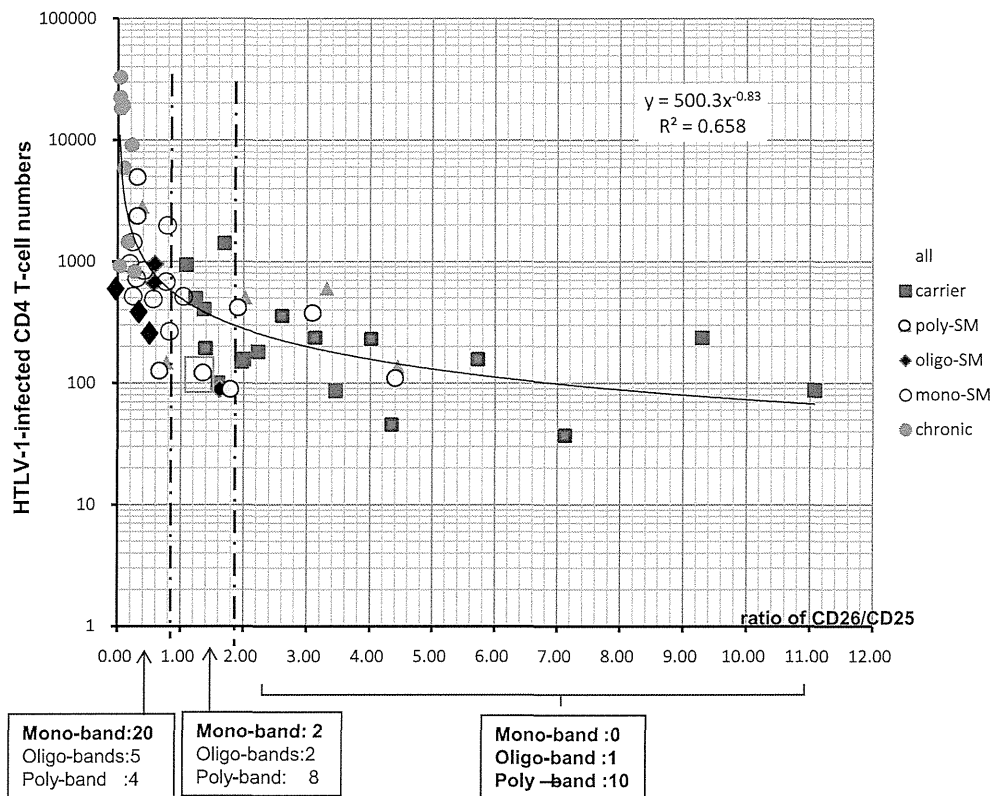

Fig. 6 Three parameter "twin dot-plot graph" between HTLV-1-infected CD4 T-cell numbers and the ratio of CD25/CD26, and the carrier or disease subtypes. Samples with the same band pattern showed a tendency to gather in the same areas bordered by the CD25/

26 ratio lines, such as most samples with monoclonal band (momo-band) in an area within 0.00–1.00 of the X-axis, and most samples with smears (poly-band) in an area within 1.00–11.00 of the X-axis

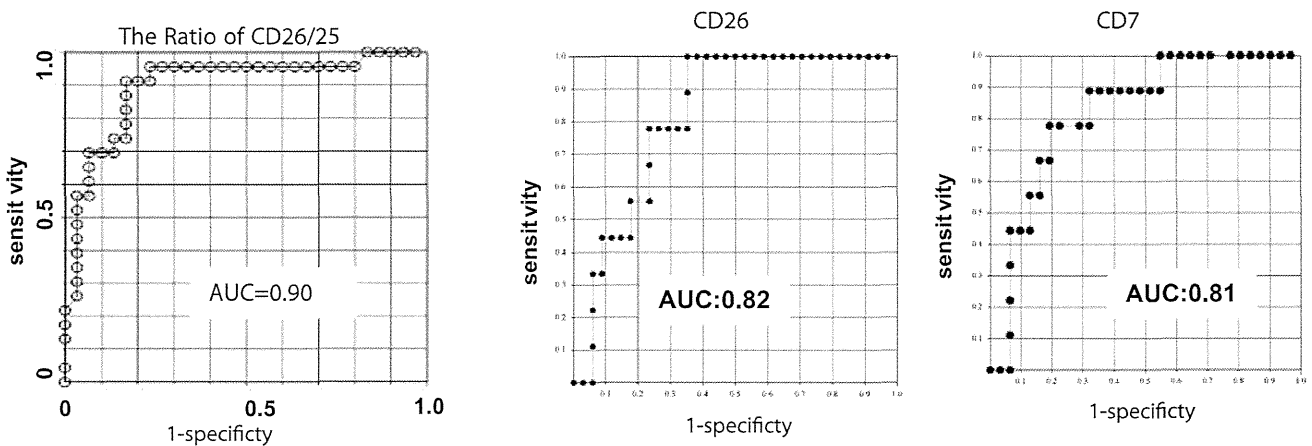**(A)** Distribution graph
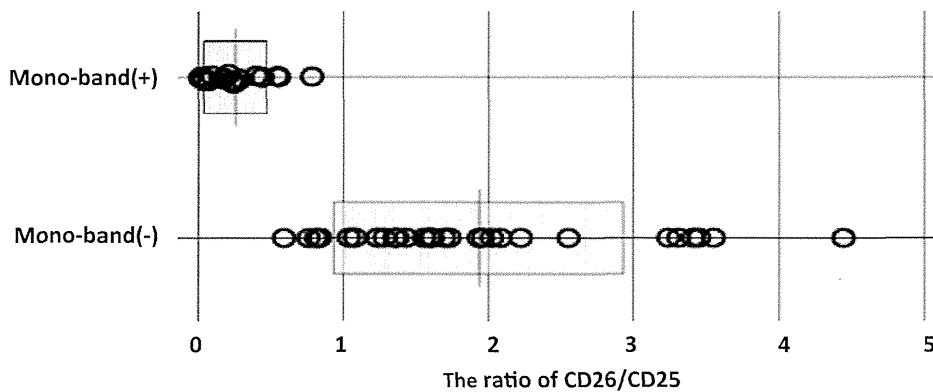
(B) ROCAnalysis

Fig. 7 **a** The distribution graph of each sample with or without a monoclonal band. The median values of mono-band (+) or (−) were 0.32 and 1.98. **b** The receiver operating curve (ROC) analysis

Correlation between down-regulation of CD26 antigens and SBH features

It is known that CD7 and CD26 antigens are lost in chronic and acute ATL cells. The present study revealed that the loss of CD26 antigens was initiated early in the pattern-2 or -3 stages. To confirm whether $CD4^{(+)}$ $CD25^{(+)}$ $CCR4^{(+)}$ cells were concurrently expressed, a four-color staining flow cytometric method for CD4, CD25, CCR4 and CD26 was used (Fig. 8). $CD4^{(+)}$ $CD25^{(+)}$ $CCR4^{(+)}$ cells (P1 square) were 1% or less, of which 75% (0.3% of total CD4 cells) were CD26 negative and 25% were CD26 positive in a healthy individual seronegative for HTLV-1 (case 1). That is, the $CD25^{(-)}/CD26^{(+)}$ ratio was 3.0. On the other hand, the ratio in common carriers and SM subtypes was about 3.0–10.0 (cases 2–4) and 10 or more (cases 5–9), respectively. This phenomenon regarding the loss of CD26 antigens was observed in other ATL cells [20].

Discussion

More than 35 years have passed since ATL was found and HTLV-1 was identified as its causative virus several years later. After that, a better molecular understanding of ATL pathology has been advancing. However, at the forefront of clinical practice, many problematic issues, such as a correct diagnosis of smoldering ATL, discrimination from unusual carriers with a percentage of ATL-like cells and promising therapeutic strategies, remain unclear. Recently, understanding of ATL pathology has deepened, but there is no point of contact between clinical and molecular aspects.

The results of the present study revealed that SM was heterogeneous in clonally related SBH features (mainly clone size) and lymphocyte subset profiles. We here designated such cases as monoclonal, oligoclonal and polyclonal smoldering (SM) subtypes. Although there was no difference in clinical manifestations, increase of only
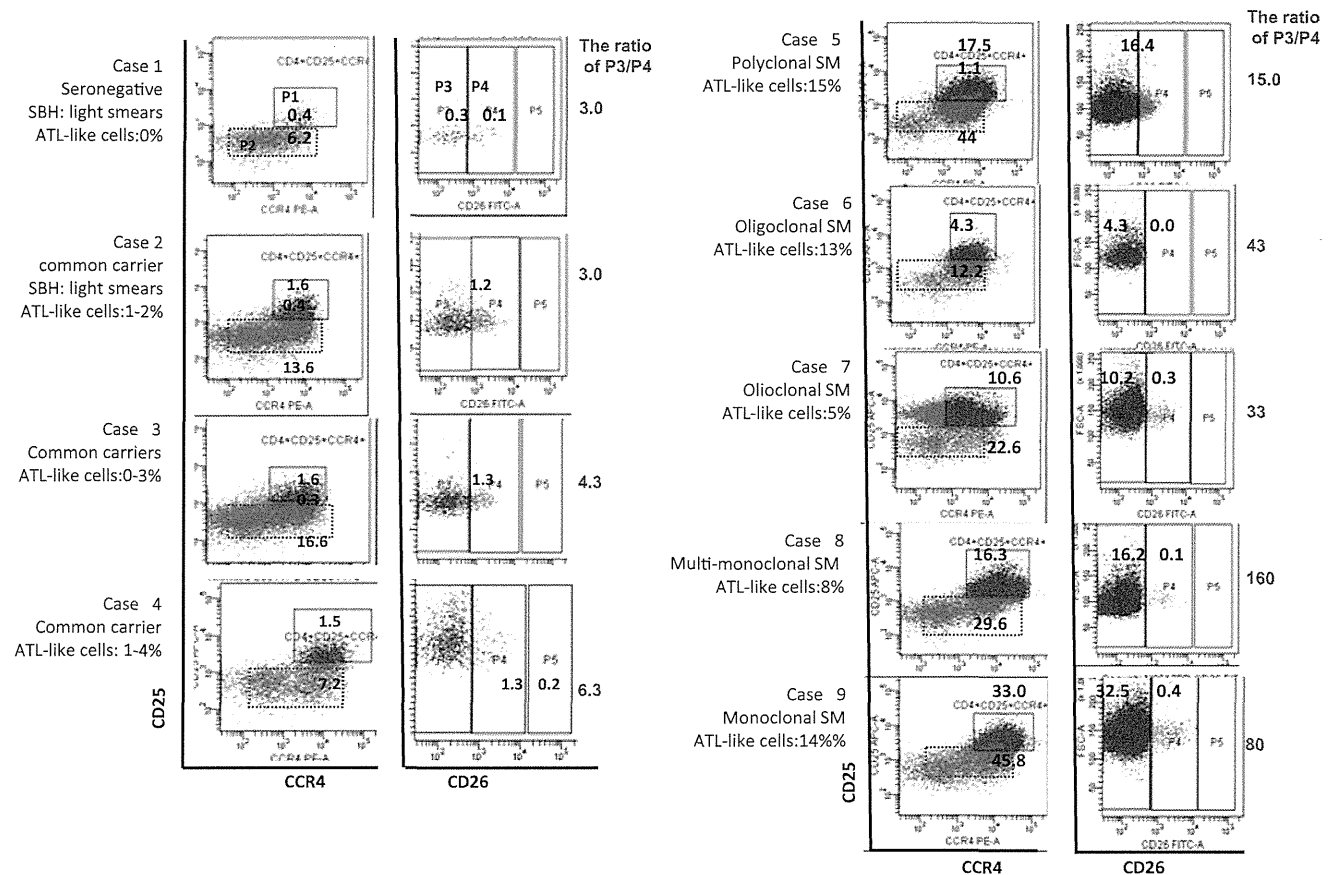
Fig. 8 Four-color flow cytometry for CD4, CD25, CCR4 and CD26. After CD4 gating, gating-CD4 T-cells were developed into a cytograph (a CCR4 = X-axis, and CD25 = Y-axis), and then P1 gating cells were developed into a cytograph (b CD26 down-regulation positive (*blue* P3 area meaning loss of CD26 antigen) or negative (*red* P4 area)

HTLV-1 provirus-carrying cells with a phenotype of $CD4^{+}CD25^{+}CCR4^{+}CD26^{-}$ was characteristic, regardless of the stable lymphocyte counts. Moreover, the ratio of CD26/CD25 was defined to be useful as an indicator of the grade of advance into ATL. Such findings were observed partially in unusual carriers with oligoclonal bands. These suggest that the expansion of leukemic clone begins in the unusual carrier stage and reaches large clone detected by SBH analysis in the SM stage. Thus, continuous changes of all ATL-related biomarkers would be explained by growing leukemic clonal cell population [15]. This is easily understood by a diagram shown in Figs. 3 and 4, which was derived from the increase of absolute CD4 T-cells infected by HTLV-1. The SM period seems to oncologically mean one of the turning points for multi-step leukemogenesis of ATL.

Now, it is interesting to develop such a subtype manifestations. Although clinical over-diagnosis cannot be completely neglected, there are in fact such cases with a highly dense smear for Eco-R1 genomic fragments and internal bands for Pst-1 genomic fragments, like the case in Fig. 2. As a possibility, a cluster of small clones may work co-operatively to develop SM manifestations. Subsequently, this appears to give rise to frequently multiclonal ATL and genomic diversity of leukemic clones [16].

Another interest is the behavior of CCR4, CD7 and CD26. So far, little is known about CD26 associated with ATL pathology. CD26/dipeptide peptidase IV (DPPIV), which is an antigenic enzyme expressed on the surface of most cell types, suppresses the development of cancer and tumors. CD26 plays an important role in tumor biology, and is useful as a marker for various cancers [17–19]. Now, why would down-regulation of CD26 first occur? The down-regulation preceding the increase in HTLV-1-infected CD4 T-cells may be indispensable to an environment for growing immature ATL cells. On the other hand, down-regulation of CD7, a glycoprotein member of the immunoglobulin (Ig) superfamily, is also one of the most commonly seen antigenic aberrations in T-lymphoproliferative disorders, but there is no specificity for malignant cell types, including a variety of reactive conditions [20, 21]. The changes in expression of CCR4 and CD26 may be the results of transformation.

Furthermore, to reveal other roles of CD26, statistical methodology and a dot plot were used. Consequently, two twin dot-plot graphs of CD25 versus CD26, and HTLV-1-CD4 T-cell number versus the ratio of CD26/CD25 revealed that the ratio of CD26/CD25 is useful as a surrogate marker for the prediction of the provirus clonal status. When the COV of the ratio is 1.04, the diagnostic validity is 87.0% in sensitivity and 83% in specificity. However, the ratio of CD26/CD25 in polyclonal SM was widely distributed, indicating that the polyclonal SM was distinctive from the other two subtypes of oligo- and monoclonal SM. This simple predictive method, alternative to the SBH test which is time- and labor-consuming, may be practically useful for screening in rapid turn-around test or epidemiological mass test.

Finally, using four-color flow cytometry, the usefulness of the CD26 antigen monitor was verified in actual cases. The antigen status was evaluated as the ratio of $CD26^{(-)}$ versus $CD26^{(+)}$ within a fraction of $CD4^{(+)}CD25^{(+)}CCR4^{(+)}$ cells. The ratio went up with increases in the $CD4^{(+)}CD25^{(+)}$ $CCR4^{(+)}$ cell populations, reflecting occult ATL cells or transforming cells. In the present study, the border line of the ratio between carriers and patients with SM was about 10.0. That is, if the $CD26^{(-)}$:$CD26^{(+)}$ ratio is 10 or more, the case is predicted to be smoldering ATL.

Taken together, the present study showed that smoldering ATL was heterogeneous in a clone size and the quality of its constituent cells. This suggests that it is relevant to classify the current smoldering ATL into two subtypes of SM with or without a monoclonal band. Indeterminate HTLV-1 carriers and smoldering ATL can be discriminated according to the patterns of SBH densitometer images and CD26 antigen status. Moreover, CD26 is expected to be used as a novel biomarker for prediction of clonal bands and discrimination of carriers or SM subtypes. CD26 may become one of the central molecules in understanding the early leukemogenic process.

Conflict of interest The authors have no conflict of interest.

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MICROBIOLOGY

Species tropism of HIV-1 modulated by viral accessory proteins

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Human immunodeficiency virus type 1 (HIV-1) is tropic and pathogenic only for humans, and does not replicate in macaque monkeys routinely used for experimental infections. This specially narrow host range (species tropism) has impeded much the progress of HIV-1/ acquired immunodeficiency syndrome (AIDS) basic research. Extensive studies on the underlying mechanism have revealed that Vif, one of viral accessory proteins, is critical for the HIV-1 species tropism in addition to Gag-capsid protein. Another auxiliary protein Vpu also has been demonstrated to affect this HIV-1 property. In this review, we focus on functional interactions of these HIV-1 proteins and species specific-restriction factors. In addition, we describe an evolutional viewpoint that is relevant to the species tropism of HIV-1 controlled by the accessory proteins.

Keywords: HIV-1, species tropism, accessory protein, Vif, Vpu

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is strictly adapted to humans, and cause disease-inducing persistent infection only in humans (Nomaguchi et al., 2008). This property is unique among primate immunodeficiency viruses, and represent one of the most evident and important viral characteristics to understand the biology/molecular biology of HIV-1. Of numerous primate immunodeficiency viruses so far identified (Kirchhoff, 2009; Sharp and Hahn, 2011), HIV-1 with an extremely limited host range exhibits exceptionally high replication ability, transmissibility, and pathogenicity in sensitive host humans. For basic HIV-1 researchers, it would be final goal to realize the basis/mechanism underlying these properties by experimental approaches.

Primate immunodeficiency viruses can be divided into three groups based on their genome structure in the central regions (Kirchhoff, 2009; Fujita et al., 2010; Sharp and Hahn, 2011). While viruses of HIV-1 type contain *vpr* and *vpu* genes, viruses of HIV-2 type carry *vpx* and *vpr* genes in tandem (**Figure 1**). The other simian immunodeficiency viruses (SIVs), the prototype virus, have only the *vpr* gene in the corresponding genomic region. HIV-1 is believed to emerge from the prototype virus via SIV-mon/mus/gsn (isolated from the mona, mustached, and greater spot-nosed monkeys), SIVcpz (isolated from the chimpanzees), and SIVgor (isolated from the gorilla) through mutational and recombinational events. SIVmon/mus/gsn is known to recombine with SIVrcm (isolated from the red-capped mangabey monkey) to generate SIVcpz (for genome structures, see, **Figure 1**). SIVcpz served as parental virus for HIV-1 (M and N) and SIVgor (and finally for HIV-1 P).

The biological and molecular biological bases for species tropism of HIV-1 should reside in the above outlined evolutional

processes. In particular, the so-called accessory proteins encoded by extra genes are important. Each virus group has a unique set of the accessory proteins in terms of their combinations and of their activities. Therefore, studies on viral accessory proteins are also meaningful for understanding viral evolution by cross-species transmission.

VIRAL AND CELLULAR DETERMINANTS FOR HIV-1 SPECIES TROPISM

Our early studies have already suggested the possible viral determinants and viral replication stage involved in the HIV-1 species tropism described above (Shibata et al., 1991, 1995; Shibata and Adachi, 1992). By the use of numerous chimeric molecular clones between HIV-1 and dual-tropic (tropic for human and monkey cells) SIVmac (isolated from the macaque monkey), we have claimed in essence, together with a work on the cyclophilin A (CypA; Dorfman and Gottlinger, 1996), that Gag-capsid (CA) and a viral protein(s) encoded by the central genomic region of HIV-1 are the determinants. We also have showed that HIV-1 is replication-incompetent in monkey cells because a certain replication step(s) before/during reverse transcription, other than the viral entry into cells, does not proceed normally. Subsequent extensive studies by us and others have clearly indicated that the interactions of Gag-CA/CypA, Gag-CA/tripartite motif (TRIM) proteins, and Vif/apolipoprotein B mRNA-editing enzyme-catalytic (APOBEC) proteins are major determinants for the HIV-1 species tropism (Nomaguchi et al., 2008, 2011; Nakayama and Shioda, 2012; Sakuma and Takeuchi, 2012) as summarized in **Table 1**. Gag-CA, CypA, and TRIM proteins have been described in detail in two articles in the Research Topic of this journal (Nakayama and Shioda, 2012; Sakuma and Takeuchi, 2012).
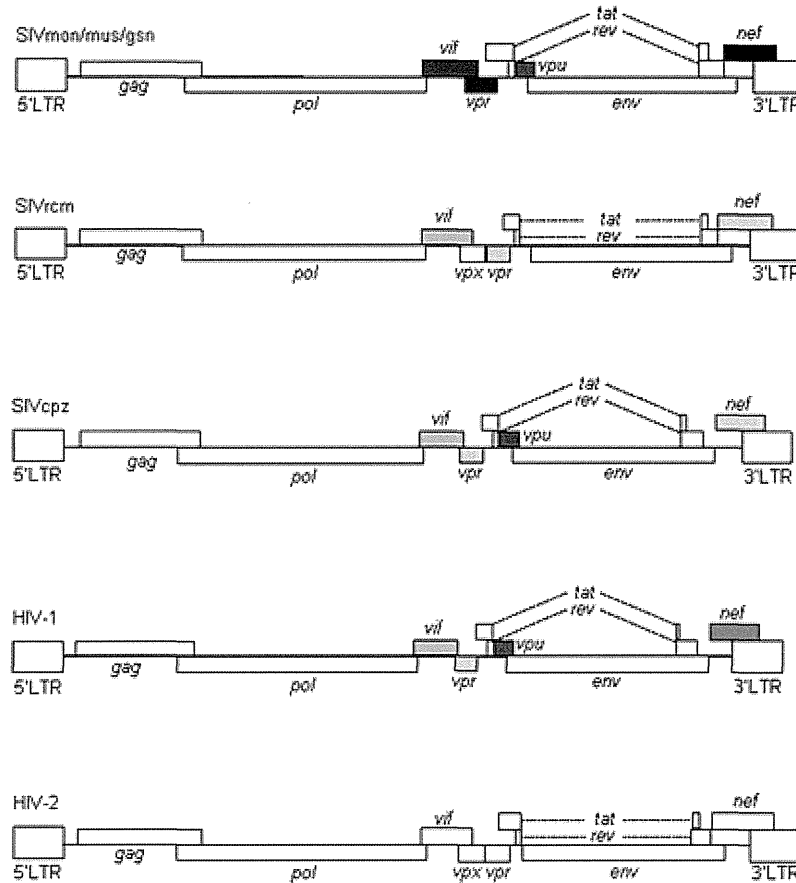
FIGURE 1 | Genome organization of primate immunodeficiency viruses. Various proviral genomes are schematically shown. As indicated by colored boxes, the *vpr* and *vpu* genes of SIVcpz/HIV-1 came from those of SIVrcm and SIVmon/mus/gsn, respectively. Also, the *vif* genes of SIVcpz/HIV-1 originated from that of SIVrcm. In addition, as shown by colored boxes, HIV-1 *nef* gene is similar to but distinct from SIVcpz *nef* gene. HIV-1 *nef* gene is different from those of SIVmon/mus/gsn, SIVrcm, and HIV-2 as indicated. For virus designations, see text.

Table 1 | Major viral and cellular determinants for HIV-1 species tropism.

Virus	Cell	Viral replication step affected
Gag-CA	CypA	
Gag-CA	TRIM5α	Uncoating (early phase)
Gag-CA	TRIMCyp	Uncoating (early phase)
Vif	APOBEC3G	Reverse transcription (early phase)
	APOBEC3F	Reverse transcription (early phase)
Vpu	Tetherin/BST-2	Virion release (late phase)

For details, see references (Nakayama and Shioda, 2012; Sakuma and Takeuchi, 2012) for Gag-CA, and Figures 3 and 4 for Vif/Vpu.

ACCESSORY PROTEINS OF PRIMATE IMMUNODEFICIENCY VIRUSES

All primate immunodeficiency viruses encode a number of extra proteins (Vif, Vpx, Vpr, Vpu, and Nef) in addition to regulatory (Tat and Rev) and structural (Gag, Pol, and Env) proteins (**Figure 1**). Structural proteins are common to all retroviruses, but the regulatory and accessory proteins are unique to the complex primate lentiviruses and not found in the other simple mammalian retroviruses. Regulatory Tat and Rev proteins are trans-activators for transcription and for the expression of late viral proteins, respectively. While the regulatory and structural proteins are essential for viral replication, the extra proteins, unfairly generically called "accessory," are dispensable under certain circumstances. However, in some cells, some of them are essential and the others are quite critical/important for optimal viral replication as illustrated for ΔVif and ΔVpu viruses (viruses that lack Vif or Vpu) in **Figure 2**. Another point to be mentioned here is relating to Vpr/Vpx proteins. Although Vpr and Vpx are genetically very similar (Khamsri et al., 2006), some primate immunodeficiency viruses bear two of them as described above (Fujita et al., 2010). Furthermore, the other viruses have Vpr only. What about the functional relationship of the two proteins? At present, the function of Vpr/Vpx is least well understood relative to that of the other accessory proteins (Malim and Emerman, 2008; Fujita et al., 2010). **Table 2** summarizes the important information regarding these accessory proteins so
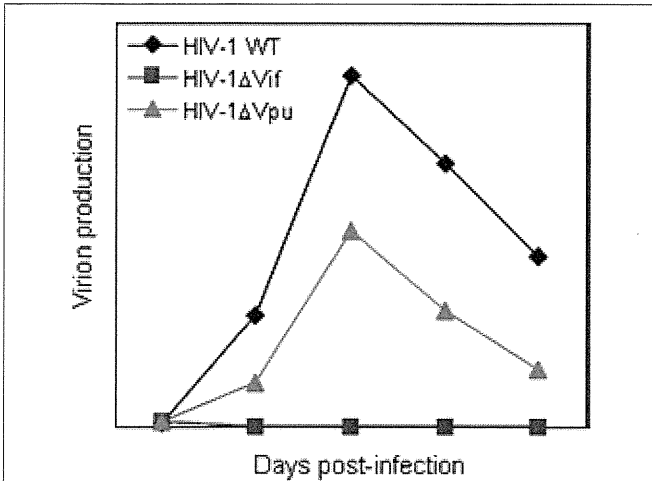
FIGURE 2 | A schema of replication kinetics by HIV-1 wild-type and mutant viruses. Viral growth properties in cells are illustrated based on numerous infection experiments in our laboratory. WT, wild-type.

Table 2 | Accessory proteins of primate immunodeficiency viruses.

Viral Proteins	Major functions for viral replication reported so far
Vif	Neutralize APOBEC3G/F. Essential for viral replication in natural target cells.
Vpx	Degrade SAMHD1/APOBEC3A. Critical for viral replication in natural target cells.
Vpr	Important for viral replication in macrophages (HIV-1).
Vpu	Down-regulate Tetherin/BST-2. Important for viral replication in CD4-positive cells.
Nef	Down-regulate cell surface molecules (CD4, MHC-I etc.).

far reported. In total, it is fairly reasonable to believe that the accessory proteins are regulators to optimize viral replication and persistence *in vivo* thereby enhancing viral transmission between individuals.
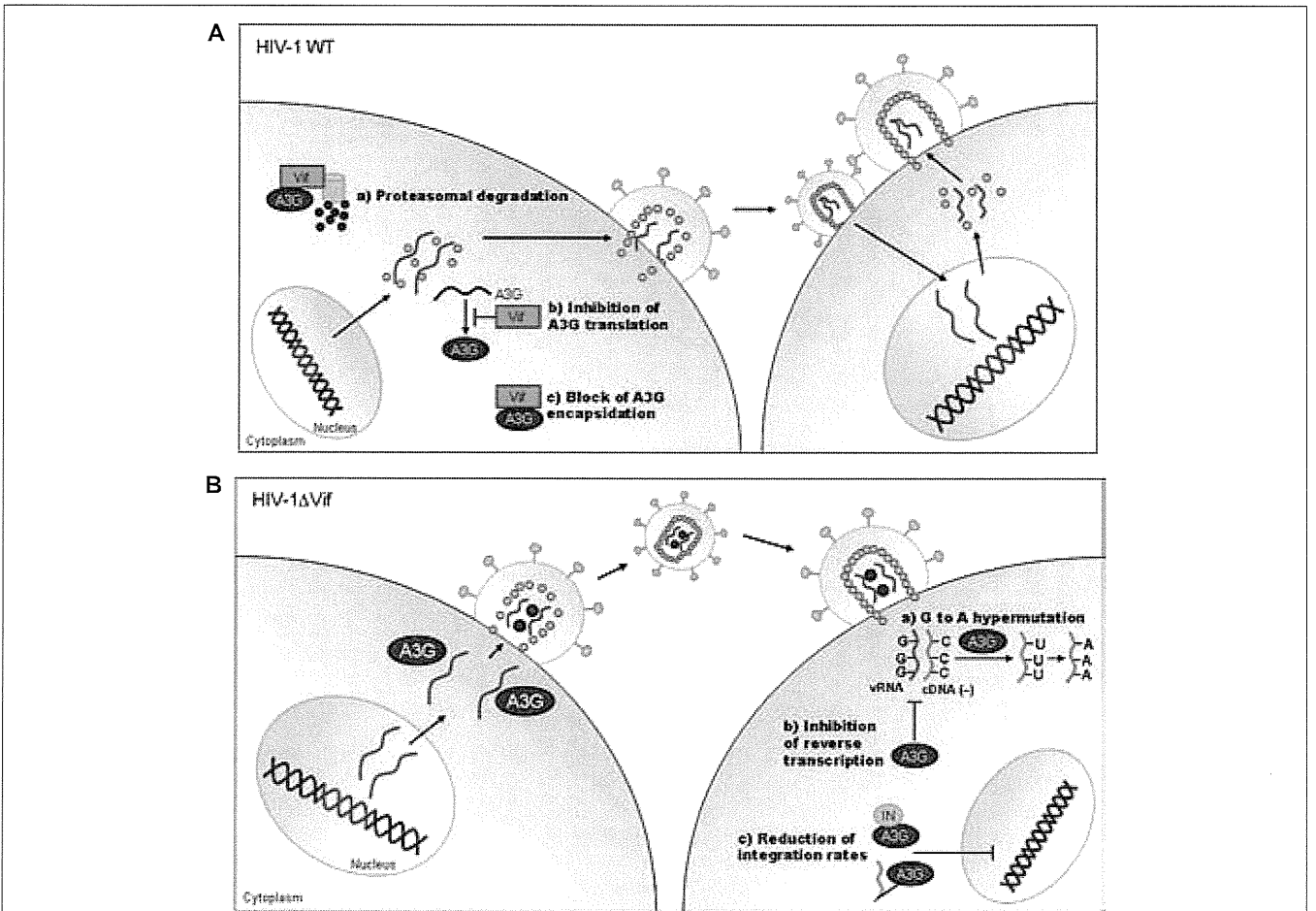

FIGURE 3 | HIV-1 replication and APOBEC3G. On the basis of results reported so far, the action mechanism of Vif is depicted. Replication process for wild-type (WT) and ΔVif mutant viruses are schematically shown on the basis of previously reported review articles (Holmes et al., 2007; Huthoff and Towers, 2008; Strebel et al., 2009). A3G, APOBEC3G; IN, viral integrase protein.

Vif AND Vpu PROTEINS

Vif protein is essential for viral replication in natural target cells such as CD4-positve lymphocytes and macrophages. Recent identification of its cellular object for attack (Sheehy et al., 2002) has clearly revealed the biological and biochemical bases for the growth property of ΔVif virus in natural target cells. This finding (identification of a family of APOBEC3 proteins, cellular cytidine deaminases, as potent inhibitors of HIV-1 replication in primary cells) has also contributed much to establish the concept of "the restriction factor" to well understand virus–cell interaction (Malim and Emerman, 2008; Sato et al., 2012). Of the APOBEC3 family proteins, APOBEC3G and APOBEC3F (Kitamura et al., 2011) strongly inhibit viral replication in the absence of Vif (Figure 3). Although HIV-1 Vif can abrogate the activities of human APOBEC3, it cannot do so against monkey APOBEC3. In contrast, SIVmac Vif can neutralize the antiviral activity of APOBEC3 of both origins. Finally, it has been demonstrated that Vif and APOBEC3 are the major determinants for the HIV-1 species tropism by constructing macaque-tropic HIV-1 (HIV-1mt) and monitoring the HIV-1mt growth

property in various genetic contexts of macaques (Hatziioannou et al., 2006, 2009; Kamada et al., 2006; Igarashi et al., 2007; Thippeshappa et al., 2011).

Vpu protein, unique to viruses of the HIV-1 group (Figure 1), modulates viral replication in human CD4-positive cell lines and primary cells. Mutant HIV-1 without Vpu (ΔVpu virus) grows poorly relative to wild-type virus. Recently, a cellular protein named Tetherin (also called BST-2) has been identified as a restriction factor against HIV-1 and is antagonized by Vpu (Neil et al., 2008; Van Damme et al., 2008). Vpu down-regulates the Tetherin from cell surface, and thereby promotes extracellular production of progeny virions (Malim and Emerman, 2008; Arias et al., 2011; Sato et al., 2012). The baseline mechanism for this action of Vpu is well studied as shown in Figure 4. Here, it must be attentive that the anti-Tetherin activity of Vpu is host species-specific as observed for Vif. HIV-1 Vpu acts against human but not (or poorly) macaque Tetherins (Sauter et al., 2009, 2010). Although the biological effect of Vpu is much milder than that of Vif as judged by the growth kinetics of mutant viruses (Figure 2), Vpu may be critical for interspecies transmission

FIGURE 4 | HIV-1 replication and Tetherin. On the basis of results reported so far, the action mechanism of Vpu is depicted. Replication process for wild-type (WT) and ΔVpu mutant viruses are schematically shown on the basis of previously reported review articles (Tokarev et al., 2009; Douglas et al., 2010; Evans et al., 2010). TGN, trans-Golgi network.