24 Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol* 1998; **72**: 8150–7.
25 Kobayashi S, Nakano K, Watanabe E *et al.* CADM1 expression and stepwise downregulation of CD7 are closely associated with clonal expansion of HTLV-I-infected cells in adult T-cell leukemia/lymphoma. *Clin Cancer Res* 2014; **20**: 2851–61.
26 Sasaki D, Imaizumi Y, Hasegawa H *et al.* Overexpression of enhancer of zeste homolog 2 with trimethylation of lysine 27 on histone H3 in adult T-cell leukemia/lymphoma as a target for epigenetic therapy. *Haematologica* 2011; **96**: 712–9.
27 Smith MR, Greene WC. Identification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes. *Genes Dev* 1990; **4**: 1875–85.
28 Tsuji T, Sheehy N, Gautier VW, Hayakawa H, Sawa H, Hall WW. The nuclear import of the human T lymphotropic virus type I (HTLV-1) tax protein is carrier- and energy-independent. *J Biol Chem* 2007; **282**: 13875–83.
29 Lauth M, Bergström A, Shimokawa T, Toftgård R. Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. *Proc Natl Acad Sci USA* 2007; **104**: 8455–60.
30 Blair HJ, Tompson S, Liu YN *et al.* Evc2 is a positive modulator of Hedgehog signalling that interacts with Evc at the cilia membrane and is also found in the nucleus. *BMC Biol* 2011; **9**: 14.
31 Ego T, Ariumi Y, Shimotohno K. The interaction of HTLV-1 Tax with HDAC1 negatively regulates the viral gene expression. *Oncogene* 2002; **21**: 7241–6.
32 Kamoi K, Yamamoto K, Misawa A *et al.* SUV39H1 interacts with HTLV-1 Tax and abrogates Tax transactivation of HTLV-1 LTR. *Retrovirology* 2006; **3**: 5.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. HTLV-1 *HBZ* does not affect EVC expression.

Fig. S2. EVC knockdown reduces ATL cell proliferation.

Methods S1. Including: details of clinical samples; and primer sequences used in the present study.

bjh research paper

Japan Clinical Oncology Group (JCOG) prognostic index and characterization of long-term survivors of aggressive adult T-cell leukaemia-lymphoma (JCOG0902A)

Takuya Fukushima,[1] Shogo Nomura,[2] Masanori Shimoyama,[3] Taro Shibata,[2] Yoshitaka Imaizumi,[4] Yoshiyuki Moriuchi,[5] Takeaki Tomoyose,[6] Kimiharu Uozumi,[7] Yukio Kobayashi,[8] Noriyasu Fukushima,[9] Atae Utsunomiya,[10] Mitsutoshi Tara,[11] Kisato Nosaka,[12] Michihiro Hidaka,[13] Naokuni Uike,[14] Shinichiro Yoshida,[15] Kazuo Tamura,[16] Kenji Ishitsuka,[16] Mitsutoshi Kurosawa,[17] Masanobu Nakata,[18] Haruhiko Fukuda,[2] Tomomitsu Hotta,[3] Kensei Tobinai[8] and Kunihiro Tsukasaki[19]

[1]Laboratory of Haematoimmunology, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Nishihara-cho, [2]JCOG Data Centre, Multi-institutional Clinical Trial Support Centre, National Cancer Centre, Tokyo, [3]Multicentre-institutional Clinical Trial Support Centre, National Cancer Centre, Tokyo, [4]Department of Haematology, Atomic Bomb Disease and Hibakusha Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, [5]Department of Haematology, Sasebo City General Hospital, Sasebo, [6]Division of Endocrinology, Diabetes and Metabolism, Haematology, Rheumatology (Second Department of Internal Medicine), Graduate School of Medicine, University of the Ryukyus, Nishihara-cho, [7]Department of Haematology and Immunology, Kagoshima University Hospital, Kagoshima, [8]Department of Haematology, National Cancer Centre Hospital, Tokyo, [9]Division of Haematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga, [10]Department of Haematology, Imamura Bun-in Hospital, Kagoshima, [11]Department of Haematology, Kagoshima City Hospital, Kagoshima, [12]Department of Haematology, Kumamoto University of Medicine, Kumamoto, [13]Department of Internal Medicine, National Hospital Organization Kumamoto Medical Centre, Kumamoto, [14]Department of

Summary

This study evaluated the clinical features of 276 patients with aggressive adult T-cell leukaemia-lymphoma (ATL) in 3 Japan Clinical Oncology Group (JCOG) trials. We assessed the long-term survivors who survived >5 years and constructed a prognostic index (PI), named the JCOG-PI, based on covariates obtained by Cox regression analysis. The median survival time (MST) of the entire cohort was 11 months. In 37 patients who survived >5 years, no disease-related deaths in 10 patients with lymphoma-type were observed in contrast to the 10 ATL-related deaths in other types. In multivariate analysis of 193 patients, the JCOG-PI based on corrected calcium levels and performance status identified moderate and high risk groups with an MST of 14 and 8 months respectively (hazard ratio, 1·926). The JCOG-PI was reproducible in an external validation. Patients with lymphoma-type who survived >5 years might have been cured. The JCOG-PI is valuable for identifying patients with extremely poor prognosis and will be useful for the design of future trials combining new drugs or investigational treatment strategies.

Keywords: adult T-cell leukaemia-lymphoma, Japan Clinical Oncology Group trials, long-term survivors, prognostic index.

First published online 14 June 2014
doi:10.1111/bjh.12962

Haematology, National Hospital Organization Kyushu Cancer Centre, Fukuoka, [15]*Department of Haematology, National Hospital Organization Nagasaki Medical Centre, Omura,* [16]*Department of Medicine, Division of Medical Oncology, Haematology and Infectious Diseases, Fukuoka University, Fukuoka,* [17]*Department of Haematology, National Hospital Organization Hokkaido Cancer Centre, Sapporo,* [18]*Department of Haematology, Sapporo Hokuyu Hospital, Sapporo, and* [19]*Department of Haematology National Cancer Centre Hospital East, Kashiwa, Japan*

Received 14 January 2014; accepted for publication 10 April 2014
Correspondence: Takuya Fukushima, MD, Laboratory of Haematoimmunology, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan.
E-mail: fukutaku@med.u-ryukyu.ac.jp

Adult T-cell leukaemia-lymphoma (ATL) is a distinct peripheral T-lymphocytic malignancy associated with human T-cell lymphotropic virus type I (HTLV-1) (Uchiyama *et al*, 1977; Poiesz *et al*, 1980; Hinuma *et al*, 1981; Miyoshi *et al*, 1981; Yoshida *et al*, 1982). Classification of clinical subtypes into acute, lymphoma, chronic and smouldering was proposed based on prognostic factors, clinical features and the natural history of the disease (Shimoyama, 1991). Patients with aggressive ATL (i.e., acute, lymphoma and unfavourable chronic types) have frequently been treated as a subtype of aggressive non-Hodgkin lymphoma (NHL), whereas those with indolent ATL (i.e., favourable chronic and smouldering types) have been managed as a subtype of chronic lymphoid leukaemia (Shimoyama, 1994; Tobinai & Watanabe, 2004). Aggressive ATL typically has a very poor prognosis compared with aggressive B-cell lymphomas, such as diffuse large B-cell lymphoma and peripheral T-cell lymphoma excluding ATL (The International Non-Hodgkin's Lymphoma Prognostic Factor Project's, 1993; Shimoyama, 1994; Gallamini *et al*, 2004; Watanabe *et al*, 2010). In the 1980's, patients with aggressive ATL were reported to have a median survival time (MST) of approximately 8 months, with a 2-year survival rate of <5% because of the multidrug-resistant phenotype of their malignant tumour cells, rapid proliferation of the tumour cells, a large tumour burden with multi-organ failure, hypercalcaemia, and/or frequent opportunistic infections (Lymphoma Study Group, 1991; Shimoyama, 1991, 1994; Tobinai & Watanabe, 2004).

The Japan Clinical Oncology Group (JCOG)-Lymphoma Study Group (LSG) has conducted consecutive clinical trials to improve the survival of patients with ATL. Earlier trials (JCOG7801, JCOG8101, and JCOG8701) revealed poor prognosis of ATL compared with other aggressive NHLs (Shimoyama *et al*, 1988; Tobinai *et al*, 1994). Furthermore, the disappointing results with conventional chemotherapies in the 1980s and the proposal for a subtype classification of ATL led us to conduct clinical trials with new agents that exclusively targeted aggressive ATL. The first phase II trial, JCOG9109 (1991–1993), evaluated combination chemotherapy with deoxycoformycin, an inhibitor of adenosine deaminase, which had been effective as a single agent against relapsed or refractory ATL (Tobinai *et al*, 1992). However, the results were disappointing with an MST of 7 months, similar to the findings of previous JCOG-LSG trials (Tsukasaki *et al*, 2003). The next phase II trial, JCOG9303 (1994–1996), evaluated the chemotherapy regimen VCAP-AMP-VECP (LSG15) against aggressive ATL. This dose-intensified multi-agent chemotherapy consisted of vincristine, cyclophosphamide, doxorubicin (DXR) and prednisone (PSL) for VCAP, DXR, ranimustine and PSL for AMP, and vindesine, etopside, carboplatin and PSL for VECP, supported by granulocyte colony-stimulating factor and intrathecal (IT) prophylaxis with methotrexate (MTX) and PSL. This phase II trial showed promising results, with complete remission (CR) and overall response rates of 36% and 81%, respectively, and an MST of 13 months at the expense of haematological and other toxicities (Yamada *et al*, 2001). Based on these results, we proceeded to the phase III trial JCOG9801 (1998–2003), which compared a modified VCAP-AMP-VECP regimen (shortened from 7 to 6 courses), to which cytarabine was added to the IT prophylaxis, *versus* CHOP (cyclophosphamide, DXR, vincristine and PSL)-14 supported by granulocyte

colony-stimulating factor and IT prophylaxis identical to the former regimen. The CR and 3-year overall survival (OS) were higher in the modified VCAP-AMP-VECP arm than in the CHOP-14 arm (40% vs. 25% and 24% vs. 13% respectively), suggesting that the former is a more effective regimen at the expense of greater toxicity for patients with newly diagnosed aggressive ATL (Tsukasaki *et al*, 2007).

Through these 3 JCOG trials for patients with aggressive ATL, the 5-year OS was improved, from 5% in the 1980's to 15% in the 1990s. To characterize the long-term survivors of aggressive ATL and to develop a new prognostic index (PI) for the disease, we performed a combined analysis (JCOG0902A) of all the patients enrolled in the 3 JCOG trials.

Methods

Study population

A total of 276 patients who were registered in the 3 JCOG trials described above were enrolled in this study (Yamada *et al*, 2001; Tsukasaki *et al*, 2003, 2007). Some patients did not receive anti-viral therapy using interferon-alpha and zidovudine because these drugs for ATL was not covered by the National Health Insurance in Japan. The eligibility criteria for the 3 JCOG trials were detailed in previous reports (Yamada *et al*, 2001; Tsukasaki *et al*, 2003, 2007). Briefly, patients were eligible to participate if they had aggressive ATL (i.e., acute, lymphoma, or unfavourable chronic type) with no prior chemotherapy, were aged 15–69 years and had preserved organ functions, no proven central nervous system (CNS) involvement and a performance status (PS) of 0–3 or 4 due to hypercalcaemia caused by ATL. The diagnosis of ATL was made based on seropositivity for HTLV-1 antibody and histologically and/or cytologically proven peripheral T-cell malignancy. Monoclonal integration of HTLV-1 provirus was analysed in 104 of 276 patients studied. Among these 104 patients, integration was detected in 100 patients and not detected in four patients.

The PI for the JCOG trials, which we refer to as the JCOG-PI, was constructed from the data of patients who participated in these trials (training set) and was then applied to an external validation set. The external validation set consisted of 136 patients who had not participated in prior JCOG studies but had received anthracycline-containing regimens as initial chemotherapy at three sites (Nagasaki University Hospital, Nagasaki Medical Centre, and Sasebo City General Hospital) under the remit of the JCOG-LSG. These patients were a subset of those from a previous retrospective study (Katsuya *et al*, 2012) and their OS and corrected calcium levels were reviewed.

Data and analysis sets

The endpoint of this study was OS, defined as the duration between registration to each JCOG trial and death from any cause or censored at the last follow up in living patients. For the validation data set, we substituted the date of treatment initiation for the date of registration.

Candidate covariates were sex, age, Eastern Cooperative Oncology Group (ECOG) PS, B symptoms, clinical stage, liver involvement, lactate dehydrogenase, blood urea nitrogen (BUN), corrected calcium levels, serum total protein, serum albumin, white blood cell count, total (normal and abnormal) lymphocyte count, neutrophil count and platelet count. We excluded the treatment regimen from the covariates because our aim was to create an index that could stratify the patients' prognosis and be applicable to future clinical trials evaluating various promising regimens. Cut-off values were determined clinically by dividing the continuous biological and laboratory test variables into no more than three categories. The data of 193 patients with a complete set of candidate covariates were used for the training set (Fig 1).

The protocol of this study was reviewed and approved by the JCOG Protocol Review Committee.

Statistical analysis

Patients who survived >5 years were categorized according to ATL subtype (acute, lymphoma or unfavourable chronic types). In addition, to evaluate the ATL-related death events for each subtype, a disease-specific mortality curve was estimated, for only those patients who survived >2 years, by means of a competing risks framework (Kalbfleisch & Prentice, 2002). The proportion of patients who survived >5 and >10 years was calculated to evaluate the association between long-term survival and CR (including CR unconfirmed) for initial treatment. The proportion of cases with

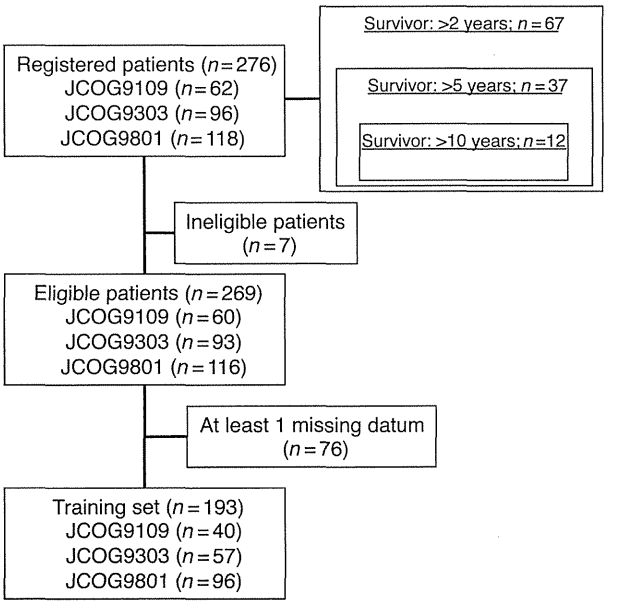

Fig 1. Patient disposition of the training set.

CNS involvement was compared among the JCOG trial regimens in an exploratory evaluation of the efficacy of prophylactic IT treatment. The prophylactic IT treatments against CNS involvement were: none in JCOG9109, MTX and PSL in JCOG9303, and MTX, cytarabine and PSL in both regimens in JCOG9801. Confidence intervals (CIs) for all the above proportions were computed using the Clopper–Pearson method (Clopper & Pearson, 1934).

Analyses for the development and validation of the JCOG-PI were performed according to a pre-specified analysis plan. The JCOG-PI consisted of risk groups that were developed using Cox's proportional hazards model. Before constructing the JCOG-PI, covariates with several definitions were selected for those with the smallest Akaike's Information Criteria (Akaike, 1973) on univariate analysis. Next, we verified the correlations between covariates to avoid multi-colinearity. Stepwise Cox regression analysis was then performed to identify unfavourable prognostic factors for constructing the JCOG-PI. The entry criterion was $P < 0{\cdot}20$ and the removal criterion was $P > 0{\cdot}15$.

The maximum number of risk group strata was set at three, based on the opinions of JCOG-LSG members who commented that too many strata were impractical for evaluating risk. The risk group was divided with patients equally distributed. The log-rank test was used to assess the discrepancy between the risk groups and the Kaplan–Meier method was applied to estimate OS.

All statistical analysis was performed using SAS Release 9·1 (SAS Institute, Inc, Cary, NC, USA). All reported P values are two-sided and $P < 0{\cdot}05$ was considered statistically significant.

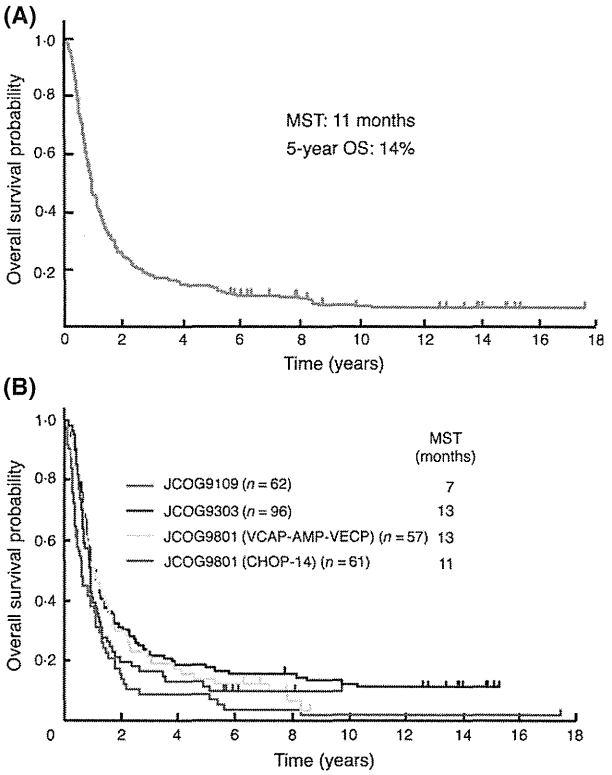

Fig 2. Overall survival (OS) of all registered patients in 3 Japan Clinical Oncology Group (JCOG) trials and according to treatment regimens. (A) OS of all 276 registered patients. Median survival time (MST) and the 5-year OS were 11 months and 14%, respectively.(B) OS according to different treatment regimens. MST was 7 months in JCOG9109, 13 months in JCOG9303, 13 months in VCAP-AMP-VECP of JCOG9801 and 11 months in CHOP-14 of JCOG9801.

Results

Patient characteristics

A total of 276 patients were registered in the 3 trials (JCOG9109, $n = 62$; JCOG9303, $n = 96$; and JCOG9801, $n = 118$) from 58 institutions in Japan. The MST and the 5-year OS of all patients were 11 months and 14% respectively (Fig 2A). The OS of each treatment regimen during the long follow up reconfirmed the findings of each original report (Fig 2B) (Yamada *et al*, 2001; Tsukasaki *et al*, 2003, 2007). Clinical characteristics are shown in Table I.

Long-term survivors according to subtype and initial response

The disease-specific mortality curve of patients who survived >2 years according to subtype is presented in Fig 3. Among the 37 patients (acute, $n = 22$; lymphoma, $n = 8$; unfavourable chronic, $n = 7$) who survived >5 years, there were no ATL-related deaths in lymphoma type, which was in contrast to the 10 ATL-related deaths in the acute and unfavourable chronic types after 5 years.

Of the 276 patients, 88 (32%) achieved CR with initial treatment. Of these 88 patients, 24 (27%) patients had survived >5 years and 11 (13%) patients had survived >10 years. Of the remaining 188 patients who did not achieve CR, 13 (17%) patients who survived >5 years and only 1 (0·5%) patient survived >10 years.

CNS involvement by treatment regimen

CNS involvement was 1·6% (95% CI, 0·04–8·7) in JCOG9109, 6·3% (95% CI, 2·3–13·1) in JCOG9303, and 3·5% (95% CI, 0·4–12·1) in the VCAP-AMP-VECP arm and 8·2% (95% CI, 2·7–18·1) in the CHOP-14 arm of JCOG9801. No significant differences in the proportion of CNS involvement were observed among the regimens.

Development of the PI

In univariate analyses, three covariates showed significant associations with OS, namely PS, corrected calcium level and serum total protein (all $P < 0{\cdot}05$; Table II). Stepwise Cox regression analysis returned three unfavourable prognostic

Table I. Clinical characteristics of 15 covariates in all 276 registered patients.

		JCOG9109 (n = 62)	JCOG9303 (n = 96)	JCOG9801 (n = 118)	Total (n = 276)
Initial date of registration		November 1991	January 1994	July 1998	
Final date of registration		July 1993	December 1996	October 2003	
Number of sites		30	20	27	49
Sex	Male/female	38/24	54/42	61/57	153/123
Age, years	≥20, <30	0	1	0	1
	≥30, <40	2	7	6	15
	≥40, <50	14	29	20	63
	≥50,<60	27	24	44	95
	≥60, <70	19	35	48	102
PS	0/1	23/22	19/25	49/46	91/93
	2/3/4/NE	7/9/1/0	17/9/8/18	18/4/1/0	42/22/10/18
B symptoms	+/−/NE	22/36/4	39/57/0	45/73/0	106/166/4
Stage	I/II/III/IV	1/4/8/49	2/6/14/74	0/4/8/106	3/14/30/229
Liver invasion	+/−	10/52	20/76	25/93	55/221
LDH, iu/l	<−1 × ULN/>	9/53	10/86	20/98	39/237
BUN, mmol/l	<−1 × ULN/>/NE	47/14/1	80/15/1	107/11/0	234/40/2
Corrected Ca, mmol/l	<2·75/≥/NE	49/9/4	75/16/5	93/25/0	217/50/9
Serum protein, g/l	<60/≥/NE	18/44/0	27/69/0	30/87/1	75/200/1
Albumin g/l	<35/35–40/≥40/NE	18/26/15/3	35/39/18/4	28/64/26/0	81/129/59/1
WBC ($\times 10^9$/l)	<3/≥	48/14	77/19	104/14	229/47
Lymphocytes ($\times 10^9$/l)*	<4/4–15/≥15/NE	28/16/14/4	54/19/23/0	64/33/20/1	146/68/57/5
Neutrophils ($\times 10^9$/l)	<8/≥/NE	49/12/1	75/21/0	94/24/0	218/57/1
Platelets ($\times 10^9$/l)	<150/≥	16/46	19/77	19/99	54/222

B symptoms: fever, night sweats, and weight loss.
JCOG, Japan Clinical Oncology Group; ECOG PS, Eastern Cooperative Oncology Group performance status; Ca, calcium level; WBC, white blood cell count; ULN, upper limit of normal; NE, not evaluated.
*total (normal + abnormal) lymphocyte count.

factors associated with OS, namely a high, corrected calcium level, high PS (2–4), and the existence of B symptoms, although the third factor was not statistically significant (Table II). Table II also presents the results of the model when the two significant factors of corrected calcium and ECOG PS were included. The hazard ratios (HRs) estimated by this model were 1·574 (95% CI, 1·088–2·277; P = 0·016) for corrected calcium and 1·554 (95% CI, 1·120–2·157; P = 0·008) for ECOG PS.

The four categories consisting of the two prognostic factors (corrected calcium level and PS) were combined into a dichotomous PI, named the JCOG-PI, by considering its potential for clinical use. Similarly, we constructed a dichotomous PI including B symptoms with two prognostic factors. We excluded B symptoms from further assessment because the Akaike Information Criteria of JCOG-PI (1537·8) was smaller than that of PI (1545·6).

According to the JCOG-PI, the MST and 5-year OS were 14 months and 18% in patients with both corrected calcium <2·75 mmol/l and a PS of 0 or 1 (moderate-risk group) and were 8 months and 4% in patients with corrected calcium ≥2·75 mmol/l and/or a PS of 2–4 (high-risk group) respectively (Fig 4A). The HR and 95% CI were 1·926 and 1·423–2·606 respectively (P < 0·0001).

External validation

Nine patients in the validation set of 136 patients had missing corrected calcium or PS data, resulting in 127 evaluable patients (Fig 5). The median and longest follow-up periods were 9 months and 97 months, respectively. The HR was 2·138 (95% CI, 1·414–3·233, P = 0·0003) with an MST of 18 months and 6 months in the moderate- and high-risk groups respectively and JCOG-PI showed good reproducibility (Fig 4B).

Discussion

In this first prospective analysis of a large cohort of aggressive ATL patients from prospective clinical trials conducted after the clinical subtype classification of ATL was introduced, we constructed the JCOG-PI based on corrected calcium level and PS and validated it with external data. The ascertained discrepancy was stronger among the external validation set. In addition, OS of high-risk patients was worse in the external validation set than in the training set, probably reflecting poor organ functions and other unfavourable prognostic factors in patients not participating in clinical trials. The OS of the moderate-risk patients was better in the

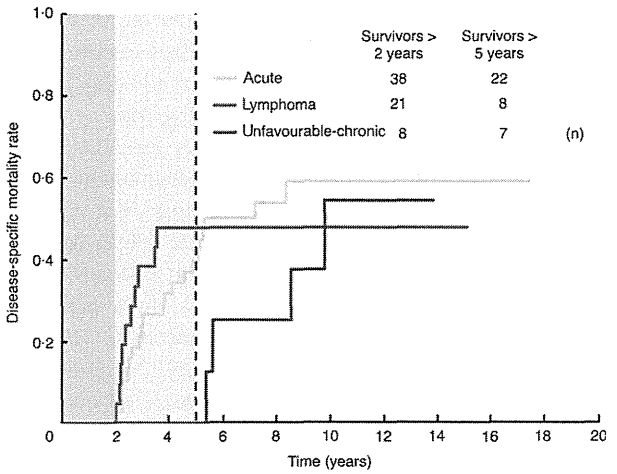

Fig 3. ATL-related deaths of patients who survived >2 years according to subtype. Among the 37 patients who survived >5 years, there were no ATL-related deaths in lymphoma type in contrast to the 10 ATL-related deaths in other types after 5 years.

external validation set than in the training set, possibly reflecting recent advances in treatment, including chemotherapy and allogeneic haematopoietic stem cell transplantation (allo-HSCT).

In our analysis of patients who survived >5 years, no ATL-related deaths occurred in those with lymphoma type, which is in contrast to the ATL-related deaths seen among patients with acute or unfavourable chronic type (Fig 3). This suggests that about 10% of patients with lymphoma type survived >5 years, most of whom might have been cured. Although abnormalities of comparative genomic hybridization might differ between acute and lymphoma types (Oshiro *et al*, 2006), the difference in clinical course between lymphoma type and acute or unfavourable chronic type remains unclear, and further analyses on the molecular and biological features of these types are needed.

Of the 276 patients studied, 20 received an allo-HSCT. The 5-year OS rate of these patients was 40%, compared with 12% in patients who did not undergo transplantation

Table II. Results of univariate and multivariate analyses in the training set (n = 193).

Factor		Univariate analysis HR (95%CI)	P value	Pre-planned multivariate analysis (AIC = 1545·6) HR (95%CI)	P value	Model used for constructing JCOG-PI (AIC = 1537·8) HR (95%CI)	P value
Ca, mmol/l	<2·75	Ref		Ref		Ref	
	≥2·75	1·742 (1·214–2·498)	0·002	1·688 (1·156–2·466)	0·007	1·574 (1·088–2·277)	0·016
ECOG PS	0–1	Ref		Ref		Ref	
	2–4	1·680 (1·219–2·314)	0·001	1·493 (1·073–2·078)	0·018	1·554 (1·120–2·157)	0·008
B symptoms	−	Ref		Ref			
	+	1·249 (0·926–1·685)	0·145	1·288 (0·945–1·755)	0·109		
Sex	Male	Ref					
	Female	0·999 (0·743–1·342)	0·994				
Age, years	<60	Ref					
	≥60	1·108 (0·818–1·502)	0·504				
Stage	I–II	Ref					
	III–IV	1·293 (0·682–2·451)	0·429				
Liver invasion	−	Ref					
	+	1·238 (0·867–1·768)	0·241				
LDH, iu/l	≤ULN	Ref					
	>1 × ULN	1·325 (0·840–2·091)	0·226				
BUN, mmol/l	≤ULN	Ref					
	>1 × ULN	1·332 (0·871–2·036)	0·184				
Serum protein, g/l	<60	Ref					
	≥60	0·642 (0·457–0·901)	0·010				
Lymphocytes, $\times 10^9$/l	<4	Ref					
	4–14·9 (vs. <4)	1·110 (0·785–1·570)	0·553				
	≥15 (vs. <4)	1·102 (0·747–1·626)	0·626				
Neutrophils, $\times 10^9$/l	<8	Ref					
	≥8	1·271 (0·888–1·817)	0·189				
Platelets, $\times 10^9$/l	<150	Ref					
	≥150	0·900 (0·626–1·294)	0·569				

AIC, Akaike's Information Criteria; JCOG, Japan Clinical Oncology Group; PI, Prognostic index; HR, hazard ratio; CI, confidence interval; Ref, reference; ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; BUN, blood urea nitrogen.

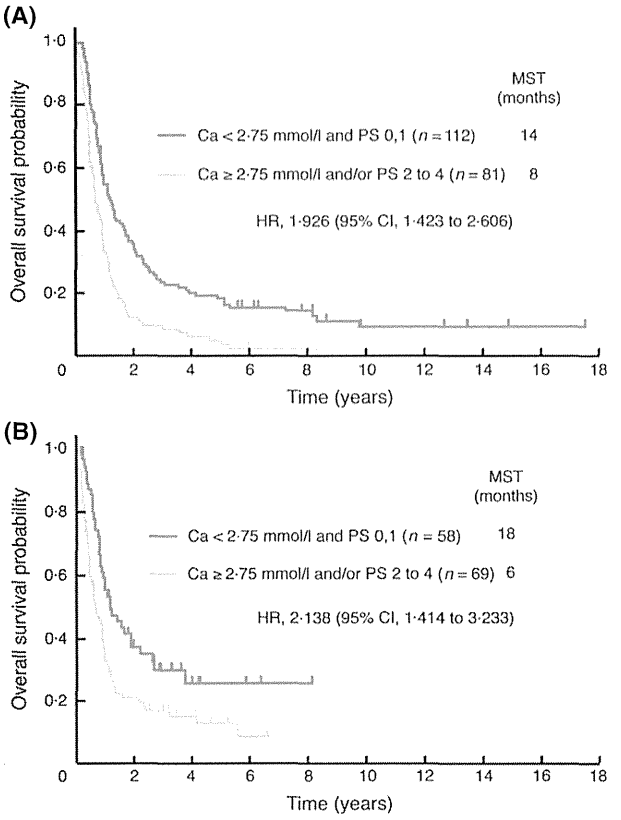

Fig 4. Overall survival of the patients in the training set and in the external validation set according to the JCOG-PI. (A) OS in the training set. The median survival time (MST) and 5-year OS were 14 months and 18% in moderate-risk group (blue line) and were 8 months and 4% in high-risk group (yellow line), respectively (B) OS in the validation set. The MST of 18 months and 6 months in the moderate- (blue line) and high-risk (yellow line) groups, respectively, and JCOG-PI showed good reproducibility.

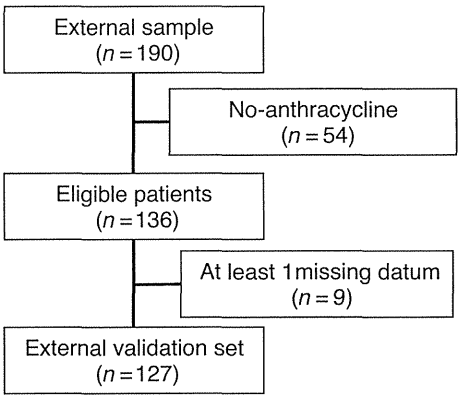

Fig 5. Patient disposition of the external validation set.

(data not shown). However, it was too difficult to evaluate the efficacy of allo-HSCT in our cohort because the disease status at transplantation and the duration from registration to transplantation were rather heterogeneous and the transition to allo-HSCT was time-dependent. To adjust this time-dependent causality, periodical data collection of, for example, indicators of treatment and time-dependent confounders, is necessary. The causal relationship between allo-HSCT and OS should be evaluated in a future prospective trial.

Several reports have revealed risk factors for ATL. In a prospective randomized trial against NHL parsimonious conducted between 1981 and 1983, Shimoyama *et al* (1988) demonstrated that poor PS and high lactate dehydrogenase levels were poor prognostic factors in patients with advanced T-cell lymphoma/leukaemia, including ATL. In a Japanese nationwide survey of 854 patients, a multivariate analysis identified major prognostic indicators of ATL as poor PS, high lactate dehydrogenase levels, age ≥40 years, >3 involved lesions and hypercalcaemia (Lymphoma Study Group, 1991). These factors were then used to construct a risk model. Additional factors reportedly associated with poor prognosis, as determined by multivariate analyses, include thrombocytopenia (Yamada *et al*, 1997), eosinophilia (Utsunomiya *et al*, 2007), bone marrow involvement (Takasaki *et al*, 2007), high interleukin (IL)5 and IL10 serum levels (Inagaki *et al*, 2006), C-C chemokine receptor 4 (CCR4) expression (Ishida *et al*, 2003), lung resistance-related protein (Ohno *et al*, 2001), *TP53* mutation (Tawara *et al*, 2006) and *CDKN2A* deletion (Yamada *et al*, 1997). Specific to chronic-type ATL, multivariate analysis has identified high lactate dehydrogenase levels, high blood urea nitrogen levels and low albumin levels as poor prognostic factors in several retrospective analyses (Shimoyama, 1994).

Recently, an ATL-PI consisting of Ann Arbour clinical stage, PS, age, serum albumin level and soluble IL2 receptor level was used to identify three risk groups for patients with acute and lymphoma types of ATL (Katsuya *et al*, 2012). However, in that study, both the ATL-PI and the risk grouping in the 1980's were constructed based on the results of questionnaires collected retrospectively; hence the treatments used were diverse and the prognostic factors might not have been evaluated homogeneously, in contrast to present study based on the three prospective trials (Lymphoma Study Group, 1991; Katsuya *et al*, 2012).

In the present study, monoclonal integration of HTLV-1 was not detected in four of 104 patients analysed. It was previously demonstrated that about 20% of patients with lymphoma-type ATL did not have monoclonal integration of HTLV-1, by Southern blot analysis, when investigating lymph node specimens (Ohshima *et al*, 1998). From this aspect, the possibility that a fraction of patients with the lymphoma type in the present study had non-ATL-peripheral T-cell lymphoma cannot be completely excluded. Further studies are required to differentiate lymphoma-type ATL from non-ATL-peripheral T-cell lymphoma by analysing monoclonal integration of the HTLV-1 provirus by Southern blot analysis or integration site-specific polymerase chain reaction.

In this study, the median age of 56 years in the training set was notably younger than that in other recent reports and that of the average population of patients with ATL. The

population investigated in the present study represents a selection of fairly young and physically fit patients with preserved organ functions. Although we expected to define a favourable prognosis group in the international PI for aggressive NHL, which consists mostly of diffuse large B-cell lymphoma, the difference in the OS between the two risk groups was small. This finding was similar to a recent retrospective nationwide survey in Japan of all patients with acute or lymphoma type at each institute (Katsuya *et al*, 2012). Therefore, the JCOG-PI could not be used to identify patients with aggressive ATL who could be treated with intensive chemotherapy alone and spared from more intensive therapy, such as allo-HSCT, as is the case with the ATL-PI (Katsuya *et al*, 2012). However, we did manage to identify patients with extremely poor prognosis despite undergoing intensive chemotherapy in clinical trials. These patients might be candidates for future trials that combine new agents or investigational strategies.

Recently, the results of several phase I and II trials using a defucosylated anti-CCR4 antibody for relapsed patients with aggressive ATL have demonstrated clinically meaningful antitumour activity and an acceptable toxicity profile (Yamamoto *et al*, 2010; Ishida *et al*, 2012a). Moreover, allo-HSCT with myeloablative and reduced intensity conditioning for patients with aggressive ATL has been reported to cure diseases associated with the graft-*versus*-ATL effect, despite the high transplant-related mortality (Hishizawa *et al*, 2010; Ishida *et al*, 2012b; Kanda *et al*, 2012). To further improve patient outcomes, two trials are ongoing in Japan: a phase II trial of VCAP-AMP-VECP followed by allo-HSCT with myeloablative conditioning for patients aged <55 years with aggressive ATL (JCOG 0907), and a randomized phase II trial of VCAP-AMP-VECP with or without anti-CCR4 antibody (Jo *et al*, 2013).

In conclusion, patients with lymphoma-type ATL who survived >5 years might have been cured, which is in contrast to long-term survivors with acute or unfavourable chronic type. The JCOG-PI, based on corrected calcium levels and PS, is a simple and valuable tool for identifying patients with aggressive ATL having extremely poor prognosis in clinical trials, and it will be useful for the design of future studies combining new drugs or investigational strategies.

Acknowledgements

The authors are grateful to all the physicians and data managers at the institutions of Japan Clinical Oncology Group (JCOG) – Lymphoma Study Group (LSG). The authors thank all the members of the JCOG Data Centre. This work was supported by the National Cancer Centre Research and Development Fund Nos. 23-A-16 and 23-A-17, Grants-in-Aid Nos. 2S-1, 5S-1, 8S-1, 11S-1, 14S-1, 17S-1, 20S-1, 20S-6, 1-1, 4-5, 7-29, 9-10, 15-11, 16-12, 19-8 and 21-6-3 and a grant (H23-gan rinsho-ippan-022) for the Cancer Research from the Ministry of Health, Labour and Welfare of Japan (1990 to present), for the Second-Term Ten-Year Strategy for Cancer Control from the Ministry of Health and Welfare (1994 to 2004) and for Basic Research from the Science and Technology Agency (1991 to 1993).

Authorship

T.F., M.S, H.F., K. T. and K.T. designed the study and wrote the paper. T.H. designed the study. S.N. and T.S. designed the study, analysed data and wrote the paper. Y.I., Y.M., T.T., K.U., Y.K., N.F., A.U., M.T., K.N., M.H., N.U., S.Y., K.T., K.I., M.K. and M.N. collected data and reviewed the paper.

Disclosure

The authors report no potential conflict of interest.

References

Akaike, H. (1973) Information theory and an extension of the maximum likelihood principle. In: *Proceedings of the 2nd International Symposium on Information theory* (ed. by B.N. Petrov & F. Caski), pp. 267–281. Akademinai, Budapest.

Clopper, C.J. & Pearson, E.S. (1934) The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika*, **26**, 404–413.

Gallamini, A., Stelitano, C., Calvi, R., Bellei, M., Mattei, D., Vitolo, U., Martelli, M., Brusamolino, E., Iannitto, E., Zaja, F., Cortelozzo, S., Rigacci, L., Devizzi, L., Todeschini, G., Santini, G., Brugiatelli, M., Federico, M. & Linfomi, I.I. (2004) Peripheral T-cell lymphoma unspecified (PTCL-U): a new prognostic model from a retrospective multicentric clinical study. *Blood*, **103**, 2474–2479.

Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K.I., Shirakawa, S. & Miyoshi, I. (1981) Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proceedings of the National Academy of Sciences of the United States of America*, **78**, 6476–6480.

Hishizawa, M., Kanda, J., Utsunomiya, A., Taniguchi, S., Eto, T., Moriuchi, Y., Tanosaki, R., Kawano, F., Miyazaki, Y., Masuda, M., Nagafuji, K., Hara, M., Takanoshi, M., Kai, S., Suzuki, R., Kawase, T., Matsuo, K., Nagamura-Inoue, T., Kato, S., Sakamaki, H., Morishima, Y., Okamura, J., Ichinohe, T. & Uchiyama, T. (2010) Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study. *Blood*, **116**, 1369–1376.

Inagaki, A., Ishida, T., Ishii, T., Komatsu, H., Iida, S., Ding, J., Yonekura, K., Takeuchi, S., Takatsuka, Y., Utsunomiya, A. & Ueda, R. (2006) Clinical significance of serum Th1-, Th2- and regulatory T cells-associated cytokines in adult T-cell leukemia/lymphoma: high interleukin-5 and -10 levels are significant unfavorable prognostic factors. *International Journal of Cancer*, **118**, 3054–3061.

Ishida, T., Utsunomiya, A., Iida, S., Inagaki, H., Takatsuka, Y., Kusumoto, S., Takeuchi, G., Shimizu, S., Ito, M., Komatsu, H., Wakita, A., Eimoto, T., Matsushima, K. & Ueda, R. (2003) Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clinical Cancer Research*, **9**, 3625–3634.

Ishida, T., Joh, T., Uike, N., Yamamoto, K., Utsunomiya, A., Yoshida, S., Saburi, Y., Miyamoto,

T., Takemoto, S., Suzushima, H., Tsukasaki, K., Nosaka, K., Fujiwara, H., Ishitsuka, K., Inagaki, H., Ogura, M., Akinaga, S., Tomonaga, M., Tobinai, K. & Ueda, R. (2012a) Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study. *Journal of Clinical Oncology*, **30**, 837–842.

Ishida, T., Hishizawa, M., Kato, K., Tanosaki, R., Fukuda, T., Taniguchi, S., Eto, T., Takatsuka, Y., Miyazaki, Y., Moriuchi, Y., Hidaka, M., Akashi, K., Uike, N., Sakamaki, H., Morishima, Y., Kato, K., Suzuki, R., Nishiyama, T. & Utsunomiya, A. (2012b) Allogeneic hematopoietic stem cell transplantation for adult T-cell leukemia-lymphoma with special emphasis on preconditioning regimen: a nationwide retrospective study. *Blood*, **120**, 1734–1741.

Jo, T., Ishida, T., Takemoto, S., Suzushima, H., Uozumi, K., Yamamoto, K., Uike, N., Saburi, Y., Nosaka, K., Utsunomiya, A., Tobinai, K., Fujiwara, H., Ishitsuka, K., Yoshida, S., Taira, N., Moriuchi, Y., Imada, K., Miyamoto, T., Tomonaga, M. & Ueda, R. (2013) Randomized phase II study of mogamulizumab (KW-0761) plus VCAP-AMP-VECP (mLSG15) versus mLSG15 alone for newly diagnosed aggressive adult T-cell leukemia-lymphoma (ATL). *Journal of Clinical Oncology*, **31**, 519s, abstract 8506.

Kalbfleisch, J.D. & Prentice, R.L. (2002) *The Statistical Analysis of Failure Time Data*, 2nd edn. John Wiley & Sons Inc, New York, NY.

Kanda, J., Hishizawa, M., Utsunomiya, A., Taniguchi, S., Eto, T., Moriuchi, Y., Tanosaki, R., Kawano, F., Miyazaki, Y., Masuda, M., Nagafuji, K., Hara, M., Takanashi, M., Kai, S., Atsuta, Y., Suzuki, R., Kawase, T., Matsuo, K., Nagamura-Inoue, T., Kato, S., Sakamaki, H., Morishima, Y., Okamura, J., Ichinohe, T. & Uchiyama, T. (2012) Impact of graft-versus-host disease on outcome after allogeneic hematopoietic cell transplantation for adult T-cell leukemia: a retrospective cohort study. *Blood*, **119**, 2141–2148.

Katsuya, H., Yamanaka, T., Ishitsuka, K., Utsunomiya, A., Sasaki, H., Hanada, S., Eto, T., Moriuchi, Y., Saburi, Y., Miyahara, M., Sueoka, E., Uike, N., Yoshida, S., Yamashita, K., Tsukasaki, K., Suzushima, H., Ohno, Y., Matsuoka, H., Jo, T., Suzumiya, J. & Tamura, K. (2012) Prognostic index for acute- and lymphoma-type adult T-cell leukemia/lymphoma. *Journal of Clinical Oncology*, **30**, 1635–1640.

Lymphoma Study Group (1991) Major prognostic factors of patients with adult T-cell leukemia-lymphoma: a cooperative study-Lymphoma Study Group (1984-1987). *Leukemia Research*, **15**, 81–90.

Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K. & Hinuma, Y. (1981) Type C virus particles in a cord T-cell line derived by co-cultivating normal cord leukocytes and human leukaemic T cells. *Nature*, **294**, 770–771.

Ohno, N., Tani, A., Uozumi, K., Hanada, S., Furukawa, T., Akiba, S., Sumizawa, T., Utsunomiya, A., Arima, T. & Akiyama, S. (2001) Expression of functional lung resistance-related protein predicts poor outcome in adult T-cell leukemia. *Blood*, **98**, 1160–1165.

Ohshima, K., Suzumiya, J., Sato, K., Kanda, M., Sugihara, M., Haraoka, S., Takeshita, M. & Kikuchi, M. (1998) Nodal T-cell lymphoma in an HTLV-1-endemic area: proviral HTLV-1 DNA, histological classification and clinical evaluation. *British Journal of Haematology*, **101**, 703–711.

Oshiro, A., Tagawa, H., Ohshima, K., Karube, K., Uike, N., Tashiro, Y., Utsunomiya, A., Masuda, M., Takasu, N., Nakamura, S., Morishima, Y. & Seto, M. (2006) Identification of subtype-specific genomic alterations in aggressive adult T-cell leukemia/lymphoma. *Blood*, **107**, 4500–4507.

Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.A., Minna, J.D. & Gallo, R.C. (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proceedings of the National Academy of Sciences of the United States of America*, **77**, 7415–7419.

Shimoyama, M. (1991) Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma: a report from the Lymphoma Study Group (1984-87). *British Journal of Haematology*, **79**, 428–437.

Shimoyama, M. (1994) Chemotherpy of ATL. In: *Adult T-Cell Leukemia* (ed. by Takatsuki, K.), pp. 221–237. Oxford University Press, Oxford, United Kingdom.

Shimoyama, M., Ota, K., Kikuchi, M., Yunoki, K., Konda, S., Takatsuki, K., Ichimaru, M., Ogawa, M., Kimura, I. & Tominaga, S. (1988) Chemotherapeutic results and prognostic factors of patients with advanced non-Hodgkin's lymphoma treated with VEPA or VEPA-M. *Journal of Clinical Oncology*, **6**, 128–141.

Takasaki, Y., Iwanaga, M., Tsukasaki, K., Kusano, M., Sugahara, K., Yamada, Y., Kamihira, S., Ikeda, S. & Tomonaga, M. (2007) Impact of visceral involvement and blood cell count abnormalities on survival in adult T-cell leukemia/lymphoma (ATLL). *Leukemia Research*, **31**, 751–757.

Tawara, M., Hogerzeil, S.J., Yamada, Y., Yakasaki, Y., Soda, H., Hasegawa, H., Murata, K., Ikeda, S., Imaizumi, Y., Sugahara, K., Tsuruda, K., Tsukasaki, K., Tomonaga, M., Hirakata, Y. & Kamihira, S. (2006) Impact of p53 aberration on the progression of adult T-cell leukemia/lymphoma. *Cancer Letters*, **234**, 249–255.

The International Non-Hodgkin's Lymphoma Prognostic Factor Project (1993) A predictive model for aggressive non-Hodgkin's lymphoma: the International Non-Hodgkin's Lymphoma Prognostic Factors Project. *New England Journal of Medicine*, **329**, 987–994.

Tobinai, K. & Watanabe, T. (2004) Adult T-cell leukemia-lymphoma. In: *Clinical Oncology* (ed. by M.D. Abeloff, J.O. Armitage, J.E. Niederhuber & M.C.B. Kastan), 3rd edn, pp. 3109–3130. Elsevier Churchill Livingstone, Philadelphia, PA

Tobinai, K., Shimoyama, M., Inoue, S., Takayasu, S., Kikuni, C., Kozuru, M., Oda, S. & Nakajima, H. (1992) Phase I study of YK-176 (2′-deoxycoformycin) in patients with adult T-cell leukemia-lymphoma. The DCF Study Group. *Japanese Journal of Clinical Oncology*, **22**, 164–171.

Tobinai, K., Shimoyama, M., Minato, K., Shirakawa, S., Kobayashi, T. & Hotta, T. (1994) Japan Clinical Oncology Group phase II trial of second-generation "LSG4 protocol" in aggressive T- and B-lymphoma: a new predictive model for T- and B-lymphoma. *Proceedings, American Society of Clinical Oncology*, **13**, 378a.

Tsukasaki, K., Tobinai, K., Shimoyama, M., Kozuru, M., Uike, N., Yamada, Y., Tomonaga, M., Araki, K., Kasai, M., Takatsuki, K., Tara, M., Mikuni, C. & Hotta, T.; Lymphoma Study Group of the Japan Clinical Oncology Group (2003) Deoxycoformycin-containing combination chemotherapy for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study (JCOG9109). *International Journal of Hematology*, **77**, 164–170.

Tsukasaki, K., Utsunomiya, A., Fukuda, H., Shibata, T., Fukushima, T., Takatsuka, Y., Ikeda, S., Masuda, M., Nagoshi, H., Ueda, R., Tamura, K., Sano, M., Momita, S., Yamaguchi, K., Kawano, F., Hanada, S., Tobinai, K., Shimoyama, M., Hotta, T. & Tomonaga, M.; Japan Clinical Oncology Group Study JCOG9801 (2007) VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. *Journal of Clinical Oncology*, **25**, 5458–5464.

Uchiyama, T., Yodoi, J., Sagawa, K., Takatsuki, K. & Uchino, H. (1977) Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood*, **50**, 481–492.

Utsunomiya, A., Ishida, T., Inagaki, A., Ishii, T., Yano, H., Komatsu, H., Iida, S., Yonekura, K., Takeuchi, S., Takatsuka, Y. & Ueda, R. (2007) Clinical significance of a blood eoshinophilia in adult T-cell leukemia/lymphoma: a blood eosinophilia is a significant unfavorable prognostic factor. *Leukemia Research*, **31**, 915–920.

Watanabe, T., Kinoshita, T., Itoh, K., Yoshimura, K., Ogura, M., Kagami, Y., Yamaguchi, M., Kurosawa, M., Tsukasaki, K., Kasai, M., Tobinai, K., Kaba, H., Mukai, K., Nakamura, S., Ohshima, K., Hotta, T. & Shimoyama, M. (2010) Pretreatment total serum protein is a significant prognostic factor for the outcome of patients with peripheral T/natural killer-cell lymphomas. *Leukaemia & Lymphoma*, **51**, 813–821.

Yamada, Y., Hatta, Y., Murata, K., Sugawara, K., Ikeda, S., Mine, M., Maeda, T., Hirakata, Y., Kamihira, S., Tsukasaki, K., Ogawa, S., Hirai, H., Koeffler, H.P. & Tomonaga, M. (1997) Deletions of p15 and/or p16 genes as a poor-prognosis factor in adult T-cell leukemia. *Journal of Clinical Oncology*, **15**, 1778–1785.

Yamada, Y., Tomonaga, M., Fukuda, H., Hanada, S., Utsunomiya, A., Tara, M., Sano, M., Ikeda, S., Takatsuki, K., Kozuru, M., Araki, K., Kawano, F., Niimi, M., Tobinai, K., Hotta, T. & Shimoyama, M. (2001) A new G-CSF supported combination chemotherapy, LSG15, for adult T-

cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *British Journal of Haematology*, **113**, 375–382.

Yamamoto, K., Utsunomiya, A., Tobinai, K., Tsukasaki, K., Uike, N., Uozumi, K., Yamaguchi, K., Yamada, Y., Hanada, S., Tamura, K., Nakamura, S., Inagaki, H., Ohshima, K., Kiyoi, H., Ishida, T., Matsushima, K., Akinaga, S., Ogura, M., Tomonaga, M. & Ueda, R. (2010) 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. *Journal of Clinical Oncology*, **28**, 1591–1598.

Yoshida, M., Miyoshi, I. & Hinuma, Y. (1982) Isolation and characterization of retrovirus from cell lines of human adult T cell leukemia and its implication in the disease. *Proceedings of the National Academy of Sciences of the United States of America*, **79**, 2031–2035.

HTLV-1 induces a Th1-like state in CD4+CCR4+ T cells

Natsumi Araya,[1] Tomoo Sato,[1] Hitoshi Ando,[1] Utano Tomaru,[2] Mari Yoshida,[3] Ariella Coler-Reilly,[1] Naoko Yagishita,[1] Junji Yamauchi,[1] Atsuhiko Hasegawa,[4] Mari Kannagi,[4] Yasuhiro Hasegawa,[5] Katsunori Takahashi,[1] Yasuo Kunitomo,[1] Yuetsu Tanaka,[6] Toshihiro Nakajima,[7,8] Kusuki Nishioka,[7] Atae Utsunomiya,[9] Steven Jacobson,[10] and Yoshihisa Yamano[1]

[1]Department of Rare Diseases Research, Institute of Medical Science, St. Marianna University School of Medicine, Kanagawa, Japan. [2]Department of Pathology, Hokkaido University Graduate School of Medicine, Hokkaido, Japan. [3]Institute for Medical Science of Aging, Aichi Medical University, Aichi, Japan. [4]Department of Immunotherapeutics, Tokyo Medical and Dental University, Graduate School, Tokyo, Japan. [5]Department of Neurology, St. Marianna University School of Medicine, Kanagawa, Japan. [6]Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan. [7]Institute of Medical Science and [8]Center for Clinical Research, Tokyo Medical University, Tokyo, Japan. [9]Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Japan. [10]Viral Immunology Section, Neuroimmunology Branch, National Institutes of Health, Bethesda, Maryland, USA.

Human T-lymphotropic virus type 1 (HTLV-1) is linked to multiple diseases, including the neuroinflammatory disease HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T cell leukemia/lymphoma. Evidence suggests that HTLV-1, via the viral protein Tax, exploits CD4+ T cell plasticity and induces transcriptional changes in infected T cells that cause suppressive CD4+CD25+CCR4+ Tregs to lose expression of the transcription factor FOXP3 and produce IFN-γ, thus promoting inflammation. We hypothesized that transformation of HTLV-1–infected CCR4+ T cells into Th1-like cells plays a key role in the pathogenesis of HAM/TSP. Here, using patient cells and cell lines, we demonstrated that Tax, in cooperation with specificity protein 1 (Sp1), boosts expression of the Th1 master regulator T box transcription factor (T-bet) and consequently promotes production of IFN-γ. Evaluation of CSF and spinal cord lesions of HAM/TSP patients revealed the presence of abundant CD4+CCR4+ T cells that coexpressed the Th1 marker CXCR3 and produced T-bet and IFN-γ. Finally, treatment of isolated PBMCs and CNS cells from HAM/TSP patients with an antibody that targets CCR4+ T cells and induces cytotoxicity in these cells reduced both viral load and IFN-γ production, which suggests that targeting CCR4+ T cells may be a viable treatment option for HAM/TSP.

Introduction

The flexibility of the CD4+ T cell differentiation program that underlies the success of the adaptive immune response has recently been implicated in the pathogeneses of numerous inflammatory diseases (1–3). The majority of CD4+ T lymphocytes belong to a class of cells known as Th cells, so called because they provide help on the metaphorical immune battlefield by stimulating the other soldiers — namely, B cells and cytotoxic T lymphocytes — via secretion of various cytokines. Interestingly, there is also a minority group of CD4+ T cells with quite the opposite function: Tregs actively block immune responses by suppressing the activities of CD4+ Th cells as well as many other leukocytes (4). Tregs are credited with maintaining immune tolerance and preventing inflammatory diseases that could otherwise occur as a result of uninhibited immune reactions (5). Thus, the up- or downregulation of certain CD4+ T cell lineages could disrupt the carefully balanced immune system, threatening bodily homeostasis.

The plasticity of CD4+ T cells, particularly Tregs, makes CD4+ T cell lineages less clean-cut than they may originally appear. CD4+ T cells are subdivided according to various lineage-specific chemokine receptors and transcription factors they express, as well as the cytokines they produce (6). Th1 cells, for example, can be identified by expression of CXC motif receptor 3 (CXCR3) and T box transcription factor (T-bet; encoded by *TBX21*) and are known to secrete the proinflammatory cytokine IFN-γ (6). While both have been known to express CC chemokine receptor 4 (CCR4) and CD25, Th2 cells and Tregs can usually be distinguished from each other by their expression of GATA-binding protein 3 (GATA3) and forkhead box p3 (FOXP3), respectively (6, 7). CCR4 is coexpressed in the majority of CD4+FOXP3+ cells and in virtually all CD4+CD25+FOXP3+ cells, making it a useful — albeit not fully specific — marker for Tregs (8, 9). FOXP3 is a particularly noteworthy marker because its expression is said to be required for Treg identity and function (10). In fact, *Foxp3* point mutations are reported to cause fatal multiorgan autoimmune diseases (11). Even partial loss of FOXP3 expression can disrupt the suppressive nature of Tregs, representing one of several pathways by which even fully differentiated Tregs can reprogram into inflammatory cells (12). There have been several reports of Tregs reprogramming in response to proinflammatory cytokines such as IL-1, IL-6, IL-12, and IFN-γ (12, 13); it is thought that this reprogramming may have evolved as an adaptive mechanism for dampening immune suppression when protective inflammation is necessary (12). However, this same plasticity can lead to pathologically chronic inflammation, and several autoimmune diseases have been associated with reduced FOXP3 expression and/or Treg function, including multiple sclerosis, myasthenia gravis, and type 1 diabetes (14, 15).

Of the roughly 10–20 million people worldwide infected with human T-lymphotropic virus type 1 (HTLV-1), up to 2%–3% are affected by the neurodegenerative chronic inflammatory dis-

Conflict of interest: The authors have declared that no conflict of interest exists.
Submitted: January 17, 2014; **Accepted:** May 8, 2014.
Reference information: *J Clin Invest.* 2014;124(8):3431–3442. doi:10.1172/JCI75250.

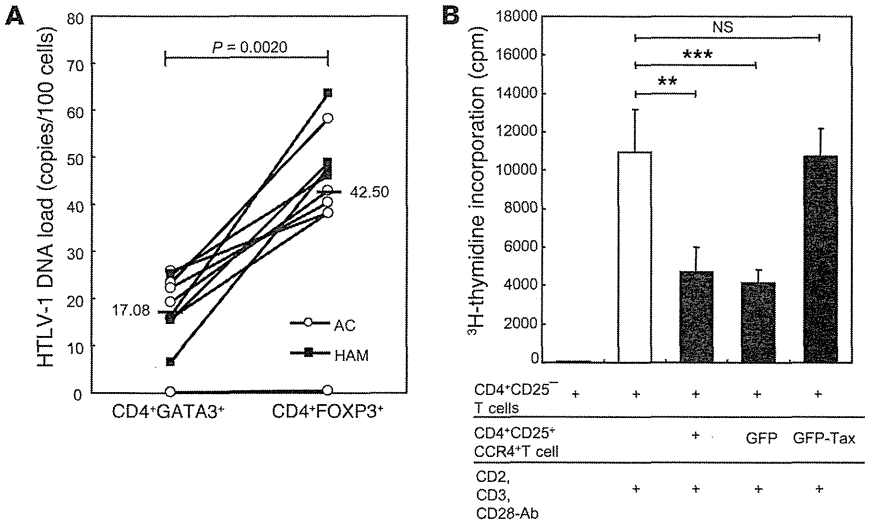

Figure 1. HTLV-1 mainly infects Tregs and inhibits their regulatory function. (**A**) Higher HTLV-1 proviral DNA load in $CD4^+FOXP3^+$ cells (Tregs) compared with $CD4^+GATA3^+$ cells ($P = 0.0020$, Wilcoxon test) from asymptomatic carriers (AC; $n = 6$) and HAM/TSP patients ($n = 4$). PBMCs were FACS sorted, and proviral load was measured using quantitative PCR. Horizontal bars represent the mean value for each set. (**B**) Loss of regulatory function in Tax-expressing $CD4^+CD25^+CCR4^+$ cells (Tregs). $CD4^+CD25^-$ T cells from an HD were stimulated with CD2, CD3, and CD28 antibodies and cultured alone or in the presence of equal numbers of $CD4^+CD25^+CCR4^+$ T cells, GFP lentivirus–infected HD $CD4^+CD25^+CCR4^+$ T cells, or GFP-Tax lentivirus–infected HD $CD4^+CD25^+CCR4^+$ T cells. As a control, $CD4^+CD25^-$ T cells alone were cultured without any stimulus. Proliferation of T cells was determined using ^{3}H-thymidine incorporation by adding ^{3}H-thymidine for 16 hours after 4 days of culture. All tests were performed in triplicate. Data are mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA followed by Tukey test for multiple comparisons.

ease HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). The main other condition associated with the retrovirus is adult T cell leukemia/lymphoma (ATLL), a rare and aggressive cancer of the T cells. HAM/TSP represents a useful starting point from which to investigate the origins of chronic inflammation, because the primary cause of the disease — viral infection — is so unusually well defined. HAM/TSP patients share many immunological characteristics with FOXP3 mutant mice, including multiorgan lymphocytic infiltrates, overproduction of inflammatory cytokines, and spontaneous lymphoproliferation of cultured $CD4^+$ T cells (16–18). We and others have proposed that HTLV-1 preferentially infects $CD4^+CD25^+CCR4^+$ T cells, a group that includes Tregs (7, 19). Samples of $CD4^+CD25^+CCR4^+$ T cells isolated from HAM/TSP patients exhibited low FOXP3 expression as well as reduced production of suppressive cytokines and low overall suppressive ability — in fact, these $CD4^+CD25^+CCR4^+FOXP3^-$ T cells were shown to produce IFN-γ and express Ki67, a marker of cell proliferation (19). The frequency of these IFN-γ–producing $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP patients was correlated with disease severity (19). Finally, evidence suggests that the HTLV-1 protein product Tax may play a role in this alleged transformation of Tregs into proinflammatory cells in HAM/TSP patients: transfecting Tax into $CD4^+CD25^+$ cells from healthy donors (HDs) reduced FOXP3 mRNA expression, and Tax expression in $CD4^+CD25^+CCR4^+$ cells was higher in HAM/TSP versus ATLL patients despite similar proviral loads (19, 20). Therefore, we hypothesized that HTLV-1 causes chronic inflammation by infecting $CD4^+CD25^+CCR4^+$ T cells and inducing their transformation into Th1-like, IFN-γ–producing proinflammatory cells via intracellular Tax expression and subsequent transcriptional alterations including but not limited to loss of endogenous FOXP3 expression.

In this study, we first sought to discover the detailed mechanism by which Tax influences the function of $CD4^+CD25^+CCR4^+$ T cells. We used DNA microarray analysis of $CD4^+CD25^+CCR4^+$ T cells from HAM/TSP patients to identify *TBX21*, known as a master transcription factor for Th1 differentiation, as a key intermediary between Tax expression and IFN-γ production. We demonstrated that Tax, in concert with specificity protein 1 (Sp1), amplified *TBX21* transcription and subsequently IFN-γ production. Next, we established the presence of Th1-like $CD4^+CCR4^+$ T cells in the CSF and spinal cord lesions of HAM/TSP patients. The majority of these $CD4^+CCR4^+$ T cells coexpressed CXCR3 as well as T-bet and IFN-γ. Finally, we investigated the therapeutic potential of an anti-CCR4 monoclonal antibody with antibody-dependent cellular cytotoxicity (ADCC) (21). Applying this antibody in vitro diminished the proliferative capacity of cultured PBMCs and reduced both proviral DNA load and IFN-γ production in cultured CSF cells as well as PBMCs. In conclusion, we were able to elucidate a more detailed mechanism for the pathogenesis of HAM/TSP and use our findings to suggest a possible therapeutic strategy.

Results

HTLV-1 preferentially infects Tregs and alters their behavior via Tax. Experiments were conducted to determine which among $CD4^+CD25^+CCR4^+$ T cells were infected by HTLV-1, and how the infection influenced their functionality. Analysis of fluorescence-activated cell sorting (FACS)-sorted PBMCs obtained from asymptomatic carriers ($n = 6$) as well as HAM/TSP patients ($n = 4$) revealed that Tregs ($CD4^+FOXP3^+$) carried much higher proviral loads than Th2 cells ($CD4^+GATA3^+$) ($P = 0.0020$; Figure 1A). As it is well established that each infected cell contains only 1 copy of the HTLV-1 provirus (22, 23), these results indicate that a larger proportion of $FOXP3^+$ than $GATA3^+$ $CD4^+$ T cells are infected. As expected, proliferation of $CD4^+CD25^-$ cells after stimulation, as measured by ^{3}H-thymidine incorporation, was suppressed upon coculture with $CD4^+CD25^+CCR4^+$ cells, including Tregs ($n = 3$, $P < 0.01$; Figure 1B). However, after being transduced with lentiviral vector expressing GFP-Tax, the $CD4^+CD25^+CCR4^+$ cells no longer suppressed cell proliferation; conversely, cells transduced with the control vector expressing only GFP retained full suppressive function ($P < 0.001$; Figure 1B).

The HTLV-1 protein product Tax induces IFN-γ production via T-bet. Experiments were conducted to determine if and how Tax affects IFN-γ production in infected T cells. First, the existence of a functional link between *Tax* and *IFNG* was established by using the

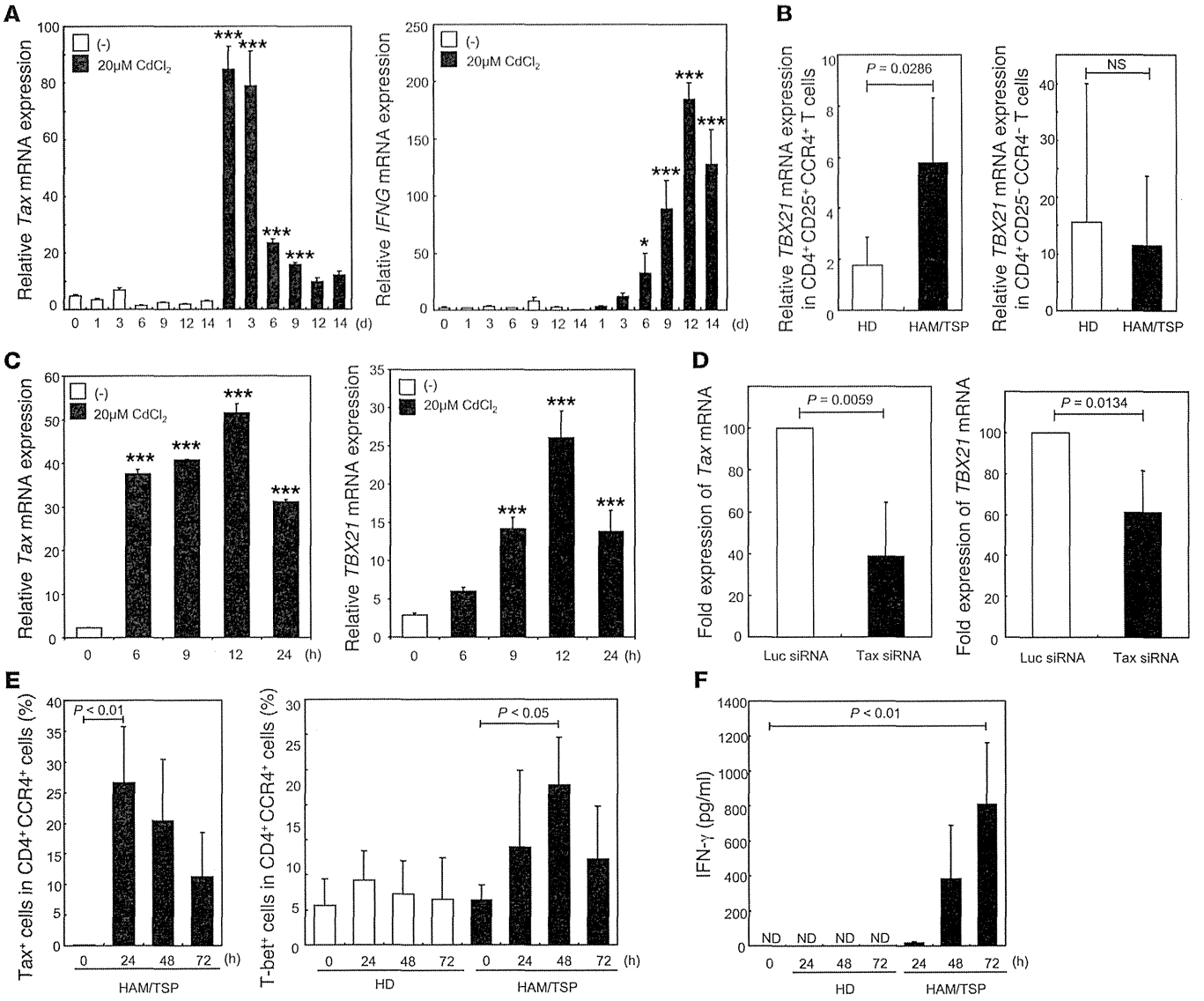

Figure 2. Tax induces IFN-γ production via T-bet. (**A**) Tax-dependent *IFNG* mRNA expression in JPX-9 cells. Experiments were performed in triplicate. (**B**) Elevated *TBX21* mRNA expression in $CD4^+CD25^+CCR4^+$ T cells from HAM/TSP patients relative to HDs ($n = 4$ per group). (**C**) Tax-dependent *TBX21* mRNA expression in JPX-9 cells. Experiments were performed in triplicate. (**D**) Reduced *TBX21* mRNA expression after silencing Tax in $CD4^+CD25^+CCR4^+$ T cells from HAM/TSP patients. PBMCs from HAM/TSP patients ($n = 5$) were FACS sorted, transfected with either Luc or Tax siRNA, and incubated for 24 hours. (**E** and **F**) Tax expression correlated with T-bet expression and IFN-γ production in $CD4^+CCR4^+$ T cells from HAM/TSP patients. $CD4^+CCR4^+$ T cells isolated from HDs and HAM/TSP patients ($n = 4$ per group) were cultured before being stained for Tax and T-bet protein and analyzed using FACS. IFN-γ production in the culture medium was measured using a CBA assay. ND, not detectable. All data are mean ± SD. *P* values were calculated using (**A** and **C**) 1-way ANOVA followed by Dunnett test for multiple comparisons, (**B**) Mann-Whitney *U* test, (**D**) paired *t* test, or (**E** and **F**) Friedman test followed by Dunn test for multiple comparisons. $^*P < 0.05$, $^{***}P < 0.001$ vs. time point 0.

JPX-9 cell line possessing a stably integrated $CdCl_2$-inducible *Tax* construct and measuring *IFNG* mRNA expression. Inducing *Tax* expression with $CdCl_2$ periodically over 2 weeks yielded a steady rise in *IFNG* expression (Figure 2A). Although there was clearly a correlation between Tax and IFN-γ expression, the *IFNG* expression level was not proportional to that of *Tax*, and the steepest rise in the former was delayed several days after the steepest rise in the latter. Thus, we suspected that expression of 1 or more additional genes may represent an important middle step on the pathway linking Tax and IFN-γ production. DNA microarray results revealed that expression of *TBX21*, which is known to be associated with IFN-γ production, was elevated in $CD4^+CD25^+CCR4^+$ cells from the HAM/TSP patient, but not the ATLL patient, compared with the HD (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI75250DS1). *TBX21* mRNA expression, measured via real-time RT-PCR, was elevated in $CD4^+CD25^+CCR4^+$ cells, but not $CD4^+CD25^-CCR4^-$ cells, from HAM/TSP patients compared with HDs (Figure 2B). A direct correlation between *Tax* and *TBX21* mRNA expression was then established using the JPX-9 cell line, as described above (Figure 2C). Silencing the *Tax* gene with siRNA in $CD4^+CD25^+CCR4^+$ cells from HAM/TSP patients reduced *TBX21* as well as *Tax* expression (Figure 2D). Similarly,

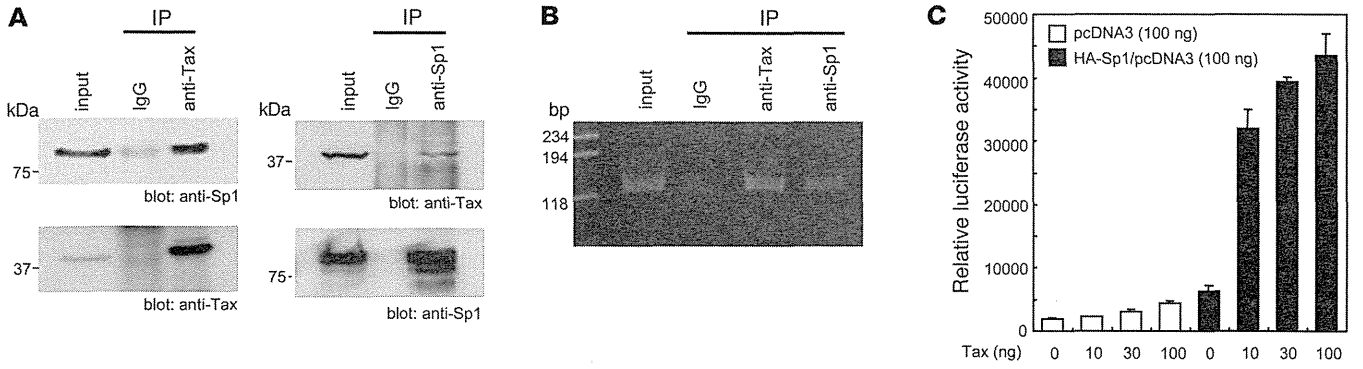

Figure 3. Tax and Sp1 cooperatively enhance *TBX21* promoter activity. (**A**) Co-IP of endogenous Tax and Sp1. Nuclear extracts from MT-2 cells were immunoprecipitated with anti-Tax or anti-Sp1 antibodies or with normal IgG as a control, then immunoblotted with anti-Tax or anti-Sp1 antibodies as indicated. (**B**) Tax bound to the *TBX21* promoter in vivo. ChIP assay using anti-Tax antibody followed by primers encompassing the *TBX21* promoter region (-179 to -59) was performed on genomic DNA isolated from MT-2 cells. DNA (input) and IP with anti-Sp1 served as positive controls, and normal IgG served as a negative control. (**C**) Coactivation of *TBX21* promoter by Sp1 and Tax. HEK293 cells were transfected with 100 ng of *TBX21*-Luc reporter plasmid or Sp1 expression plasmid, as well as 0–100 ng of Tax expression plasmid as indicated. Values were normalized to β-galactosidase activity as an internal control. Data are mean ± SD.

elevation of *Tax* expression via transduction of a GFP-Tax construct into $CD4^+CD25^+CCR4^+$ cells from a HD increased expression of *TBX21* as well as *Tax* (Supplemental Figure 2). Thus, a functional relationship between *Tax* and *TBX21* was confirmed. Finally, among $CD4^+CCR4^+$ cells from HAM/TSP patients, the appearance of Tax^+ cells was associated with a rise in the percentage of $T\text{-}bet^+$ cells, which was associated with a delayed but roughly proportional rise in the amount of IFN-γ protein (Figure 2, E and F). The production of Tax versus T-bet in these $CD4^+CCR4^+$ cells from HAM/TSP patients was compared at 0 versus 48 hours of culturing. At 0 hours, the overwhelming majority of the $CD4^+CCR4^+$ cells were both Tax^- and $T\text{-}bet^-$; by 48 hours, a substantial presence of $Tax^+T\text{-}bet^+$ cells had emerged, and there were very few $T\text{-}bet^+$ cells that were not also Tax^+ (Supplemental Figure 3).

Tax in concert with Sp1 induces TBX21 transcription. Experiments were conducted to investigate the mechanism by which Tax may be involved in *TBX21* transcription in HTLV-1–infected T cells. First, co-IP reactions were performed using nuclear extracts from the HTLV-1–infected MT-2 T cell line to confirm a suspected interaction between endogenous Tax and Sp1, which is known to both form a complex with Tax and to activate *TBX21* transcription (24, 25). Precipitation with anti-Tax or anti-Sp1 antibodies yielded bands corresponding to both Tax and Sp1, whereas precipitation with the nonspecific IgG antibody as the negative control yielded neither band (Figure 3A), thus demonstrating the existence of a Tax-Sp1 complex in HTLV-1–infected T cells. Second, a ChIP assay using primers encompassing the *TBX21* promoter region (-179 to -59) was performed on the MT-2 cells to confirm the suspected interaction between this Tax-Sp1 complex and the *TBX21* promoter. Precipitation with anti-Tax or anti-Sp1, but not IgG, yielded a PCR product corresponding to the *TBX21* promoter (Figure 3B), which suggests that a Tax-Sp1 complex does bind to the *TBX21* promoter site. Finally, a reporter assay was performed using cells transfected with *TBX21*-Luc, a luciferase reporter plasmid containing the *TBX21* promoter region, to confirm a functional relationship among Tax, Sp1, and *TBX21* transcription. Cotransfection with Sp1 resulted in elevated luciferase activity compared with transfection with the reporter alone, and addition of Tax heightened this effect in a concentration-dependent manner (Figure 3C). These findings suggested that Tax, in concert with Sp1, induces *TBX21* transcription.

HTLV-1–infected Th1-like $CCR4^+$ cells are in the CNS of HAM/TSP patients. We next sought to confirm that HTLV-1–infected $CCR4^+$ T cells infiltrate the spinal cords of HAM/TSP patients and exhibit Th1-like traits, such as T-bet and IFN-γ production. Fluorescent immunohistochemical staining of tissue sections from HAM/TSP patient spinal cord lesions revealed the presence of abundant $CCR4^+$ cells infiltrating around the small blood vessels and coexpressing T-bet and IFN-γ (Figure 4A and Supplemental Figure 4). Further investigation revealed that these $CCR4^+$ cells also expressed CXCR3, the marker for Th1 cells (6). It should be noted that both IFN-γ and CXCR3 expression are reported to be induced by T-bet expression (6). Immunofluorescent staining was also used to demonstrate the existence of HTLV-1–infected $CCR4^+$ cells in the CSF of HAM/TSP patients (Figure 4B). $CCR4^+CXCR3^+$ cells were numerous among cells isolated from the CSF of HAM/TSP patients, representing 73.90% of $CD4^+$ cells isolated from a representative patient (Figure 4C) and 63.63% ± 6.73% of $CD4^+$ cells isolated from all patients ($n = 8$; Figure 4D). However, nearly all of these $CD4^+CCR4^+CXCR3^+$ cells were negative for Ki67, a marker of cell proliferation, in the CSF of the HAM/TSP patients (93.94% ± 2.07%, $n = 3$; Figure 4E). The majority of these $CD4^+CCR4^+CXCR3^+$ cells were also $CD25^+$ (70.16% ± 14.08%, $n = 3$, Supplemental Figure 5), confirming the existence of a substantial $CD4^+CD25^+CCR4^+CXCR3^+$ cell population in the CSF of HAM/TSP patients. Importantly, $CD4^+CCR4^+CXCR3^+$ cells did not make up the majority of PBMCs in HAM/TSP patients nor in HDs; in fact, such cells were very few (HAM/TSP, 3.65% ± 1.96%, $n = 8$; HD, 6.88% ± 3.09%, $n = 4$; Figure 4D). PBMCs were also isolated from ATLL patients for comparison, and $CD4^+CCR4^+CXCR3^-$ cells made up the overwhelming majority (83.03% ± 18.61%, $n = 5$; Supplemental Figure 6).

CCR4 shows potential as a molecular target for HAM/TSP immunotherapy. Analysis of HTLV-1 proviral DNA load in subpopulations of $CD4^+$ PBMCs from HAM/TSP patients confirmed that $CCR4^+$ cells were heavily infected, compared with less than

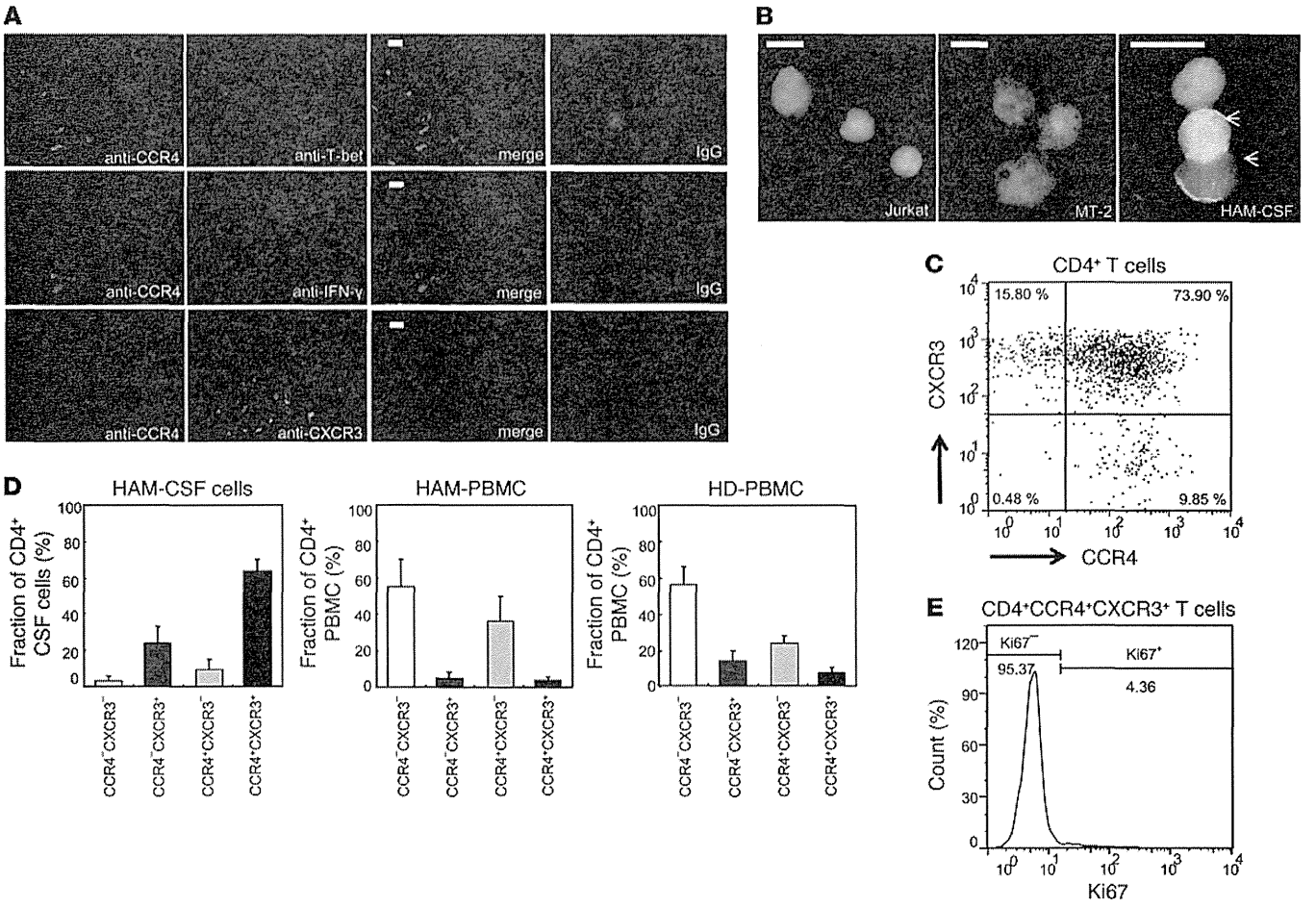

Figure 4. HTLV-1–infected Th1-like CCR4⁺ cells invade the CNS of HAM/TSP patients. (**A**) Detection of CCR4⁺ cells expressing T-bet, IFN-γ, and CXCR3 infiltrating the spinal cord of a HAM/TSP patient. Representative images show immunofluorescent codetection of CCR4 with T-bet, IFN-γ, and CXCR3, as well as the merged images, in thoracic spinal cord sections. Rabbit and goat IgG antibody served as a negative control. Scale bars: 20 μm. (**B**) Presence of HTLV-1–infected CCR4⁺ cells in HAM/TSP patient CSF. Representative images show immunofluorescence-FISH codetection of CCR4 (green) and HTLV-1 provirus (red) in Jurkat cells (uninfected control), MT-2 cells (infected control), and CSF cells from the patients. Arrows denote red provirus signal in the CSF sample. Scale bars: 20 μm. (**C**) CD4⁺ T cells in HAM/TSP patient CSF were mostly CCR4⁺CXCR3⁺. A dot plot of CCR4 and CXCR3 expression in CD4⁺ gated cells isolated from the CSF of a representative HAM/TSP patient is shown. (**D**) CD4⁺CCR4⁺CXCR3⁺ cells were numerous in CSF, but not elevated in peripheral blood, of HAM/TSP patients. Graphs show the percentages of $CCR4^-CXCR3^-$, $CCR4^-CXCR3^+$, $CCR4^+CXCR3^-$ and $CCR4^+CXCR3^+$ T cells among CD4⁺ PBMCs and CSF cells from HAM/TSP patients (n = 8) and PBMCs from HDs (n = 4). Analysis was performed using FACS. Data are mean ± SD. (**E**) Proliferation was not observed in CD4⁺CCR4⁺CXCR3⁺ cells from HAM/TSP patient CSF. The rate of Ki67 expression, a marker for cell proliferation, is shown for CD4⁺CCR4⁺CXCR3⁺ gated cells from the CSF of a representative HAM/TSP patient.

1% of $CCR4^-$ cells (n = 7; Figure 5A). To predict the efficacy of a CCR4⁺ cell–targeting cytotoxic antibody as a treatment for HAM/TSP, PBMCs were isolated from patients (n = 9) and analyzed after being cultured with and without the defucosylated chimeric anti-CCR4 monoclonal antibody KM2760 (21) or, for comparison, the steroid therapy prednisolone (PSL). Addition of 1 μg/ml KM2760 significantly reduced the percentage of CCR4⁺ cells, as measured after 7 days (P = 0.0039; Figure 5B). As little as 0.1 μg/ml KM2760 was necessary to reduce the HTLV-1 DNA load (P < 0.05), whereas PSL had no significant impact (Figure 5C). Use of 1 μg/ml of either KM2760 or PSL was sufficient to suppress spontaneous proliferation of the PBMCs, as measured by ^{3}H-thymidine incorporation (P < 0.05 and P < 0.01, respectively; Figure 5D) as well as IFN-γ production (P < 0.05 and P < 0.001, respectively; Figure 5E). Similar results were observed in experiments using cells isolated from the CSF of HAM/TSP patients (n = 8): cultures to which 1 μg/ml of KM2760 had been added exhibited reduced HTLV-1 DNA load (P = 0.0078; Figure 5F) and IFN-γ production (P = 0.0391; Figure 5G). Certain samples shown in Figure 5G did not exhibit this reduction in IFN-γ production; those samples had particularly low cell counts (0.33–2.00 cells/μl), yielding less reliable data. Despite the presence of those lower-quality samples, statistical significance was still established for the sample group as a whole.

Discussion

Previously, we hypothesized that HTLV-1 gives rise to HAM/TSP by altering the behavior of infected cells via Tax expression to yield a new population of Th1-like proinflammatory cells (26). Evidence indicated that a significant portion of this population might be Tregs, as suggested by the CD4⁺CD25⁺CCR4⁺ expres-

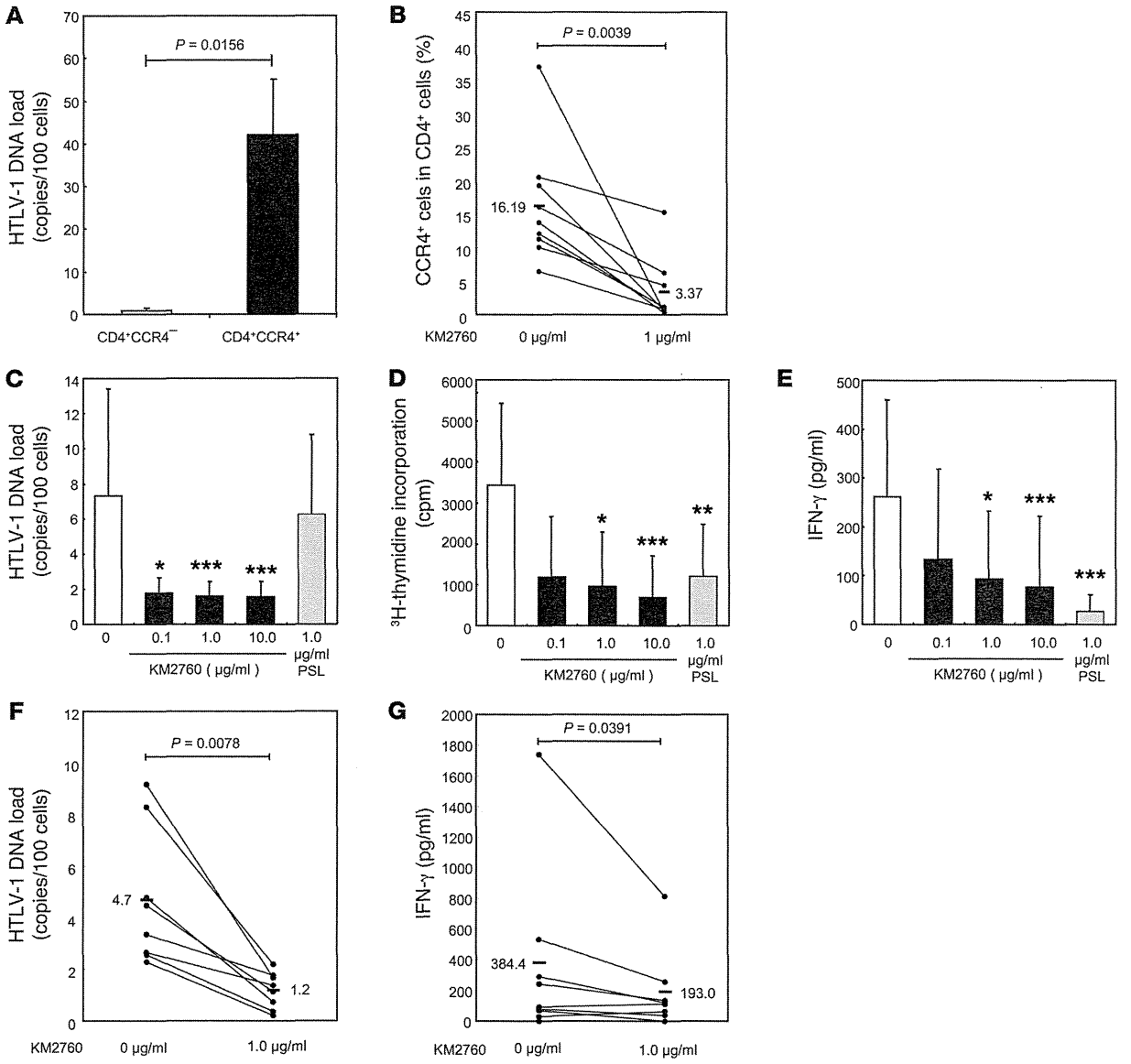

Figure 5. CCR4 shows potential as a molecular target for HAM/TSP immunotherapy. (**A**–**G**) Cells isolated from HAM/TSP patients were sorted via FACS (**A**; $n = 7$) or cultured for 7 days under the following conditions: PBMCs were cultured with various concentrations of KM2760 or 1 μg/ml PSL (**B**–**E**; $n = 9$), and CSF cells were cultured with 1 μg/ml KM2760 (**F** and **G**; $n = 8$). (**A**, **C**, and **F**) HTLV-1 proviral DNA loads were measured using quantitative PCR. (**D**) Degree of spontaneous proliferation was assessed by measuring ^{3}H-thymidine incorporation. (**E** and **G**) IFN-γ production in the culture media was evaluated using CBA assays. HTLV-1 resided in $CD4^+CCR4^+$ rather than $CCR4^-$ cells among PBMCs (**A**), and KM2760 treatment effectively targeted these cells (**B**). Consequently, KM2760 treatment successfully reduced HTLV-1 proviral DNA load (**C**), suppressed spontaneous proliferation (**D**), and decreased IFN-γ production (**E**) in PBMC cultures as well as reducing HTLV-1 DNA load (**F**) and IFN-γ production (**G**) in CSF cell cultures derived from HAM/TSP patients. (**A** and **C**–**E**) Data are mean ± SD. (**B**, **F**, and **G**) Thick horizontal bars represent mean value for all patients; line segments represent individual patients. Statistical analyses were performed using Friedman test followed by Dunn test for multiple comparisons (**C**–**E**) or Wilcoxon test (**A**, **B**, **F**, and **G**). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. untreated control.

sion profile (19). We suspected that these infected cells may infiltrate the CNS and trigger an inflammatory positive feedback loop, ultimately leading to chronic spinal cord inflammation (27). In the present study, we provided concrete evidence to support these theories on HAM/TSP pathogenesis, with a particular emphasis on the mechanism by which Tax can induce a proinflammatory phenotype intracellularly via transcriptional regulation.

There is strong evidence to support the conclusion that a substantial portion of the Treg population in HAM/TSP patients is infected with HTLV-1 (28, 29). In a previous study, we demonstrated that $CD4^+CD25^+CCR4^+$ cells were the main reservoir for HTLV-1 in HAM/TSP patients (19), but that expression profile is not exclusive to Tregs. Our present observation that $CD4^+$ T cells positive for FOXP3, a well-established marker for Tregs (10), were more thoroughly infected than the $GATA3^+$ subgroup (Figure 1A) strengthens the argument that Tregs may be the main viral reservoir. It remains debatable whether the virus preferentially infects these cells, promotes their survival (30), or even induces the expression of these

markers. One report postulates that HTLV-1 preferentially infects $CCR4^+$ cells by upregulating CCL22 to encourage cell-to-cell transfer via chemotactic attraction (31). More research is necessary to determine the true mechanism by which infected $CCR4^+$ and $FOXP3^+$ cells become so abundant in HAM/TSP patients.

We demonstrated that the suppressive ability of $CD4^+CD25^+$ $CCR4^+$ cells that characterizes Treg function was impaired by expression of the Tax protein, encoded in the pX region of the HTLV-1 genome (Figure 1B). Prior evidence indicates that Tax may exert these effects via downregulation of FOXP3 expression (20, 32). Transgenic mice expressing Tax exhibit reduced $CD4^+CD25^+FOXP3^+$ Tregs (33) and develop arthritis (34), and transgenic rats expressing HTLV-1 env-pX develop destructive arthropathies, Sjogren syndrome, vasculitis, and polymyositis (35). Collectively, these observations suggest that Tax expression can lead to inflammatory disease by weakening immune tolerance and disrupting homeostasis.

It has long been suspected that in addition to reducing FOXP3 expression, Tax may have the ability to induce IFN-γ production, thereby converting once-suppressive cells into proinflammatory cells. Indeed, intracellular Tax expression has been associated with the rapid upregulation of IFN-γ in infected cells, and researchers have theorized that this upregulation may contribute to the pathogenesis of HTLV-1–associated inflammatory disorders, including HAM/TSP (19, 36, 37). Here we showed at the mRNA level that *Tax* expression stimulated *IFNG* expression; moreover, the effect appeared delayed (Figure 2A), in a manner suggestive of 1 or more intermediate steps in the pathway, rather than direct transcriptional activation. Several candidate pathways have been proposed— such as via NF-κB, STAT1, or STAT5 — but none have been confirmed experimentally (38, 39).

We provided convincing evidence that Tax induces IFN-γ production in infected cells indirectly by amplifying the effects of Sp1 binding to — and increasing the activity of — the *TBX21* promoter: the resulting amplification of T-bet expression was responsible for the rise in IFN-γ production.

T-bet is said to be a Th1-specific T box transcription factor that controls the expression of the hallmark Th1 cytokine, IFN-γ (6). *TBX21*-deficient mice exhibit greater resistance to a variety of inflammatory and autoimmune diseases than their wild-type counterparts (40). Thus, it has been of interest that elevated *TBX21* levels have been found in the PBMCs of HAM/TSP patients (41). We showed that *TBX21* expression was elevated in the $CD4^+CD25^+CCR4^+$ cells of HAM/TSP patients, but not ATLL patients (Figure 2B and Supplemental Figure 1), which suggests that this trait is specific to HAM/TSP pathogenesis. Furthermore, we interpreted the lack of elevation in $CD4^+CD25^-CCR4^-$ cells to indicate that elevated *TBX21* is characteristic of infected cells. Importantly, we clearly demonstrated for the first time that Tax induced T-bet expression (Figure 2, C and E, and Supplemental Figures 2 and 3). Moreover, we showed that this pathway was active in $CD4^+CD25^+CCR4^+$ cells of HAM/TSP patients by silencing Tax expression and observed a corresponding reduction in *TBX21* expression; in the reverse scenario, inducing Tax expression in otherwise-normal $CD4^+CD25^+CCR4^+$ cells from HDs resulted in heightened *TBX21* expression (Figure 2D and Supplemental Figure 2). Finally, we confirmed that this correlation extended to protein production and clearly showed how Tax induces T-bet and subsequently IFN-γ production over time in culture (Figure 2E).

Tax has been reported to stably bind Sp1, a known positive transcriptional regulator of *TBX21* (25, 42). More specifically, interaction with Tax is thought to increase the DNA binding activity of Sp1 (42). Here we used co-IP with samples from the HTLV-1–infected MT-2 cell line to show that endogenous Tax interacted with Sp1 (Figure 3A). Subsequently, ChIP assays revealed that both Sp1 and Tax associated with the *TBX21* promoter region (Figure 3B), a novel finding that supports our theory that Tax and Sp1 together activate *TBX21* transcription. Finally, we showed that in the absence of Sp1, Tax had no significant effect on *TBX21* expression; however, in the presence of Sp1, Tax induced *TBX21* expression in a concentration-dependent manner (Figure 3C). This finding further substantiates our claim that Tax does not directly bind the promoter, but rather acts via Sp1. It should be noted that Tax may induce *TBX21* expression via multiple pathways: it has been reported that Tax enhances STAT1 gene expression in HTLV-1–transformed T cell lines and $CdCl_2$-stimulated JPX-9 cells (38), which suggests that Tax may also induce *TBX21* expression indirectly via STAT1.

The presence of T cell infiltrates in the CNS, indicative of spinal cord inflammation, is a well-known feature of HAM/TSP. Researchers have worked to characterize these cells over the years; together, their findings suggest that the infiltrates are dominated by $CD4^+$ T cells with relatively high proviral loads and elevated Tax and IFN-γ expression (43–45). We hypothesized that a substantial portion of the infiltrate may be made up of infected $CD4^+CCR4^+$ T cells exhibiting Th1-like properties, including IFN-γ production. We used immunohistochemistry to investigate this theory and were able to establish the presence of $CD4^+CCR4^+CXCR3^+$T-$bet^+IFN\text{-}\gamma^+$ cells in spinal cord tissue and HTLV-1–infected $CCR4^+$ cells in the CSF of HAM/TSP patients (Figure 4, A and B). We used FACS analysis to confirm that $CD4^+CCR4^+CXCR3^+$ cells made up the majority of the $CD4^+$ T cells in the HAM/TSP patient CSF (Figure 4C). For the sake of continuity between this and our previous study (19), we also confirmed that the majority of these $CD4^+CCR4^+CXCR3^+$cellswerealso$CD25^+$(SupplementalFigure5), further suggestive of a Treg identity.

We interpret the observation that these $CD4^+CCR4^+CXCR3^+$ cells were virtually nonexistent among PBMCs in HAM/TSP patients (Figure 4D) to mean that the cells had migrated to the CNS, leaving few behind in the periphery. The surprising observation that the Ki67 marker for cell proliferation was negative in the overwhelming majority of $CD4^+CCR4^+CXCR3^+$ cells in the CSF (Figure 4E) signifies that the cells are indeed proliferating elsewhere and subsequently migrating to the CNS. It has in fact been said that HTLV-1–infected cells may be extraordinarily capable of crossing the blood-brain barrier (46). Due to the high proportion of CCR4 positivity among HTLV-1–infected cells (19), the high proviral load in the CSF of HAM/TSP patients (47), and the elevated levels of CCL22 in HAM/TSP patient peripheral blood (30), one might hypothesize that the infected cells migrate across the blood-brain barrier in response to chemokine ligands of CCR4, namely CCL22. However, we found that the CSF of HAM/TSP patients contained only negligible amounts of CCL22, instead favoring the CXCR3 ligand CXCL10 (48). We now postulate that

$CD4^+CCR4^+CXCR3^+$ T cells and other $CXCR3^+$ cells may migrate to the CNS via chemotaxis induced by CXCL10 secreted by astrocytes in the CNS. Previously, we showed that these astrocytes produce CXCL10 in response to IFN-γ, and these levels are further amplified by the invading $CXCR3^+$ cells (27). Together, these findings indicate that a positive feedback loop involving the recruitment of proinflammatory cells to the CNS is the source of chronic inflammation in HAM/TSP, and that the original trigger is the migration of IFN-γ–producing HTLV-1–infected cells to the CNS. Where these proinflammatory cells are primarily proliferating, and why they proliferate at different rates in different settings, are questions to be addressed in future studies.

Our findings in this and previous studies imply that targeting $CCR4^+$ cells could constitute an effective treatment for HAM/TSP. Indeed, this strategy is already in play for ATLL patients, the majority of whom suffer from $CCR4^+$ T cell–derived cancers (7). The humanized defucosylated anti-CCR4 monoclonal antibody KW-0761, which has been shown to induce CCR4-specific ADCC, has been approved as a treatment for ATLL (49, 50). The observation that the majority of infected $CD4^+$ PBMCs in HAM/TSP patients were $CCR4^+$ (Figure 5A) suggests that an anti-CCR4 antibody with ADCC properties might be used to effectively treat HAM/TSP patients as well. Steroids are currently the standard of care for HAM/TSP patients, but this approach is not considered optimal: as with many nonspecific treatments, the effectiveness is limited, and the side effects are numerous (51). Here we compared the effects of the defucosylated chimeric anti-CCR4 monoclonal antibody KM2760 (21) with those of the steroid PSL on ex vivo cultures of cells from HAM/TSP patients. Although PSL had more potent effects per microgram, both treatments successfully reduced cell proliferation and IFN-γ production (Figure 5, D, E, and G). In addition, even a small dose of the antibody effectively reduced proviral load, whereas PSL treatment had no significant effect (Figure 5, C and F). These findings support the main premise of this paper, namely, that $CCR4^+$ cells are major viral reservoirs and producers of IFN-γ. Our study is the first to test the effects of such an antibody-based treatment on cells from HAM/TSP patients; the results were promising, and a clinical trial investigating the in vivo effectiveness in HAM/TSP patients is now underway. Importantly, our research indicates that even if the antibody does not cross the blood-brain barrier, it could be therapeutically effective against spinal cord inflammation by eliminating the proinflammatory $CCR4^+$ cells in the peripheral blood that would have migrated to the CNS.

Until very recently, there had been no reports of T cell character changing from suppressive to inflammatory in response to internal transcriptional alterations induced intracellularly by viral products. There have been many reports of Tregs transforming in the presence of inflammation due to the influence of cytokines, including instances where FOXP3 expression is lost and even cases where IFN-γ production is gained (12, 13). The only report of a similar phenomenon occurring via an intracellular virus-induced pathway states that the HTLV-1 basic leucine zipper (HBZ) gene product can reduce the expression of FOXP3 in HBZ-transgenic mouse Tregs (52). Here we showed for the first time that the HTLV-1 virus can similarly affect gene expression in human cells, inducing IFN-γ production, as well as reduce suppressive function. Collectively, the research to date suggests that HTLV-1 may preferentially infect $CCR4^+$ cells, including Tregs, and induce transcriptional changes via Tax that not only reduce FOXP3 expression, but also induce T-bet expression and consequently IFN-γ production, yielding a proinflammatory immune imbalance. While there is considerable evidence to support this theory, further experiments are necessary to prove that this pathway is indeed the origin of HAM/TSP chronic inflammation. However, here we have directly shown that the HTLV-1 protein product Tax can induce the expression of the Th1 master transcription factor T-bet, which certainly implies that HTLV-1 is capable of activating inherent plasticity in T cells and shifting their gene expression profiles toward a Th1-like state.

Methods

Patient selection and sample preparation. The study included HTLV-1–noninfected HDs ($n = 8$, 4 male and 4 female; mean age, 36 yr), asymptomatic carriers ($n = 6$, 4 male and 2 female; mean age, 56 yr), ATLL patients ($n = 6$, 2 male and 4 female; mean age, 68 yr), and HAM/TSP patients ($n = 31$, 9 male and 22 female; mean age, 61 yr). Diagnosis of ATLL was based on the criteria established by Shimoyama (53). HTLV-1 seropositivity was determined by a particle agglutination assay (Serodia-HTLV-1) and confirmed by Western blot (SRL Inc.). HAM/TSP was diagnosed according to WHO guidelines (54).

Samples of PBMCs were prepared using density gradient centrifugation (Pancoll; PAN-Biotech) and viably cryopreserved in liquid nitrogen (Cell Banker 1; Mitsubishi Chemical Medience Corp.). CSF samples were taken from 17 HAM/TSP patients. CSF cells were isolated by centrifugation and cryopreserved in the aforementioned freezing medium until use. Thoracic spinal cord tissue samples from 1 HAM/TSP patient were obtained postmortem, fixed in 10% formalin, and embedded in paraffin.

Antibodies. For FACS studies, labeled anti-CD3 (UCHT1), anti-CD4 (OKT4), anti-GATA3 (TWAJ), and anti-FOXP3 (PCH101) were purchased from eBioscience, and labeled anti-CCR4 (1G1), anti-CD25 (BC96), anti-CXCR3 (1C6), anti-T-bet (4B10), and anti-Ki67 (B56) were purchased from BD Biosciences. For IP studies, anti-Sp1 (PEP2) and normal IgG were purchased from Santa Cruz Biotechnology Inc., and anti-Tax (Lt-4) was prepared as described previously (55). For immunofluorescence studies, anti-CCR4, anti-IFN-γ, and anti-CXCR3 were purchased from Abcam; anti–T-bet was purchased from Santa Cruz Biotechnology Inc.; and Alexa Fluor 488– and Alexa Fluor 594–conjugated secondary antibodies were purchased from Invitrogen. Kyowa Hakko Kirin Co. Ltd. provided KM2760, a chimeric anti-CCR4 IgG1 monoclonal antibody (21).

Plasmids. The *TBX21*-Luc reporter gene plasmid was constructed as described previously (25). The 100-bp promoter fragment (–101 to –1) in the 5′-flanking region of the human *TBX21* gene was obtained by PCR using human PBMC genomic DNA as the template. Primers used for PCR were 5′-CGCCTCGAGGGCGGGGTGGGGCGAGGCGG-3′ and 5′-CCCAAGCTTCTGTCACTAGAGTCGCAGCGCTTT-3′. The amplified PCR product was digested with XhoI/HindIII and cloned into pPicaGene-Basic vector II (Toyo-ink), which yielded *TBX21*-Luc. Creation of the human Sp1 construct with HA-tag added to the N terminus was accomplished via real-time RT-PCR amplification of human PBMC cDNA with the following primers: Sp1 forward, 5′-CGCGAATTCATGAGCGACCAAGATCACTCCATGGA-3′; Sp1 reverse, 5′-CGCCTCGAGTCAGAAGCCATTGCCACTGATATTAATGGAC-3′. The amplified fragment was digested with EcoRI/XhoI and

subcloned into HA-tagged pcDNA3 (Invitrogen). Tax construct with FLAG-tag added to the N terminus was prepared via PCR amplification of template DNA (56) with the following primers: Tax forward, 5′-CGCGAATTCATGGCCCACTTCCCAGGGTTT-3′; Tax reverse, 5′-CGCCTCGAGTCAGACTTCTGTTTCACGGAAATGTTTTTC-3′. The amplified fragment was digested with EcoRI/XhoI and subcloned into FLAG-tagged pcDNA3. The plasmid HTLV-1 provirus (pUC/HTLV-1) was provided by T. Watanabe (University of Tokyo, Tokyo, Japan) (57). A lentiviral vector, CSIICMV, was used as a null expression vector for lentiviral infection (provided by H. Miyoshi, RIKEN BioResource Center, Tsukuba, Japan) (58). CSIICMV/GFP and CSIICMV/GFP-Tax, which express GFP and GFP fused Tax, were constructed by inserting digested GFP and GFP-Tax from pEGFP (Clontech) and pEGFP-Tax, respectively, into CSIICMV.

Flow cytometric analysis. PBMCs and CSF cells were immunostained with various combinations of the following fluorescence-conjugated antibodies that tag cell surface markers: CD3 (UCHT1), CD4 (OKT4), CD25 (BC96), CCR4 (1G1), CXCR3 (1C6). In some experiments, cells were fixed with a staining buffer set (eBioscience), then intracellularly stained with antibodies against T-bet (4B10), FOXP3 (PCH101), and GATA3 (TWAJ). Cells were stained with a saturating concentration of antibody in the dark (4°C, 30 minutes) and washed twice before analysis using FACSCalibur or LSR II (BD Biosciences). Data were processed using FlowJo software (TreeStar). For cell sorting, JSAN (Bay Bioscience) was used, and the purity exceeded 95%.

Cell isolation. $CD4^+CD25^+CCR4^+$ cells, $CD4^+CD25^-CCR4^-$ cells, $CD4^+GATA3^+$ cells, and $CD4^+FOXP3^+$ cells were separated by FACS sorting. $CD4^+$ T cells were isolated from PBMCs using negative selection with magnetic beads (MACS $CD4^+$ T cell isolation kit; Miltenyi Biotec). $CD4^+CCR4^-$ or $CD4^+CCR4^+$ cells were then isolated from these $CD4^+$ T cells using positive selection with anti-CCR4 Ab (1G1) and rat anti-mouse IgG1 microbeads (Miltenyi Biotec).

Cell culture conditions. HEK293 cells were cultured in MEM (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated FBS (Gibco, Invitrogen) and 1% penicillin/streptomycin (P/S) (Wako Pure Chemical Industries). HEK293T cells were cultured in DMEM–high glucose (Sigma-Aldrich) supplemented with 10% FBS and 1% P/S. Jurkat, MT-2, and JPX-9 cells were cultured in RPMI 1640 medium (Wako Pure Chemical Industries) supplemented with 10% FBS and 1% P/S. JPX-9 is a subline of Jurkat carrying Tax under the control of the metallothionein promoter (provided by M. Nakamura, Tokyo Medical and Dental University, Tokyo, Japan) (59), by which Tax expression is inducible by the addition of 20 μM $CdCl_2$ (Nacalai Tesque Inc.). PBMCs, $CD4^+CCR4^+$ cells, $CD4^+CD25^+CCR4^+$ cells, and $CD4^+CD25^-CCR4^-$ cells isolated from HDs or HAM/TSP patients were cultured in RPMI 1640 medium supplemented with 5% human AB serum (Gibco, Invitrogen) and 1% P/S.

Gene expression profiling and analyses. For transcriptional profiling, $CD4^+CD25^+CCR4^+$ T cells from a HAM/TSP patient, an ATLL patient, and an HD were separated using FACS sorting. Total RNA was prepared using ISOGEN (Nippon gene) following the manufacturer's recommendations. RNA was amplified and labeled with cyanine 3 (Cy3) using an Agilent Quick Amp Labeling Kit, 1-color (Agilent Technologies), following the manufacturer's instructions. For each hybridization, Cy3-labeled cRNA were fragmented and hybridized to an Agilent Human GE 4x44K Microarray (design ID 014850). After washing, microarrays were scanned using an Agilent DNA microarray scanner. Intensity values of each scanned feature were quantified using Agilent feature extraction software (version 9.5.3.1), which performs background subtractions. All data were analyzed using GeneSpring GX software (Agilent Technologies). There were a total of 41,000 probes on Agilent Human GE 4x44K Microarray (design ID 014850), not including control probes. Microarray data were deposited in GEO (accession no. GSE57259).

Real-time PCR and real-time RT-PCR. The HTLV-1 proviral DNA load was measured using ABI Prism 7500 SDS (Applied Biosystems) as described previously (19). For real-time RT-PCR analysis, total RNA isolation and cDNA synthesis were performed as described previously (19). Real-time PCR reactions were carried out using TaqMan Universal Master Mix (Applied Biosystems) and Universal Probe Library assays designed using ProbeFinder software (Roche Applied Science). ABI Prism 7500 SDS was programmed to have an initial step of 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. The primers used were as follows: *TBX21*, 5′-TGTGGTCCAAGTTTAATCAGCA-3′ (forward) and 5′-TGACAGGAATGGGAACATCC-3′ (reverse) (probe no. 9; Roche Applied Science); *Tax*, 5′-ATACAACCCCCAACATTCCA-3′ (forward) and 5′-TTTCGGAAGGGGGAGTATTT-3′ (reverse) (probe no. 69; Roche Applied Science). The primers and probes for detecting *Tax*, *IFNG*, and *GAPDH* mRNA were described previously (19). Relative quantification of mRNA was performed using the comparative Ct method using GAPDH as an endogenous control. For each sample, target gene expression was normalized to the expression of GAPDH, calculated as $2^{-(Ct[target]-Ct[GAPDH])}$.

Virus preparation and cell infection. 293T cells (1×10^6) plated in 100-mm dishes were cotransfected with the appropriate lentiviral-GFP or lentiviral–GFP-Tax expression vector (17 μg), vesicular stomatitis virus G expression vector VSV-G (pMD.G) (5 μg), rev expression vector pRSVRev (5 μg), and gag-pol expression vector pMDLg/pRRE (12 μg) (60) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 4 hours, cells were washed 3 times with PBS, 5 ml of new medium was added, and cells were incubated for 48 hours. Culture supernatants were harvested and filtered through 0.45-μm pore size filters. Lentivirus was concentrated approximately 40-fold by low centrifugation at 6,000 *g* for 16 hours and resuspended in 2 ml RPMI 1640 medium. Freshly isolated $CD4^+CD25^+CCR4^+$ T cells were activated using Treg Suppression Inspector (Anti-Biotin MACSiBead Particles preloaded with biotinylated CD2, CD3, and CD28 antibodies) according to the manufacturer's protocol (Miltenyi Biotec). After being cultured for 36 hours, cells were transduced with equal amounts of the GFP or GFP-Tax lentivirus (MOI 15), followed by centrifugation for 1 hour at 780 *g*, 32°C. After being cultured for 4 hours at 32°C, cells were washed with culture medium and cultured in round-bottomed 96-well plates at 37°C.

Treg suppression assay. A study was conducted to compare the capacities of GFP versus GFP-Tax lentivirus–infected $CD4^+CD25^+CCR4^+$ T cells to suppress cell proliferation. T cell samples were taken from HDs, and 5×10^4 $CD4^+CD25^-$ T cells were stimulated with the Treg Suppression Inspector (see above) according to the manufacturer's instructions. These cells were then cocultured with 5×10^4 GFP lentivirus–infected $CD4^+CD25^+CCR4^+$ T cells or GFP-Tax lentivirus–infected $CD4^+CD25^+CCR4^+$ T cells. After culturing for 4 days, cell proliferation was measured using a ^3H-thymidine incorporation assay as described previously (19).