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RESEARCH ARTICLE

Clinical trial of raltegravir, an integrase inhibitor, in HAM/TSP

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Abstract

Objective: Human T-cell lymphotropic virus 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic, progressive myelopathy. A high proviral load (PVL) is one of the main risk factors for HAM/TSP. Recently, it was shown that raltegravir could inhibit cell-free and cell-to-cell transmission of HTLV-1 in vitro. Given the substantial clinical experience in human immunodeficiency virus infection and its excellent safety profile, this agent may be an attractive therapeutic option for HAM/TSP patients. **Methods**: Sixteen subjects with HAM/TSP received raltegravir 400 mg orally twice daily in an initial 6-month treatment phase, followed by a 9-month post-treatment phase. HTLV-1 PVLs were assessed using droplet digital PCR from the PBMCs every 3 months, and from the CSF at baseline, month 6, and month 15. We also evaluated the ability of raltegravir to regulate abnormal immune responses in HAM/TSP patients. **Results**: While a downward trend was observed in PBMC and/or CSF PVLs of some patients, raltegravir overall did not have any impact on the PVL in this HAM/TSP patient cohort. Clinically, all patients' neurological scores and objective measurements remained relatively stable, with some expected variability. Immunologic studies showed alterations in the immune profiles of a subset of patients including decreased $CD4^{+}CD25^{+}$ T cells and spontaneous lymphoproliferation. **Interpretation**: Raltegravir was generally well tolerated in this HAM/TSP patient cohort. A subset of patients exhibited a mild decrease in PVL as well as variations in their immune profiles after taking raltegravir. These findings suggest that raltegravir may be a therapeutic option in select HAM/TSP patients. **Clinical Trial Registration Number**: NCT01867320.

Introduction

Human T-cell lymphotropic virus 1 (HTLV-1) is a human retrovirus and causes persistent infection in humans. HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic, progressive, neurological disease, which is clinically characterized by progressive lower extremity weakness, spasticity, and bladder/bowel sphincter dysfunction.[1,2] HAM/TSP is associated with perivascular inflammatory T cell infiltrates in the brain and spinal cord.[3] A higher HTLV-1 proviral load (PVL) and high level of HTLV-1-specific antibodies are also detected in peripheral blood and cerebrospinal fluid (CSF) of HAM/TSP patients.[1,2,4] Thus, infiltration of inflammatory cells including HTLV-1-infected cells into the central nervous system associated with a chronically activated immune responses against HTLV-1 have been suggested to underlie the pathogenesis of HAM/TSP. To date, no therapy has been shown to significantly modify the long-term disability associated with HAM/TSP.

An elevated HTLV-1 PVL has been suggested to be the main risk factor for developing HAM/TSP in HTLV-1-infected subjects.[5] Although HTLV-1 PVL varies widely among HTLV-1-infected subjects and remains relatively

stable within each subject, a higher HTLV-1 PVL is frequently observed in the blood of HAM/TSP patients compared to asymptomatic carriers (ACs) and is particularly increased in the cells from CSF.[4] Elevated PVL was correlated with increased HTLV-1 *tax* mRNA expression and Tax-specific $CD8^+$ T cells in HAM/TSP patients.[6] The main reservoir of HTLV-1, $CD4^+CD25$ (IL-2 receptor α chain)$^+$ T cells, were shown to express HTLV-1 *tax* mRNA at significantly higher levels than in $CD4^+CD25^-$ T cells and produce various cytokines including IFN-γ.[7,8] It has also been reported that $CD4^+CD25^+$ T cells were significantly higher in the CSF of HAM/TSP patients, compared to ACs and healthy controls, which was also significantly correlated with HTLV-1 PVL and the presence of antibody secreting B cells in the CSF of HAM/TSP patients.[9] Therefore, reduction in the HTLV-1 PVL by use of antiretrovirals may prevent immune dysregulation in HAM/TSP patients thereby modulating or reducing progression and severity of disease.

Raltegravir is the integrase inhibitor which, in combination with other antiretroviral medications, is used in both treatment-naive and treatment-experienced patients with human immunodeficiency virus 1 (HIV-1).[10] In HTLV-1, it has been reported that raltegravir efficiently blocked cell-to-cell HTLV-1 infection, integration, and immortalization in vitro.[11,12] A small case study reported that two HAM/TSP patients treated with raltegravir showed a transient decline of HTLV-1 PVL in PBMCs.[13] Given the substantial clinical experience with its use in HIV-1 infection and its safety profile, raltegravir might be considered as a therapeutic option for HAM/TSP patients, either alone or in combination with other antiretroviral treatment.

In this single-center, single-arm, open-label pilot trial, 16 HAM/TSP patients received raltegravir at 400 mg orally twice daily in an initial 6-month treatment phase, followed by an additional 9-month post-treatment phase. The goals of the study were to evaluate the safety and tolerability of raltegravir and the ability to reduce HTLV-1 PVL and to regulate immune responses in HAM/TSP patients following treatment with raltegravir.

Methods

Patients and treatment plan

Eighteen patients with clinically defined HAM/TSP by the WHO criteria were enrolled into the clinical trial of raltegravir in HAM/TSP (NCT01867320). The trial schema is summarized in Figure 1. The patients received raltegravir 400 mg orally twice daily in an initial 6-month treatment phase, followed by a 9-month post-treatment phase. Patients were evaluated clinically at each study time point (baseline/month 0, month 3, month 6, month 9, and month 15), with full neurological evaluations and clinical measures [Expanded Disability Status Scale (EDSS),[14] Scripps Neurologic Rating Scale (SNRS),[15] Timed 25 Foot Walk,[16] Insituto de Pesquisa Clinica Evandro Chagas scale (IPEC),[17] Ambulation Index (AI),[18] 9-Hole Peg Test[19]] performed at each clinic visit. Of the 18 patients, 2 patients withdrew prior to the end of the treatment period, 1 due to skin reaction (possibly related to treatment) and 1 due to inability to travel for study visits (not related to treatment), and therefore both patients were not included in the final trial analysis. In addition, 27 healthy volunteers (NINDS protocol 13-N-0149) and 13 HAM/TSP patients (NINDS protocol 98-N-0047) were included for comparison of the immunological markers and coefficient of variation of the longitudinal HTLV-1 PVL, respectively. Blood samples were taken at each study visit; PBMCs were isolated by Ficoll-Hypaque (Lonza) centrifugation and cryopreserved in liquid nitrogen until use. CSF was obtained by lumbar puncture at baseline, month 6, and month 15 time points of the trial; the CSF cells were collected within an hour by centrifugation of CSF samples and stored in −80 °C freezer until use or freshly used for flow cytometric analysis. The study protocol (13-N-0135) was reviewed and approved by the National Institute of Neurological Disorders and Stroke Institutional Review Board. Prior to study inclusion, written informed consent was obtained from all the participants in accordance with the Declaration of Helsinki. Details on this clinical trial are recorded at clinicaltrials.gov (NCT01867320).

HTLV-1 PVL

HTLV-1 PVL was measured using droplet digital PCR (ddPCR; Bio-Rad) as previously described.[20] DNA was extracted from the PBMC and CSF cell pellets using a DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. Primers and probes specific for HTLV-1 *tax* gene and human ribonuclease P protein subunit 30 (*RPP30*) was used. All samples were tested in duplicate, and PVL is reported as the average of the two measurements.

HTLV-1 tax/HBZ mRNA expression

HTLV-1 *tax* and *HBZ* mRNA was measured using ddPCR (Bio-Rad) as previously described.[21] RNA was extracted from fresh-frozen PBMCs or cultured PBMCs for 20 h using a RNAeasy plus mini kit (Qiagen) and cDNA was synthesized from extracted RNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems). The primers and probes specific for HTLV-1 *tax* and *HBZ* and hypoxanthine phosphoribosyl transferase (*HPRT*) were used. All

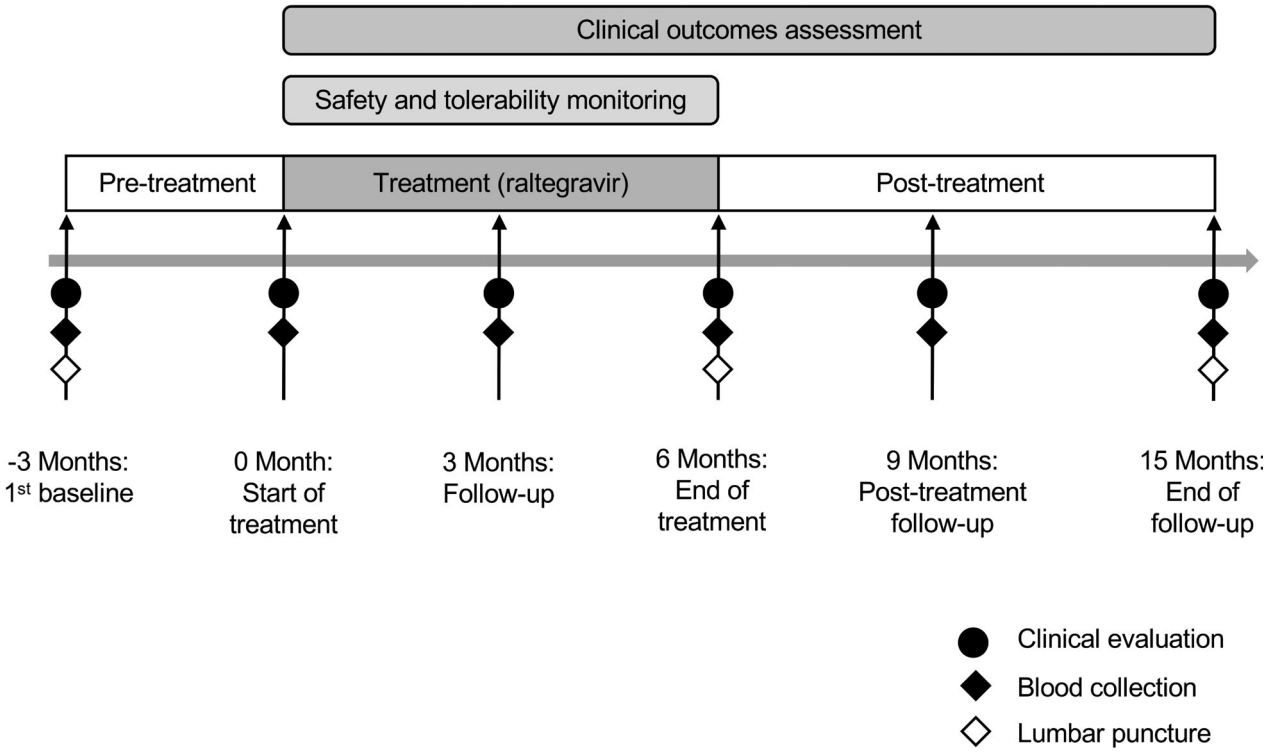


Figure 1. Schema of clinical trial of raltegravir for HAM/TSP patients.

samples were tested in duplicate, and normalized values of HTLV-1 *tax* mRNA or *HBZ* mRNA to *HPRT* mRNA are reported as the average of the two measurements.

Flow cytometry

For analysis of peripheral blood lymphocyte and CSF lymphocyte populations, EDTA-treated whole blood or CSF cells were stained with CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD27, CD45, CD45RA, CD56, CD122, CXCR5, IgD, HLA-DR (all from BD Biosciences), and FoxP3 (eBiosciences), as previously described.[9]

For detections of HTLV-1 Tax and IFN-γ, PBMCs of HAM/TSP patients were cultured for 20 h and then were stained with antibodies for CD3, CD4, CD8, and CD25 (all from BD Biosciences) as previously reported.[9] After treatment with fixation/permeabilization solution, the cells were stained with antibodies for Tax (Lt-4) and IFN-γ (BD Biosciences). All flow cytometric analysis was performed using an LSR II (BD Biosciences). The data were analyzed using FlowJo 10.5 software (FlowJo LLC).

Lymphoproliferation assays

Lymphoproliferation assay using [^{3}H]-thymidine was performed as previously described.[22] PBMCs were cultured in triplicate and pulsed after 3–5 days of culture with 1 μCi [^{3}H]-thymidine. The average cpm from each of the wells was plotted. Lymphoproliferation assay was also performed using CFSE (CellTrace™ CFSE cell proliferation kit; Invitrogen) as previously described.[23] After culturing for 4 and 5 days, the cells were stained with antibodies against CD3, CD4, CD8, and CD25 (all from BD Biosciences). The data were acquired on an LSRII flow cytometer (BD Biosciences) and were analyzed using FlowJo 10.5 software (FlowJo LLC).

Statistics

One-way ANOVA with mixed effect analysis was used to compare: HTLV-1 PVL, HTLV-1 *tax*/*HBZ* mRNA, spontaneous lymphoproliferation, $CD25^+$ cell frequency in blood and CSF $CD4^+$ and $CD8^+$ T cells, $CD122^+$ cell frequency in CSF $CD8^+$ T cells of HAM/TSP patients, and antibody secreting B cells in CSF of HAM/TSP patients. A paired *t*-test was used to compare: spontaneous $CD4^+$ T-cell proliferation between baseline and month 6 time points, $CD122^+$ cell frequency in blood $CD8^+$ T cells, $CD25^+$ cell frequency in CSF $CD4^+$ T cells, Tax and Tax-induced IFN-γ expression in cultured PBMCs of HAM/TSP patients between baseline and at month 15 of the trial. A Pearson correlation test was used for compare

Table 1. Demographics of HAM/TSP patients for raltegravir trial.

Patient No.	Age (year)	Gender	Race/Ethnicity	Disease duration (year)	HTLV-1 PVL (%)	
					PBMC[1]	CSF
HAM #1	53	F	Black/African American	8.4	20.50	45.06
HAM #2	54	F	Black/African American	1.3	15.34	25.84
HAM #3	38	F	Black/African American	1.2	10.92	50.95
HAM #4	48	F	Black/African American	25.2	10.63	29.73
HAM #5	75	M	Black/African American	9.8	18.37	25.70
HAM #7	68	M	White	10.1	16.60	40.44
HAM #8	53	F	White	4.6	0.79	7.48
HAM #9	66	F	Black/African American	4.1	11.57	20.36
HAM #10	63	M	Black/African American	20.2	13.72	51.68
HAM #12	42	F	Black/African American	6.6	12.76	15.55
HAM #13	60	M	White	20.6	20.73	23.17
HAM #14	55	M	Black/African American	3.5	33.40	58.53
HAM #15	39	F	Hispanic	18.3	18.84	23.00
HAM #16	48	F	Hispanic	17.4	10.11	32.13
HAM #17	40	F	Black/African American	14.0	27.75	34.42
HAM #18	54	M	Hispanic	7.1	17.07	27.34

[1]Average of two baselines (month 0 and up to 3 months before the treatment).

between HTLV-1 PVL in PBMCs and *tax* mRNA expression in cultured PBMCs of HAM/TSP patients. All statistical analysis was performed using Prism (GraphPad software).

Results

Patients' characteristics

Of the 18 HAM/TSP patients enrolled into the clinical trial of raltegravir, 16 HAM/TSP patients completed the treatment period with at least one post-treatment visit. Fourteen patients had a slowly progressive course of neurologic disease and two patients had rapid progression. The demographic characteristics of the study population are summarized in Table 1. Mean age of the study population was 53.5 years. The ratio of female to male was 10:6 and patients were predominantly African American. Mean disease duration of the study population was 10.8 years (Table 1).

Safety and clinical effects of raltegravir

Overall, the medication was well tolerated, with very few significant adverse events thought to be related to treatment. While on the study medication, a number of study participants (HAM #2, 3, 4, 7, 9, 10, 14, 16, 17) noted subjective improvements in a variety of symptoms at month 3 and/or month 6 time points, including increase in energy, ease with walking, improved spasticity, urination, and constipation. However, most objective clinical measurements were overall stable or showed clinical variations and slow progression typical of HAM/TSP patients (Table 2). Several patients showed very mild improvements in IPEC scores over the course of the trial (HAM #3, 5, 16, 17), and a few patients showed improved walking times during or after treatment (HAM #2, 4, 10, 16), but these findings are likely related to typical clinical fluctuations seen in HAM/TSP patients and may not be attributable to medication effect. No patients significantly worsened clinically during the trial, with the exception of one patient whose functional status declined after an unrelated hospitalization (HAM #13).

HTLV-1 PVL in HAM/TSP patients with raltegravir

We analyzed HTLV-1 PVL in PBMCs and CSF cells of the 16 HAM/TSP patients pre- and post-administration of raltegravir. Before treatment, HTLV-1 PVL was detected in the range of 0.79%–33.40% and 7.48%–58.53% in PBMC and CSF cells of the HAM/TSP patients, respectively (Table 1). As reported,[4] HTLV-1 PVL in CSF cells was much higher than the PVL in the PBMCs of each patient at pretreatment. Figure 2 shows the group analysis of HTLV-1 PVL in PBMCs and CSF cells (Fig. 2A and B, respectively) of the 16 HAM/TSP patients during the trial. There was no statistical change of HTLV-1 PVL in the group analysis of either PBMCs or CSF cells during the trial, suggesting that HTLV-1 PVL remained relatively stable in HAM/TSP patients after raltegravir treatment. However, analysis of individual

Table 2. Clinical parameters of HAM/TSP patients at baseline and post-treatment of raltegravir.

Patient No.	Time course	EDSS	SNRS	T25-FW	IPEC	AI	9HPT Right	9HPT Left
HAM #1	Baseline	6.0	67	13.4	14	4.0	18.6	19.1
	Month 3	6.0	67	13.5	13	4.0	18.6	18.3
	Month 6	6.0	67	14.3	14	4.0	16.9	19.1
	Month 9	6.0	67	14.5	14	4.0	18.2	17.9
	Month 15	6.0	67	14.1	14	4.0	15.8	18.9
HAM #2	Baseline	6.5	57	33.1	22	6.0	19.1	20.7
	Month 3	6.5	60	39.2	18	6.0	19.6	19.6
	Month 6	6.5	60	39.5	23	6.0	17.9	21.0
	Month 9	6.5	54	33.1	23	6.0	19.2	20.6
	Month 15	6.5	57	33.5	22	6.0	19.8	21.2
HAM #3	Baseline	6.0	70	23.5	12	5.0	22.3	21.3
	Month 3	6.0	74	22.7	11	5.0	19.8	18.6
	Month 6	6.0	74	26.2	11	5.0	19.2	20.7
	Month 9	6.0	74	18.3	11	4.0	19.2	21.8
	Month 15	6.0	74	21.2	11	5.0	19.7	19.9
HAM #4	Baseline	6.5	61	20.7	15	6.0	23.6	26.8
	Month 3	6.5	61	21.6	15	6.0	20.4	23.3
	Month 6	6.5	64	21.4	15	6.0	22.2	23.5
	Month 9	6.5	73	16.8	15	6.0	NA	NA
	Month 15	6.5	73	19.3	15	6.0	18.7	23.5
HAM #5	Baseline	6.0	56	5.3	13	2.0	21.4	25.3
	Month 3	6.0	56	6.0	13	2.0	19.3	26.2
	Month 6	6.0	56	6.1	13	2.0	19.8	23.3
	Month 9	6.0	56	6.5	13	2.0	21.2	23.9
	Month 15	6.0	68	6.3	12	2.0	19.4	24.7
HAM #7	Baseline	6.0	76	9.0	12	4.0	21.8	22.8
	Month 3	6.0	76	11.2	12	4.0	22.0	24.6
	Month 6	6.0	76	10.1	14	4.0	21.1	25.2
	Month 9	6.0	76	7.9	13	4.0	22.2	28.9
	Month 15	NA	NA	NA	NA	NA	NA	NA
HAM #8	Baseline	6.0	79	15.5	13	4.0	24.7	25.6
	Month 3	6.0	79	14.3	13	4.0	22.8	23.8
	Month 6	6.0	79	14.6	13	4.0	23.8	23.1
	Month 9	6.0	79	15.1	13	4.0	24.7	24.5
	Month 15	6.0	79	14.7	13	4.0	24.3	24.9
HAM #9	Baseline	8.0	65	NA	25	8.0	24.6	27.8
	Month 3	8.0	55	NA	25	8.0	21.8	23.2
	Month 6	8.0	55	NA	25	8.0	21.5	21.7
	Month 9	8.0	55	NA	25	8.0	20.2	23.1
	Month 15	8.0	55	NA	25	8.0	20.5	21.3
HAM #10	Baseline	6.0	41	38.4	14	5.0	28.7	25.9
	Month 3	6.0	44	26.7	14	5.0	28.0	30.8
	Month 6	6.0	48	43.7	14	5.0	29.5	32.0
	Month 9	6.0	48	37.1	14	5.0	31.5	35.1
	Month 15	6.0	48	33.4	14	5.0	29.1	38.2
HAM #12	Baseline	7.0	62	NA	22	8.0	21.8	22.2
	Month 3	7.0	60	NA	22	8.0	26.0	31.4
	Month 6	7.0	60	NA	22	8.0	NA	26.8
	Month 9	7.0	60	NA	22	8.0	21.9	26.0
	Month 15	7.0	56	NA	23	8.0	25.5	27.5
HAM #13	Baseline	6.5	42	NA	19	6.0	28.1	24.7
	Month 3	6.5	42	NA	19	6.0	24.4	31.6
	Month 6	6.5	42	NA	19	6.0	28.0	24.7
	Month 9	7.0	42	NA	22	6.0	27.4	25.7
	Month 15	7.0	42	NA	22	6.0	34.0	29.4

(*Continued*)

Table 2 Continued.

Patient No.	Time course	EDSS	SNRS	T25-FW	IPEC	AI	9HPT Right	9HPT Left
HAM #14	Baseline	8.0	51	NA	23	8.0	19.8	20.4
	Month 3	8.0	51	NA	23	8.0	18.2	19.7
	Month 6	8.0	51	NA	23	8.0	18.8	19.3
	Month 9	8.0	51	NA	23	8.0	20.9	21.2
	Month 15	8.0	51	NA	24	8.0	16.6	21.1
HAM #15	Baseline	6.5	58	17.2	17	6.0	25.0	25.5
	Month 3	6.5	58	16.7	19	6.0	20.7	21.3
	Month 6	6.5	58	17.2	19	6.0	21.3	23.0
	Month 9	6.5	58	18.2	20	6.0	21.3	22.9
	Month 15	6.5	58	17.5	20	6.0	18.5	19.3
HAM #16	Baseline	6.5	66	33.5	17	6.0	26.6	29.2
	Month 3	6.5	66	30.0	17	6.0	25.2	27.1
	Month 6	6.5	66	12.7	16	5.0	26.1	29.6
	Month 9	6.5	66	19.1	16	5.0	24.1	24.5
	Month 15	6.5	66	26.5	16	6.0	22.0	26.0
HAM #17	Baseline	7.0	55	62.0	17	6.0	24.2	27.1
	Month 3	7.0	58	37.5	19	7.0	22.9	23.0
	Month 6	7.0	55	47.0	17	7.0	22.7	23.4
	Month 9	7.0	55	48.9	17	7.0	21.5	23.4
	Month 15	7.0	66	53.5	17	7.0	21.2	23.4
HAM #18	Baseline	6.0	80	10.6	13	4.0	19.5	22.4
	Month 3	6.0	80	11.0	13	4.0	19.8	20.6
	Month 6	6.0	80	10.3	13	4.0	22.1	21.5
	Month 9	6.0	80	9.5	13	4.0	19.5	21.4
	Month 15	6.0	80	12.2	13	4.0	21.3	21.3

NA, not applicable; EDSS, Expanded Disability Status Scale; SNRS, Scripps Neurologic Rating Scale; T25-FW, Timed 25-Foot Walk; IPEC, Insituto de Pesquisa Clinica Evandro Chagas; AI, Ambulation Index; 9HPT, Nine-Hole Peg Test.

patients showed a differential reduction of HTLV-1 PVL in some patients (Table 3). Based on the percentage changes of HTLV-1 PVL in the 6-month treatment phase compared to the baseline, we classified HAM/TSP patients into three groups: patients with a decreased HTLV-1 PVL into group 1, stable HTLV-1 PVL into group 2, and patients with increased HTLV-1 PVL into group 3 (Fig. 2C and D). The cut-off value for change of HTLV-1 PVL was determined as 12.19% based on the coefficient of variation of the HTLV-1 PVL in PBMCs of 13 HAM/TSP patients observed in our natural history study (NINDS protocol 98-N-0047) ranging from 1 to 9 years. As shown in Figure 2C, eight HAM/TSP patients (group 1) demonstrated a reduction of HTLV-1 PVL in PBMC at month 3 and/or month 6 of raltegravir treatment (Fig. 2C). Six patients in group 2 had stable HTLV-1 PVL over this study period while only two HAM/TSP patients showed an increased HTLV-1 PVL in PBMCs during treatment (group 3) (Fig. 2C). Analysis of HTLV-1 PVL in CSF at month 6 of raltegravir treatment showed that 4 HAM/TSP patients trended toward a decrease in PVL (group 1) while seven HAM/TSP patients appeared relatively stable (group 2) (Fig. 2D). Five HAM/TSP patients showed increased HTLV-1 PVL in the CSF (group 3) (Fig. 2D). Of note, some HAM/TSP patients also demonstrated a reduction of HTLV-1 PVL in PBMC and/or CSF during the post-treatment period. In this subset analysis, there was no correlation within any of these groups between CSF and PBMC proviral load (data not shown). Collectively, these results demonstrated that a downward trend in HTLV-1 PVL, particularly in PBMCs, was observed in a subset of treated HAM/TSP patients even though group analysis showed no overall reduction of HTLV-1 PVL in both PBMCs and CSF cells with raltegravir therapy.

HTLV-1 mRNA expression in PBMC of HAM/TSP patients with raltegravir

Two HTLV-1 genes, *tax* and *HBZ*, have been shown to play important roles in the pathogenesis of HAM/TSP.[24] To determine if raltegravir affected HTLV-1 mRNA expression in HAM/TSP patients, we examined and compared HTLV-1 mRNA expressions, *tax* and *HBZ*, in PBMCs of HAM/TSP patients at pretreatment, at month 6, and month 15 of raltegravir treatment. Although

HTLV-1 *tax* mRNA is rarely detectable directly in fresh PBMCs of HTLV-1-infected individuals, HAM/TSP patients are known to have spontaneously increase *tax* mRNA expression in PBMCs after ex vivo culture without any exogenous stimulators.[6] Interestingly, group analysis of *tax* mRNA expression after such ex vivo culture showed a significant inhibition in PBMCs of HAM/TSP patients after treatment that was sustained at the 15 month time point (Fig. 3A). This inhibition appeared specific for HTLV-1 *tax* mRNA since no such inhibition of the HTLV-1 *HBZ* mRNA was seen. Unlike *tax* mRNA, *HBZ* mRNA is ubiquitously expressed in HTLV-1-infected cells and in PBMCs of HAM/TSP patients.[25,26] Consistent with these previous studies, the expression of *HBZ* mRNA was detected in uncultured PBMCs of all the HAM/TSP patients (Fig. 3B). However, in contrast to *tax* mRNA expression (Fig. 3A), group analysis of *HBZ* mRNA expression did not show any significant change in fresh, uncultured PBMCs of HAM/TSP patients after treatment with raltegravir (Fig. 3B). Consistent with previous reports,[6] *tax* mRNA expression correlated with HTLV-I PVL in HAM/TSP patients at baseline and at month 6 of raltegravir treatment (Fig. 3C).

We also examined if there is any difference in *tax* and *HBZ* mRNA expression among the three HAM/TSP groups based on the percentage change of HTLV-1 PVL in the 6-month treatment phase compared to baseline. Interestingly, while there was no significant difference of the percentage change of *tax* mRNA expression in HAM/TSP patients (Fig. 3D), a downward trend in the percentage change of *HBZ* mRNA expression was observed in group 1 compared to group 2 or 3 (Fig. 3E). In addition to *tax* mRNA expression, we assessed Tax protein and IFN-γ expression in $CD4^+CD25^+$ T cells, a major reservoir of HTLV-1, in ex vivo PBMC culture of HAM/TSP patients at baseline, at month 6, and month 15 of the trial. Tax expression was significantly decreased in $CD4^+CD25^+$ T cells of HAM/TSP patients at month 15 compared to the baseline (Fig. 3F). Concomittant with this decrease in Tax protein expression, there was also a significant reduction of Tax-induced IFN-γ expression in $CD4^+CD25^+$ T cells of HAM/TSP patients at month 15 compared to the baseline (Fig. 3G). These results demonstrated that raltegravir had a selective inhibitory effect on the expression of HTLV-1 mRNA, especially *tax* mRNA, from PBMCs of HAM/TSP patients.

Immunological changes in peripheral blood and CSF of HAM/TSP patients treated with raltegravir

Several types of immune cell dysregulations in peripheral blood and CSF have been reported in HAM/TSP including increased activated T cells, effector T cells, and antibody secreting B cells.[27] Some immunological markers of HAM/TSP patients at baseline and post-treatment of raltegravir are summarized in Table 3. As shown in Figure 4A, as expected, a higher frequency of $CD4^+CD25^+$ T cells was detected in both peripheral blood and CSF of HAM/TSP patients before raltegravir treatment compared to control ranges of blood and CSF of 27 healthy volunteers (25%–75% percentile) highlighted in gray (Fig. 4A). Importantly, group analysis demonstrated that the frequency of $CD4^+CD25^+$ T cells significantly decreased in both peripheral blood and CSF of HAM/TSP patients after treatment with raltegravir, particularly at month 15 (Fig. 4A). It has been reported that antibody secreting B cells are elevated in CSF of HAM/TSP patients and that this was significantly correlated with increased $CD4^+CD25^+$ T cells in the CSF.[9] In 15 HAM/TSP patients, antibody secreting B cells were detected in the CSF of HAM/TSP patient, except for one HAM/TSP patient (HAM #9), before treatment (mean 4.873 $\pm$ standard deviation 4.504; Table 3). Interestingly, after treatment with raltegravir, antibody secreting B cells gradually decreased in CSF of HAM/TSP patients and was sustained at the 15-month time point ($n = 15$; Fig. 4B). Moreover, 10 HAM/TSP patients had no detectable antibody secreting B cells at month 6 and/or 15 (Table 3). The subset analysis of CSF B cells was not done in one HAM/TSP patient pre- and post-raltegravir treatment due to low B-cell number in the CSF (HAM#4; Table 3).

In addition, the frequency of $CD8^+CD25^+$ T cells was also significantly decreased in peripheral blood of HAM/TSP patients although not in the CSF after treatment with raltegravir (Fig. 4C). Group analysis also demonstrated a

Figure 2. HTLV-1 PVL in HAM/TSP patients with raltegravir. (A) Group analysis of HTLV-1 PVL in PBMCs of 16 HAM/TSP patients. Closed circles represent median of HTLV-1 PVL and error bars represent range. (B) Group analysis of HTLV-1 PVL in CSF cells of 16 HAM/TSP patients. Closed circles represent median of HTLV-1 PVL and error bars represent range. (C) Classification of HAM/TSP patients into three groups based on change of HTLV-1 PVL in PBMCs during the treatment. Group 1: Decreased HTLV-1 PVL; Group 2: Stable (no significant change); Group 3: increased HTLV-1 PVL. (D) Classification of HAM/TSP patients into three groups based on change of HTLV-1 PVL in CSF cells during the treatment. Group 1: Decreased HTLV-1 PVL; Group 2: No significant change; Group 3: increased HTLV-1 PVL. Cut off value (12.19%) was determined based on coefficient of variation of PBMC HTLV-1 PVL of 13 HAM/TSP patients with chronic infection in natural history of 1–9 years.

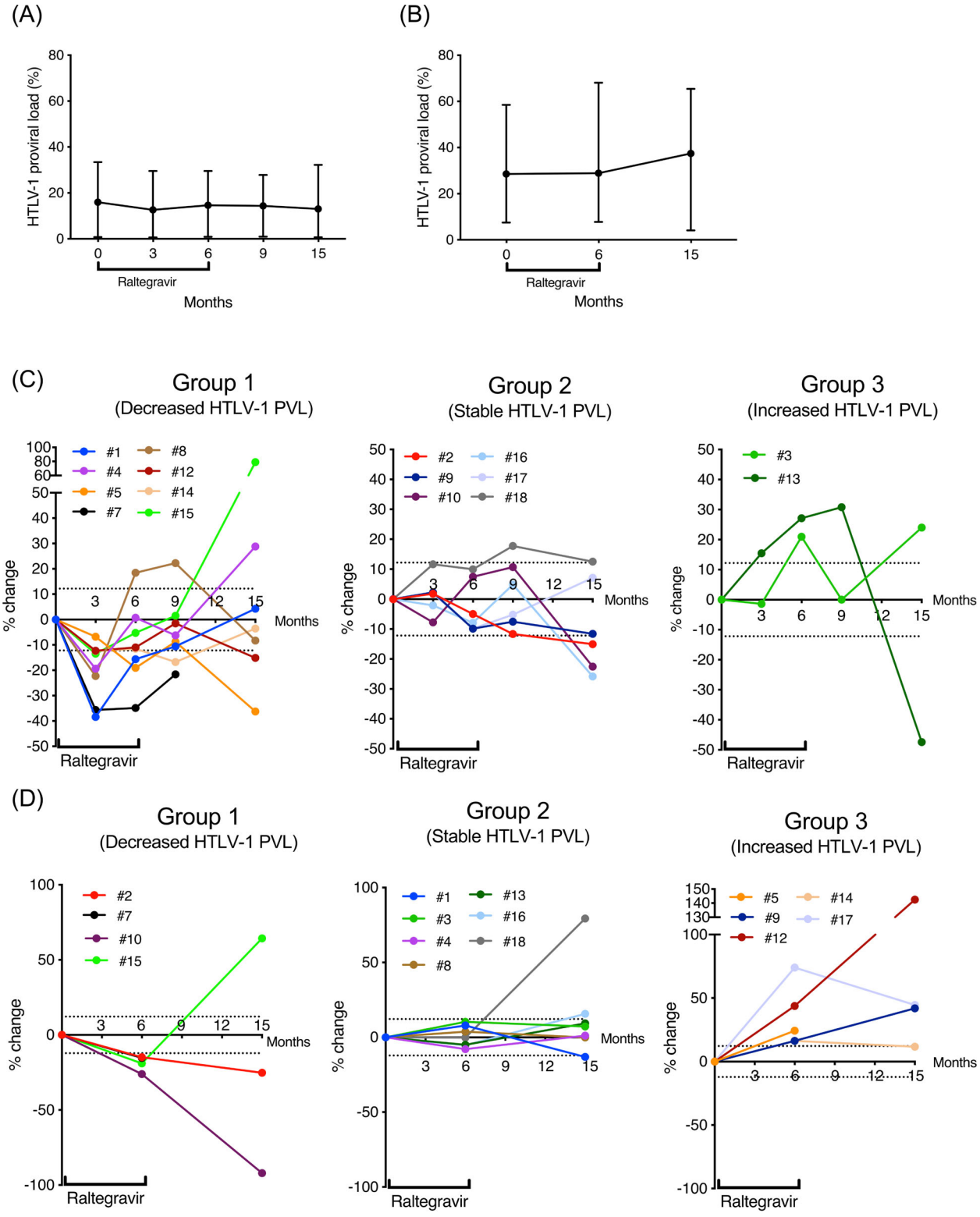


Table 3. HTLV-1 PVL and immunological markers of HAM/TSP patients at baseline and post-treatment of raltegravir.

Patient No.	Time course	HTLV-1 PVL (%)		$CD4^+CD25^+$ T cells (%)		$CD8^+CD25^+$ T cells (%)		$CD8^+CD122^+$ T cells (%)		Antibody secreting B cells (%)
		PBMC[1]	CSF	PBMC[1]	CSF	PBMC[1]	CSF	PBMC[1]	CSF	CSF
HAM #1	Baseline	20.50	45.06	16.25	12.20	3.47	1.64	57.90	63.20	5.43
	Month 6	17.30	48.61	16.60	12.90	5.60	1.32	30.60	67.20	6.20
	Month 15	21.37	39.14	7.96	2.80	2.67	0.40	60.00	59.00	0.81
HAM #2	Baseline	15.34	25.84	5.14	10.10	0.64	1.67	37.05	ND	8.57
	Month 6	14.58	22.00	6.31	8.76	0.86	0.98	29.30	77.60	9.09
	Month 15	13.04	19.32	7.68	5.68	0.40	0.94	27.10	80.70	5.13
HAM #3	Baseline	10.92	50.95	11.55	27.40	0.76	2.41	37.70	88.80	16.60
	Month 6	13.21	56.24	13.20	31.00	0.89	5.14	41.60	94.00	10.00
	Month 15	13.54	54.66	6.44	19.90	0.77	7.40	26.20	89.10	4.79
HAM #4	Baseline	10.63	29.73	3.97	5.07	0.78	0.23	48.45	74.90	(-)
	Month 6	10.70	27.40	8.63	6.24	0.87	0.54	53.50	81.80	(-)
	Month 15	13.69	30.07	4.09	3.76	0.43	0.57	30.60	68.00	(-)
HAM #5	Baseline	18.37	25.70	10.70	8.09	5.75	0.72	38.35	62.20	1.79
	Month 6	14.87	31.95	12.30	7.18	4.39	1.20	27.60	76.30	0.00
	Month 15	11.71	ND	2.48	ND	0.63	ND	21.70	ND	ND
HAM #7	Baseline	16.60	40.44	7.01	7.85	1.19	2.10	46.25	89.60	1.56
	Month 6	10.81	34.27	7.76	7.21	1.40	1.56	39.80	81.00	0.00
	Month 15	ND	ND	ND	ND	ND	ND	ND	ND	ND
HAM #8	Baseline	0.79	7.48	2.13	2.25	0.22	0.66	34.15	57.20	1.67
	Month 6	0.93	7.76	1.09	2.01	0.09	0.46	28.30	55.80	2.17
	Month 15	0.72	7.47	1.14	2.98	0.17	0.90	26.00	54.20	0.00
HAM #9	Baseline	11.57	20.36	4.52	5.14	0.65	0.14	39.00	71.70	0.00
	Month 6	10.42	23.69	2.73	4.57	0.49	0.57	28.10	65.90	0.00
	Month 15	10.22	28.89	2.34	3.31	0.34	0.58	32.80	63.50	(-)
HAM #10	Baseline	13.72	51.68	3.90	1.67	0.21	0.45	34.55	60.00	0.92
	Month 6	14.74	38.19	4.37	5.84	0.31	0.62	41.60	58.10	0.00
	Month 15	10.62	4.10	2.53	3.74	0.17	0.55	24.60	60.20	(-)
HAM #12	Baseline	12.76	15.55	4.44	3.52	0.64	0.26	45.85	58.00	1.64
	Month 6	11.36	22.36	4.17	3.21	0.83	0.29	57.40	64.10	0.00
	Month 15	10.83	37.71	1.25	1.83	0.18	0.87	40.20	52.10	1.33
HAM #13	Baseline	20.73	23.17	16.59	13.70	2.78	3.11	49.10	82.00	8.33
	Month 6	26.35	22.02	20.80	6.33	3.48	0.77	47.90	55.00	0.00
	Month 15	10.90	25.33	10.10	9.72	0.64	1.42	46.80	52.00	(-)
HAM #14	Baseline	33.40	58.53	15.20	18.30	2.37	4.74	45.40	81.30	8.33
	Month 6	29.57	68.10	13.30	4.74	2.42	0.88	40.20	60.80	0.00
	Month 15	32.21	65.40	13.70	12.20	1.95	3.11	52.20	61.20	0.00
HAM #15	Baseline	18.84	23.00	7.93	14.10	1.06	2.41	51.70	84.80	8.70
	Month 6	17.85	18.64	6.98	2.09	0.95	0.42	42.00	55.60	0.00
	Month 15	33.74	37.80	3.06	9.67	0.36	1.36	38.10	57.10	0.00
HAM #16	Baseline	10.11	32.13	7.64	14.60	0.42	2.72	32.70	69.00	4.00
	Month 6	9.31	30.43	5.17	7.75	0.40	0.82	35.50	53.90	11.50
	Month 15	7.50	37.16	4.95	9.07	0.80	1.74	31.90	45.90	5.41
HAM #17	Baseline	27.75	34.42	5.05	1.84	0.73	0.41	61.60	83.60	1.39
	Month 6	25.00	59.88	5.83	13.40	0.69	1.90	49.00	92.50	3.28
	Month 15	29.72	49.75	2.70	6.18	0.22	2.44	51.30	60.20	2.38
HAM #18	Baseline	17.07	27.34	6.12	12.50	0.44	2.35	38.95	66.90	4.17
	Month 6	18.77	27.27	11.60	13.10	0.98	1.57	62.00	89.30	2.15
	Month 15	19.20	49.03	12.30	16.30	0.77	2.38	39.90	75.40	0.00

(-): It was not able to analyze due to low B cell number. ND, Not done.

[1]Baseline: Average of two baselines (month 0 and up to 3 months before the treatment).

decreased frequency in CD122(IL-15 receptor β chain)$^+$ cells in both peripheral blood and CSF CD8$^+$ T cells of HAM/TSP patients after treatment with raltegravir (Fig. 4D). However, other biological markers of HAM/TSP patients including CD4/CD8 ratio, T-cell subsets (effector/memory (CD45RA$^-$CD27$^-$) T cells, effector (CD45RA$^+$CD27$^-$) T cells, memory folicular helper (CD45RA$^-$CXCR5$^+$) CD4$^+$ T cells and FoxP3 expression), and NK cell subsets (CD56^{bright} and CD56^{dim}), did not show any significant changes during the trial (data not shown). In addition, there was no significant difference of these immunological markers among the three HAM/TSP groups based on the percentage change of HTLV-1 PVL in the 6-month treatment phase compared to baseline (data not shown). Collectively, these results demonstrated alterations in the immune profiles of HAM/TSP patients with raltegravir, most notably decreased activated T cells and antibody secreting B cells in peripheral blood and/or CSF.

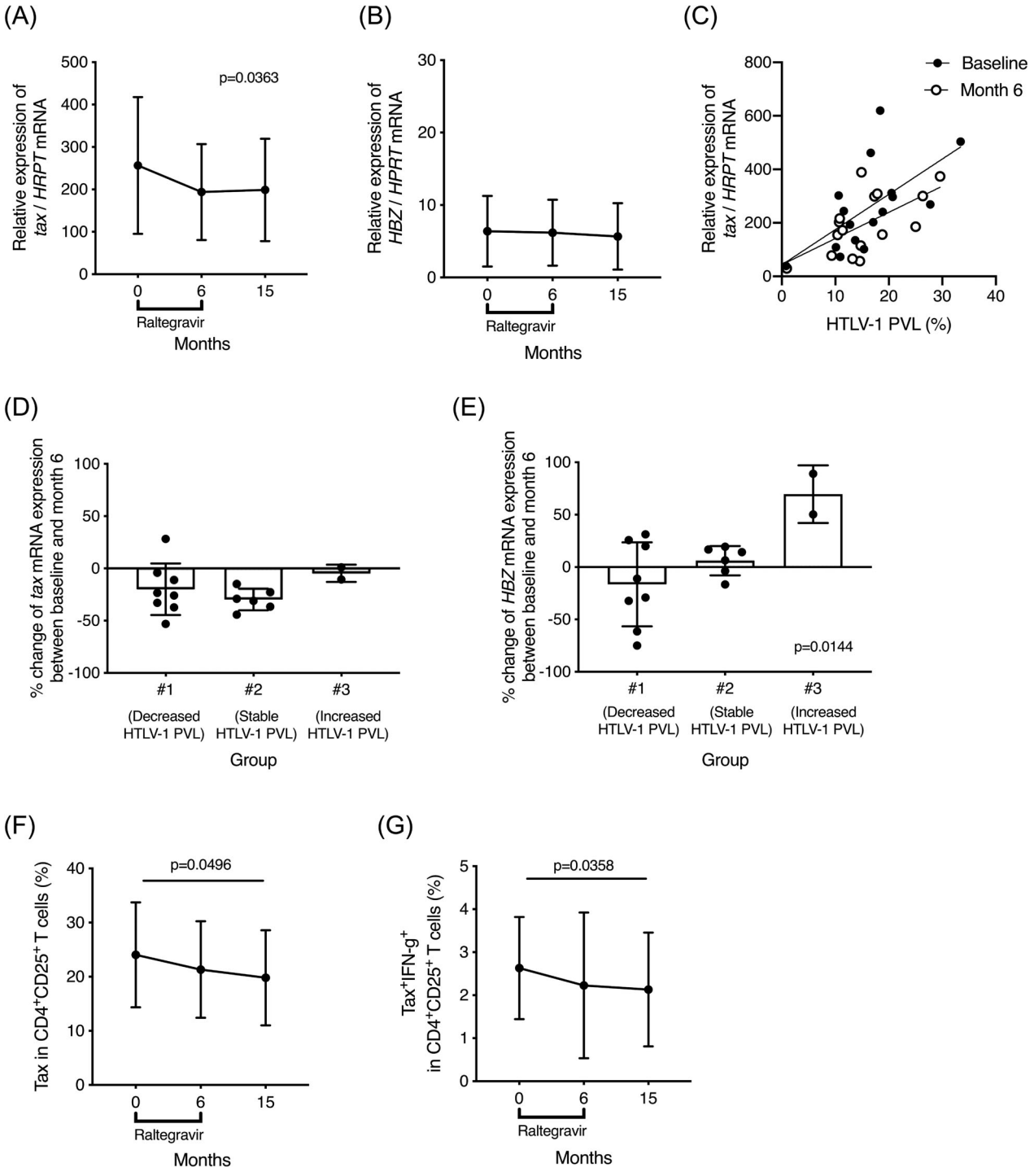
Spontaneous lymphoproliferation in HAM/TSP patients with raltegravir

Spontaneous lymphoproliferation is a well-established measure of ex vivo T-cell activation for HTLV-1-infected subjects induced by several factors including increased IL-2 and IL-15 associated with HTLV-1 gene expression.[28,29] Given the observed inhibitory effects in this trial of raltegravir on HTLV-1 mRNA expression and activated T cells, we asked whether treatment with raltegravir would effect HTLV-1-associated spontaneous lymphoproliferation. As shown in Figure 5A, group analysis of the 16 HAM/TSP patients demonstrated a significant reduction in [^{3}H]-thymidine uptake by spontaneous proliferating PBMC compared to their baseline values at month 6 of raltegravir treatment that was maintained at month 15 of the trial (Fig. 5A). In addition, a CFSE assay was used to assess spontaneous proliferating PBMC.[23] Figure 5B shows a representative dot plot of T-cell proliferation in a HAM/TSP patient (HAM #14) cultured at day 4 using CFSE in which both CD4$^+$ and CD8$^+$ T cells can clearly be seen to proliferate at the pretreatment time point. At month 6 of raltegravir treatment (Fig. 5B), there was a reduction in proliferation of both CD4$^+$ and CD8$^+$ T cells. Group analysis demonstrated that spontaneous proliferation of CD4$^+$ T cells, as well as CD4$^+$CD25$^+$ T cells, was significantly inhibited in cultured PBMC of HAM/TSP patients at month 6 of raltegravir treatment, although the reduction of CD8$^+$ T-cell proliferation did not reach significance (Fig. 5C). By month 15, proliferation of CD4$^+$ and CD4$^+$CD25$^+$ T cells were back to baseline. These CFSE results support the observation that treatment with raltegravir reduced HAM/TSP spontaneous proliferation particularly in activated CD4$^+$CD25$^+$ T cells, suggesting that raltegravir might inhibit T-cell activation through modulation of HTLV-1 *tax* mRNA expression.

Discussion

HAM/TSP is associated with high levels of HTLV-1 PVL, most notably in the CSF that drive a variety of immune functions associated with the pathogenesis of this disorder.[30] Therefore, reduction in HTLV-1 PVL may help infected individuals by ameliorating such abnormal immune dysregulatory mechanisms and subsequent development of disease. Since it had been reported that raltegravir, a clinically approved integrase inhibitor, efficiently blocked cell-to-cell HTLV-1 infection, integration, and immortalization in vitro,[11–13] we assessed the effects of raltegravir in HAM/TSP patients in a 6-month treatment phase, followed by a 9-month post-treatment phase, on HTLV-1 PVL and the associated immune activation markers. Clinically, neurological scores and objective measurement remained stable in all patients during the 6-month treatment phase, with some expected variability. There was no correlation of clinical change with HTLV-1 PVL. No patients showed any significant related toxicity or manifested unusually progressive disease during the trial.

Quantification of HTLV-1 PVL is one of the best tools for confirmation and evaluation of disease development in HTLV-1-infected individuals, although HTLV-1 PVL in PBMCs varies widely between individuals and remains relatively stable within individuals over time.[31,32] In our study, group analysis demonstrated no significant changes of HTLV-1 PVL in either PBMCs or CSF cells of HAM/TSP patients during the trial. However, data from individual patients showed a differential reduction of HTLV-1 PVL in a subset of patients after raltegravir treatment, even during the post-treatment period, suggesting a potential low viral suppression effect of raltegravir on HTLV-1 PVL in certain HAM/TSP patients. Consistent with a previous report that *HBZ* mRNA expression is correlated with HTLV-1 PVL in HAM/TSP patients,[26] the group of HAM/TSP patients that demonstrated a reduction of HTLV-1 PVL (group 1) also showed a reduction in *HBZ* mRNA expression. This supports our results in which raltegravir reduced HTLV-1 PVL in a subset of HAM/TSP patients (Fig. 2). Unexpectedly, measures of HTLV-1 *tax* mRNA expression showed a significant reduction in PBMCs of HAM/TSP patients at month 6 of raltegravir treatment, which was sustained at month 15 of the trial. It is well known that *tax* mRNA and Tax protein is spontaneously expressed in a subset of HTLV-1-infected PBMCs of HTLV-1-infected individuals after



short-term ex vivo culture.[6,33] Recent high-throughput analysis of genomic integration sites of HTLV-1 determine the pattern of Tax expression of the provirus and the clonal abundance and pathogenic potential of infected T-cell clones in vivo.[34,35] This analysis surprisingly revealed that there was no significant difference in HTLV-1 clonality between HAM/TSP patients and ACs and that high HTLV-1 PVL in HAM/TSP patients was correlated with a large number of distinct HTLV-1-infected T-cell clones with unique insertion sites, and not with the extent

of clonal proliferation of a few infected T-cell clones in the host.[34] In addition, the majority of spontaneous Tax-expressing cells corresponded to a large number of distinct and less expanded viral clones, rather than a small number of clonally expanded viral clones in HAM/TSP patients.[35] Therefore, our results suggest that inhibitory effect of raltegravir on *tax* mRNA expression may result in a reduction of less expanded viral clones in HAM/TSP patients leading to a reduction of HTLV-1 PVL in a subset of HAM/TSP patients. While HTLV-1 transmission occurs primarily via cell-to-cell contact such as formation of a virological synapse,[36] it has been reported that HTLV-1 LTR DNA circles, indicators of an active HTLV-1 replication, are detected at low levels in PBMCs of symptomatic carriers and patients with ATLL and HAM/TSP, strongly suggesting that HTLV-1 viral replication is maintained during infection.[37] Since little is known about HTLV-1 proviral latency including reactivation associated with new infection in HAM/TSP patients, further studies are needed to evaluate this mode of HTLV-1 transmission and to determine if raltegravir can function to prevent integration of virus into uninfected cells in vivo.

Our results demonstrated that while raltegravir did not dramatically reduce the HTLV-1 PVL, it may instead modulate HTLV-1 *tax* mRNA expression as well as the T-cell activation that characterize HAM/TSP. In HIV-1, raltegravir effectively prevents integration of HIV-1 proviral DNA into the host cell genome, and clinical trials of raltegravir in HIV-1-infected adults showed significant decline of plasma HIV-1 RNA levels and increase of $CD4^+$ T-cell counts in the raltegravir-treated group.[10] In HAM/TSP patients, increased HTLV-1 PVL, viral expression and T-cell activation have been reported.[6–9] Specifically, $CD4^+CD25^+$ T cells contain high levels of HTLV-1 proviral DNA, highly express HTLV-1 *tax* mRNA, and produce various cytokines including IFN-γ.[7,8] HAM/TSP patients have higher frequencies of $CD4^+CD25^+$ T cells in the CSF as well as in the peripheral blood compared to ACs and healthy controls, which also significantly correlated with increased HTLV-1 PVL and dysregulation of the other immune cells, such as $CD8^+$ T cells and B cells in HAM/TSP.[9,38] During treatment with raltegravir, HAM/TSP patients showed a reduction of $CD4^+CD25^+$ T cells in both peripheral blood and CSF that was sustained at month 15 of the trial. Downward trends in antibody secreting B cells and activated $CD8^+$ T cells in the CSF were also observed in raltegravir treated HAM/TSP patients. Importantly, *tax* mRNA, Tax protein, and Tax-induced IFN-γ expressions were decreased in ex vivo PBMC culture of HAM/TSP patients at month 15 compared to baseline. In addition, inhibition of spontaneous lymphoproliferation was detected in HAM/TSP patients during the 6-month treatment with raltegravir and/or post-treatment period. In ex vivo PBMC culture of HAM/TSP patients, increase in plus-strand transcription of HTLV-1 provirus, especially HTLV-1 Tax gene products, induce various cellular genes including the NF-κB and CREB/ATF pathway and the common γ chain family of cytokines such as IL-2/IL-2 receptor and IL-15/IL-15 receptor.[25,39] Increased expressions of critical immune mediators directly contribute to both $CD4^+$ and $CD8^+$ T-cell activation and proliferation observed in HAM/TSP patients. Therefore, our findings suggest that a decrease in plus-strand transcription associated with raltegravir treatment may modulate T-cell activation and proliferation in HAM/TSP patients, but further studies would be required to determine how raltegravir affect these cell populations as well as virus infection in HAM/TSP patients during treatment phase and also at post-treatment period.

Collectively, the results in this relatively short-term trial of raltegravir demonstrated marked reductions in spontaneous lymphoproliferation, a marker of T-cell activation characteristic of HAM/TSP patients in all treated patients. While group statistics showed no significant inhibition of HTLV-1 proviral load during the trial, subset analysis of individual patients demonstrated differential inhibition of HTLV-1 PVL in some patients in either PBMC or CSF. There was no correlation of inhibition of CSF PVL with reduced PBMC PVL. Although this trial was not powered

Figure 3. HTLV-1 mRNA expression in PBMC of HAM/TSP patients with raltegravir. (A) Group analysis of *tax* mRNA expression in PBMCs of HAM/TSP patients after ex vivo culture for 20 h. Error bars represent standard deviation. (B) Group analysis of *HBZ* mRNA expression in PBMCs of HAM/TSP patients at pre-culture. Error bars represent standard deviation. (C) Correlation of HTLV-1 PVL in PBMC and HTLV-1 *tax* mRNA expression in PBMC of HAM/TSP patients at baseline and at month 6 of raltegravir treatment. Closed circles and opened circles represent the data at baseline ($R = 0.6168$, $P = 0.0109$) and at month 6 of raltegravir treatment ($R = 0.6206$, $P = 0.0103$), respectively. (D) Percentage change in *tax* mRNA expression among the three HAM/TSP groups based on the percentage changes of HTLV-1 PVL in the 6-month treatment phase compared to the baseline. Error bars represent standard deviation. (E) Percentage change in *HBZ* mRNA expression among the three HAM/TSP groups based on the percentage changes of HTLV-1 PVL in the 6-month treatment phase compared to the baseline. Error bars represent standard deviation. (F) Group analysis of HTLV-1 Tax protein expression in $CD4^+CD25^+$ T cells of HAM/TSP patients after ex vivo PBMC culture for 20 h. Error bars represent standard deviation. (G) Group analysis of HTLV-1 Tax and IFN-γ expressions in $CD4^+CD25^+$ T cells of HAM/TSP patients after ex vivo PBMC culture for 20 h. Error bars represent standard deviation.

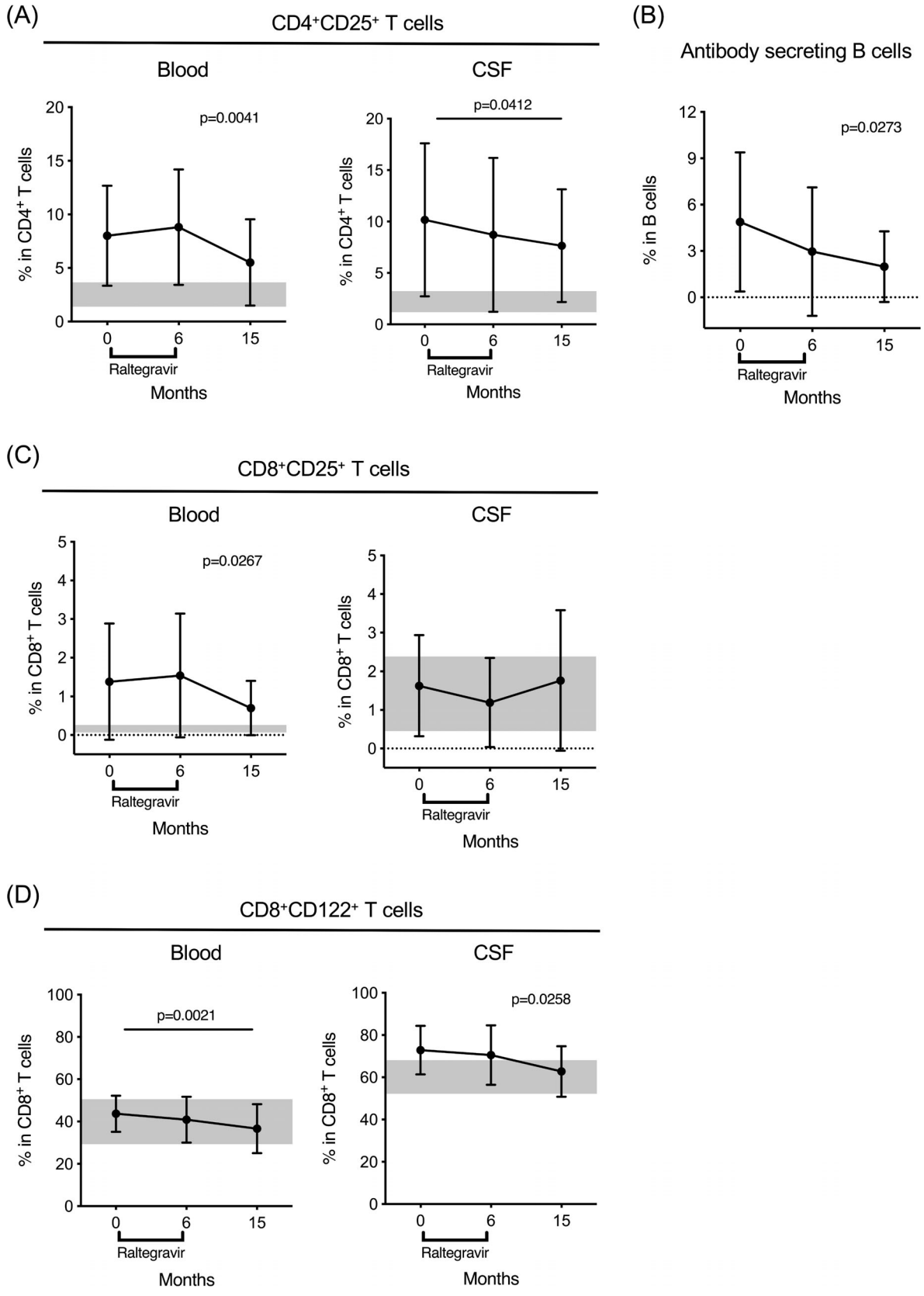


Figure 4. Immunological changes in peripheral blood and CSF of HAM/TSP patients with raltegravir. (A) Group analysis of frequencies of $CD25^+$ cells in blood and CSF $CD4^+$ T cells of HAM/TSP patients. (B) Group analysis of frequencies of antibody secreting B cells in CSF B cells of HAM/TSP patients. (C) Group analysis of frequencies of $CD25^+$ cells in blood and CSF $CD8^+$ T cells of HAM/TSP patients. (D) Group analysis of frequencies of $CD122^+$ cells in blood and CSF $CD8^+$ T cells of HAM/TSP patients. Error bars represent standard deviation. In (A), (C), (D), the control ranges of blood and CSF of 27 healthy volunteers (25%–75% percentile) are highlighted in gray.

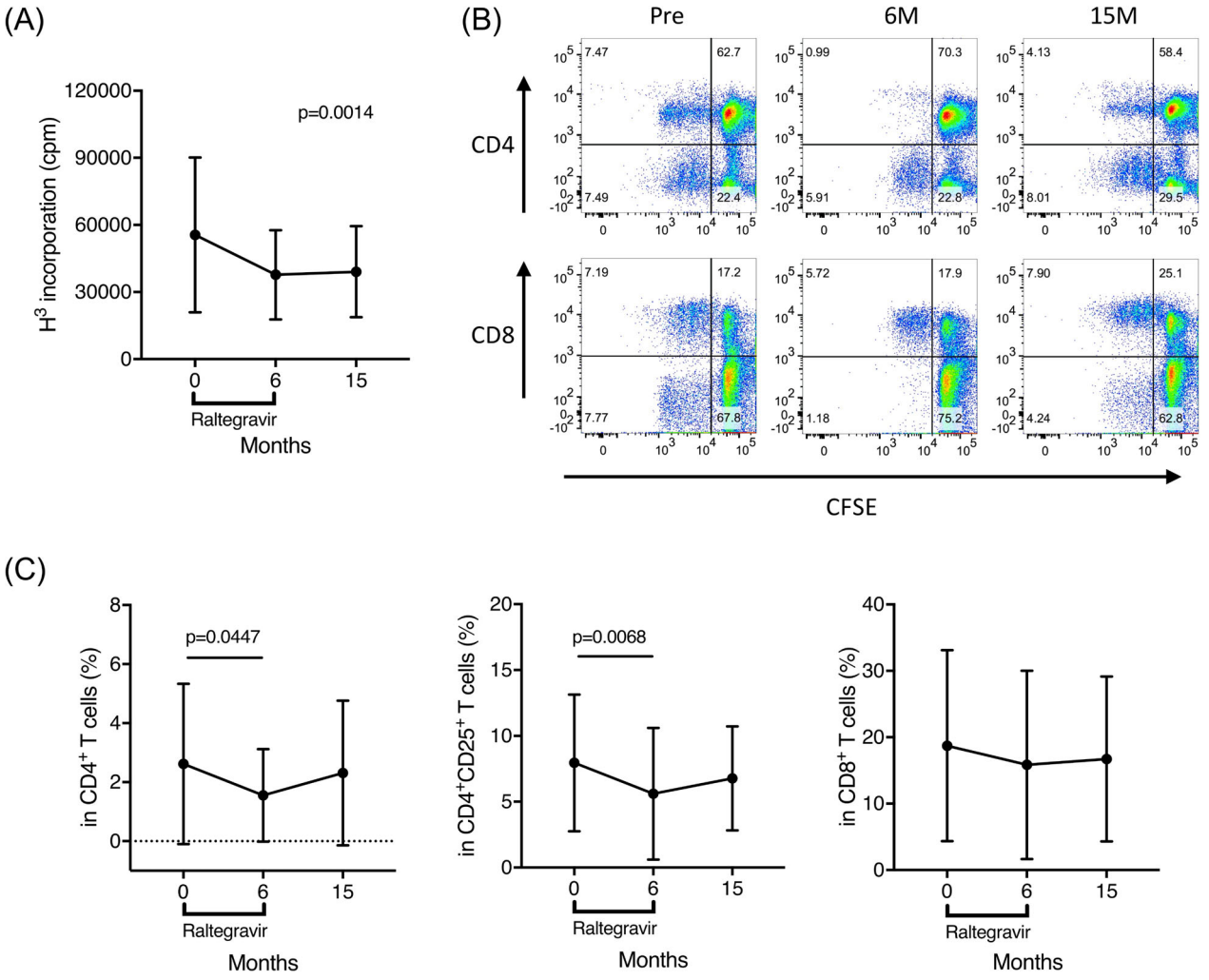


Figure 5. Spontaneous lymphoproliferation in HAM/TSP patients with raltegravir. (A) Group analysis of spontaneous lymphoproliferation in PBMCs of HAM/TSP patients at ex vivo culture of Day 4. Error bars represent standard deviation. (B) Representative dot plot of CFSE staining in $CD4^+$ and $CD8^+$ T cells of a HAM/TSP patient (HAM #14) after ex vivo culture of Day 4. (C) Group analysis of spontaneous $CD4^+$, $CD4^+CD25^+$, and $CD8^+$ T cell proliferation in PBMCs of HAM/TSP patients at ex vivo culture of Day 4. Error bars represent standard deviation.

for clinical efficacy, raltegravir was well tolerated and no patient progressed during the 6-month treatment period and 9-month follow-up. It is therefore reasonable to consider longer duration trials with raltegravir to determine if sustained clinical benefit including limiting progression of disease could be achieved. Since HTLV-1 infection is associated with a variety of clinical manifestations with varying rates of progression,[40] it is of interest to determine the effects of raltegravir in HAM/TSP patients with different courses of disease progression and in HTLV-1-infected individuals who may not yet have fully developed HAM/TSP. Moreover, future therapeutic studies including antiviral compounds combined with immunomodulatory therapies may be needed to maximize effective responses in patients with chronic virus-associated neuroinflammatory disease such as HAM/TSP.

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Author Contributions

BJB, SA, JD, RM, IC, and JO coordinated clinical work and patient care. YE-A AV, NN, and SN performed the experimental works. YE-A performed statistical analysis. YE-A, BJB, and JO contributed to discussion and paper writing. SJ supervised the project and contributed to discussion and writing.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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