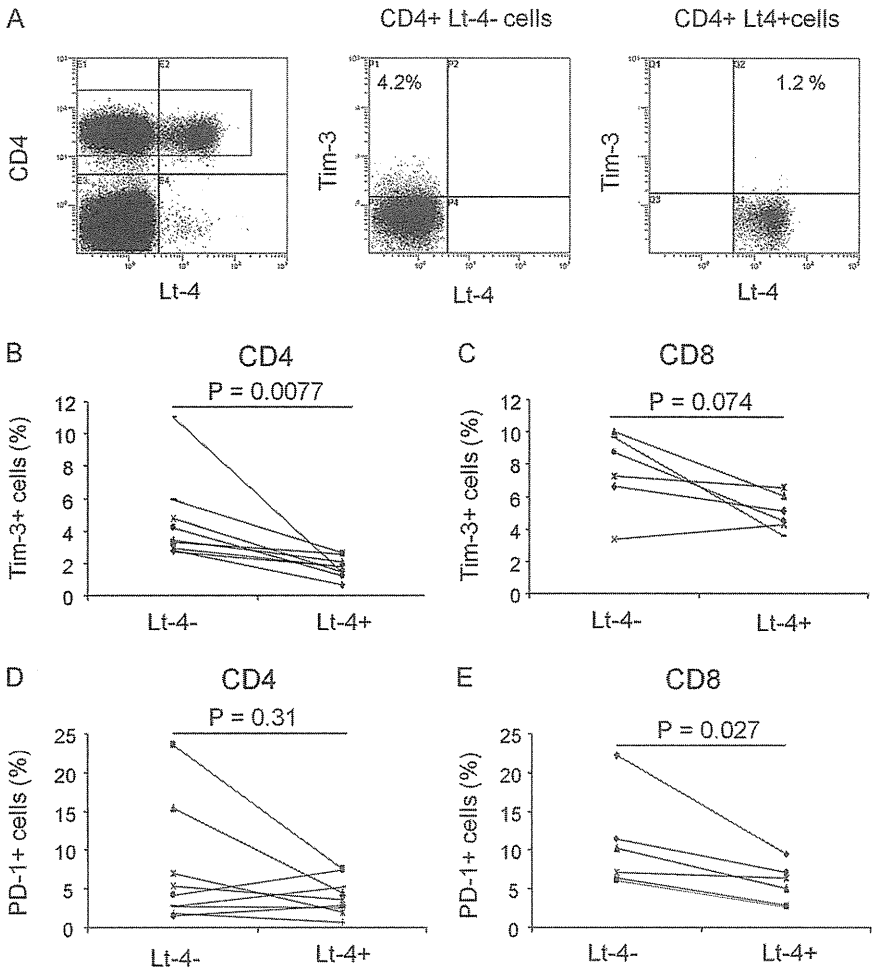

Figure 6. Low expression of Tim-3 on HTLV-I–infected cells. PBMCs from 9 HAM/TSP patients were cultured for 12 hours. Cells were double stained for the intracellular HTLV-I Tax protein, using the Lt-4 antibody, and the cell surface Tim-3 or PD-1. (*A*) After gating on $CD4^+$ cells, expression of Tim-3 was analyzed in either $CD4^+Lt\text{-}4^+$ or $CD4^+Lt\text{-}4^-$ cells. Representative data from a HAM/TSP patient show low percentage of $Tim\text{-}3^+$ cells in $CD4^+Lt\text{-}4^+$ cells in comparison to $CD4^+Lt\text{-}4^-$ cells. (*B, D*) Combined data from 9 HAM/TSP patients show significantly lower Tim-3 expression in $CD4^+Lt\text{-}4^+$ cells than in $CD4^+Lt\text{-}4^-$ cells. No significant difference in PD-1 expression between both groups was found by Wilcoxon signed-rank test. (*C, E*) Combined data from 6 HAM/TSP patients show that Tim-3 expression tended to be lower in $CD8^+Lt\text{-}4^+$ cells than in $CD8^+Lt\text{-}4^-$ cells and that PD-1 expression was significantly lower in $CD8^+Lt\text{-}4^+$ cells than in $CD8^+Lt\text{ -}4^-$ cells, by Wilcoxon signed-rank test.

NOTE: PBMCs: peripheral blood mononuclear cells; HAM/TSP: HTLV-I–associated myelopathy/tropical spastic paraparesis; HTLV-I: human T-lymphotropic virus type I; Tim-3: T cell immunoglobulin and mucin domain-containing molecule-3; PD-1: programmed cell death-1.

Furthermore, when we reanalyzed the frequency or MFI of $CD107a^+$ cells within the Tax $tetramer^+$ cell population, we could not detect a significant difference between HAM/TSP patients and asymptomatic carriers (Figure 5*D*). Also, we could not detect a significant correlation between the frequency of $CD107a^+$ cells and HTLV-I proviral load (data not shown).

Low Expression of Tim-3 on $CD4^+$ HTLV-I–infected Cells

To assess Tim-3 expression on HTLV-I–infected cells, we cultured PBMCs from 9 HAM/TSP patients for 12 hours in order to induce the expression of the HTLV-I Tax protein [30]. After harvesting, Tax protein was simultaneously detected with Tim-3 or PD-1 (Figure 6*A*). We observed that Tim-3 expression was significantly lower in Tax^+ $CD4^+$ cells ($Lt\text{-}4^+$ cells) than in Tax^-CD4^+ cells ($P = .0077$ [Figure 6*B*]). On the contrary, we observed no significant differences in PD-1 expression between Tax^+CD4^+ and Tax^-CD4^+ cells ($P = .31$ [Figure 6*D*]). In addition, we assessed the expression of Tim-3 or PD-1 in infected $CD8^+$ cells from 6 cases that showed a reasonable percentage of infected $CD8^+$ cells. We found that Tim-3 expression tended to be lower in Tax^+CD8^+ cells than in Tax^-CD8^+ cells ($P = .074$ [Figure 6*C*]), whereas PD-1 expression was significantly lower in Tax^+CD8^+ cells than in Tax^-CD8^+ cells ($P = .027$ [Figure 6*E*]). No significant correlations were observed between the MFI of Lt-4–positive cells and the frequency or

MFI of Tim-3 or PD-1 within $CD4^+$ or $CD8^+$ cells (data not shown). The percentage of infected $CD4^+$ and $CD8^+$ cells in asymptomatic carriers or tetramer$^+CD8^+$ cells in HAM/TSP patients was too small to assess Tim-3 or PD-1 expression.

CONCLUSIONS

We found that the proportion of Tim-3^+ cells within $CD4^+$ and $CD8^+$ T cell populations of HTLV-I–infected individuals (both HAM/TSP patients and asymptomatic carriers) is significantly lower than in healthy controls (Figure 1). This reduction was much clearer in Tax-specific CTLs because the frequency of Tim-3–expressing cells in CTLs was lower than in the total $CD8^+$ population of infected individuals (Figures 2*B–D*). In addition, Tim-3^+ cell frequency in HTLV-I Tax-specific CTLs was significantly lower than in CMV-specific CTLs from HAM/TSP patients (Figure 2*B*). Interaction of Tim-3 with its ligand, galectin-9, regulates Th1 cell responses by promoting the death of IFN-γ–producing Th1 cells, suggesting that Tim-3 may play a role in suppressing Th1-mediated immune responses [18]. Our results showing that the frequency of Tim-3^+ cells is reduced within $CD4^+$ and $CD8^+$ T cells in HTLV-I infection strongly suggest that the Th1/Tc1 immune response is not negatively regulated by Tim-3 in HTLV-I infection. Rather, immune cells such as HTLV-I–specific CTLs may be resistant to cell death through the Tim-3/galectin-9 pathway [18]. In this sense, the increased number of Tim-3^- HTLV-I Tax-specific CTLs may contribute to the control of viral replication. In the present study, we found that IFN-γ production was decreased in $CD8^+$ cells and HTLV-I Tax-specific CTLs that expressed Tim-3 as compared with their Tim-3^- counterparts in HAM/TSP patients (Figure 4). In addition, CD107a expression was lower in Tim-3^+ HTLV-I Tax-specific CTLs from HAM/TSP patients (Figure 5). These results indicate that Tim-3 identifies a subset of CTLs with impaired production of cytokines and cytolytic activity. The decreased expression of Tim-3 in HTLV-I infection is in marked contrast to other chronic viral infections such as HIV and HCV infections, where Tim-3 expression is increased in T cells, including the virus-specific CTLs [19, 20]. It would be of interest to determine whether Tim-3 expression is also reduced in other chronic viral infections and to clarify the mechanisms underlying Tim-3 down-regulation in HTLV-I infection.

Interestingly, our data demonstrated that Tim-3 and CD107a expression in HTLV-I Tax-specific CTLs was not significantly different between HAM/TSP patients and asymptomatic carriers (Figures 2*F* and 5*D*); however, Tim-3 MFI was higher in asymptomatic carriers than in HAM/TSP patients. Our data suggest that the killing activity of the CTLs is not different between the 2 groups. Controversially, others have reported that CD107a expression is lower in HTLV-I Tax-specific CTLs from HAM/TSP patients than from asymptomatic carriers, and that CTL function is impaired in HAM/TSP patients as compared with asymptomatic carriers [24]. This controversy may result from differences in sample type and procedures, including the gating for tetramer$^+$ cells after antigen stimulation. To address this issue, more detailed analyses of HTLV-I–specific CTL function in HAM/TSP patients and asymptomatic carriers would be necessary to ascertain whether differences could define the clinical condition.

In this study, we found that PD-1 expression levels on T cells of HAM/TSP patients and asymptomatic carriers were not different from those of healthy controls. However, we observed that PD-1 expression was significantly higher in HTLV-I Tax-specific CTLs than in CMV-specific CTLs (Figure 3*E*) and significantly higher in HTLV-I Tax-specific CTLs from asymptomatic carriers than from HAM/TSP patients. This result is in partial agreement with a previous study on HTLV-I infection, in which a marked increase of PD-1 expression was found in HTLV-I Tax-specific CTLs from both asymptomatic carriers and ATL patients as compared with CMV- and EBV-specific CTLs [31]. We found that IFN-γ production was higher in $CD8^+$ cells and HTLV-I Tax-specific CTLs that expressed PD-1 as compared with their PD-1^- counterparts in HAM/TSP patients (Figures 4*E* and 4*F*). In addition, CD107a expression was higher in PD-1^+ HTLV-I Tax-specific CTLs of HAM/TSP patients (Figure 5*C*). These results indicate that PD-1^+ HTLV-I Tax-specific CTLs are capable of producing proinflammatory cytokines and have high cytolytic activity during HTLV-I infection. An increase in IFN-γ production by PD-1^+ T cells has been recently shown in simian immunodeficiency virus (SIV) infection and in an animal model of autoimmune nephritis [32, 33]. Interestingly, PD-1^+ cells were predominantly detected within $CD107a^+$ antigen-specific T cells in SIV infection [34]. In this context, it is proposed that the primary mechanism by which PD-1 affects $CD8^+$ T cell function involves regulation of cell proliferation and survival [32, 35]. Our results suggest that HTLV-I Tax-specific CTLs exhibit an increased expression of PD-1, albeit a reduced expression of Tim-3. This is in marked contrast to other chronic viral infections such as HIV and HCV infections, in which both PD-1 and Tim-3 are expressed at high levels in the virus-specific CTLs [19, 20]. Double staining for Tim-3 and PD-1 revealed that these are expressed by distinct populations of $CD8^+$ T cells in HIV infection and that the predominance of either Tim-3^+PD-1^- or Tim-3^-PD-1^+ cells in HIV-specific CTLs differs among individuals [19]. At the same time, CMV- and HCV-specific CTLs are predominantly Tim-3^-PD-1^+ and Tim-3^+PD-1^+, respectively [20]. In our study, the average percentage of PD-1^+ cells in HTLV-I Tax-specific CTLs was 65.9% and 22.3% in carriers and HAM/TSP patients, respectively (Figure 3*C*); the average percentage of Tim-3^+ cells was 3.5% and 3.2%, respectively (Figure 2*F*), suggesting that the majority of the CTLs expressing T cell exhaustion molecules has a Tim-3^-PD-1^+ phenotype. Taken together, these results suggest that PD-1 and Tim-3 may have a distinct function in regulating immune responses in HTLV-1 infection.

We observed that HTLV-I Tax-expressing cells show a significant reduction in Tim-3 expression as compared with $Tax^{-}CD4^{+}$ cells and that Tim-3 expression in $Tax^{+}CD8^{+}$ cells tends to be lower than in $Tax^{-}CD8^{+}$ cells. $Tax^{+}CD8^{+}$ cells also showed significantly lower PD-1 expression (Figure 6). This suggests that HTLV-I–infected cells may be resistant to cell death through the Tim-3/galectin-9 pathway. HTLV-I Tax combines a positive effect on cell cycle with a negative effect on apoptosis through transactivation of several host genes [36]. It would be of interest to further investigate whether Tax might regulate Tim-3 expression.

In summary, we demonstrated that the expression of the negative immune regulator Tim-3, but not of PD-1, is reduced on HTLV-I Tax-specific CTLs, $CD4^{+}$, and $CD8^{+}$ T cells in both HAM/TSP patients and asymptomatic carriers. Moreover, we showed that Tim-3^{+}, but not PD-1^{+}, cells produce less IFN-γ and exhibit low cytolytic activity within the CTL population. Tim-3 expression and CTL cytolytic activity were not different between HAM/TSP patients and asymptomatic carriers. In addition, $CD4^{+}$ HTLV-I Tax-expressing cells showed a significant reduction in Tim-3 expression as compared with $Tax^{-}CD4^{+}$ cells. These results suggest that HTLV-I Tax-specific CTLs preserve their cytolytic activity, thereby controlling viral replication.

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Brief report

Kinetics and intracellular compartmentalization of HTLV-1 gene expression: nuclear retention of HBZ mRNAs

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Human T-cell leukemia virus type 1 (HTLV-1) codes for 9 alternatively spliced transcripts and 2 major regulatory proteins named Tax and Rex that function at the transcriptional and posttranscriptional levels, respectively. We investigated the temporal sequence of HTLV-1 gene expression in primary cells from infected patients using splice site-specific quantitative RT-PCR. The results indicated a two-phase kinetics with the tax/rex mRNA preceding expression of other viral transcripts. Analysis of mRNA compartmentalization in cells transfected with HTLV-1 molecular clones demonstrated the strict Rex-dependency of the two-phase kinetics and revealed strong nuclear retention of HBZ mRNAs, supporting their function as noncoding transcripts. Mathematical modeling underscored the importance of a delay between the functions of Tax and Rex, which was supported by experimental evidence of the longer half-life of Rex. These data provide evidence for a temporal pattern of HTLV-1 expression and reveal major differences in the intracellular compartmentalization of HTLV-1 transcripts. (Blood. 2011;117(18):4855-4859)

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia-lymphoma (ATLL) and tropical spastic paraparesis/HTLV-1–associated myelopathy (TSP/HAM). HTLV-1 uses several strategies for controlling the expression of its genome, including the production of 9 alternatively spliced transcripts (Figure 1A).[1-6] Production of plus-strand transcripts is controlled by Tax at the level of transcription and by Rex at the level of nucleo-cytoplasmic export of unspliced and partially spliced mRNAs.[7,8] Regulation of the minus-strand HBZ transcripts, which lack elements responsive to Rex, remains to be determined.

Current models suggest that plus-strand HTLV-1 mRNAs are expressed with a distinct timing during the course of the viral life cycle, with a switch from early (Rex-independent) to late (Rex-dependent) transcripts. Although early studies showed a qualitative switch among classes of HTLV-1 mRNAs (multiply spliced vs unspliced),[9-12] detection of this phenomenon with quantitative transcript-specific methods has proven difficult.[13]

To answer this question we used quantitative RT-PCR to quantify proviral expression during the spontaneous proviral reactivation observed in cells from infected patients. The results demonstrated a "two-phase" expression pattern. Using transfection of HTLV-1 molecular clones and subcellular RNA fractionation we demonstrated the Rex-dependency of the two-phase kinetics and determined the compartmentalization of the individual mRNAs, showing that more than 90% of the HBZ mRNAs were retained in the nucleus. Mathematical modeling[14] revealed the importance of a delay of Rex function compared with Tax, which was supported by experimental evidence of delayed accumulation and longer half-life of Rex.

Methods

Samples from HTLV-1–infected patients

Peripheral blood mononuclear cells (PBMCs) from ATLL and TSP/HAM patients were purified as in.[15] Patients are described in supplemental Table 1 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). All samples were obtained from patients after informed consent in accordance with the Declaration of Helsinki, with approval from the Imperial College and King's College hospitals (London) Institutional Rreview Boards.

Plasmids, cells, and transfections

Plasmid pBS1–2-3 consists of the tax/rex cDNA (exons 1, 2, and 3 flanked by the 5′ and 3′LTRs, from infectious molecular clone CS-HTLV-1[16]) inserted in pBluescript (Stratagene). Plasmid ACH-Rex knockout (KO) was

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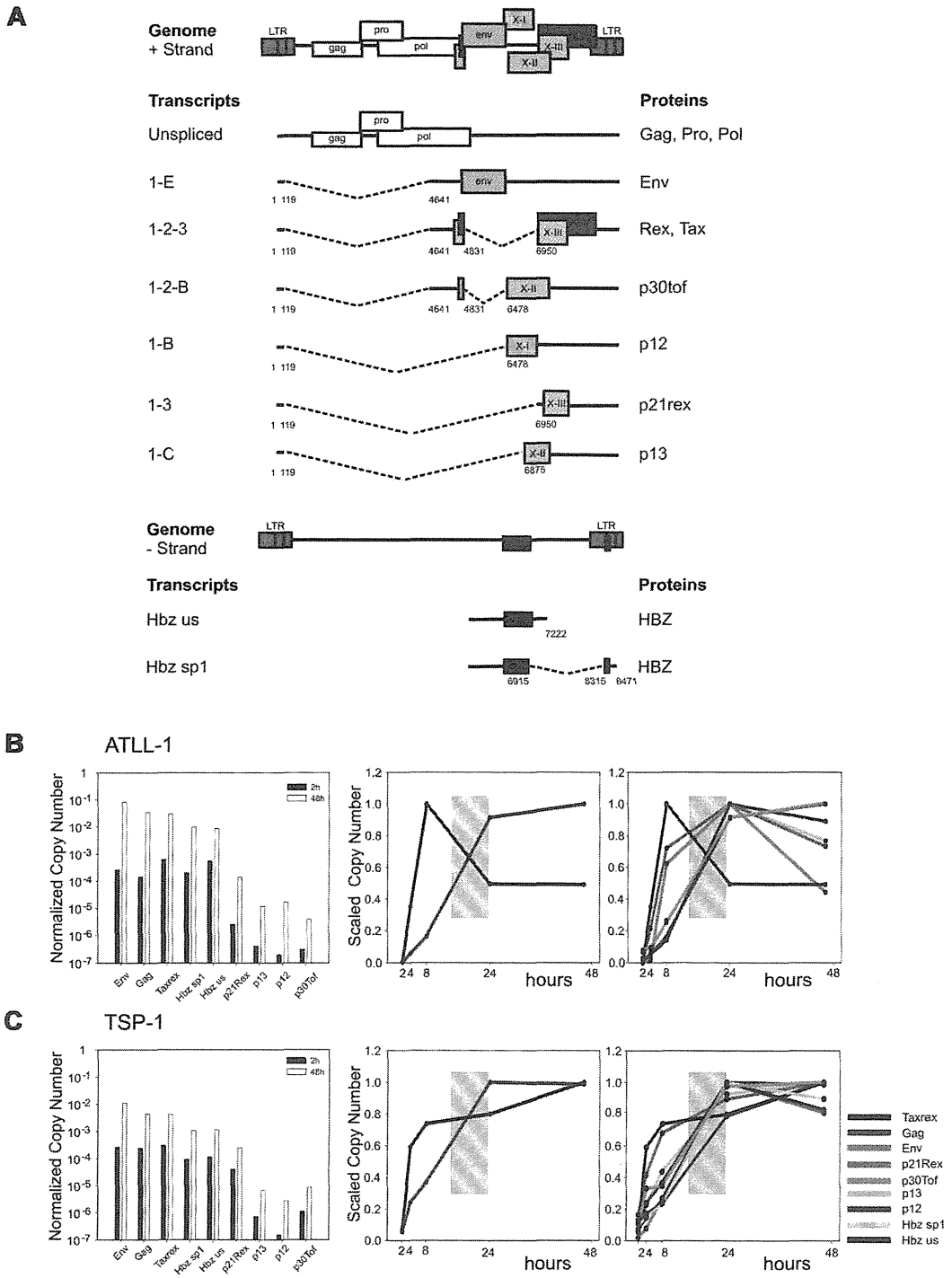

Figure 1. Temporal analysis of HTLV-1 expression in PBMCs from infected patients. (A) Structure and coding potential of plus- and minus-strand HTLV-1 mRNAs. (B-C) Bar graphs (left panels) show the Normalized Copy Numbers (NCN) of the indicated mRNAs after 2 hours (black bars) and 48 hours (white bars) of culture in vitro measured in representative ATLL and TSP/HAM patients; data on all patients studied are shown in supplemental Figure 1. NCN values were calculated by dividing the absolute copy number of each transcript by the absolute copy number of the 18S rRNA. Line graphs show the variation in the *tax/rex* and *gag* mRNAs (middle panels) and in all measured transcripts (right panels). Lines corresponding to *HBZ* mRNA are not shown for patient ATLL-1 because of insufficient material in the 8- and 24-hour time points. Scaled Copy Numbers (SCN) are plotted over a 48-hour time period (ie, at 2, 4, 8, 24, and 48 hours after depletion of CD8-positive cells and culture; cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin). SCN values were calculated by dividing the NCN of each transcript at each time point by the maximum NCN value measured for that mRNA during the time course experiment. mRNAs are indicated by colors as shown in panel C right.

derived from the HTLV-1 molecular clone ACH[17] by digestion with SphI followed by removal of 3′ overhangs (including the Rex initiation codon) with T4 DNA polymerase and religation. Transfections were performed in the HeLa-derived cell line HLtat,[18] chosen for its high transfection efficiency.

Quantitative RT-PCR

RNA of PBMCs from infected patients and transfected cells was extracted and viral transcripts were quantitated as detailed in supplemental Table 2.

Analysis of Tax and Rex expression

The time course of Tax and Rex expression was analyzed as described in Figure 2.

Results and discussion

Temporal analysis of HTLV-1 expression in PBMCs from infected patients

Although postulated based on the function of Tax and Rex,[7] a temporal switch in HTLV-1 expression has not been demonstrated with quantitative transcript-specific methods. To investigate this possibility, we set up an ex vivo virus reactivation model based on the depletion of $CD8^+$ T-cells from unstimulated PBMCs isolated from HTLV-1–infected patients, which reveals a sharp up-regulation of viral expression in the remaining PBMCs.[15] Samples from 6 patients with TSP/HAM and 3 patients with ATLL were analyzed (supplemental Table 1). Splice-junction-specific Real-Time RT-PCR was used to measure the abundance (normalized copy number, NCN) and timing (scaled copy number, SCN) of expression of HTLV-1 transcripts (supplemental Table 2).

Figure 1 (B-C left) shows the NCN of the different mRNAs after 2 and 48 hours of culture in vitro (black bars and white bars, respectively) in representative cases of ATLL (patient ATLL-1) and TSP/HAM (patient TSP-1). Data on all patients studied are shown in supplemental Figure 1. Expression of all transcripts was substantially up-regulated on culture in vitro. The most abundant plus-strand transcripts were tax/rex, gag and env, followed by p21rex, p30tof, p13 and p12; the minus-strand (HBZ) transcripts were readily detected.

Analysis of the timing of expression (SCN, Figure 1B-C middle) over a 48-hour time period showed that tax/rex was the earliest transcript followed by a rise in gag expression whose curve intersected that of tax/rex between 8 and 24 hours (indicated by a gray box in the figure), suggesting an "early-late" switch in HTLV-1 gene expression. Analysis of the SCN of all mRNAs (Figure 1B-C right) confirmed the "early-late" switch (gray box) and suggested a distinct temporal sequence of expression among the "late" mRNAs. The p21Rex mRNA was also detected as an early transcript in most samples, although its expression profile did not follow that of tax/rex in all the patients examined (see supplemental Figure 1).

Rex-dependence of the "two-phase" kinetics and nuclear retention of HBZ transcripts

The abundance and timing of expression of the HTLV-1 mRNAs were further investigated in cells transfected with the infectious HTLV-1 molecular clone ACH. This system permitted quantitation of transcripts in the cytoplasmic and nuclear fractions, which was not possible with patient samples because of limited amounts of material. Using a Rex knock-out derivative of ACH (ACH-Rex KO, Figure 2B right) we also tested the Rex-dependence of the two-phase expression kinetics.

Figure 2A (left) shows NCN in the cytoplasmic and nuclear fractions 24 hours after transfection of ACH. The most abundant plus-strand transcripts were tax/rex and gag, followed by env and p21rex; p12, p13, and p30tof were expressed at lower levels. The plus-strand transcripts showed similar partition in the nucleus and cytoplasm; in contrast the HBZ NCN was over 10-fold higher in the nucleus than in the cytoplasm.

The timing of expression was investigated by calculating "Export Ratios" over 48 hours (Figure 2B left). Consistent with results obtained from patient PBMCs (see preceding paragraph), ACH showed a two-phase expression kinetics with "early" tax/rex expression (measured as a sharp increase in export ratio) followed by a rise in the export ratios of the gag and env mRNAs. Importantly, the two-phase kinetics was abolished in cells expressing ACH-Rex KO (Figure 2B middle), demonstrating the critical role of Rex in regulating these kinetics. The export ratios of HBZ transcripts remained remarkably low throughout the time course and were not affected by Rex (Figure 2B left and middle). The nuclear retention of HBZ transcripts was also confirmed in the infected cell line C91PL[19] (Figure 2A right). Although the significance of the nuclear retention of HBZ mRNAs remains to be understood, we propose that it might favor viral persistence by reducing HBZ translation thereby reducing exposure of the infected cell to the HBZ-specific host $CD8^+$ T-cell response[20,21] while allowing its function as a noncoding transcript driving T-cell proliferation.[22]

Kinetics of Tax and Rex protein turnover

Mathematical modeling (supplemental Figure 2) underscored the importance of a delay in Rex function compared with Tax in the observed expression kinetics. These considerations led us to investigate the time course of Tax and Rex expression from plasmid pBS1–2-3, which expresses the full-length mature tax/rex mRNA (Figure 2C), and from the ACH molecular clone (Figure 2D). Flow cytometry analyses showed a relative accumulation of Rex at later time points (32, 48 hours) resulting in a progressive rise in the Rex/Tax ratio (right panels).

Consistent with these observations, a comparison of the half-lives of Tax and Rex expressed from pBS1–2-3 after treatment with cycloheximide revealed a slower rate of degradation of Rex compared with Tax (Figure 2E), with half-lives of 19.6 hours and 6.6 hours, respectively; similar half-lives were measured in ACH-transfected cells (data not shown). These findings provide experimental grounds for the delay in Rex function postulated in the mathematical model and suggest a posttranslational control of Tax and Rex activity.

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Authorship

Contribution: F.R. and I.C. carried out transfections, immunoblotting and real-time RT-PCR assays; M.S.B. carried out flow cytometry analyses; G.P.T. provided patient samples and was responsible for all aspects of diagnosis and clinical management of the patients; C.R.M.B., F.T., and S.J. helped design and set up the assays to measure HTLV-1 mRNAs in cells from infected

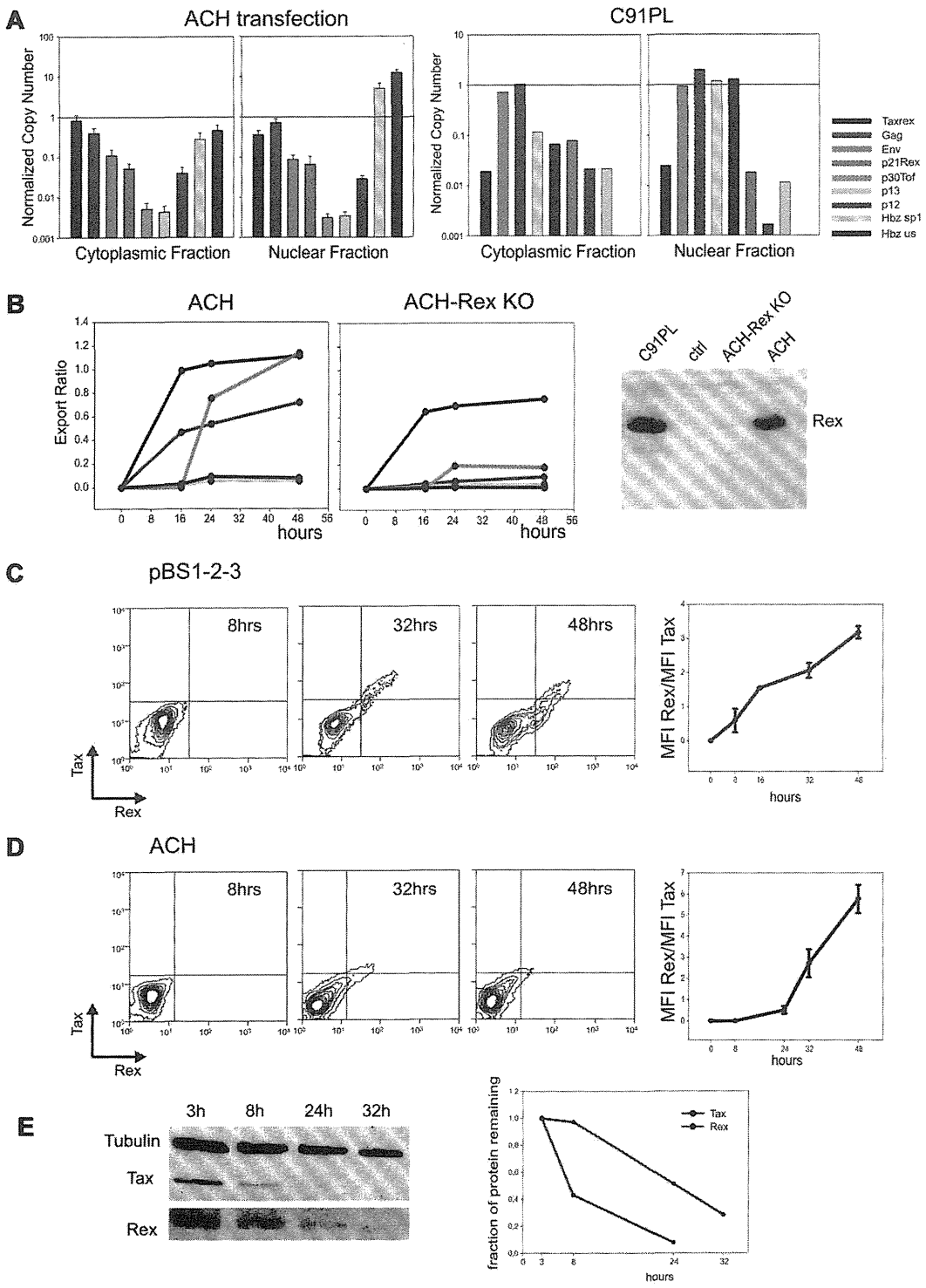

Figure 2. Kinetics and intracellular compartmentalization of HTLV-1 mRNAs; temporal analysis of Tax and Rex protein turnover. (A left) NCN of all HTLV-1 mRNAs in the cytoplasmic and nuclear fractions 24 hours after transfection of HLtat cells with wild-type HTLV-1 molecular clone ACH using Fugene6 (Roche; mean of 3 experiments, standard error bars). NCN values were determined by dividing the absolute copy number of each transcript by the absolute copy number of the *GAPDH* mRNA. (Right) NCN of all HTLV-1 mRNAs in the cytoplasmic and nuclear fractions of the chronically infected cell line C91PL.[19] (B) Kinetic analysis of the nucleo-cytoplasmic export of the *tax/rex*, *gag*, *env* and *HBZ* mRNAs expressed from ACH (left) and ACH-Rex-KO (middle) in transfected HLtat cells. RNA was extracted from nuclear and cytoplasmic fractions using the Paris Kit (Ambion). "Export Ratios" were calculated as the ratio between cytoplasmic and total NCN over a time course of 48 hours (harvesting at 0, 16, 24, and 48 hours). The right panel shows Western blot analysis to detect Rex protein (see description in panel E); results verified that the ACH-Rex-KO does not express Rex. (C) Kinetics of Tax and Rex protein expression in HLtat cells from plasmid pBS1–2-3, which contains the viral 5′ and 3′ LTRs and expresses the full-length *tax/rex* mRNA (including all coding and noncoding regions). Cultures were harvested at 8, 16, 32, and 48 hours after transfection. Cells were fixed in 3.7% formaldehyde-PBS, permeabilized in 0.2% Triton-PBS, blocked with 3% BSA (bovine serum albumin)–PBS and then incubated for 1 hour with mouse anti-Tax monoclonal antibody[23] (1:100, in PBS-1.5% BSA) and rabbit anti-Rex polyclonal antibody[24] (1:500, in PBS-1.5% BSA). Cells were next incubated for 1 hour with Alexa 633-conjugated goat anti–mouse and Alexa 488–conjugated chicken anti–rabbit antibodies (Molecular Probes) diluted 1:1000 in PBS-1.5% BSA. Tax and Rex protein expression was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson) equipped with 633-nm Helium-Neon and 488-nm Argon lasers. Alexa 633 and Alexa 488 fluorescent signals were analyzed using the FL4 (661 ± 16 nm) and the FL1 (530 ± 30 nm) detection lines, respectively. Data are represented as equal probability plots. The line graph (right) shows mean and standard error values of Rex/Tax

Figure 2. (continued) fluorescence intensity (mean fluorescence value X number of positive events) ratios measured in 3 independent experiments. (D) Kinetics of Tax and Rex protein expression in HLtat cells from the infectious HTLV-1 molecular clone ACH. Cultures were harvested at 8, 24, 32, and 48 hours after transfection. Cells were processed and analyzed as described for panel C. Data are represented as equal probability plots. The line graph (right) shows mean and standard error values of Rex/Tax fluorescence intensity (mean fluorescence value X number of positive events) ratios measured in 3 independent experiments. (E) Degradation rates of the Tax and Rex protein expressed from pBS1–2-3 after blocking protein synthesis. HLtat cells transfected with pBS1–2-3 were treated with 10μM cycloheximide 24 hours after transfection and harvested in "disruption buffer" (Paris kit; Ambion) at 3, 8, 24, and 32 hours after cycloheximide treatment. Lysates were subjected to SDS-PAGE and electrotransferred to Hybond-C Extra (GE Healthcare). Blots were incubated with mouse anti-Tax monoclonal antibody (1:500), rabbit anti-Rex polyclonal antibody (1:5000) and mouse anti-tubulin monoclonal antibody (1:2000) in PBS-3% BSA-0.05% Tween followed by a horseradish peroxidase-conjugated anti–mouse or anti–rabbit antibody (Pierce) diluted 1:5000 in 2% milk (Roche)–PBS-0.05% Tween. Blots were developed using chemiluminescence reagents (Supersignal, Pierce) and immunoreactive bands were visualized and quantified using a BioRad ChemiDoc XRS imager. The left panel shows a composite of this Western blot analysis to detect the Tax, Rex and Tubulin signals. Data were normalized by dividing Tax and Rex signals by the tubulin signal and scaled against the value at 3 hours; resulting numbers, which represented the fraction of protein remaining, were plotted in the graph on the right. Protein half-life was estimated by fitting a linear decay model to the data, assuming a constant degradation rate.

patients; D.M.D. and V.C. designed the experiments and prepared the manuscript; G.M.T. and A.C. developed the mathematical models of HTLV-1 expression; Y.T. provided Tax-specific antibodies; and all authors contributed to the analysis and interpretation of the data.

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Review Article

Double control systems for human T-cell leukemia virus type 1 by innate and acquired immunity

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Human T-cell leukemia virus type 1 (HTLV-1) is the causative retrovirus of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1-specific T-cell responses elicit antitumor and antiviral effects in experimental models, and are considered to be one of the most important determinants of the disease manifestation, since they are activated in HAM/TSP but not in ATL patients. The combination of low T-cell responses and elevated HTLV-1 proviral loads are features of ATL, and are also observed in a subpopulation of HTLV-1 carriers at the asymptomatic stage, suggesting that these features may be underlying risk factors. These risks may potentially be reduced by vaccination to activate HTLV-1-specific T-cell responses. HAM/TSP and ATL patients also differ in their levels of HTLV-1 mRNA expression, which are generally low *in vivo* but slightly higher in HAM/TSP patients. Our recent study indicated that viral expression in HTLV-1-infected T-cells is suppressed by stromal cells in culture through type-I IFNs. The suppression was reversible after isolation from the stromal cells, mimicking a long-standing puzzling phenomenon in HTLV-1 infection where the viral expression is very low *in vivo* and rapidly induced *in vitro*. Collectively, HTLV-1 is controlled by both acquired and innate immunity *in vivo*: HTLV-1-specific T-cells survey infected cells, and IFNs suppress viral expression. Both effects would contribute to a reduction in viral pathogenesis, although they may potentially influence or conflict with one another. The presence of double control systems for HTLV-1 infection provides a new concept for understanding the pathogenesis of HTLV-1-mediated malignant and inflammatory diseases. (*Cancer Sci* 2011; 102: 670–676)

It has been three decades since the discovery of human T-cell leukemia virus type 1 (HTLV-1) as the causative retrovirus of adult T-cell leukemia (ATL).[1,2] ATL develops during middle age or later mainly in a small portion of vertically HTLV-1-infected populations.[3,4] HTLV-1 also causes HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in another small population of infected individuals.[5,6] Some other inflammatory diseases such as uveitis and arthritis are also associated with HTLV-1 infection.[7,8] New therapeutic approaches such as hematopoietic stem cell transplantation (HSCT),[9,10] an antibody therapy targeting CCR4,[11] and antiviral therapy with interferon-alpha and zidovudin[12] partly improved the prognosis of ATL. However, ATL still shows high mortality, and HAM/TSP remains to be an intractable disease.

Enormous amounts of research findings have been accumulated regarding the virus-mediated pathogenesis. HTLV-1 Tax, a virus-encoded regulatory gene product, mediates cell activation, proliferation and resistance to apoptosis by transactivation through NF-κB, cAMP response element binding protein (CREB) and serum response factor (SRF), and by inactivation of tumor suppressors,[13–15] which would be involved in leukemogenesis and inflammation in HTLV-1 infection. Another minus-strand HTLV-1-encoded gene product, HTLV-1 basic leucine zipper factor (HBZ), is continuously expressed in infected cells *in vivo* regardless of the disease and may also be involved in the growth ability of infected cells.[16]

However, many unsolved questions still remain regarding the pathogenesis of HTLV-1 infection, for example, how the same virus causes totally different diseases such as ATL and HAM/TSP, why only small portions of HTLV-1-infected populations develop diseases, and why it takes more than 40 years to develop ATL. The answers to these questions would provide hints for predicting disease risks as well as aiding the development of prophylactic and therapeutic strategies.

HTLV-1-specific T-cell responses that contribute to antiviral and antitumor surveillance could be one of the most important determinants of the diseases. In fact, HTLV-1-specific T-cells are activated in HAM/TSP but not in ATL.[17–19] Oral HTLV-1 infection induces T-cell tolerance to HTLV-1 and increased proviral loads,[20,21] consistent with the epidemiological finding that vertical HTLV-1 infection is one of the risk factors for ATL.[3] Therefore, the individual status of HTLV-1-specific T-cell responses is expected to be an indicator of risk for ATL.[22] Although the pathological significance of HTLV-1-specific T-cells in HAM/TSP remains controversial,[23,24] advantages for HLA-A02-positive individuals in protection against HAM/TSP have been reported, and interpreted through the association of this HLA with strong CTL responses to a major epitope of HTLV-1 Tax.[25]

Elevation of proviral loads is also a risk factor for ATL. Given the fact that HTLV-1-specific CTLs have antiviral effects, these CTLs are likely to be one of the determinants of proviral loads.[26] However, proviral loads are also increased in HAM/TSP patients, and the correlations between proviral loads and HTLV-1-specific T-cell responses vary among studies,[27,28] suggesting the presence of additional factors for determining individual proviral loads.

Another curious finding in HTLV-1 infection is the scarcity of viral antigen expression in the peripheral blood, although the viral mRNA is barely expressed.[29] The transcription of HTLV-1 is mainly regulated by CRE-like repeats in the HTLV-1 LTR.[30] Involvement of inducible cAMP early repressor (ICER) and transducers of regulated CREB 2 (TORC2) in the inhibition of HTLV-1 transactivation has been suggested.[31,32] However, the mechanism involved in suppressing viral expression only *in vivo* has remained obscure. It is a paradox that HTLV-1 Tax contributes to the pathogenesis while Tax protein is undetectable *in vivo*. Expression of HBZ in the absence of Tax may partly

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explain the growth advantage of infected cells,[33] but not all of HTLV-1-mediated leukemogenesis. In addition, it does not make sense that Tax-specific T-cell responses are maintained if Tax is not expressed *in vivo*. The paradox will remain until the state of viral expression and the mechanisms for suppressing HTLV-1 expression *in vivo* are clarified.

We recently found that innate immune responses, especially type-I interferons (IFNs), suppress HTLV-1 expression.[34] This integrates the issue of viral expression and the host defense system against HTLV-1, which includes innate immunity as well as acquired immunity. The presence of double control systems explains some of the paradox in persistent HTLV-1 infection, and adds new aspects to the pathogenesis of HTLV-1-mediated diseases.

Control of HTLV-1 by HTLV-1-specific T-cell responses

Antitumor surveillance by HTLV-1-specific T-cells. $CD8^+$ HTLV-1-specific CTL responses are found in many HAM/TSP patients and asymptomatic carriers (AC), but rarely in ATL patients.[17–19,35,36] These CTLs kill HTLV-1-infected cells *in vitro*, and mainly recognize HTLV-1 Tax.[18,37] The HTLV-1 envelope is also a popular target, especially for $CD4^+$ CTLs.[38] Other viral antigens, including polymerase,[39] ROF (p12) and TOF (p30/p13),[40] and HBZ,[41] have also been shown to be targets of CTLs. Elimination of $CD8^+$ cells among PBMCs from HAM/TSP patients induces HTLV-1 expression during subsequent cell culture,[42] clearly indicating that $CD8^+$ HTLV-1-specific CTLs contribute to the control of HTLV-1-infected cells.

A series of animal model experiments indicated that HTLV-1-specific T-cell responses limit the expansion of HTLV-1-infected cells *in vivo*. Oral HTLV-1 infection induced insufficiency of HTLV-1-specific T-cell responses in rats, and the HTLV-1 proviral loads were inversely correlated with HTLV-1-specific T-cell responses.[21] Re-immunization of these rats with mitomycin C-treated HTLV-1-infected cells restored HTLV-1-specific T-cell responses and reduced the proviral loads[43] (Fig. 1). In another rat model of HTLV-1-induced tumors, the otherwise fatal HTLV-1-infected lymphomas in T-cell-deficient rats were eradicated by transfer of T-cells from syngeneic rats that had been vaccinated with a Tax-encoding DNA or peptides corresponding to a major epitope for Tax-specific CTLs.[44,45]

Recent clinical reports have indicated that HTLV-1-carrying recipients after liver transplantation developed ATL under the administration of immunosuppressants.[46,47] In contrast, Tax-specific CTL responses were strongly activated in some ATL patients who obtained complete remission after HSCT, but were not observed in the same patients before transplantation.[48] These findings suggest that HTLV-1-specific T-cells, including Tax-specific CTLs, play important roles in antitumor surveillance against HTLV-1 leukemogenesis.

Insufficient HTLV-1-specific T-cell responses as a potential risk for ATL. Most HTLV-1-infected individuals are asymptomatic, and only about 5% develop ATL and <1% develop HAM/TSP.[3,49] The epidemiological risk factors for ATL include vertical transmission and increases in the number of abnormal lymphocytes or HTLV-1 proviral loads.[3,50,51] HTLV-1 proviral loads are also elevated in HAM/TSP patients.[52]

Immunological studies have suggested that insufficiency in host T-cell responses against HTLV-1 might be another risk factor for ATL.[22] A small-scale survey measuring Tax protein-specific IFN-γ production revealed a wide variety in the strengths of HTLV-1-specific T-cell responses among HTLV-1 carriers.[53] The combinations of HTLV-1-specific T-cell responses and proviral loads categorize HTLV-1 carriers into the following four groups: (i) low proviral loads with HTLV-1-specific T-cell responses; (ii) elevated proviral loads with HTLV-1-specific T-cell responses; (iii) low proviral loads with low T-cell responses; and (iv) elevated proviral loads with low T-cell responses (Fig. 2).

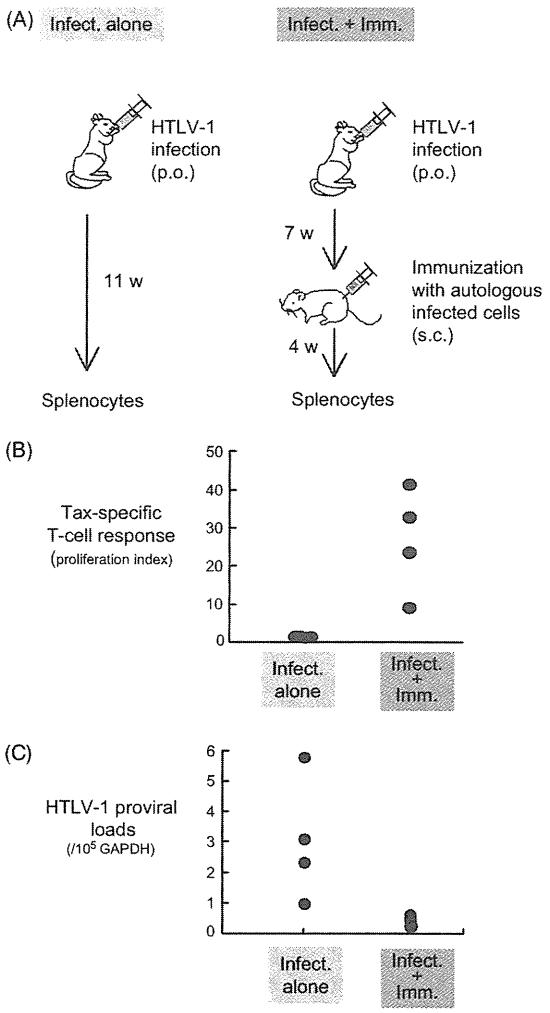

Fig. 1. Recovery of human T-cell leukemia virus type 1 (HTLV-1)-specific T-cell responses and reduction of proviral loads by re-immunization. Eight rats orally infected with HTLV-1 were divided into two groups. (A) One group was left untreated (Infect. alone) and the other was subcutaneously immunized with mitomycin C-treated HTLV-1-infected syngeneic rat T-cells (Infect. + Imm.) at 4 weeks. Spleen T-cells were harvested at 7 weeks after infection. (B,C) T-cells from the re-immunized rats (Infect. + Imm.) show elevated levels of Tax-specific T-cell proliferative responses (B) and lower proviral loads (C), compared with untreated rats (Infect. alone).[43]

Regarding these groups, ATL patients exhibit elevated proviral loads with low T-cell responses, while many, but not all, HAM/TSP patients show elevated proviral loads with high HTLV-1-specific T-cell responses. ACs are found in all four categories. It is noteworthy that small subgroups of ACs and smoldering ATL patients share a common feature with ATL patients. This indicates that the insufficiency of HTLV-1-specific T-cell responses is not merely the result of malignancy but is an underlying problem before the stage without apparent lymphoproliferation. Further follow-up studies are required to clarify whether the extent of the combination of elevated proviral loads with low T-cell responses could be a diagnostic indicator for risk of ATL.

Dissociation between proviral loads and T-cell responses. Although HTLV-1-specific T-cells have the potential to control infected cells, there are no clear correlations between

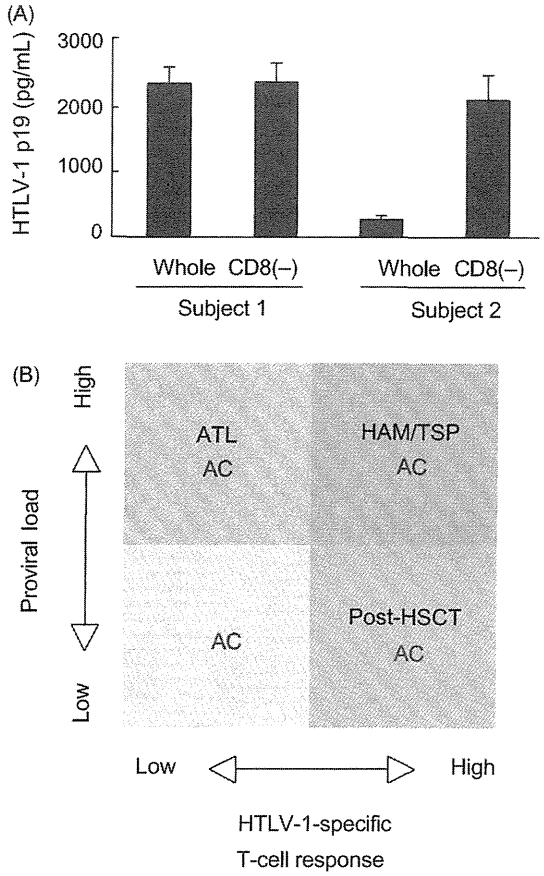

Fig. 2. Diversities in Tax-specific T-cell responses and dissociation with proviral loads in human T-cell leukemia virus type 1 (HTLV-1)-infected individuals. (A) Diversity in $CD8^+$ T-cell functions in two representative HTLV-1-infected individuals at the asymptomatic stage. Abundant amounts of HTLV-1 p19 were produced in PBMC cultures with or without $CD8^+$ T-cells in subject 1, but only after $CD8^+$ T-cell depletion in subject 2.[53] (B) A general image for the categories of HTLV-1-infected individuals at various stages according to the combinations of HTLV-1-specific T-cell responses (*x*-axis) and proviral loads (*y*-axis) is shown schematically. AC, asymptomatic carriers; ATL, adult T-cell leukemia; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; HSCT, hematopoietic stem cell transplantation.

proviral loads and HTLV-1-specific T-cell responses among HTLV-1-infected individuals. This is not surprising because both the proviral loads and T-cell responses are high in HAM/TSP patients. The proviral loads may be negatively correlated with T-cell responses only within an individual but not among individuals. Several other reports have indicated various findings concerning this issue. For example, a study measuring IFN-γ-producing $CD8^+$ HTLV-1-specific CTLs indicated a positive correlation with proviral loads in HAM/TSP patients but not in ACs,[28] while a study evaluating $CD8^+$ CTL function by *ex vivo* clearance of infected cells showed negative correlations with low proviral loads within an AC or a HAM/TSP group,[42] and another study indicated an association of higher frequency of tetramer-binding Tax-specific CTLs with low proviral loads in ACs.[27] Such inconsistent results suggest the presence of certain other determinants of proviral loads in addition to HTLV-1-specific CTLs.

The HTLV-1 proviral loads reflect the number of infected cells in the peripheral blood. Expansion of HTLV-1-infected cells *in vivo* occurs through both *de novo* infection and proliferation of infected cells.[54] The number of $CD4^+$ $FoxP3^+$ cells,[55] the frequency of iNKT cells,[56] or MHC-I favorable for HBZ-specific T-cell responses[41] have been suggested to influence HTLV-1 proviral loads.

In HTLV-1-infected rats, however, the proviral loads are inversely correlated with HTLV-1-specific T-cell responses.[21] One reason for the discrepancy between humans and rats may be the genetic heterogeneity in humans. It appears that, under the homogeneous genetic background in the experimental rat system, the influence of insufficient HTLV-1-specific T-cell responses may appear more clearly than in humans, allowing *de novo* infection and proliferation of HTLV-1-infected cells *in vivo*. The dissociation of proviral loads and HTLV-1-specific T-cell responses in humans suggests that additional determinants of proviral loads may vary genetically among individuals. As described in the next section, we suppose that innate immunity could be a candidate for this effect.

Control of HTLV-1 by innate immunity

Status of HTLV-1 expression *in vivo*. Since HTLV-1-specific antibodies and T-cells are maintained in HTLV-1-infected individuals, viral expression must occur somewhere *in vivo*. This notion is further supported by the emergence of Tax-specific CTL responses in HTLV-1-uninfected donor-derived hematopoietic systems reconstituted in recipient ATL patients after HSCT.[48,57] However, HTLV-1 mRNA but not viral proteins are detectable in PBMCs freshly isolated from HTLV-1-infected individuals. The levels of HTLV-1 mRNA are higher in HAM/TSP patients than in ACs,[58] but viral proteins are still undetectable. Only a few reports have indicated HTLV-1 protein expression *in situ*.[59]

HTLV-1 expression in ATL cells immediately after isolation from the peripheral blood is very low, and becomes significantly induced after culture for some hours *in vitro*.[60,61] This phenomenon is observed in about one half of ATL patients regardless of the disease severity.[62] Viral induction after *in vitro* culture does not occur in the other one half of ATL patients, probably because of genetic and epigenetic changes in the viral genome.[63–65] Rapid induction of viral expression after *in vitro* culture has also been observed in PBMCs from HAM/TSP patients and ACs,[66] indicating that there must be a common mechanism for transiently suppressing HTLV-1 expression *in vivo* regardless of the diseases.

Suppression of HTLV-1 expression by type-I IFN responses. Recently, we found that type-I IFN responses are involved in the suppression of HTLV-1 expression.[34] When HTLV-1-infected T-cell line cells were co-cultured with stromal cells such as epithelial cells and fibroblasts, HTLV-1 mRNA and proteins were markedly decreased in HTLV-1-infected cells. Similarly, induction of HTLV-1 expression in cultures of primary ATL cells was also suppressed by co-culture with stromal cells. Type-I IFNs were involved in the stromal cell-mediated suppression of HTLV-1 expression, because it was partly neutralized by anti-IFN-α/β receptor antibodies. Since efficient HTLV-1 expression is dependent on transactivation of its own LTR by Tax protein,[30,67] limitation of this protein below a certain level will lead to the maintenance of HTLV-1 expression at low levels. Stromal cells reduced viral expression via type-I IFNs, but did not reduce cell growth and even supported it by unknown mechanisms.[34,68]

It has been reported that plasmacytoid dendritic cells (pDCs), a major producer of type-I IFNs, are susceptible to HTLV-1 infection.[69,70] In ATL patients, pDCs are decreased in number and also lack the ability to produce IFN-α.[69] A recent report indicated that pDCs generate type-I IFNs mainly through TLR7 recognition of HTLV-1 RNA.[71] The precise mechanisms of the HTLV-1-mediated IFN responses remain to be clarified.

In addition to recombinant IFN-α and IFN-β, recombinant IFN-γ was also capable of reducing HTLV-1 expression to

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lesser extents in HTLV-1-infected cell lines.[34,72] Participation of type-II IFN-producing cells other than stromal cells in HTLV-1 suppression *in vivo* is also conceivable.

Potential involvement of type-I IFNs in HTLV-1 suppression *in vivo*. In *in vitro* experiments, co-cultured stromal cells suppressed viral expression in HTLV-1-infected cells. Interestingly, when infected cells were re-isolated from the co-cultures, viral expression was restored to the original level over the following 48 h (Fig. 3).[34] This observation shows a striking similarity to the rapid induction of HTLV-1 expression in freshly isolated ATL cells after culture *in vitro*.

Involvement of type-I IFN responses in the suppression of HTLV-1 expression *in vivo* was confirmed using interferon regulatory factor-7-KO mice, which are deficient in most type-I IFN responses. Viral expression in HTLV-1-infected cells was significantly suppressed when the infected cells were intraperitoneally injected into WT mice but not into interferon regulatory factor-7-KO mice.[34]

It is speculated that the levels of viral expression in HTLV-1-infected lymphocytes may differ among various tissues depending upon the strength of IFN responses. Thus far, there is little information regarding HTLV-1 expression in various tissues. In transgenic mice with an HTLV-1 LTR-driven construct of the pX gene, expression of the transgene was only observed in limited organs including the central nervous system, eyes, salivary glands and joints.[73] It is intriguing that all of these tissues are involved in human inflammatory diseases related to HTLV-1 infection. Such coincidences suggest the involvement of HTLV-1 gene expression in the pathogenesis of these inflammatory diseases.

Double control of HTLV-1 by innate and acquire immunity

Relationship between acquired and innate immune control in HTLV-1 infection. At the primary infection, type-I IFNs generally play a critical role in limiting viral replication, and have positive effects on antigen presentation by activating DCs, inducing type-II IFN, and upregulating MHC-I, which subsequently augments T-cell responses.[74] However, the role of type-I IFNs in the chronic phase of viral infection may not always be positive. In HIV-1 infection, type-I IFNs may be a progressive factor for the disease by accelerating T-cell exhaustion.[75]

Suppression of HTLV-1 expression by type-I IFNs may reduce the efficacy of T-cell-mediated surveillance against HTLV-1-infected cells, because T-cells require viral proteins for recognition. On the contrary, if the IFN-mediated suppressive system is insufficient, HTLV-1-specific T-cell responses will be activated in response to viral antigens.

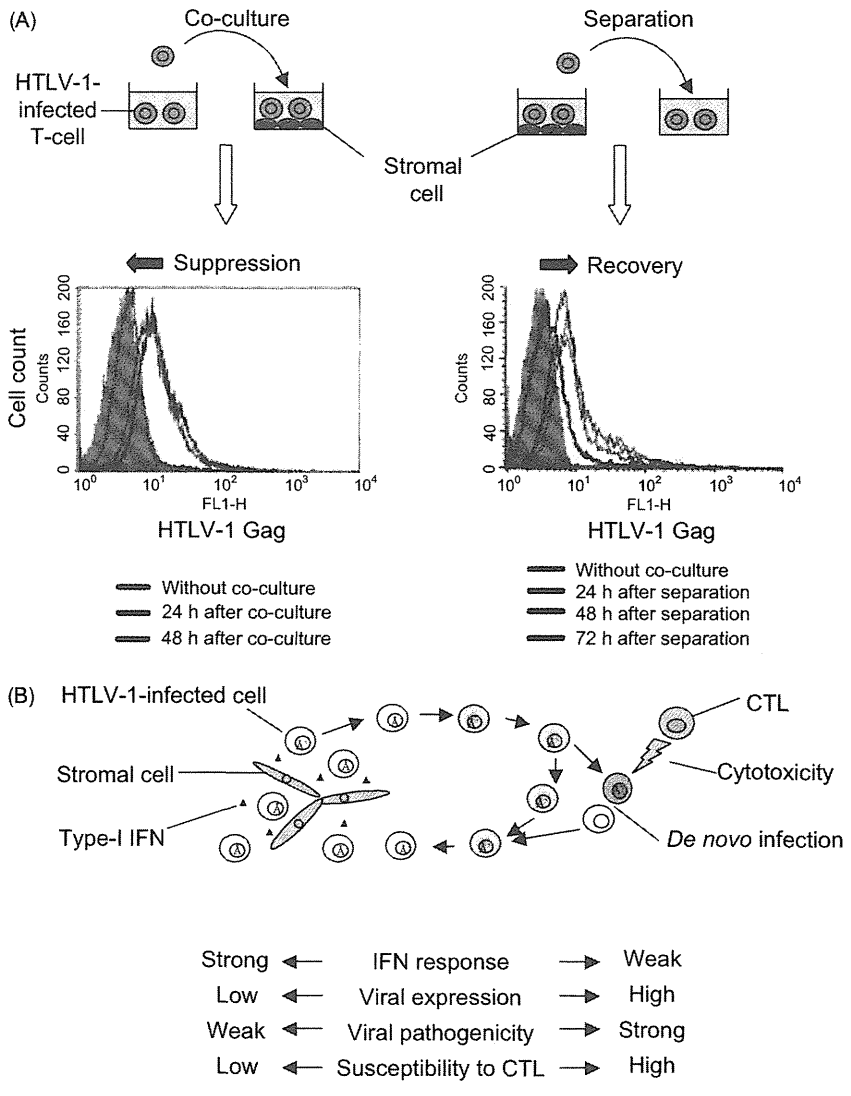

Fig. 3. Reversible suppression of human T-cell leukemia virus type 1 (HTLV-1) expression by innate immunity. (A) When IL-2-dependent HTLV-1-infected cells are co-cultured with 293T cells, intracellular HTLV-1 Gag proteins in the infected cells are decreased within 48 h (left panel). When the infected cells are re-isolated and further cultured on their own, Gag expression is recovered within 48 h (right panel).[34] (B) Scheme of the presumed status of HTLV-1-infected cells *in vivo*. Viral expression (indicated as pink) would be suppressed in tissues with strong IFN responses (left) and increased in tissues with weak IFN responses (right). CTL function, if any, is only effective upon viral expression, resulting in an infected cell reservoir without viral expression (left) and a T-cell surveillance system with low efficiency (right).

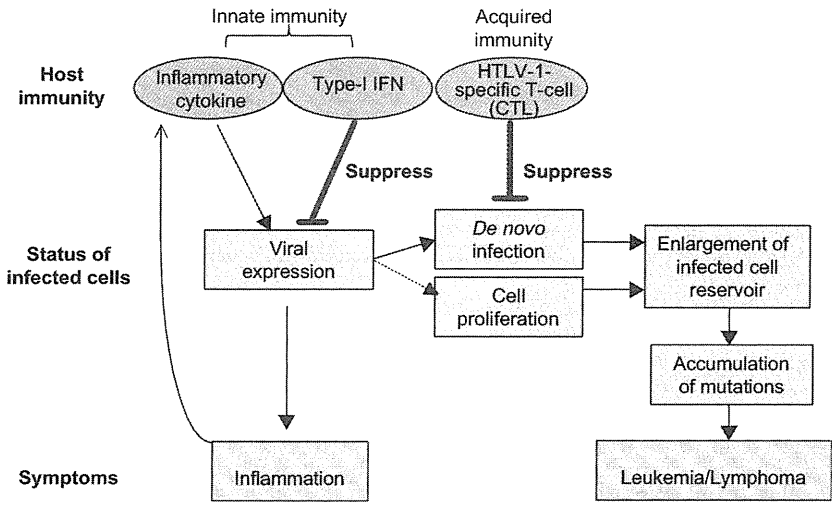

Fig. 4. Hypothetical relationships among the host immunity, status of human T-cell leukemia virus type 1 (HTLV-1)-infected cells and symptoms. HTLV-1-infected cells are controlled by at least two systems: type-I IFNs (innate immunity) and HTLV-1-specific T-cells (acquired immunity). The former suppress viral expression and the latter kill infected cells. An increase in viral expression would accelerate inflammation, increase the number of infected cells through *de novo* infection and activate HTLV-1-specific T-cells that determine an equilibrium level of proviral load within an individual. Viral expression may be a positive, but not absolute, factor for cell proliferation. When the viral expression is well controlled, the viral pathogenesis will proceed slowly, and may not be apparent until infected cell clones with a malignant phenotype finally emerge from the enlarged infected cell reservoir. Without proper T-cell responses, the emergence of such clones may occur earlier, because they would have more chance to survive.

The relationship between innate and acquired immunity may also differ among tissues. In tissues with strong IFN responses, viral expression in the infected cells would be suppressed and CTLs would ignore these cells. However, in tissues with weak IFN responses, infected cells would express viral antigens to be recognized by CTLs (Fig. 3). These presumptions can explain the status of HTLV-1-infected cells *in vivo*, which comprises a large reservoir of infected cells without viral expression and a low-efficiency surveillance system by CTLs that can only work on limited occasions.

Potential relationship between disease manifestation and innate and acquired host immunity in HTLV-1 infection. Although suppression of HTLV-1 expression may partly interfere with the efficacy of T-cell immunity, it may contribute to a slowing down of the Tax-mediated pathogenesis, tumorigenesis and inflammation (Fig. 4). In a rat model, shRNA-mediated suppression of Tax in HTLV-1-transformed cells rendered these cells resistant to Tax-specific CTLs but also reduced their ability for tumorigenesis *in vivo*.[76] Continuous suppression of HTLV-1 expression in humans may have a similar decelerating effect against Tax-mediated tumorigenesis. This might be a reason why it takes so long for ATL to develop. So long as the viral expression is well controlled, the viral pathogenesis may not be apparent until malignant cell clones finally come through the process of clonal evolution in the infected cell reservoir. Without proper T-cell responses, the emergence of such clones may occur earlier, because they would have more chance to survive.

HAM/TSP patients show elevated levels of viral expression for an unknown reason. Increased levels of inflammatory cytokines could be either a cause or a result of this phenomenon. The involvement of HTLV-1 proviral integration sites in transcription units in elevated viral expression has also been suggested.[77] An experimental rat model of HAM/TSP using a certain WKAH strain exhibits increased Tax mRNA expression in the spinal cord without T-cell infiltration,[78] suggesting that viral expression is a primary event while T-cell responses are not. Further studies revealed that this particular rat strain contains mutations in the promoter region of the IL-12 receptor, which potentially lead to reduced IFN-γ production in the spinal cord.[72] The associations of genetic factors related to the IFN system with HAM/TSP patients have remained obscure. Very recently, a gene expression profiling study indicated that expression of suppressor of cytokine signaling 1 (SOCS1) is upregulated in HAM/TSP patients and ACs, and is positively correlated with high HTLV-1 mRNA loads.[79]

Conclusions

HTLV-1 is controlled by both acquired and innate immunity. HTLV-1-specific T-cells contribute to antitumor surveillance, and type-I IFNs contribute to silencing viral expression. The presence of the double control systems with partial conflicts would explain some of the puzzles in HTLV-1 infection, such as the transient suppression of viral expression *in vivo*, apparently reciprocal occurrence of ATL and HAM/TSP, inconsistent correlations of proviral loads with T-cell responses, and a long incubation period.

Insufficient T-cell responses are regarded as a risk factor for ATL, and vaccines that augment HTLV-1-specific T-cell responses would be beneficial in reducing the risk in a subpopulation of HTLV-1 carriers exhibiting insufficient T-cell responses and elevated proviral loads.

Innate immune responses in HTLV-1 infection should be further investigated, because they could be another important determinant of disease manifestation and represent therapeutic targets in HTLV-1-related diseases.

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Functional impairment of Tax-specific but not cytomegalovirus-specific CD8$^+$ T lymphocytes in a minor population of asymptomatic human T-cell leukemia virus type 1-carriers

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Abstract

Background: Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in a small percentage of infected individuals. ATL is often associated with general immune suppression and an impaired HTLV-1-specific T-cell response, an important host defense system. We previously found that a small fraction of asymptomatic HTLV-1-carriers (AC) already showed impaired T-cell responses against the major target antigen, Tax. However, it is unclear whether the impaired HTLV-1 Tax-specific T-cell response in these individuals is an HTLV-1-specific phenomenon, or merely reflects general immune suppression. In this study, in order to characterize the impaired HTLV-1-specific T-cell response, we investigated the function of Tax-specific CD8$^+$ T-cells in various clinical status of HTLV-1 infection.

Results: By using tetramers consisting of HLA-A*0201, -A*2402, or -A*1101, and corresponding Tax epitope peptides, we detected Tax-specific CD8$^+$ T-cells in the peripheral blood from 87.0% of ACs (n = 20/23) and 100% of HAM/TSP patients (n = 18/18) tested. We also detected Tax-specific CD8$^+$ T-cells in 38.1% of chronic type ATL (cATL) patients (n = 8/21), although its frequencies in peripheral blood CD8$^+$ T cells were significantly lower than those of ACs or HAM/TSP patients. Tax-specific CD8$^+$ T-cells detected in HAM/TSP patients proliferated well in culture and produced IFN-γ when stimulated with Tax peptides. However, such functions were severely impaired in the Tax-specific CD8$^+$ T-cells detected in cATL patients. In ACs, the responses of Tax-specific CD8$^+$ T-cells were retained in most cases. However, we found one AC sample whose Tax-specific CD8$^+$ T-cells hardly produced IFN-γ, and failed to proliferate and express activation (CD69) and degranulation (CD107a) markers in response to Tax peptide. Importantly, the same AC sample contained cytomegalovirus (CMV) pp65-specific CD8$^+$ T-cells that possessed functions upon CMV pp65 peptide stimulation. We further examined additional samples of two smoldering type ATL patients and found that they also showed dysfunctions of Tax-specific but not CMV-specific CD8$^+$ T-cells.

Conclusions: These findings indicated that Tax-specific CD8$^+$ T-cells were scarce and dysfunctional not only in ATL patients but also in a limited AC population, and that the dysfunction was selective for HTLV-1-specifc CD8$^+$ T-cells in early stages.

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Background

Human T-cells leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive $CD4^+$ T-cell malignancy, adult T-cell leukemia (ATL)[1,2]. As many as 10 million individuals are thought to be infected worldwide, in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa[3]. Unlike human immunodeficiency virus (HIV), the majority of HTLV-1-infected individuals are clinically asymptomatic during their lifetime. However, approximately 5% develop ATL, and another 2-3% develop a variety of chronic inflammatory diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[4-8].

HTLV-1-specific cytotoxic T-lymphocytes (CTLs) are thought to play a pivotal role in containing the proliferation of HTLV-1-infected T-cells[9,10]. Tax is known to be the dominant target antigen for HTLV-1-specific CTLs[10-13], and a high frequency of Tax-specific CTLs can be detected in HAM/TSP patients and some asymptomatic HTLV-1 carriers (ACs)[10-14]. However, ATL patients show general immune suppression[15], reduced frequency and dysfunction of Tax-specific CTLs[16,17]. Regulatory T cell (Treg)-like function of $FoxP3^+$ ATL cells and diminished function of dendritic cells may be involved in the immune suppression in ATL patients [18,19], but the precise mechanism is not yet clarified. We previously demonstrated that a fraction of ACs also exhibit reduced T-cell responses against Tax protein [20]. These observations suggest that the reduced HTLV-1-specific T-cell response might be an underlying risk of ATL development, but not the result of ATL. However, it is unknown how the function of HTLV-1-specific $CD8^+$ T-cells becomes impaired in a small percentage of ACs and whether its dysfunction is specific for HTLV-1 antigen or due to general immune suppression.

During chronic stage of infection with several viruses, such as HIV and hepatitis C virus (HCV), virus-specific CTLs gradually lose their cytotoxic activity, the ability to proliferate and secrete a diverse profile of cytokines, ultimately leading to exhaustion, anergy or even deletion of these cells[21-26]. Programmed death-1 (PD-1), a negative regulator in the CD28 superfamily, has recently been shown to be highly expressed on virus-specific T-cells during many chronic viral infections[27-29]. It has also been reported that the interaction of PD-1 with PD-ligand 1 (PD-L1) negatively regulates cytokine production and proliferation of T-cells[30,31]. A previous report indicates that PD-1 is up-regulated on the dominant Tax-specific CTLs in ATL patients and ACs and that immune regulation through the PD-1/PD-L1 pathway may be involved in the dysfunction of HTLV-1-specific CTLs in ATL patients[32].

Studies on memory T-cell differentiation have shown that phenotype, function, and homeostasis of memory T-cells vary for different persistent virus infections[33]. Central memory T-cells (T_{CM}; $CD45RA^-CCR7^+$) are elicited by non-persisting virus that provide transient antigen stimulation, such as in Influenza virus infection. In contrast, effector memory T-cells (T_{EM}; $CD45RA^-CCR7^-$) predominate when relatively high levels of antigen persist, such as in HIV infection. Terminally differentiated memory (T_{Diff}; $CD45RA^+CCR7^-$) can be seen when antigen persists at a low level, such as in cytomegalovirus (CMV) infection. In HTLV-1 infection, it has been reported that dominant Tax-specific CTLs in HAM/TSP patients consist of T_{EM} and T_{Diff} compartments[34].

We previously identified some major epitopes recognized by HTLV-1-specific CTLs in infected individuals carrying HLA-A2, -A11, or -A24[12,35,36]. These allowed us to monitor HTLV-1-specific CTLs and analyze their functions *ex vivo*, by using antigen/HLA tetrameric complexes. In this study, we demonstrate that IFN-γ production and proliferative capacity of tetramer-binding Tax-specific $CD8^+$ T-cells were severely impaired not only in ATL patients but also in a minor population of asymptomatic HTLV-1 carriers (ACs). Importantly, the T-cell dysfunction at the asymptomatic stage was selective for HTLV-1 but not for CMV antigen. In addition, severely impaired HTLV-1-specific but not CMV-specific $CD8^+$ T-cells responses were also observed in patients diagnosed as smoldering ATL, the clinical condition of which is close to that of AC. The dysfunction of HTLV-1-specific $CD8^+$ T-cells in an early clinical stage implies HTLV-1-specific immune suppressive mechanism might be an underlying risk for ATL.

Results

Incidence and frequency of Tax-specific $CD8^+$ T-cells in ACs, and HAM/TSP and cATL patients

In 23 ACs and 18 HAM/TSP and 21 cATL patients carrying HLA-A2, -A11 and/or -A24 alleles, we evaluated the frequencies of Tax-specific $CD8^+$ T-cells by using cognate Tax/HLA tetramers (Figure 1 and Table 1). Tax-specific $CD8^+$ T-cells were detected in 87.0% of ACs and all HAM/TSP patients tested. In contrast, only 38.1% of cATL patients have detectable frequencies of Tax-specific $CD8^+$ T-cells (Table 1). Figure 1B shows that the average frequency of Tax-specific $CD8^+$ T-cells in the $CD8^+$ T-cells of cATL patients (n = 21, 0.90% range: 0%-9.45%) was significantly lower than that in ACs (n = 23, 2.37%, range: 0%-8.23%, $P = 0.0023$). HAM/TSP patients had the highest average frequency of Tax-specific $CD8^+$ T-cells among the three groups (n = 18, 8.88%, range: 1.86%-29.9%, $P = 0.0001$; vs. AC, $P < 0.0001$; vs. cATL patients), which is consistent with

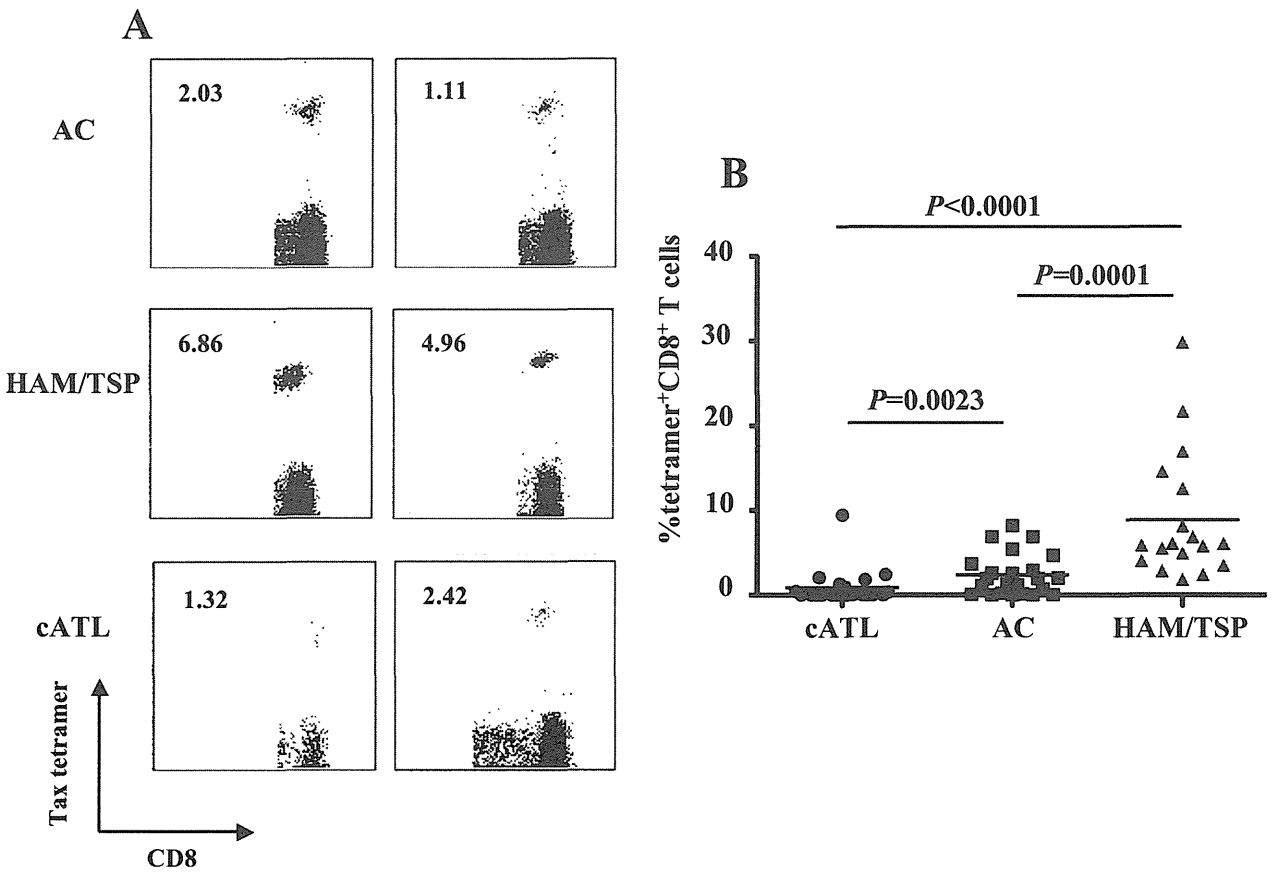

Figure 1 Incidence and frequency of Tax-specific CD8$^+$ T-cells in ACs, and HAM/TSP and cATL patients. (A) Whole blood or PBMCs from AC (top), and HAM/TSP (middle) and cATL (bottom) patients were stained with Tax/HLA tetramer. Number indicates the percentage of tetramer$^+$ cells in CD8$^+$ T-cells. (B) The percentage of Tax-tetramer$^+$ CD8$^+$ T-cells in AC (n = 23), and HAM/TSP (n = 18) and cATL (n = 21) patients. *P* value was determined by the Mann-Whitney *U* test. Horizontal bars indicate the average percentage of Tax-tetramer$^+$ CD8$^+$ T-cells for the group.

previous reports [10,17,37]. It is of note that Tax-specific CD8$^+$ T-cells are detectable even in cATL patients, although the frequency is very low.

Impaired cell proliferation and IFN-γ production of Tax-specific CD8$^+$ T-cells in cATL but not HAM/TSP patients

We next examined IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in HAM/TSP and cATL patients (Figure 2A). Intracellular IFN-γ staining showed that Tax-specific CD8$^+$ T-cells in all HAM/TSP patients tested produced IFN-γ when stimulated with Tax peptide (Figure 2A). Tax-specific CD8$^+$ T-cells in those HAM/TSP patients proliferated regardless of stimulation with Tax peptide (Figure 2B). In contrast to HAM/TSP patients, IFN-γ production from Tax-specific CD8$^+$ T-cells in a cATL patient was hardly detectable even when stimulated with Tax peptide (4.8%, Figure 2A). In the same donor, Tax-specific CD8$^+$ T-cells

Table 1 The number of blood samples with detectable Tax-specific CD8$^+$ T-cells in all samples tested in this study

Tax/HLA tetramers used in this study	Disease Status		
	AC	HAM/TSP	cATL
HLA-A*0201/Tax11-19	12/14[1]	7/7	2/11
HLA-A*1101/Tax88-96	4/4	4/4	3/5
HLA-A*2402/Tax301-309	13/15	13/13	5/16
No. of tetramer$^+$ samples/total no. of blood samples[2]	20/23 (87.0%)	18/18 (100%)	8/21 (38.1%)

[1] No. of samples with detectable Tax-specific CD8$^+$ T-cells/total no. of samples carrying each HLA allele. When the frequency of tetramer$^+$ cells was more than 0.04% of CD8$^+$ T-cells, the sample was regarded as detectable.

[2] In case Tax-specific CD8$^+$ T-cells was detectable by either tetramer in a sample carrying two of three HLA-A alleles above, the sample was regarded as positive.

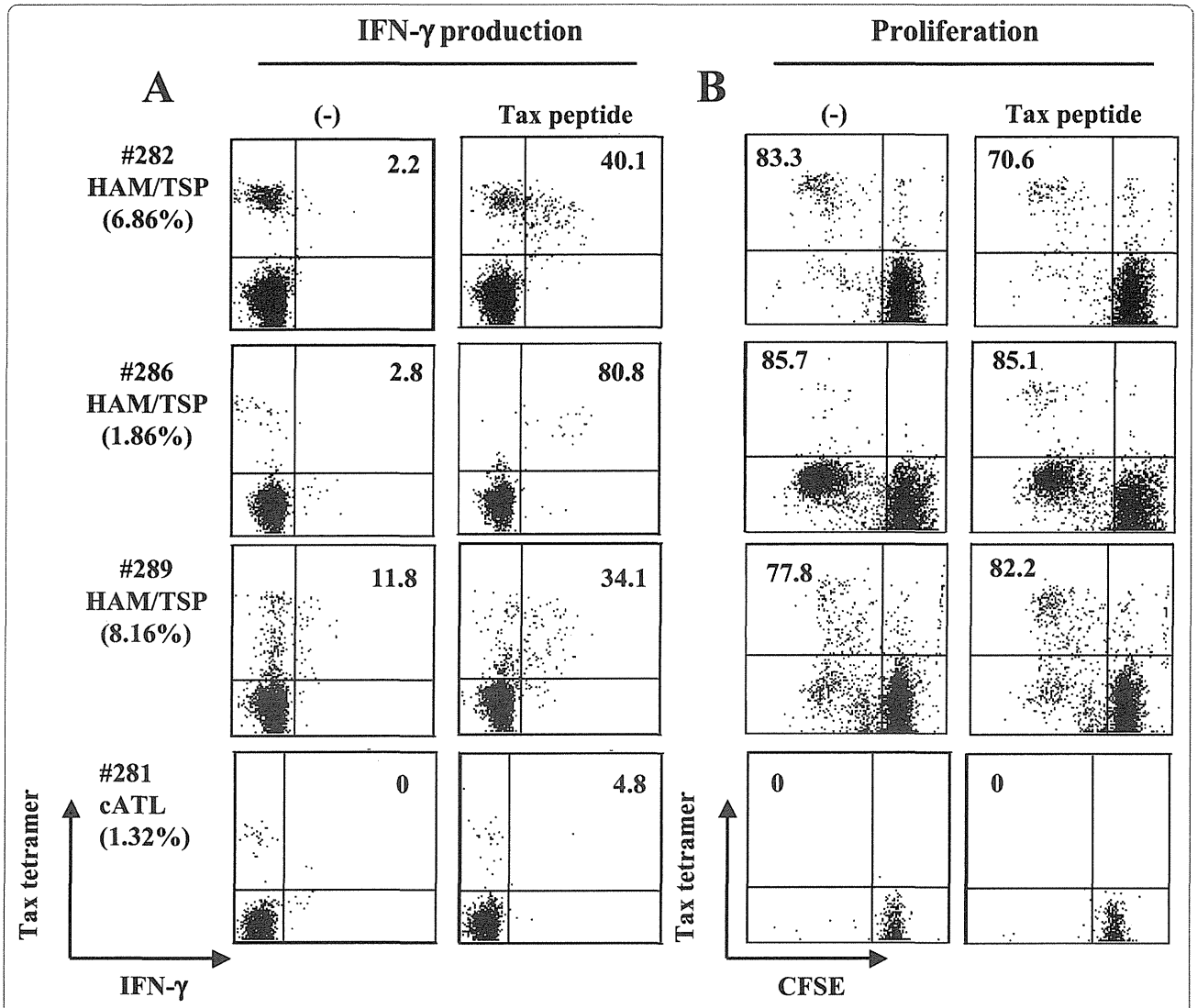

Figure 2 IFN-γ production and proliferation of Tax-specific CD8$^+$ T-cells in HAM/TSP and cATL patients. (A) PBMCs from HAM/TSP and cATL patients were stimulated with or without 10 μM Tax peptide for 6 hrs. The number indicates the percentage of IFN-γ-producing cells in tetramer$^+$ cells. (B) For CFSE-based T-cell proliferation, CFSE-labeled PBMCs from HAM/TSP and cATL patients were cultured in the presence or absence of 100 nM Tax peptide for 6 days. The number indicates the percentage of dividing (CFSE^{low}) cells in tetramer$^+$ cells. The percentage of tetramer$^+$ cells among CD8$^+$ T cells in fresh blood is indicated in parenthesis under the patient ID.

could be detected in fresh blood (1.32%) and after 6 hrs incubation as shown in Figure 2A, but not after 6 day-culture, suggesting that Tax-specific CD8$^+$ T-cells in this cATL patient had no proliferative capacity (Figure 2B). We tested PBMC from four other cATL patients who had detectable Tax-specific CD8$^+$ T-cells, but none of them showed proliferation of Tax-specific CD8$^+$ T-cells by either the CFSE-based proliferation assay or 13-day culture (Additional file 1). Collectively, these results indicate that Tax-specific CD8$^+$ T-cells from most cATL patients are impaired in their capacities to proliferate and produce IFN-γ.

Diversity in the IFN-γ production and cell proliferation of Tax-specific CD8$^+$ T-cells in ACs

Our recent studies using the GST-Tax protein-based assay demonstrated that the extent of Tax-specific T-cell responses varied widely in ACs[20]. We then evaluated proliferation and/or IFN-γ production of tetramer-binding Tax-specific CD8$^+$ T-cells in 14 ACs (Table 2). Representative data on 4 of 14 ACs are shown in Figures 3A and 3B. In 3 ACs (#251, #313, and #360), Tax-specific CD8$^+$ T-cells produced IFN-γ and proliferated in response to Tax peptide (Figures 3A and 3B). Similarly to HAM/TSP samples, a large proportion of Tax-