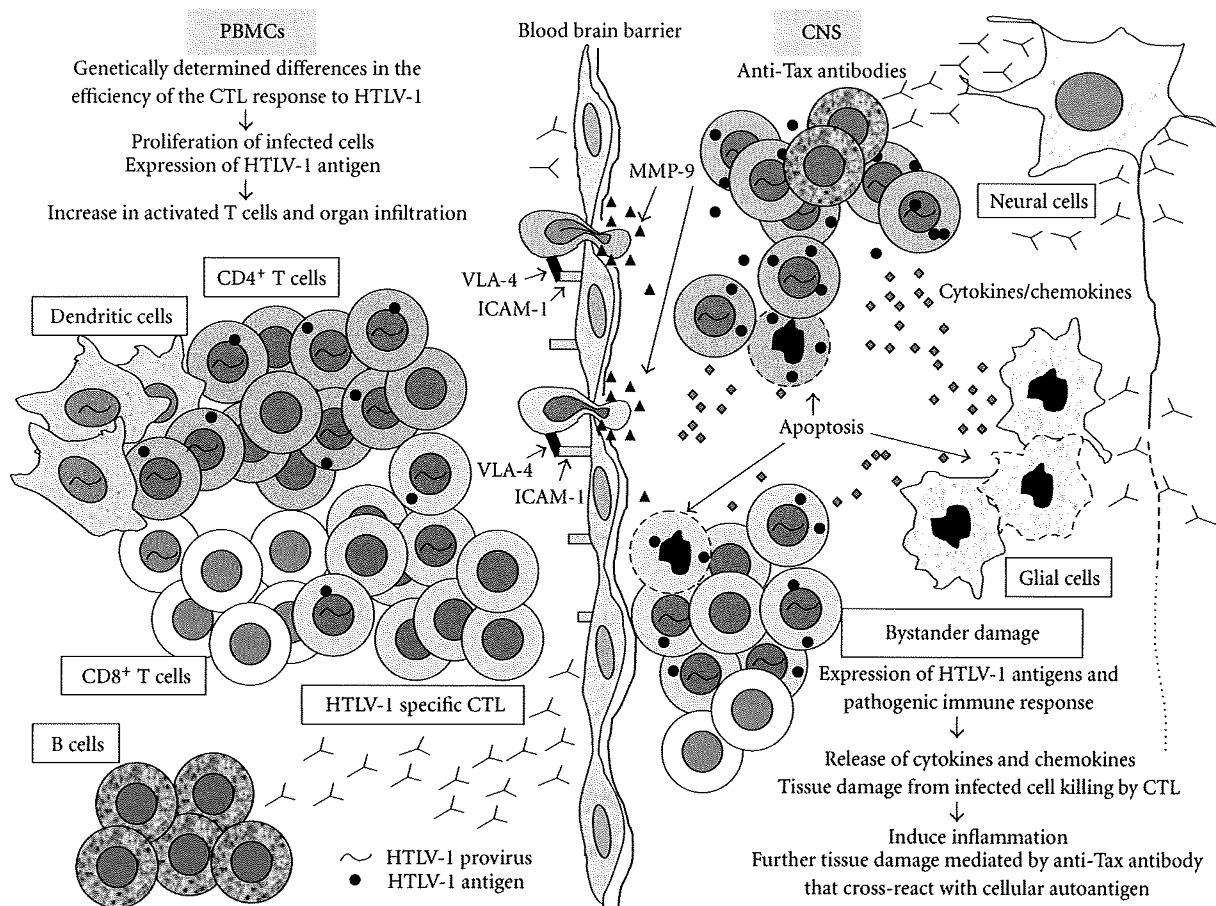


FIGURE 1: *Hypothesis for the pathogenesis of Human T-cell leukemia virus type 1- (HTLV-1-)associated myelopathy/tropical spastic paraparesis (HAM/TSP).* In patients with HAM/TSP, genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression, leading in turn to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development. It is also possible that the immunoglobulin G specific to HTLV-1-Tax, which cross-reacts with heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1), is associated with subsequent inflammation following initial tissue damage.

plays a critical part in limiting HTLV-1 replication in vivo and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk for developing HAM/TSP. However, since the frequency of HTLV-1-specific $CD8^+$ T cells was significantly elevated in HAM/TSP patients than in ACs [64, 65] and these cells have the potential to produce proinflammatory cytokines [66], there is a debate on the role of HTLV-1-specific-$CD8^+$ T cells, that is, whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP or whether the dominant effect of such cells in vivo is protective against disease, although these two mechanisms are not mutually exclusive. Recently, Sabouri et al. reported that a frequency of $CD8^+$ T cells that were negative for costimulatory molecules such as CD27, CD28, CD80, CD86, and CD152 was significantly higher in patients with HAM/TSP than in age-matched uninfected controls, but there was no such difference between ACs and uninfected controls [67]. They also found a significantly lower frequency of $perforin^+$ cells and granzyme $B^+$ cells in the $CD8^+$ T cells in HTLV-1-infected subjects than in uninfected controls, although there was no significant difference between patients with HAM/TSP and ACs. Furthermore, the lytic capacity of HTLV-1-specific CTL between HAM/TSP and ACs estimated by CD107a mobilization assay showed the significantly lower CD107a staining in HTLV-1-specific CTL in HAM/TSP than ACs. These findings suggest that patients with HAM/TSP have a high frequency of HTLV-1-specific $CD8^+$ T cells with poor lytic capacity, whereas ACs has a lower frequency of cells with high lytic capacity.

*4.6. Dendritic Cells (DCs).* Dendritic cells are antigen-presenting cells which play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from HAM/TSP patients were infected with HTLV-1 [68], and the development of HAM/TSP is associated with rapid maturation of DCs [69]. One of the hallmarks of HTLV-1 infection is the in vitro proliferation of PBMCs when cultured in the absence of exogenous antigen or mitogen, referred to as spontaneous

lymphocyte proliferation (SLP), and in HAM/TSP patients, the levels of SLP reflect the severity of the disease [70, 71]. Interestingly, depletion of DCs from the HAM/TSP patient's PBMCs abolishes SPL while supplementing DCs, but not B cells nor macrophages restore proliferation [68]. DC dependent mechanism of SLP was further supported by data showing that antibodies to MHC class II, CD86, and CD58 can block SPL [72]. Recently, Jones et al. had demonstrated that human-derived both myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1, and HTLV-1-infected DCs can rapidly transfer virus to autologous primary $CD4^+$ T cells [73]. Furthermore, in contrast to the previous report that $CD4^+CD25^+$ T cells are responsible for the stimulation of Tax-specific $CD8^+$ T cells [74], it was recently demonstrated that, compared to the $CD4^+CD25^+$ T cells, the DCs are the major cell type responsible for the generation and maintenance of the Tax-specific $CD8^+$ T cells both in vitro and in vivo [75]. These findings suggest that the interaction of DCs with HTLV-1 is also crucial for the pathogenesis of HAM/TSP.

*4.7. The Other Reservoirs of HTLV-1.* Previous studies have indicated that only a small proportion of the monocyte-macrophage lineage cells are infected with HTLV-1 in peripheral blood [76] and that there has been no direct evidence indicating that HTLV-1-infected cells of the monocyte-macrophage lineage cells are present in the CNS [77]. However, monocyte-macrophage lineage cells may also play important roles in the pathogenesis of HAM/TSP, since it has been shown that the activation of macrophage and microglial cells within the CNS closely correlated with the proviral load within the CNS of HAM/TSP patients [78]. Meanwhile, it was also shown that a vast majority of bone marrow cells from HAM/TSP patients are positive for HTLV-1 proviral DNA but negative for viral RNA expression [79], whereas no HTLV-1 proviral DNA positive $CD34^+$ hematopoietic progenitor cells were detected in ATL patients [80]. These results suggest that HTLV-1-infected cells within the bone marrow may be a reservoir of HTLV-1 in HAM/TSP patients and play an important role in the etiology of neuroinflammation observed in HAM/TSP [77].

## 5. Conclusions

As shown in Figure 1, accumulating evidence suggests that the virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression in infected individuals, which lead to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development.

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Mini Review

# Immunogenetics of human T-cell leukemia virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

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Human T-cell leukemia virus type 1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease: the malignancy known as adult T-cell leukemia (ATL) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), whereas the vast majority of infected individuals remain asymptomatic carriers of the virus in lifetime. It is not yet fully understood why do certain individuals develop ATL or HAM/TSP, and how does HTLV-1 persist in spite of host immune response. This review focuses on the complex virus-host interactions and the cellular immune responses to HTLV-1 infection seen in HAM/TSP patients, which are important factors in determining HTLV-1 proviral load and the risk of developing disease.

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**Key words** HTLV-1, HAM/TSP, HLA, immune response, disease susceptibility

## Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection is of particular interest to the field of immunology as well as microbiology because HTLV-1 is never be eliminated from the host in spite of a vigorous cellular and humoral immune responses against the virus, but causes no disease in vast majority of infected subjects (asymptomatic carriers: AC). Since HTLV-1 infection causes two distinct intractable diseases without effective treatment known as adult T-cell leukaemia[1,2] and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[3,4], evaluation of the individual risk for developing diseases in each AC would certainly be of considerable importance especially in HTLV-1 endemic area such as southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa[5]. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction and mild sensory disturbance in the lower extremities, and the main pathological features are chronic inflamma-

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tion in the spinal cord characterized by perivascular lymphocytic cuffing and parenchymal lymphocytic infiltration. It is therefore widely assumed that the immune response against HTLV-1 causes the inflammatory spinal cord damage seen in HAM/TSP patients[6]. In this review, I shall summarize the recent work for HAM/TSP attempting to resolve outstanding question, i.e. why do some HTLV-1-infected people develop disease whereas the vast majority remains healthy in lifetime.

## HTLV-1 infection and clinical features of HAM/TSP

HTLV-1 is classified as a complex retrovirus in the genus *Deltaretrovirus* of the subfamily *Orthoretrovirinae*, and infects 10-20 million people worldwide[7]. HTLV-1 can be transmitted through sexual contact[8], injection drug use[9], and breastfeeding from mother to child[10,11]. Although HTLV-1 infection is associated with a range of non-malignant chronic inflammatory diseases in the eyes, the lungs, or the skeletal muscles[7], HAM/TSP is the best-recognized with chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction and mild sensory disturbance in the lower extremities[12]. Pathological analysis of HAM/TSP autopsy materials indicates that the disease affects the spinal cord, predominantly at the thoracic level[13-15]. Loss of myelin and axons in the lateral, anterior, and posterior columns is associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis. Clinical progression of HAM/TSP is associated with increased proviral load in individual patients, and the ratio of proviral loads in cerebrospinal fluid (CSF) cells/in peripheral blood mononuclear cells (PBMCs) is significantly associated with clinically progressive disease[16]. MHC class I tetramer analysis of lymphocytes isolated from the CSF of HAM/TSP patients showed even higher frequencies of HTLV-1 Tax11-19-specific, HLA-A*02-restricted CD8 lymphocytes compared to PBMCs[17]. Therefore, an increased proliferation or migration of HTLV-1-infected and/or HTLV-1 specific lymphocytes to the central nervous system (CNS) might be closely associated with HAM/TSP pathogenesis[18]. The presence of atypical lymphocytes (so-called "flower-cells") in peripheral blood and CSF, a moderate pleocytosis and raised protein content in CSF are typically found in HAM/TSP patients. Oligoclonal bands, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-6 and interferon (IFN)-$\gamma$, and an increased intrathecal antibody synthesis specific for HTLV-1 antigens have also been described[19].

## Risk factors for HAM/TSP

Previous population association study of 202 cases of HAM/TSP and 243 AC in Kagoshima, HTLV-1 endemic southern Japan, revealed that one of the major risk factors is the HTLV-1 proviral load. The median proviral load was more than ten times higher in HAM/TSP patients than in AC, and a high proviral load was also associated with an increased risk of progression to disease[20]. It was suggested that genetic factors such as HLA is related to the high proviral load in HAM/TSP patients and genetic relatives. Namely, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 proviral load and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predispose to HAM/TSP in the same population[21,22]. Since the function of class I HLA proteins is to present antigenic peptides to cytotoxic T lymphocytes (CTL), these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may be an important determinant of HTLV-1 proviral load and the risk of HAM/TSP. Further analysis to look at non-HLA host genetic factors revealed that non-HLA gene polymorphism also affects the risk for developing HAM/TSP. For example, the TNF-$\alpha$ promoter -863 A allele[23] and the longer CA repeat alleles of MMP-9 promoter[24] predisposed to HAM/TSP, whereas IL-10 -592 A[25], SDF-1 +801A[23] and IL-15 +191 C alleles[23] conferred protection against HAM/TSP. The polymorphisms of MMP-9 and IL-10 promoter each linked to the HTLV-1-encoded transactivator Tax mediated transcriptional activity of each gene[24,25].

Meanwhile, although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al reported the association between HTLV-1 *tax* gene variation and the risk of HAM/TSP[26]. The *tax* subgroup A that belongs to cosmopolitan subtype A was more frequently observed in HAM/TSP patients and this effect was independent of protective allele HLA-A*02. HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, *tax* subgroup B that belongs to cosmopolitan subtype B, but not against *tax* subgroup A in Japanese population[26]. Interestingly, HLA-A*02 appears not to give protection against infection with cosmopolitan subtype A in a population in Iran[27]. These findings suggest that both host genetic factors and HTLV-1 subgroup play a part in determining the risk of HAM/TSP, although the effect of HTLV-1 genotype is relatively small so the factors that determine the different outcomes of HTLV-1 infection must lie chiefly in the host.

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## Estimation of the odds for developing HAM/TSP

Based on these observations, a best-fit logistic regression equation that can be used to predict the odds of HAM/TSP has been developed[23]. Using this equation, knowledge of HTLV-1-infected individuals' ages, sex, provirus load, HTLV-1 *tax* subgroup, and genotypes at the loci HLA-A (HLA-A*02), HLA-C (HLA-Cw*08), stromal cell-derived factor (SDF)-1 (+801G/A), and TNF-$\alpha$ (-863A/C) allowed for the correct identification of 88% cases of HAM/TSP in Kagoshima cohort. To validate whether this multivariate logistic equation can be useful to identify HAM/TSP related symptom in AC, the individual odds of 181 consecutive AC were calculated and compared with their clinical parameters and laboratory findings[28]. Interestingly, although no clear difference was seen between the odds of HAM/TSP and either sex, family history of HAM/TSP or ATL, and history of blood transfusion, however, brisk patellar deep tendon reflexes, which suggest latent central nervous system compromise, and flower cell-like abnormal lymphocytes, which is the morphological characteristic of ATL cells, has found to be associated with a higher odds of HAM/TSP. These observations indicated that this best-fit logistic regression equation may be useful for detecting subclinical abnormalities in AC in Kagoshima, where HTLV-1 endemic southern Japan.

## The immune response to HTLV-1

### 1) The humoral immune response to HTLV-1

In HTLV-1 infection, anti-HTLV-1 antibody that often includes IgM is detected in all infected individuals, either AC or patients with HTLV-1-associated diseases. It has been reported that HAM/TSP patients generally had higher anti-HTLV-1 antibody titer than AC with the similar HTLV-1 proviral load[29-31]. These data suggest that there was persistent expression of HTLV-1 proteins *in vivo* and the existence of an augmented humoral immune response to HTLV-1 in HAM/TSP patients. Levin et al reported some intriguing evidence for antigen mimicry in HTLV-1 infection[32]. Namely, antibodies that recognize HTLV-1 Tax protein can cross-react with a host nuclear riboprotein hnRNP-A1. However, since the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed, and is not normally accessible to antibody attack, it is unlikely that anti-Tax antibody explains the onset or initial tissue damage of HAM/TSP. Rather, anti-Tax antibody might be associated with subsequent inflammation following initial tissue damage, which probably caused by the antiviral immune responses to HTLV-1 and induce the release of auto-antigens.

### 2) The natural killer (NK) cell response

Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of NK cells (especially the $CD3^{+}$ $CD16^{+}$ subset) than AC, although the results were not normalized with respect to the proviral load[33]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the non-polymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, synthetic tetramers of HLA-E with the HLA-G signal sequence peptide was used to identify NK cells in HAM/TSP patients[34]. The results clearly showed a lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than AC, and as in the earlier studies[33], this reduction in frequency was particularly notable in the $CD3^{+}$ cells whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^{-}$ cells between patients with HAM/TSP and AC. These results suggest that the activity of the NK or NK-like cell response was associated with the presence or absence of HAM/TSP. On the other hand, we previously reported that an uncontrolled preliminary trial by oral administration of viable *Lactobacillus casei* strain Shirota containing fermented milk for HAM/TSP patients resulted in significant increase of NK cell activity with improvements in clinical symptoms[35], suggesting that NK cells might be associated with the pathogenesis of HAM/TSP.

### 3) The regulatory T cells (Tregs)

It has been reported that HTLV-1 preferentially and persistently infects $CD4^{+}CD25^{+}$ lymphocytes *in vivo*[36], which contain the majority of the $Foxp3^{+}$ Tregs[37]. In HAM/TSP patients, the percentage of $Foxp3^{+}$ Tregs in $CD4^{+}CD25^{+}$ cells is lower than that in AC and uninfected healthy controls[38], however, the percentage of $Foxp3^{+}$ cells in the $CD4^{+}$ population tended to be higher in the HAM/TSP patients than in the AC[39]. This is probably because $CD25^{+}$ cells contain both Tregs and activated non-Tregs, and HTLV-1 infected individuals especially HAM/TSP patients increases the number of activated T cells expressing CD25. Interestingly, the percentage of $Foxp3^{+}$ Tregs positively correlated with the HTLV-1 proviral load and the CTL activity negatively correlated with the frequency of $Foxp3^{+}$ Tregs[39], suggesting that an increase in Tregs reduces CTL activity, which in turn increases the HTLV-1 proviral load.

### 4) The $CD4^{+}$ helper T cell response to HTLV-1

The HTLV-1 antigen most commonly recognized by $CD4^{+}$ T cells is the Envelope (Env) protein, in contrast with the

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immunodominance of Tax in the CD8+ T cell response. Since an HTLV-1 Env gp21 immunodominant epitope was restricted by HLA-DRB1*0101, and HLA-DRB1*0101 was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in southern Japan[21,22] and northeastern Iran[27], a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380-394 peptide was used to analyze Env-specific CD4+ T cells directly *ex vivo*[40]. The results clearly showed that the frequency of tetramer+ CD4+ T cells was significantly higher in HAM/TSP patients than AC with similar proviral load. Direct *ex vivo* analysis of tetramer+ CD4+ T cells from two unrelated DRB1*0101 positive HAM/TSP patients indicated that certain TCR V$\beta$s were utilized and antigen-specific amino acid motifs were identified in CDR3 regions from both patients. These data suggest that the observed increase in virus-specific CD4+ T cells in HAM/TSP patients, which may contribute to CD4+ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1 infected CD4+ T cells but was the result of *in vivo* selection by specific MHC-peptide complexes, as observed in freshly isolated HLA-A*0201/ Tax11-19 tetramer+ CD8+ T cells[41] and muscle infiltrating cells from HAM/TSP patients and HTLV-1 infected polymyositis patients[42].

## The cytotoxic T lymphocyte (CTL) response to HTLV-1

Previous reports indicated that the HTLV-1 specific CD8+ CTL are typically abundant, chronically activated, and mainly targeted to the viral transactivator protein Tax[6]. Also, as already mentioned, the median proviral load in PBMCs of HAM/TSP patients was more than ten times higher than that in AC, and a high proviral load was also associated with an increased risk of progression to disease[20]. Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower proviral load and a lower risk of HAM/TSP[21,22], and CD8+ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1 infected individuals[43]. These data have raised the hypothesis that the class I-restricted CD8+ CTL response plays a critical part in limiting HTLV-1 replication *in vivo*, and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk for developing HAM/TSP. However, since the frequency of HTLV-1-specific CD8+ T cells were significantly elevated in HAM/TSP patients than AC[44,45], and these cells have the potential to produce proinflammatory cytokines[46], there is a debate on the role of HTLV-1-specific-CD8+ T cells, i.e. whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP, or whether the dominant effect of such cells *in vivo* is protective against disease, although these two mechanisms are not mutually exclusive. Recently, we reported that a frequency of CD8+ T cells that were negative for costimulatory molecules such as CD27, CD28, CD80, CD86 and CD152 were significantly higher in patients with HAM/TSP than in age-matched uninfected controls, but there was no such difference between AC and uninfected controls[47]. We also found a significantly lower frequency of perforin+ cells and granzyme B+ cells in the CD8+ T cells in HTLV-1 infected subjects than in uninfected controls, although there was no significant difference between patients with HAM/TSP and AC. Furthermore, the lytic capacity of HTLV-1 specific CTL between HAM/TSP and AC estimated by CD107a mobilization assay showed the significantly lower CD107a staining in HTLV-1 specific CTL in HAM/TSP than AC. Based on these findings, we have suggested that patients with HAM/TSP have a high frequency of HTLV-1 specific CD8+ T cells with poor lytic capacity, whereas AC have a lower frequency of cells with high lytic capacity.

## Conclusions

As shown in Figure 1, the evidence summarized in this paper is consistent with the idea that virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression in infected individuals, which lead to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development.

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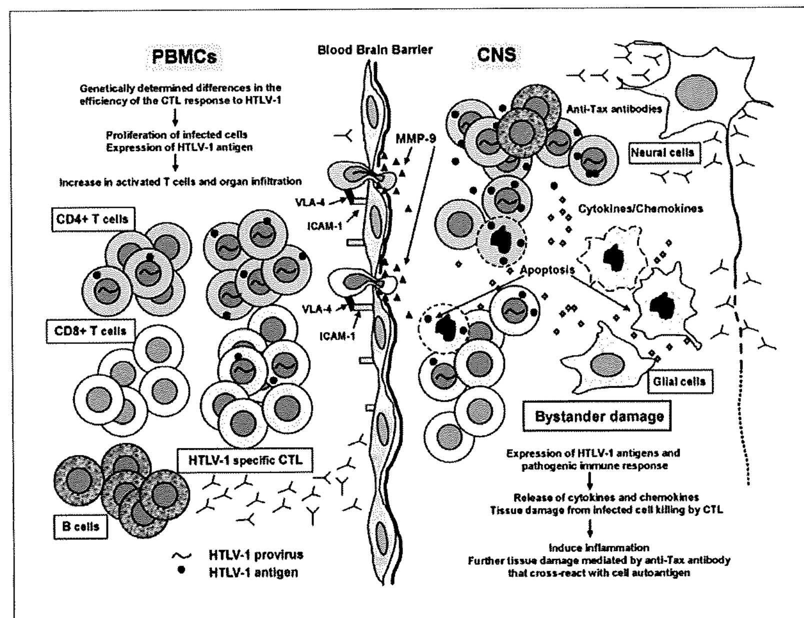


Fig.1 Hypothesis for the pathogenesis of Human T-cell leukemia virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP)

In patients with HAM/TSP, genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression, which lead to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development. It is also possible that the immunoglobulin G specific to HTLV-1-Tax, which cross-react with heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1), is associated with subsequent inflammation following initial tissue damage.

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# FoxP3$^+$ regulatory T cells are distinct from leukemia cells in HTLV-1–associated adult T-cell leukemia

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**Human T-lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATLL). It has been postulated that ATLL cells might act as regulatory T cells ($T_{regs}$) which, in common with ATLL cells, express both CD25 and FoxP3, and so contribute to the severe immune suppression typical of ATLL. We report here that the frequency of CD25$^+$ cells varied independently of the frequency of FoxP3$^+$ cells in both a cross-sectional study and in a longitudinal study of 2 patients with chronic ATLL. Furthermore, the capacity of ATLL cells to suppress proliferation of heterologous CD4$^+$CD25$^-$ cells correlated with the frequency of CD4$^+$ FoxP3$^+$ cells but was independent of CD25 expression. Finally, the frequency of CD4$^+$FoxP3$^+$ cells was inversely correlated with the lytic activity of HTLV-1-specific CTLs in patients with ATLL. We conclude that ATLL is not a tumor of FoxP3$^+$ regulatory T cells, and that a population of FoxP3$^+$ cells distinct from ATLL cells has regulatory functions and may impair the cell-mediated immune response to HTLV-1 in patients with ATLL.**
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**Key words:** FoxP3; HTLV-1; ATLL; tumor immunity; CTL

Adult T-cell leukemia/lymphoma (ATLL) is a lymphoproliferative disorder caused by the retrovirus human T-lymphotropic virus type 1 (HTLV-1).[1–3] The tumor typically consists of oligoclonal or monoclonal outgrowth of CD4$^+$CD25$^+$ T lymphocytes carrying a complete or defective provirus of HTLV-1. ATLL is classified into clinical subtypes, each with a different course and prognosis.[4,5] Four different types of ATLL have been described: acute ATLL (55% of cases), lymphoma (20% of cases), chronic (20% of cases) and the remaining 5% of cases were classified as "smoldering." The HTLV-1 Tax protein is required for the virus to transform cells in ATLL,[6] but interestingly the *tax* transcript was found in only 40% of cases of ATLL.[6] The current view is that Tax is needed early after infection to initiate transformation but is not required later to maintain the transformed phenotype of ATLL cells (reviewed in Ref. 7).

ATLL cells characteristically express high levels of the IL-2 receptor alpha chain, CD25. In addition, 3 groups observed expression of the forkhead transcription factor FoxP3 in ATLL cells.[8–10] CD25 and FoxP3 are the 2 principle markers of regulatory T cells ($T_{regs}$),[11,12] but CD25 is also expressed by activated T cells, and it has previously been demonstrated that CD25 expression is strongly induced by the HTLV-1 Tax protein.[13,14] So in HTLV-1 infection, it appears that the best current single marker of $T_{regs}$ in CD4$^+$ cells is FoxP3, and the phenotype CD4$^+$FoxP3$^+$ is increasingly used to identify a major population of $T_{regs}$.[15,16]

The coexpression of CD25 and FoxP3 in ATLL cells suggested a possible regulatory function of the leukemic cells. Two recent studies have described an apparent immunosuppressive function of ATLL cells on the proliferation of T cells.[10,17] But it remains difficult to distinguish ATLL cells from $T_{regs}$ phenotypically and to separate the populations physically because both cell types express CD25 and other cell-surface markers characteristic of $T_{regs}$.

In this study, we investigated the phenotype and function of ATLL cells. The data show that the functional FoxP3$^+$$T_{reg}$ population is distinct from the CD25$^+$ ATLL population. Furthermore, the results demonstrate an association between the frequency of CD4$^+$FoxP3$^+$ cells and the rate of CD8$^+$ T cell-mediated lysis of autologous HTLV-1–expressing cells measured in fresh PBMCs isolated from patients with ATLL, suggesting an important function of the FoxP3$^+$ population in the control of the immune response against ATLL cells.

## Material and methods

### *Subjects and cell sampling*

Peripheral venous blood samples, anticoagulated with EDTA, were donated by subjects at the National Centre for Human Retrovirology, St Mary's Hospital and King's College Hospital, London. Samples from additional ATLL patients were donated by patients attending the Department of Hematology in the University of Kumamoto, Japan. All individuals gave informed written consent, and the study was approved by the Local Research Ethics Committee of the hospital concerned. The present analysis was performed on samples classified according to the criteria of Shimoyama[5] and all were HTLV-1 seropositive. Clinical data on the patients are summarized in Table I. None of the patient in the present study had a history of opportunistic infection. PBMCs were isolated by density centrifugation on Histopaque (Sigma, UK) and cryopreserved until use. Cells were cultured in complete medium (RPMI-1640, 10% FCS, penicillin/streptomycin and L-glutamine) at 37°C in 5% $CO_2$ for 18 hr. When CD8$^+$ cell-depleted PBMCs were required, CD8$^+$ cells were removed using anti-CD8 antibody-coupled magnetic microbeads following the manufacturer's instructions (Miltenyi Biotec, Surrey, UK). The median CD8$^+$ cell depletion achieved was 97% (range 95–99%).

### *Flow cytometry*

To detect Tax and FoxP3 proteins in HTLV-1–infected cells, whole PBMCs or CD8$^+$ cell-depleted PBMCs were incubated *in vitro* for 18 hr. The cells were then surface stained with monoclonal antibodies to CD4 and CD8 (each at 15 μg/ml; Beckman Coulter, Marseille, France). Cells were then fixed and permeabilized with a commercial kit (Insight Biotechnology, Wembley,

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TABLE I – CLINICAL DETAILS OF PATIENTS STUDIED

| ATLL Type | Origin | Sex | Age | WBC | %CD25$^+$ | Surviving/died (month after diagnosis) | Treatment |
|---|---|---|---|---|---|---|---|
| Chronic | UK | M | 35 | 7.6 | 95 | Died (37.2) | CHOP IFNα + ZDV |
| Chronic | Japan | F | 56 | 53.6 | 88 | Alive (93) | Nothing |
| Chronic | UK | M | 40 | 12.9 | 86 | Alive (66) | Valporate/IFNα + ZDV |
| Chronic | UK | F | 36 | 6.8 | 66 | Alive (47) | |
| Chronic | Japan | M | 54 | 13.5 | 41 | Alive (149) | Chemotherapy + Allo PBSCT |
| Chronic | UK | F | 55 | 12 | 86 | Alive (34.1) | CHOP |
| Chronic | Japan | F | 56 | 9.6 | 19 | Died (120) | Nothing |
| Chronic | UK | F | 52 | 6.8 | 95 | Alive (7.2) | – |
| Chronic | UK | F | 64 | 5.1 | 87 | Alive (54) | Valporate/IFNα + ZDV |
| Acute | UK | F | 39 | 32.1 | 49 | Alive (36) | CHOP-Z |
| Acute | UK | F | – | 105 | 57 | Died (2.75) | IFNα + ZDV |
| Acute | UK | M | 52 | 12.2 | 40 | Alive (40.8) | IFNα + ZDV to CHOP-Z |
| Acute | Japan | F | 55 | 12.9 | 10 | Alive (29) | Chemotherapy |
| Acute | UK | F | 52 | 13.5 | 73 | Died (9) | CHOP-Z |
| Acute | Japan | M | 58 | 16.3 | 44 | Died (7) | Chemotherapy |
| Acute | UK | F | – | 104 | 98 | Died (6) | CHOP-Z |
| Acute | UK | F | – | 35.4 | 80 | Died (5) | CHOP-Z |
| Acute | Japan | F | 52 | 16.4 | 40 | Died (8) | Chemotherapy |

F: female, M: Male. Age in years at the date of diagnosis. The white blood cell count (WBC) is expressed in units of $10^9$ cells/l. %CD25$^+$ cells represents the percentage of CD25$^+$ cells in the CD4$^+$ fraction. Full details were not available on 3 patients.

UK), following the manufacturer's protocol. Finally, cells were stained intracellularly with the FITC-conjugated anti-Tax protein antibody Lt-4[18] (diluted 1/100) and anti-human FoxP3-PE antibody (clone 236A/E7; Insight Biotechnology) in permeabilization buffer (Insight Biotechnology) following the manufacturer's protocol. After staining, cells were analyzed on a Coulter Epics XL flow cytometer. Thirty thousand events were routinely collected. Viable lymphocytes were gated for further analysis using Expo32 analysis software (Beckman Coulter). For TCRVβ identification, each TCRVβ antibody was conjugated to FITC. All the anti-TCRVβ mAbs used were obtained from Immunitech (Beckman Coulter, Marseille, France).

*Proliferation assay*

CD4$^+$CD25$^-$ cells were purified with antibody-coupled magnetic microbeads following the manufacturer's instructions (Miltenyi Biotec). Cells were stained with 10 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, UK) for 10 min at 37°C. These cells were coincubated with CD25$^+$ cells isolated by antibody-coupled magnetic microbeads (Miltenyi Biotec) at a ratio of 1 CD25$^-$:2 CD25$^+$ cells. Proliferation of cells was induced by means of the $T_{reg}$ Suppression Inspector Kit from Miltenyi Biotec (beads carrying anti-CD3, anti-CD28 and anti-CD2 mAbs) following the manufacturer's instructions. After 4 days' incubation at 37°C in 5% $CO_2$, the cells were stained as previously described and analyzed using Expo32 analysis software (Beckman Coulter). For functional studies of cells that express a specific TCRVβ chain (designated here as "TCRVβn$^+$" cells), following purification of CD4$^+$ cells, the TCRVβn$^+$ population was labeled using the FITC-conjugated mAb specific to the TCRVβ chain of the ATLL clone(s) and purified with the anti-FITC Multisort kit (Miltenyi Biotec).

*CD8$^+$ cell lytic efficiency assay*

The rate (or "efficiency") of CD8$^+$ cell-mediated lysis of HTLV-1–infected cells was measured as previously described.[16,19] PBMCs were thawed, washed and then CD8$^+$ cells were positively selected (as described above) and titrated back into the CD8-depleted fraction at CD8$^+$:CD8$^-$ ratios above, below and including the physiological ratio for that individual. Cells were then cocultured at 37°C for 18 hr, harvested and stained for Tax, FoxP3, CD4 and CD8 as described above. The proportion of Tax$^+$CD4$^+$ cells surviving coculture was plotted against the proportion of CD8$^+$ cells present and a mathematical model[19] was then fitted to the data. CD8$^+$ cell lytic efficiency (expressed as % Tax-expressing CD4$^+$ cells killed/%CD8$^+$ cells/day) was calculated for each HTLV-1–infected individual tested. All assays were done in duplicate and the results are presented as the mean CD8$^+$ cell lytic efficiency.

*Statistical analysis*

Nonparametric statistical tests were used as appropriate, taking the null hypothesis and the sample size into account. The Spearman rank-order correlation coefficient was calculated when the significance of observed changes in 2 parameters across all HTLV-1–infected individuals was tested. The rate of lysis parameter was calculated with the software SPSS 12-0 for Windows.

## Results

*Frequency of FoxP3$^+$ cells did not correlate with frequency of CD25$^+$ cells in PBMCs from patients with ATLL*

We quantified the expression of FoxP3 in CD4$^+$ PBMCs from patients with ATLL, asymptomatic carriers (ACs) and uninfected individuals, after 18 hr incubation *in vitro*. The results (Fig. 1*a*) show that CD4$^+$ cells from patients with chronic ATLL expressed FoxP3 at a high frequency. PBMCs from patients with acute ATLL also had a high mean frequency of FoxP3 expression, but in such patients the range of FoxP3 expression was very wide. This FoxP3 protein expression in circulating CD4$^+$ cells in patients with different types of ATLL is consistent with previous observations on the detection of FoxP3 mRNA in patients with ATLL.[10] In patients with lymphoma, the frequency of circulating FoxP3$^+$ cells was very low.

CD25 is one of the chief markers used to quantify ATLL cells. But this marker is also frequently used as part of the phenotypic definition of $T_{regs}$. We compared, in the different categories of patients with ATLL, the percentage of FoxP3$^+$ cells with the percentage of CD25$^+$ cells, to test for a possible association between the expressions of these 2 respective markers. The results (Fig. 1*b*) showed no correlation between the frequency of CD4$^+$CD25$^+$ cells and the frequency of CD4$^+$FoxP3$^+$ cells, either in all patients with ATLL combined, or independently in chronic ATLL cases or acute ATLL cases alone. There was also no significant correlation between the frequencies of CD25 expression and FoxP3 expression in peripheral venous blood samples from cases of HTLV-1–associated lymphoma; however, the number of such cases was small, and ATLL cells in patients with lymphoma are (by definition) confined to the lymphatic circulation and are not present in the blood.

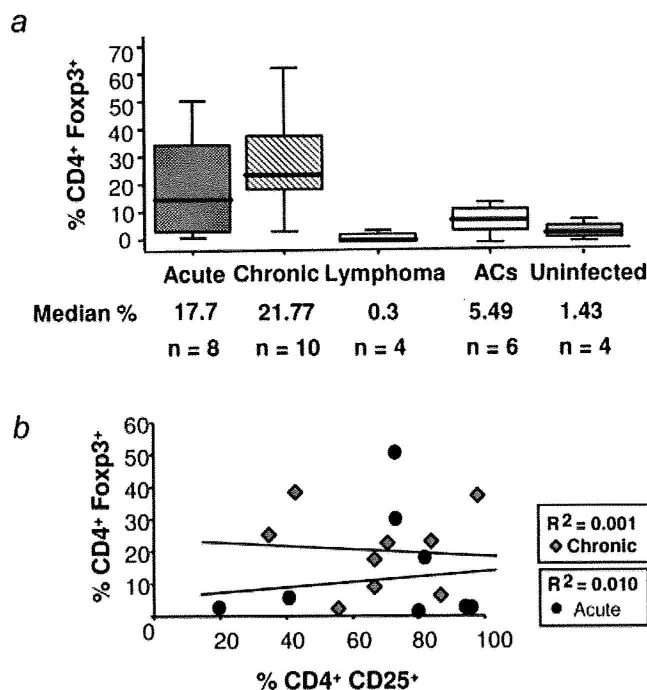



FIGURE 1 – FoxP3 and CD25 expression in patients with ATLL. (*a*) Spontaneous FoxP3 expression in CD4$^+$ cells after incubation *in vitro* for 18 hr. PBMCs were taken from patients with acute ATLL ($N$ = 8), chronic ATLL ($N$ = 10), lymphoma ($N$ = 4), asymptomatic carriers (ACs, $N$ = 4), uninfected patients ($N$ = 4). (*b*) We tested for a correlation between %FoxP3 expression in CD4$^+$ cells and %CD4$^+$CD25$^+$ cells in PBMCs from both patients with acute ATLL ($N$ = 8) and those with chronic ATLL ($N$ = 10): $p > 0.5$ in each case (2-tailed Spearman test).

*Longitudinal follow-up of $T_{reg}$ marker expression in 2 patients with chronic ATLL*

We studied 2 patients with chronic ATLL at successive time points over approximately 2 years, during which the number of ATLL cells varied in each patient. The absolute numbers of white blood cells (WBC) and the frequency of CD4$^+$CD25$^+$ and CD4$^+$FoxP3$^+$ cells are shown in Figure 2. The first patient (Fig. 2*a*) had chronic ATLL who underwent spontaneous remission. The remission occurred in March 2006 and was characterized by a decrease in the absolute number of CD4$^+$CD25$^+$ cells and WBCs. During the whole period of observation, the frequency of CD4$^+$FoxP3$^+$ cells remained approximately constant. Furthermore, at the start of the period, the absolute number of CD4$^+$CD25$^+$ cells was about 4 times greater than the number of CD4$^+$FoxP3$^+$ cells, whereas at the end of the study the size of these 2 respective populations was approximately equal.

The second patient (Fig. 2*b*) was initially asymptomatic and later developed chronic ATLL. From September 2004 to September 2005, this patient underwent treatment with sodium valproate. During the treatment period, we observed an approximately constant absolute number of CD4$^+$CD25$^+$ cells and CD4$^+$FoxP3$^+$ cells in the circulation. After the treatment, we observed an increase in the CD4$^+$CD25$^+$ cell population and WBCs, but a much smaller increase in the absolute number of CD4$^+$FoxP3$^+$ cells. At the peak of the ATLL cell number, the absolute number of CD4$^+$CD25$^+$ cells was 12-fold greater than the number of CD4$^+$FoxP3$^+$ cells. The patient changed treatment to zidovudine plus interferon-alpha at the beginning of 2006, after which there was a large decrease in the absolute number of CD4$^+$CD25$^+$ cells and WBCs, but little change in the absolute number of CD4$^+$FoxP3$^+$ cells. At the final time of sampling, this patient had approximately equal absolute numbers of CD4$^+$CD25$^+$ cells and CD4$^+$FoxP3$^+$ cells.

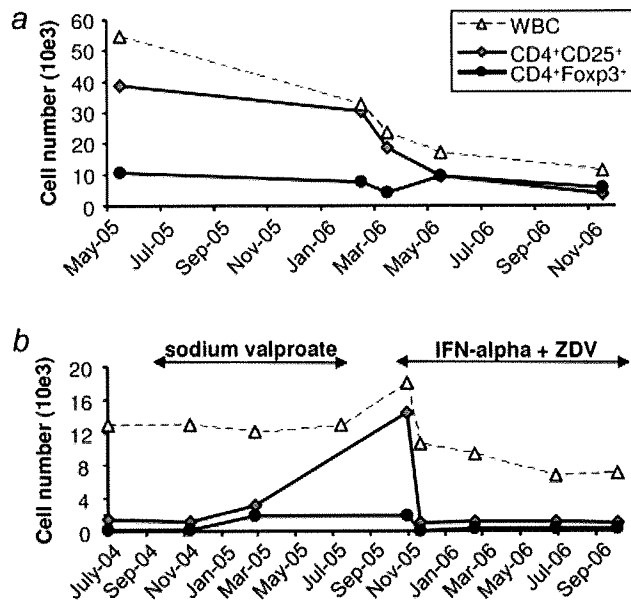



FIGURE 2 – Longitudinal follow-up of 2 patients with chronic ATLL. Each graph depicts the absolute numbers of white blood cells (WBC; white triangles), CD4$^+$CD25$^+$ cells and CD4$^+$FoxP3$^+$ cells. (*a*) In this patient, the chronic ATLL underwent spontaneous remission in March 2006. (*b*) A case of chronic ATLL diagnosed after an initially asymptomatic phase (AC). This patient began treatment with an HDAC inhibitor (sodium valproate) in September 2004; the treatment was changed to zidovudine (ZDV) plus interferon-alpha in September 2005; the patient achieved remission in February 2006.

*FoxP3 and Tax expression in PBMCs differs between subtypes of ATLL*

Staining both FoxP3 and Tax in PBMCs from patients with ATLL revealed 3 different patterns of expression respectively in the 3 different types of ATLL (Fig. 3*a*). We measured the level of Tax expression by intracellular staining in PBMCs from patients with ATLL after 18-hr incubation *in vitro* (Fig. 3*b*). It was previously reported that Tax protein is spontaneously expressed in PBMCs from a proportion of patients with ATLL after 1 day of incubation *in vitro*.[20] Tax expression was detected in PBMCs from all patients with chronic ATLL at a frequency between 0.1% and 2.1% of CD4$^+$ cells. The median frequency of Tax expression corresponded to the frequency measured in asymptomatic patients, in agreement with the previous study.[16] However, among the patients with acute ATLL, 2 distinct subgroups were identified: patients with no detectable Tax expression and patients with Tax expression. In the Tax-positive group, we observed a broad range of Tax expression, but the median frequency of Tax-positive cells was very high (39.9%) compared with that in patients with chronic ATLL or lymphoma (Fig. 3*b*). The frequency of Tax expression in patients with lymphoma was similar to the frequency observed in patients with chronic ATLL.

PBMCs from patients with acute ATLL showed a high frequency of Tax expression and a high frequency of FoxP3 expression (Figures 1*a*, 3*a* and 3*b*). Compared with patients with chronic ATLL or lymphoma patients with acute ATLL had a much greater frequency of coexpression of Tax and FoxP3. When we separated the different cell populations according to the expression of Tax, the results showed a high frequency of FoxP3 expression in the CD4$^+$Tax$^-$ population in patients with chronic ATLL, in sharp contrast with patients with acute ATLL (Fig. 3*c*). When we studied the relationship between the frequency of FoxP3 expression and the frequency of Tax expression in acute ATLL (Fig. 3*d*), we observed a strong correlation between the percentage of CD4$^+$Tax$^+$ cells and the percentage of CD4$^+$FoxP3$^+$ cells (Fig. 3*d*) or CD4$^+$FoxP3$^+$Tax$^-$ cells (data not shown). In those patients

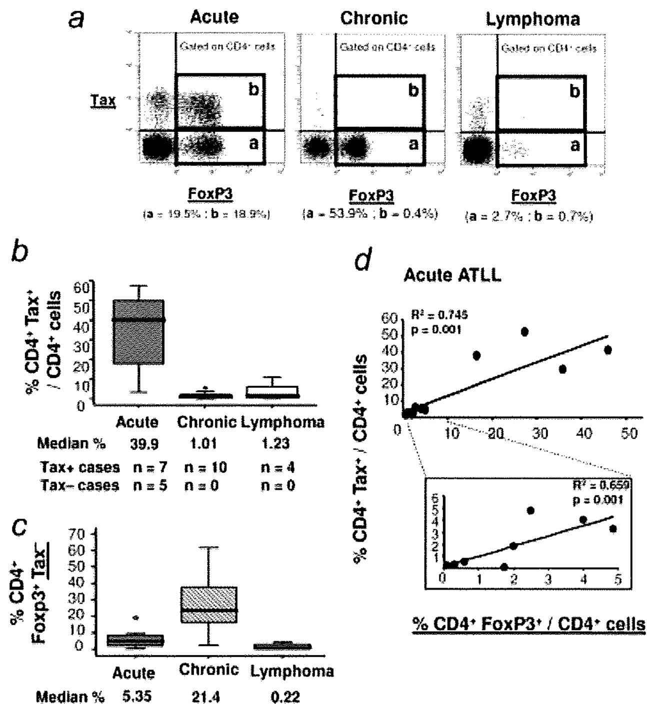


FIGURE 3 – FoxP3 and Tax expression in patients with ATLL. (*a*) Tax and FoxP3 expression in $CD4^+$ cells from representative patients with acute ATLL, chronic ATLL and lymphoma, after 18 hr incubation *in vitro*. Gate **a** represents the $CD4^+FoxP^+Tax^-$ population and gate **b** represents the $CD4^+FoxP3^+Tax^+$ population. (*b*) Percentage of $CD4^+$ cells expressing Tax in 12 patients with acute ATLL (7 with Tax expression detected and 5 with no Tax expression detected), 10 patients with chronic ATLL and 4 with HTLV-1-associated lymphoma. Tax expression was assayed at the physiological ratio of $CD4^+$:$CD8^+$ cells after 18 hr incubation *in vitro*. (*c*) FoxP3 expression in $CD4^+Tax^-$ cells. Data are shown on patients with acute ATLL ($N$ = 8), chronic ATLL ($N$ = 10) and lymphoma ($N$ = 4). (*d*) The $\%CD4^+FoxP3^+$ expression was significantly correlated with the $\%CD4^+Tax^+$ cells in patients with acute ATLL ($N$ = 12). The box shows an enlargement of the graph in the low range of $CD4^+Tax^+$ expression. *p* values were calculated by a two-tailed Spearman test.

with acute ATLL whose PBMCs expressed no detectable Tax protein, there was also an absence of detectable FoxP3 expression (Fig. 3*d* small graph). In contrast, there was no correlation between the frequency of $CD4^+Tax^+$ cells and the frequency of $CD4^+FoxP3^+$ cells in chronic ATLL (data not shown).

We considered the $CD4^+FoxP3^+Tax^+$ and $CD4^+FoxP3^+Tax^-$ populations separately in further analysis because in a previous study,[16] we demonstrated that only the $CD4^+FoxP^+Tax^-$ population had a functional effect on the control of the immune response.

### *Inhibition of $CD4^+CD25^-$ T-cell proliferation by $CD25^+$ ATLL cells and FoxP3 expression*

As explained above, it is difficult to separate $FoxP3^+$ cells from ATLL cells because the 2 populations share characteristic surface markers, in particular CD25 and CCR4. However, in this study, we identified certain patients with ATLL with a high frequency of $CD25^+$ cells but with undetectable or very low expression of FoxP3 (cf Fig. 1*b*). These patients made it possible to compare independently the effects of $FoxP3^+$ cells and $CD25^+$ (ATLL) cells in inhibiting T-cell proliferation. According to the normal phenotype definition, we refer to the $CD4^+CD25^+$ cells in these patients as ATLL cells.

To test the capacity of $CD25^+$ cells from patients with ATLL to inhibit T-cell proliferation, we purified $CD4^+CD25^-$ cells from uninfected patients and mixed them at a ratio of 1:2 with $CD25^+$ cells purified from different respective patients with ATLL. The uninfected $CD4^+CD25^-$ cells were labeled with CFSE and proliferation was induced with microbeads carrying anti-CD2, anti-CD3 and anti-CD28 antibodies. After 4 days' incubation, the CFSE content and the frequency of FoxP3 expression were analyzed. The results of a typical experiment are shown in Figure 4*a*. Each value represents the percentage of cells that did not proliferate. The results show that T-cell proliferation was inhibited more strongly (mean 57.4% non-proliferated cells) by cells from patients with ATLL with a high frequency of FoxP3 expression (acute ATLL3 and chronic ATLL) than by cells from patients with ATLL with a low frequency of Foxp3 expression (acute ATLL 1 and 2) (mean 46.6% nonproliferated cells). We then tested the hypothesis that the level of inhibition of T-cell proliferation caused by $CD25^+$ ATLL cells correlated with the frequency of FoxP3 expression in the ATLL cells. Figure 4*b* represents the results obtained from 2 independent experiments. In this experiment, we stained PBMCs from the patients with ATLL with both FoxP3 and Tax and tested for a correlation between the percentage of inhibition and the frequency of the $FoxP3^+Tax^-$ and $FoxP3^+Tax^+$ population, respectively. The results showed a strong and statistically significant correlation in each experiment in the case of the $FoxP3^+Tax^-$ population, but no correlation was observed with the $FoxP3^+Tax^+$ population.

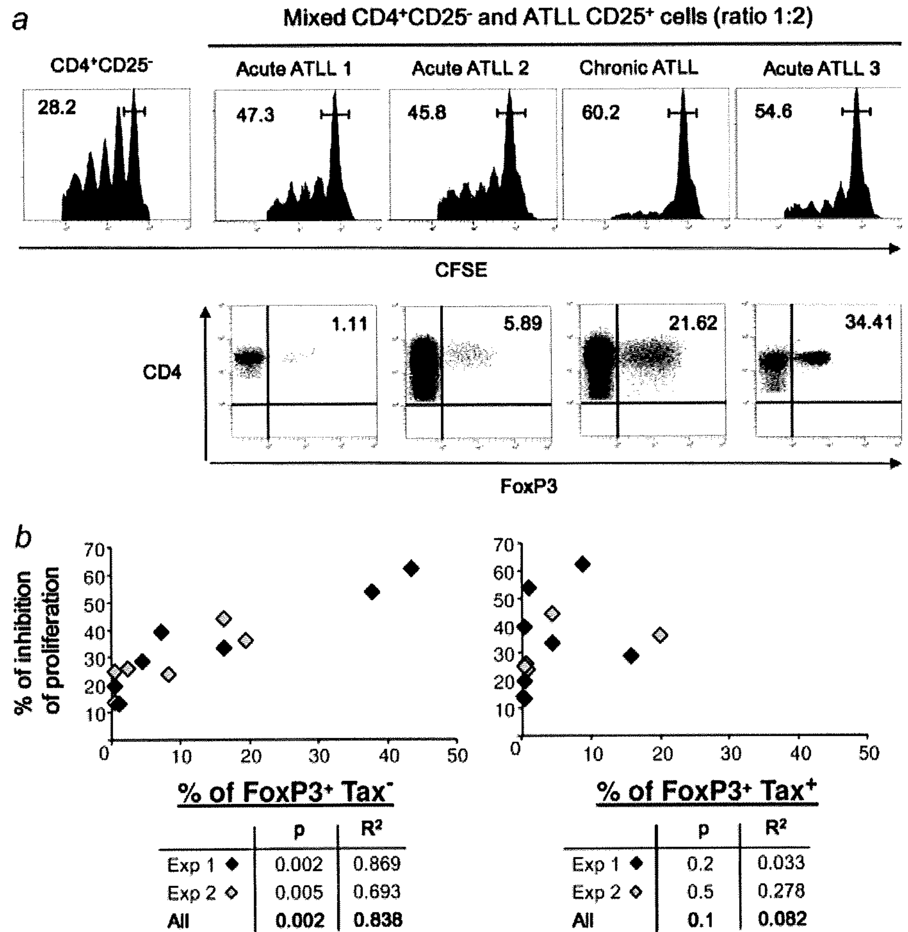
### *Identification of TCRVβ chains distinguished between ATLL clones and the main $FoxP3^+$ population*

Using a panel of 20 TCRVβ-specific monoclonal antibodies, we measured the frequency of expression of each TCRVβ chain in PBMCs from 8 patients with ATLL (Supporting Information Figure 1A). Among these 8 patients, we observed one who had a monoclonal TCRVβ expansion (ATLL#3; Supporting Information Data 1B), whereas the other patients had more than one large clone. In 5 patients with ATLL, we quantified the coexpression of FoxP3 and the respective TCRVβ chain expressed by the dominant clones. Representative results are presented in Figure 5. In each patient tested, we observed that the expanded ATLL clones, identified by expression of the respective TCRVβ chain, expressed zero or very low frequencies of FoxP3. (All data are presented in Supporting Information Figure 2).

### *$CD25^+FoxP3^+$ cells but not $CD4^+TCRV\beta n^+$ cells inhibit heterologous $CD4^+CD25^-$ T-cell proliferation*

To test the functional capacity of the $TCRV\beta n^+$ clones present in a patient with ATLL, we positively selected the $CD4^+$ cells from fresh PBMCs and then used the respective TCRVβ-specific mAb(s) to isolate the expanded T-cell clone(s). Next, we purified the $CD25^+$ cells from the remaining ($TCRV\beta n^+$-depleted) population (Fig. 6a). We then added the 3 respective cell populations ($CD4^+TCRV\beta n^+$; $CD4^+CD25^+$; $CD4^+TCRV\beta n^-CD25^-$) to $CD4^+CD25^-$ cells labeled with CFSE from an uninfected individual. A representative result is shown in Figure 6*b*. We observed that only the $CD25^+$ population caused strong inhibition of proliferation, and this $CD25^+$ population expressed a high frequency of FoxP3 and a low frequency of the respective expanded TCRVβ. In contrast, the $TCRV\beta n^+$ clones caused little inhibition of proliferation of the $CD4^+CD25^-$ population and also expressed a low frequency of FoxP3.

We reproduced this experiment with 3 different patients with ATLL (Fig. 6*c*). In each case, we observed that the inhibition of proliferation of the $CD4^+CD25^-$ population was associated with a low frequency of expression of the ATLL clone TCRVβ chain and a high frequency of FoxP3 expression. Furthermore, the $TCRV\beta n^+$ clonal population in each case caused little inhibition of proliferation of $CD4^+CD25^-$ cells similar to the degree of inhibition caused by the $CD4^+TCRV\beta n^-CD25^-$ cells.




| % of FoxP3+ Tax- | p | $R^2$ |
|---|---|---|
| Exp 1 ◆ | 0.002 | 0.869 |
| Exp 2 ◇ | 0.005 | 0.693 |
| All | **0.002** | **0.838** |

| % of FoxP3+ Tax+ | p | $R^2$ |
|---|---|---|
| Exp 1 ◆ | 0.2 | 0.033 |
| Exp 2 ◇ | 0.5 | 0.278 |
| All | **0.1** | **0.082** |

FIGURE 4 – Effect of ATLL cells and FoxP3 expression on proliferation of uninfected $CD4^+CD25^-$ cells. In each of two independent experiments, we tested the ability of $CD25^+$ ATLL cells to inhibit proliferation of $CD4^+CD25^-$ cells. In Experiment 1, effector ($CD25^+$) cells from 7 patients with ATLL were used. In Experiment 2, effector cells were used from 6 of the same patients with ATLL. In each respective experiment, responder ($CD4^+CD25^-$) cells from a different HTLV-1 seronegative subject were used. (*a*) Data on 4 representative patients with ATLL from Experiment 1. Each histogram represents the frequency of CFSE expression in $CD4^+$ cells; the number in each histogram shows the percentage of undivided cells. The number in each scatter plot shows the frequency of expression of FoxP3 in the ATLL $CD4^+$ population. (*b*) Correlation between frequency of FoxP3 expression in the $Tax^-$ or $Tax^+$ population and inhibition of T-cell proliferation. Each point represents results from a single patient with ATLL. The percentage of inhibition of proliferation was obtained using the following formula: [(% divided cells in control) − (% divided cells in sample)/(% divided cells in control)]. The *p* values were calculated by a two-tailed Spearman test.

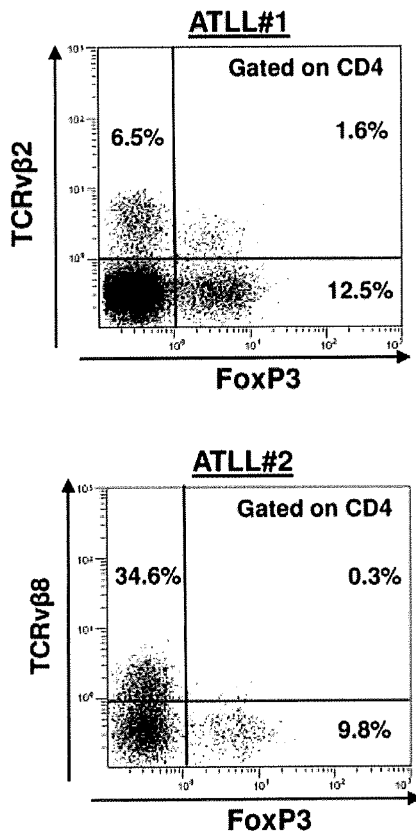



FIGURE 5 – Coexpression of TCRVβ and FoxP3 in CD4+ cells. PBMCs from 2 patients with ATLL were stained with mAbs specific to FoxP3 and 2 different TCRVβ chains. The percentage in the dot plot represents the percentage of each population gated on the $CD4^+$ population.

*$FoxP3^+$ population but not $TCRV\beta n^+$ ATLL clones inhibit proliferation of autologous $CD4^+$ T cells*

In one patient, after depletion of both the expanded $TCRV\beta n^+$ clones and the $CD25^+$ population, we labeled the $CD4^+$-depleted population with CFSE. We mixed these cells with either the respective $TCRV\beta n^+$ clones or the purified $CD25^+$ population in a ratio of 1:2. The results show (Fig. 6*d*) that the $CD25^+$ population inhibited proliferation more powerfully than did the purified $TCRV\beta n^+$ clones, despite the presence of $FoxP3^+$ cells in the $TCRV\beta n^+$ clone.

*Frequency of FoxP3 expression was negatively correlated with the rate of CTL-mediated lysis of $Tax^+$ cells*

We then tested the hypothesis that the frequency of the different populations of $FoxP3^+$ cells in PBMCs from patients with ATLL correlate with the rate of spontaneous $CD8^+$ (CTL)-mediated lysis of autologous $Tax^+$ cells. The rate of lysis was measured by the method previously described.[16,19] We purified $CD8^+$ cells from a patient with ATLL and added these cells at different frequencies to the CD8 depleted population. After 18 hr, we analyzed by flow cytometry the frequency of Tax and FoxP3 expression in each sample as previously described.[16] A mathematical model[19] was then used to quantify the per-$CD8^+$ cell rate of killing for each

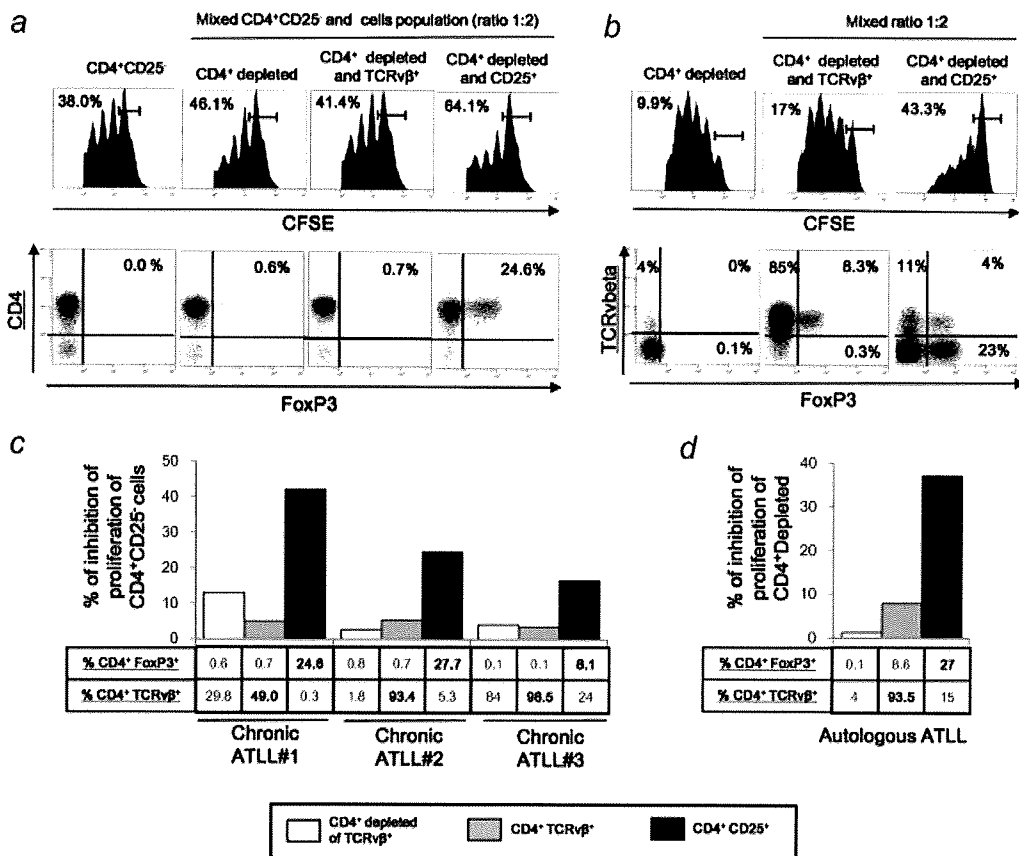



FIGURE 6 – Effect of $CD4^+TCRV\beta n^+$ clone and $CD4^+CD25^+FoxP3^+$ population on the proliferation of heterologous or autologous $CD4^+$ cells. We tested the ability of the $CD4^+TCRV\beta n^+$ or the $CD4^+CD25^+FoxP3^+$ population purified from PBMCs of patients with ATLL to inhibit the proliferation of either $CD4^+$-depleted cells from an uninfected individual (*b* and *c*) or autologous $CD4^+$-depleted ATLL cells (*d*). (*a*) Data on a representative patient with chronic ATLL. Each histogram represents the frequency of CFSE expression in $CD4^+$ cells isolated from uninfected cells, incubated for 4 days with the different cell populations indicated. The number in each histogram shows the percentage of undivided cells. The number in each scatter plot shows the frequency of expression of FoxP3 in the $CD4^+$ population. (*b*) Each histogram represents the frequency of CFSE expression in $CD4^+$ cells depleted of specific $TCRV\beta n^+$ and $CD25^+$ cells from autologous chronic ATLL patients. Cells were mixed with the different cell populations indicated and incubated for 4 days. The number in each histogram shows the percentage of undivided cells. The number in each scatter plot shows the frequency of expression of FoxP3 and specific $TCRv\beta n^+$ in the $CD4^+$ population. (*c* and *d*) Percentage of inhibition of $CD4^+$ T-cell proliferation, obtained using the following formula: [(% divided cells in control) − (% divided cells in sample)/(% divided cells in control)]. (*c*) Shows inhibition of proliferation of $CD4^+CD25^-$ cells from an uninfected individual; (*d*) shows inhibition of proliferation of autologous $CD4^+$ cells.

patient. Because we observed a saturation of the effect of $FoxP3^+$ cells on the inhibition of killing, we present the results on a logarithmic scale.

We observed a strong and statistically significant negative correlation between the frequency of FoxP3 expression and the rate of lysis of autologous $CD4^+Tax^+$ cells in unstimulated PBMCs from patients with ATLL (Fig. 7*a*). We also observed a significant negative correlation between the frequency of $CD4^+FoxP3^+Tax^-$ cells and the rate of CTL lysis (Fig. 7*b*). However, the frequency of the $CD4^+FoxP^+Tax^+$ population showed no correlation with the rate of lysis (data not shown).

## Discussion

We report here 2 principal findings. First, our observations on patients with ATLL suggest that ATLL cells themselves are distinct from a functional $CD4^+FoxP3^+$ $T_{reg}$ population. Second, in PBMCs from patients with ATLL, we detected a specific $CD8^+$ T-cell response to HTLV-1–expressing autologous lymphocytes, and this $CD8^+$ T-cell response was inversely correlated with the percentage of $CD4^+FoxP3^+$ cells.

ATLL cells characteristically express CD25 and CCR4 on the cell surface. Because ATLL cells in certain cases have also been found to express FoxP3, it has been suggested that the severe immune suppression characteristic of ATLL might result from a regulatory T-cell–like effect of ATLL cells. However, it was already established that Tax upregulates the expression of CD25 on infected cells, which complicates the attempt to identify $T_{regs}$ in HTLV-1 infection.[16] In this study, we therefore chose to quantify expression of FoxP3, which is the best currently identified single marker of the main $T_{reg}$ population.

Measurement of the frequency of FoxP3 expression in patients with ATLL and lymphoma showed that consistent with previous observations by others using RT-PCR to detect FoxP3 mRNA[10] patients with ATLL had a high frequency of FoxP3 expression in $CD4^+$ cells (Fig. 1*a*). As in our previous study of nonmalignant cases of HTLV-1 infection,[16] we distinguished 2 populations of $FoxP3^+$ cells in patients with ATLL: $CD4^+FoxP3^+Tax^+$ and $CD4^+FoxP3^+Tax^-$ cells. In patients with $Tax^+$ acute ATLL, we observed frequent coexpression of Tax and FoxP3 in $CD4^+$ cells (Fig. 3). The mean frequency of FoxP3 expression in the total $CD4^+$ population was lower in cases of acute ATLL than in chronic ATLL (Fig. 1*a*), although this difference was not statisti-

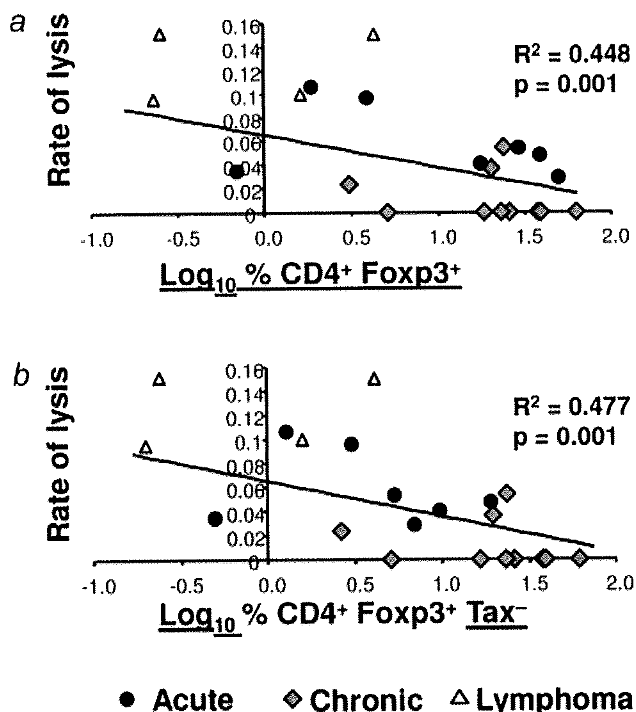



FIGURE 7 – FoxP3 control the CD8-mediated lysis against Tax$^+$ cells. Correlation between the rate of CD8$^+$ cell-mediated lysis of autologous Tax$^+$ cells in fresh PBMCs (rate of lysis) and (*a*) $\log_{10}$ (% CD4$^+$FoxP3$^+$/CD4$^+$) or (*b*) $\log_{10}$ (% CD4$^+$FoxP3$^+$Tax$^-$/CD4. *p* values were determined by a two-tailed Spearman test. Least-squares regression analysis showed (*e-g*, right-hand panels) a significant negative correlation between $\log_{10}$ (% CD4$^+$FoxP3$^+$/CD4$^+$) and the rate of CD8$^+$ cell-mediated lysis.

cally significant. Note that the cases of acute ATLL represented in Figure 1*a* are those whose PBMCs expressed detectable Tax protein. However, there was a clear distinction between these 2 subtypes of ATLL in the frequency of CD4$^+$FoxP3$^+$ cells in the Tax$^-$ population (Fig. 3*c*): this frequency was significantly lower in patients with acute ATLL than in those with chronic ATLL. Furthermore, in acute ATLL, we observed a correlation between the frequency of expression of Tax and FoxP3, and cells from patients with acute ATLL that did not express Tax also did not express detectable FoxP3 (Fig. 3*d*). It remains unclear why this correlation was found among patients with acute ATLL but not in those with chronic ATLL. The positive correlation observed in acute ATLL patients was not simply caused by Tax-induced expression of FoxP3 in the infected cell because the frequency of Tax-expressing cells correlated independently with both the frequency of FoxP3$^+$ Tax$^+$ cells and the frequency of FoxP3$^+$Tax$^-$ cells (data not shown).

By observing FoxP3 and CD25 expression in patients with either chronic or acute ATLL, we found no correlation between the percentage of CD4$^+$CD25$^+$ cells and the percentage of CD4$^+$FoxP3$^+$ cells. This observation suggests that ATLL cells, which are almost invariably CD25$^+$, do not systematically express FoxP3$^+$ (Fig. 1*b*). This conclusion was reinforced by our observations on successive samples of PBMCs from 2 patients with chronic ATLL, in whom the frequency of ATLL cells varied substantially over years (Fig. 2). The first patient had a spontaneous remission; the second patient was initially asymptomatic, then the ATLL progressed and subsequently regressed after modification of treatment. In each of these 2 patients, we observed a variation in the absolute number of CD4$^+$CD25$^+$ cells that was independent of the number of CD4$^+$FoxP3$^+$ cells.

As an independent test of the expression of FoxP3 by ATLL cells, we used TCRVβ-specific antibodies to identify the ATLL clones in fresh PBMCs. Flow cytometric analysis of cells costained for CD4, FoxP3 and the respective TCRVβ chain (Fig. 5 and Supporting Information Data 1 and 2) confirmed that the chief FoxP3$^+$ cell population was distinct from the expanded TCRVβn$^+$ (ATLL) clones.

We wished to test the hypothesis that the FoxP3$^+$ cells observed in PBMCs from patients with ATLL exert immune suppressive effects typical of FoxP3$^+$ $T_{reg}$ cells. We therefore studied the capacity of ATLL cells to inhibit proliferation of heterologous CD4$^+$ cells and the relationship of this inhibitory capacity to the frequency of FoxP3$^+$ expression (Fig. 4). We conclude that, even if the CD25$^+$ cells present in PBMCs in patients with ATLL exert an immune suppressive activity, this activity correlates with the frequency of FoxP3$^+$ expression. Moreover, we observed that the magnitude of inhibition of heterologous T-cell proliferation correlated with the frequency of FoxP3$^+$ Tax$^-$ cells but not with the frequency of FoxP3$^+$ Tax$^+$ cells. These experiments indicate that the phenotype FoxP3$^+$ Tax$^-$ remains a useful marker of $T_{reg}$ function even in ATLL.

At present, it is not possible to specifically select FoxP3$^+$ cells and to conserve their functional properties because FoxP3$^+$ is a nuclear marker and unfixed cells therefore cannot be stained for FoxP3 expression. We therefore initially used the TCRVβ-specific antibodies to select the TCRVβn$^+$ ATLL clone and subsequently isolated the CD25$^+$ cells from the resulting TCRVβn$^+$-depleted population. In this way we enriched both the ATLL population and the FoxP3$^+$ population, respectively. The results (Fig. 6) showed that only the FoxP3$^+$ fraction caused strong inhibition of proliferation of CD4$^+$ cells from an uninfected patient (Figs. 6*b* and 6*c*) and of autologous CD4$^+$ cells (Fig. 6*d*). These experiments present further evidence of the presence of an independent functional FoxP3 population in patients with ATLL.

An HTLV-1–specific cell-mediated immune response in patients with ATLL has previously been reported by other groups.[21–23] Here, we used a recently developed method to quantify the CTL response against autologous Tax-expressing cells.[19] In addition, PBMCs from a proportion of patients with ATLL have been previously demonstrated to express HTLV-1 Tax protein after short-term incubation *in vitro*. In the present study, fresh PBMCs from all patients studied with chronic ATLL or lymphoma had detectable Tax expression. In contrast, Tax expression remained undetectable even after *in vitro* incubation for 18 hr in 5 of the 12 patients with acute ATLL. We next wished to test the hypothesis that the frequency of these different populations of FoxP3$^+$ cells was correlated with the rate of CTL-mediated lysis of Tax$^+$ cells measured in PBMCs from the patients with ATLL. We observed a strong negative correlation between the percentage of CD4$^+$FoxP3$^+$ cells and the rate of lysis (Fig. 7*a*). Furthermore, this correlation was stronger (*i.e.*, there was a larger value of $R^2$) with the CD4$^+$FoxP3$^+$Tax$^-$ population than with the total CD4$^+$FoxP3$^+$ population, and there was no significant correlation between the frequency of CD4$^+$FoxP3$^+$Tax$^+$ cells and the rate of lysis (Fig. 7*b*). This result suggests that the functional $T_{reg}$ population consists of CD4$^+$FoxP3$^+$Tax$^-$ cells, whereas the CD4$^+$FoxP3$^+$Tax$^+$ cells do not appear to influence the CTL response to HTLV-1.

These observations indicate that the frequencies of functional Tax-FoxP3+Treg cells are lower in acute ATLL than in patients with chronic ATLL (Fig. 3*c*). Because we also demonstrate that the FoxP3$^+$ cells in ATLL are independent of the TCRVβ clone and retain the ability to inhibit the proliferation of CD4$^+$ T cells (Fig. 4), we hypothesize that the high frequency of CD4$^+$FoxP3$^+$Tax$^-$ cells with regulatory function observed in chronic ATLL contributes to the slow progression of the disease by suppressing ATLL cell proliferation.

This study suggests that ATLL cells themselves are distinct from a population of FoxP3$^+$ cells with functional characteristics of $T_{regs}$. Although the impact of the FoxP3$^+$ population on ATLL development and progression remains unclear, the present data