The TO subgroup strains have another nonstructural protein designated L*. By both "loss of function" and "gain of function" experiments, the L* protein has been shown to be essential for virus growth in macrophage cells (26–28), the major site of virus persistence. In addition, the anti-apoptotic activity of the L* protein in DA infection has been reported (8, 9). In the present paper, it was clearly demonstrated that the L* protein inhibits the apoptosis induced by DA L.

It has recently been reported that both nonstructural proteins L and L* are required for virus growth in a macrophage cell line (29). In this study, it was additionally demonstrated that the opposite apoptotic effects of two nonstructural proteins, L and L*, regulate TMEV-induced apoptosis; therefore, the collaboration of these two nonstructural proteins may give great effects to TMEV biological activities, especially virus persistence and demyelination. More detailed studies on these nonstructural proteins will help elucidate the pathomechanism(s) of TMEV persistence and demyelination, leading to the clarification of the pathogenesis of MS.

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Review Article

Immunopathogenesis of Human T-Cell Leukemia Virus Type-1-Associated Myelopathy/Tropical Spastic Paraparesis: Recent Perspectives

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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 only in a minority of infected individuals: 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). HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities. Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, accumulating evidence from host population genetics, viral genetics, DNA expression microarrays, and assays of lymphocyte function suggests that complex virus-host interactions and the host immune response play an important role in the pathogenesis of HAM/TSP. Especially, the efficiency of an individual's cytotoxic T-cell (CTL) response to HTLV-1 limits the HTLV-1 proviral load and the risk of HAM/TSP. This paper focuses on the recent advances in HAM/TSP research with the aim to identify the precise mechanisms of disease, in order to develop effective treatment and prevention.

1. Introduction

Human T-cell leukemia virus type-1 (HTLV-1) is a human retrovirus etiologically associated with adult T-cell leukemia (ATL) [1–3] and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4, 5]. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [6]. Cases of HAM/TSP have been reported throughout the HTLV-1 endemic areas such as Southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa [7]. Sporadic cases have also been described in nonendemic areas such as the United States and Europe, mainly in immigrants from an HTLV-1 endemic area. In contrast to HIV-1 infection, few with HTLV-1 develop disease: approximately 2%-3% of infected persons develop ATL [8] and other 0.25%–3.8% develop HAM/TSP [9–12], while the majority of infected individuals remain lifelong asymptomatic carriers (ACs). However, the ability to evaluate the individual risk of HTLV-1-associated diseases in each AC would make a significant clinical impact, especially in HTLV-1 endemic areas. During the last three decades since the discovery of HTLV-1 as the first pathogenic human retrovirus, advances in HTLV-1 research have helped us to understand the clinical features of HTLV-1 associated diseases, the virological properties of HTLV-1, and the importance of the viral, host, and environmental risk factors as well as the host immune response against HTLV-1 infection. However, the precise mechanism of disease pathophysiology is still incompletely understood, and the treatment is still unsatisfactory, because good small-animal models for studying HTLV-1 infection and its associated diseases were unavailable until recently. In this paper, we summarize the recent developments of HTLV-1 research to try to identify more precisely the pathogenetic mechanisms

of the disease in order to develop effective treatment and prevention.

2. HTLV-1 Infection and Clinical Features of HAM/TSP

2.1. Virological Aspects of HTLV-1. HTLV-1 is classified as a complex retrovirus in the genus *Deltaretrovirus* of the subfamily *Orthoretrovirinae* and infects 10–20 million people worldwide [13–15]. HTLV-1 can be transmitted through sexual contact [16], injection drug use [15], and breastfeeding from mother to child [17, 18]. For over two decades, the investigation of HTLV-1-mediated pathogenesis has been focused on Tax, an HTLV-1 encoded viral oncoprotein, since Tax has been viewed as critical for leukemogenesis because of its pleiotropic effects on both viral and many cellular genes responsible for cell proliferation, genetic instability, dysregulation of the cell cycle, and apoptosis [19]. However, Tax expression is not detected in about 60% of freshly isolated samples from ATL cases [20]. In 2002, another regulatory protein encoded in the minus or antisense strand of the virus genome, named HTLV-1 basic leucine zipper factor (HBZ), was identified [21]. The spliced form of HBZ is expressed in all ATL [22] and HAM/TSP [23] cases, and its expression is strongly correlated with the HTLV-1 proviral load (PVL) in HTLV-1-infected individuals and with disease severity in HAM/TSP patients [23]. Also, HBZ protein promotes proliferation of ATL cells and induces T-cell lymphomas in $CD4^+$ T cells by transgenic expression, indicating the possible involvement of HBZ expression in the development of ATL [22, 24]. Moreover, among the HTLV-1 encoded viral genes, only the HBZ gene sequence remains intact, unaffected by nonsense mutations and deletion [25]. These findings indicate that HBZ expression is indispensable for proliferation and survival of ATL cells and HTLV-1 infected cells, and that Tax expression is not always necessary for the maintenance of ATL [26].

2.2. Clinical and Pathological Features of HAM/TSP. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [6]. In addition to neurological symptoms, some HAM/TSP cases also exhibit autoimmune-like disorders, such as uveitis, arthritis, T-lymphocyte alveolitis, polymyositis, and Sjögren syndrome [14]. Among ACs, the lifetime risk of developing HAM/TSP, which is different among different ethnic groups, ranges between 0.25% and 4%. It has been reported that the annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times higher risk for women in both populations [9–12]. The period from initial HTLV-1 infection to the onset of HAM/TSP is assumed to range from months to decades, a shorter time than for ATL onset [11, 31]. HAM/TSP occurs both in vertically infected individuals and in those who become infected later in life (i.e., through sexual contact [almost exclusively from male to female], intravenous drug use, contaminated blood transfusions, etc.). The mean age at onset is 43.8 years, and the frequency of HAM/TSP is higher in women than in men (the male to female ratio of occurrence is 1 : 2.3) [11].

Pathological analysis of HAM/TSP autopsy materials indicates that the disease affects the spinal cord, predominantly at the thoracic level [27, 32, 33]. 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. In the cases with active-chronic lesions in the spinal cord, perivascular inflammatory infiltration with similar composition of cell subsets was also seen in the brain [28]. The peripheral nerve pathology of HAM/TSP patients with sensory disturbance showed varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineurial fibrosis [29, 30]. The presence of atypical lymphocytes (so-called "flower cells") in peripheral blood and cerebrospinal fluid (CSF), a moderate pleocytosis, and raised protein content in CSF are typically found in HAM/TSP patients. Oligoclonal immunoglobulin bands in the CSF, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and interferon (IFN)-γ, and an increased intrathecal antibody (Ab) synthesis specific for HTLV-1 antigens have also been described [34]. Clinical progression of HAM/TSP is associated with an increase in the proviral load in individual patients, and a high ratio of proviral loads in CSF cells/peripheral blood mononuclear cells (PBMCs) is also significantly associated with clinically progressive disease [35]. The clinical and pathological characteristics of HAM/TSP described above are shown in Table 1.

3. Risk Factors for HAM/TSP

3.1. Host Genetic. A previous population association study of 202 cases of HAM/TSP and 243 ACs in Kagoshima prefecture, HTLV-1 endemic Southern Japan, revealed that one of the major risk factors is the HTLV-1 PVL. The median PVL was more than ten times higher in HAM/TSP patients than in ACs, and a high PVL was also associated with an increased risk of progression to disease [36, 37]. A higher PVL in HAM/TSP patients than in ACs was observed in other endemic areas such as the Caribbean [38], South America [39], and the Middle East [40]. It was suggested that genetic factors such as the human leukocyte antigen (HLA) genotype are related to the high PVL in HAM/TSP patients and genetic relatives. In Southern Japan, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 PVL and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predisposes to HAM/TSP in the same population (Table 2) [37, 41]. Since the function of class I HLA proteins is to present antigenic peptides to 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 therefore be an important determinant of HTLV-1 PVL and the risk of HAM/TSP. In fact, it has been reported that CTL spontaneously kills autologous HTLV-1-infected

Table 1: Clinical and pathological characteristics of HAM/TSP.

Clinical characteristics		References
Onset	Insidious, slowly progressive	[11]
Major clinical symptoms	Spastic paraparesis Sphincter dysfunction Mild sensory disturbance in the lower extremities	[11]
Complications	Uveitis Arthritis T-lymphocyte alveolitis Polymyositis Sjögren syndrome	[14]
Mean age at onset	43.8 years	[11]
Male-to-female ratio	1 : 2.3 (male : female)	[11]
Laboratory data	Positive anti-HTLV-1 antibody in both serum and CSF Moderate pleocytosis and raised protein content in CSF	[11]
Pathological characteristics		**References**
Spinal cord	Loss of myelin and axons in the lateral, anterior, and posterior columns-predominantly at the thoracic level Perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis-predominantly at the thoracic level	[27]
Brain	Perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis Perivascular inflammatory infiltration and fibrosis only in the cases with active-chronic lesions in the spinal cord. The composition of cell subsets was similar both in the spinal cord and in the brain	[28]
Peripheral nerve	Varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineurial fibrosis	[29, 30]

cells *ex vivo* [42], granzymes and perforin are more highly expressed in individuals with a low PVL [43], and the lytic efficiency of the $CD8^+$ T cell response, that is, the fraction of autologous HTLV-1-expressing cells eliminated per $CD8^+$ T cell per day, was inversely correlated with both PVL and the rate of spontaneous proviral expression [44]. These findings indicate that the CTL against HTLV-1 reduces PVL and risk of HAM/TSP. Moreover, using a combination of computational and experimental approaches, MacNamara et al. recently reported that a CTL response against HBZ restricted by protective HLA alleles such as HLA-A*02 or Cw*08, but not a response to the immunodominant protein Tax, determines the outcome of HTLV-1 infection [45].

Analysis of non-HLA host genetic factors by candidate gene approaches revealed that non-HLA gene polymorphisms also affect the risk of developing HAM/TSP (Table 2). For example, the TNF-α promoter-863 A allele [47] and the longer CA repeat alleles of matrix metalloproteinase (MMP)-9 promoter [48] predisposed to HAM/TSP, whereas IL-10-592 A [49], stromal-derived factor (SDF)-1 +801A, and IL-15 +191 C alleles [47] conferred protection against HAM/TSP. The polymorphisms in the MMP-9 and IL-10 promoters were each associated with differences in the HTLV-1 Tax-mediated transcriptional activity of the respective gene [48, 49]. However, the contributions of these non-HLA genes to the pathogenesis of HAM/TSP are largely unknown, and these data have not yet been reproduced in different populations. Further candidate gene studies together with genome-wide association studies in different ethnic populations in larger sample size may provide evidence for the association of non-HLA genes with HAM/TSP pathogenesis.

3.2. HTLV-1 Genotype and Genomic Integration Site. 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 [46]. The *tax* subgroup A, which belongs to cosmopolitan subtype A, was more frequently observed in HAM/TSP patients, and this association was independent of the protective effect of the HLA 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 which belongs to cosmopolitan subtype B, but not against *tax* subgroup A in the Japanese population [46]. Interestingly, HLA-A*02 appeared not to give protection against infection with cosmopolitan subtype A in a population in Iran [40]. Moreover, the Iranian HTLV-1 strain has a Rex protein that is 20 amino acids longer than that of the Japanese strain that belongs to cosmopolitan subtype B. Experiments are now underway to compare the functions of these Rex proteins.

Table 2: Host genetic and viral factors associated with the risk of HAM/TSP.

Factor	Condition	Effect	Reference(s)
Viral factors	HTLV-1 *tax* subgroup A	Susceptible	[46]
	Proviral load	Susceptible	[36]
Host factors			
HLA	A*02	Protective	[37, 41]
	Cw*08	Protective	[41]
	B*5401	Susceptible	[41]
	DRB1*0101	Susceptible	[37]
Non-HLA	TNF-α promoter −863 A allele	Susceptible	[47]
	longer CA repeat alleles of MMP-9 promoter	Susceptible	[48]
	IL-10 promoter −592 A allele	Protective	[49]
	SDF-1 promoter +801 A allele	Protective	[47]
	IL-15 +191 C allele	Protective	[47]

Recently, to test whether the genomic integration site determines the abundance and the pathogenic potential of an HTLV-1-positive T-cell clone, Gillet et al. reported the results of high-throughput mapping and quantification of HTLV-1 proviral integration in the host genome [50]. They mapped >91,000 unique insertion sites (UISs) of the provirus from 61 HTLV-1-infected individuals in primary PBMCs and showed that a typical HTLV-1-infected host carries between 500 and 5000 UISs in 10 μg of PBMC genomic DNA. They calculated an oligoclonality index (OCI) to quantify the clonality of HTLV-1-infected cells *in vivo* and found that the OCI did not distinguish between ACs and patients with HAM/TSP and that there was no correlation between OCI and HTLV-1PVL in either ACs or HAM/TSP patients. These results indicate that the higher PVL observed in patients with HAM/TSP was attributable to a larger number of UISs but not, as previously thought, from a difference in clonality. They also obtained evidence that the abundance of established HTLV-1 clones is determined by genomic features of the host DNA flanking the provirus. Namely, HTLV-1 clonal expansion *in vivo* is favored by a proviral integration site near a region of host chromatin undergoing active transcription, or same-sense transcriptional orientation of the provirus. Negative selection of infected clones, probably by CTLs during chronic infection, favors establishment of proviruses integrated in transcriptionally silenced DNA, and this selection is more efficient in ACs than in HAM/TSP, indicating the selection of HTLV-1-infected T-cell clones with low pathogenic potential.

4. Immune Response to HTLV-1

4.1. Innate Immune Response

4.1.1. Natural Killer (NK) Cells. 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 ACs although the results were not normalized with respect to PVL [51]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, a synthetic tetramer of HLA-E with the HLA-G signal sequence peptide was used to identify NK cells in HAM/TSP patients [52]. The results showed a significantly lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than ACs, and as in the earlier studies [51], 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 ACs [52]. Recent data also suggest that the frequency of invariant NKT (iNKT) cells in the peripheral blood of HAM/TSP patients is significantly decreased when compared with healthy subjects and/or ACs [53, 54]. These findings indicate that the activity of the NK or NKT cell response was associated with the absence of HAM/TSP. Interestingly, a previous uncontrolled preliminary trial of treatment of HAM/TSP with fermented milk containing viable *Lactobacillus casei* strain Shirota resulted in a significant increase in NK cell activity, with improvements in clinical symptoms [55]. Thus, circulating NK and NKT cells might also play an important role in the disease progression and the pathogenesis of HAM/TSP. Recently, it has been reported that in addition to the previously described $CD8^+$ T-cell spontaneous proliferation [56], $CD56^+$ NK cells also spontaneously proliferated *in vitro*, and spontaneous NK cell proliferation positively correlated with HTLV-1 PVL but not with the presence of HAM/TSP [57]. A hallmark 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 [58, 59]. Most of the high SLP observed in PBMCs from HAM/TSP patients is likely to be explained by a greater spontaneous expression of the provirus and consequently a greater proliferation of responding $CD8^+$ T cells in culture [56]. The greater proviral expression may be partly attributable to the impaired function and decreased number of NK cells in HAM/TSP patients. Although further

studies are required to clarify the role of NK cells in HTLV-1 infection and HAM/TSP pathogenesis, NK cells might be also an interesting candidate for future immunotherapy.

4.1.2. Interferons. Type I interferon (IFN) is a key innate immune cytokine produced by cells in response to viral infection. The type I IFN response protects cells against invading viruses by inducing the expression of interferon-stimulated genes (ISGs), which execute the antiviral effects of IFN [60]. The ISGs then generate soluble factors including cytokines that activate adaptive immunity or directly inhibit the virus itself [61]. To date, IFN-α is not only one of the effective therapeutic agents for HAM/TSP, but also known as an only therapeutic agent whose efficacy was demonstrated in randomized placebo-controlled trials [62, 63]. However, the therapeutic benefit is small, and IFN-α is not in general use in the treatment of HAM/TSP. The combination of the antiretroviral agent zidovudine (AZT) and IFN-α is also beneficial for overall survival in smoldering and chronic (i.e. indolent) ATL [64] although its efficacy has not yet been confirmed in well-designed prospective studies. It might be interesting to analyse which ISGs are changed in the course of IFN-α treatment and the functional role of ISGs as potential targets for therapy. In PBMCs of HTLV-1-infected individuals, the level of HTLV-1 mRNA is very low, and viral protein is not detectable, but these molecules are rapidly expressed after a short time in culture *in vitro* [42]. However, the mechanisms of this phenomenon are largely unknown. Recently, it has been reported that HTLV-1 expression in HTLV-1-infected T-cells is suppressed by stromal cells, that is epithelial cells and fibroblasts, in culture through type I IFNs [65]. Namely, HTLV-1 Gag protein expression was suppressed when contacted with stromal cells and restored when separated from the stromal cells. Although neutralizing antibodies against human IFN-α/β receptor only partly abrogated this phenomenon, the results indicate that the innate immune system suppresses HTLV-1 expression *in vitro* and *in vivo*, at least through type I IFN.

4.2. Antibody Response to HTLV-1. In 2002, it was reported that antibodies that recognize HTLV-1 Tax protein can cross-react with a heterogenous-nuclear-riboprotein (hnRNP-) A1, suggesting intriguing evidence for antigen mimicry in HTLV-1 infection [66]. However, subsequent analysis using Japanese samples under fully masked conditions indicated that there was no difference in the incidence of anti-hnRNP A1 Abs between HAM/TSP and other neurological diseases [67]. It is unlikely that anti-Tax Ab explains the onset or initial tissue damage of HAM/TSP, as the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed [68] and is not normally accessible to Ab attack. Anti-Tax Ab might be associated with subsequent inflammation following initial tissue damage and disruption of blood brain barrier, which is probably caused by the antiviral immune responses to HTLV-1 and induces the release of autoantigens.

In HTLV-1 infection, HAM/TSP patients generally have a higher anti-HTLV-1 Ab titer than ACs with a similar HTLV-1 proviral load [69–71]. These anti-HTLV-1 Abs often include IgM in both ACs and patients with HAM/TSP [70, 71]. These findings 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. Although Ab responses to the immunodominant epitopes of the HTLV-1 envelope (Env) proteins were similar in all of three clinical groups (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was higher in HAM/TSP patients (71%–93%) than in ATL patients (4%–31%) or ACs (27%–37%) [72]. Among these anti-HTLV-1 antibodies, anti-EnvAb is particularly important since some anti-Env Abs have neutralizing activity against HTLV-1. Antisera raised against recombinant HTLV-1 Env polypeptides [73, 74], vaccinia virus containing HTLV-1 env gene [75, 76], immunization with neutralizing epitope peptides [77], and passive transfer of human IgG that has neutralizing activity [78, 79] were all shown to neutralize HTLV-1 infectivity. In HTLV-1 infection, the roles of HTLV-1 neutralizing Ab *in vivo* are still largely unknown. It will be interesting to examine whether HTLV-1 neutralizing Ab titres correlate with disease status and PVL in infected individuals. Since the mutation rate of HTLV-1 provirus is significantly lower than HIV-1, passive immunization with human monoclonal Ab may be beneficial and effective method to prevent HTLV-1 infection.

4.3. Cytotoxic T-Lymphocyte (CTL) Response to HTLV-1. Previous reports indicated that the HTLV-1-specific $CD8^+$ CTLs are typically abundant, chronically activated, and mainly targeted to the viral trans activator protein Tax [80]. Also, as already mentioned, the median PVL in PBMCs of HAM/TSP patients was more than ten times higher than that in ACs, and a high PVL was also associated with an increased risk of progression to disease [36, 37]. Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower PVL and a lower risk of HAM/TSP [37, 41], and $CD8^+$ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals [42]. 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. Indeed, as mentioned above (Section 3.1), MacNamara et al. [45] have shown that HLA class 1 alleles which strongly bind oligopeptides from the HBZ protein enable the host to make a more effective immune response against HTLV-1; therefore, such individuals have a lower PVL and are more likely to be asymptomatic. Moreover, another recent report showed the presence of HBZ-specific $CD4^+$ and $CD8^+$ cells *in vivo* in patients with HAM/TSP and in ACs and a significant association between the HBZ-specific $CD8^+$ cell response and asymptomatic HTLV-1 infection [81]. These findings provide strong evidence to support the hypothesis of the crucial role of CTLs and also confirm the importance of HBZ for persistent infection.

Since the frequency of HTLV-1-specific $CD8^+$ T cells was significantly higher in HAM/TSP patients than ACs [82, 83], and these cells have the potential to produce

proinflammatory cytokines [84], 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. The analysis of gene expression profiles using microarrays in circulating CD4$^+$ and CD8$^+$ lymphocytes indicated that granzymes and perforin are more highly expressed in individuals with a low PVL [43], suggesting that a strong CTL response is associated with a low PVL and a low risk of HAM/TSP. Indeed, the lytic capacity of HTLV-1-specific CTL in patients with HAM/TSP and ACs, quantified by a CD107a mobilization assay, showed significantly lower CD107a staining in HTLV-1-specific CTL in HAM/TSP than ACs [85]. Recently, it has been reported that the high CTL avidity, which is closely associated with the lytic efficiency of CTL, correlates with low PVL and proviral gene expression [44], indicating that the efficient control of HTLV-1 *in vivo* depends on the quality of CTL, which determines the position of virus-host equilibrium and also the outcome of persistent HTLV-1 infection. However, two caveats must be made here. First, a protective role and a pathogenic role of CTLs are not mutually exclusive. Indeed, there are other examples of viral infections in which the virus-specific CTLs exert both beneficial (antiviral) and detrimental (inflammatory) effects, such as lymphocytic choriomeningitis virus (LCMV) infection in the mouse [86]. Second, it is difficult to separate cause and effect in analyzing the association between T-cell attributes and the efficiency of viral control in a persistent infection at equilibrium.

4.4. CD4$^+$ Helper T-Cell Response to HTLV-1. Antiviral CD4$^+$ T-cell responses are of central importance in driving B-cell and CD8$^+$ T-cell responses *in vivo*. The most common HTLV-1 antigen recognized by CD4$^+$ T-cells is the Env protein [87, 88], in contrast with the immunodominance of Tax in the CD8$^+$ T-cell response [89–91]. At a similar PVL, patients with HAM/TSP had significantly increased frequency of virus-specific CD4$^+$ T cells compared to ACs [88, 92]. The antiviral T-helper (Th)1 phenotype is also dominant among HTLV-1-specific CD4$^+$ T cells in both ACs and patients with HAM/TSP [93], and there is a higher frequency of IFN-γ, TNF-α, and IL-2 production by CD4$^+$ T cells in patients with HAM/TSP compared to AC of a similar PVL [93, 94]. A role for CD4$^+$ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts the immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in Southern Japan [37, 41] and Northeastern Iran [40]. Accordingly, 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* [92]. The results showed that the frequency of tetramer$^+$CD4$^+$ T cells was significantly higher in HAM/TSP patients than ACs with similar PVL. Furthermore, direct ex vivo analysis of tetramer$^+$CD4$^+$ T cells from two unrelated DRB1*0101-positive HAM/TSP patients indicated that certain T-cell receptors (TCRs) Vβs were utilized and antigen-specific amino acid motifs were identified in complementarity determining region (CDR) 3 from both patients. These results 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 [95] and muscle-infiltrating cells from HAM/TSP patients and HTLV-1-infected polymyositis patients [96].

4.5. Regulatory T Cells (Tregs) in HTLV-1 Infection. Regulatory T cells (Tregs) are important mediators of peripheral immune tolerance and also play an important role in chronic viral infections. In HTLV-1 infection, it has been reported that HTLV-1 preferentially and persistently infects CD4$^+$CD25$^+$ lymphocytes *in vivo* [97], which contain the majority of the Foxp3$^+$ Tregs [98]. In HAM/TSP patients, the frequency of Foxp3$^+$ expression in CD4$^+$CD25$^+$ cells is lower than that in ACs and uninfected healthy controls [97, 99]. This is probably due to the fact that CD25 is transcriptionally induced by HTLV-1 Tax [100], which may result in the reduced proportion of Foxp3$^+$ cells in the CD4$^+$CD25$^+$ population in HTLV-1-infected individuals, especially HAM/TSP patients. It is important to note that the CD4$^+$CD25$^+$ population contains a mixture of Tregs and activated non-Tregs. Therefore, it is inappropriate to use CD25 as a marker of Tregs in HTLV-1 infection: the best current working definition of Treg phenotype is CD4$^+$Foxp3$^+$. Reports from different geographic regions indicate that the percentage of CD4$^+$Foxp3$^+$ cells is higher in the HAM/TSP patients than in ACs [101–103]. It has been reported that the high frequency of CD4$^+$Foxp3$^+$T cells in HTLV-1-infected individuals is maintained by CCL22 produced by HTLV-1-infected PBMCs [104]. The frequency of HTLV-1-negative CD4$^+$Foxp3$^+$ cells was positively correlated with the HTLV-1 proviral load [102, 105], and the CTL activity was negatively correlated with the frequency of HTLV-1-negative CD4$^+$Foxp3$^+$ cells [102], suggesting that CD4$^+$Foxp3$^+$ Tregs may impair the CTL surveillance of HTLV-1. If this is the case, activity of CD4$^+$Foxp3$^+$ cells may also determine the risk of developing HAM/TSP via increasing the HTLV-1 PVL.

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 [106], and the development of HAM/TSP is associated with rapid maturation of DCs [107]. As already mentioned, one of the hallmarks of HTLV-1 infection is the spontaneous lymphocyte proliferation (SLP). Interestingly, depletion of DCs from the HAM/TSP patient's PBMCs abolished SLP, whereas supplementing DCs restores proliferation [106]; supplementing B cells or macrophages had no effect. A DC-dependent mechanism of SLP was further supported by data showing that antibodies to MHC

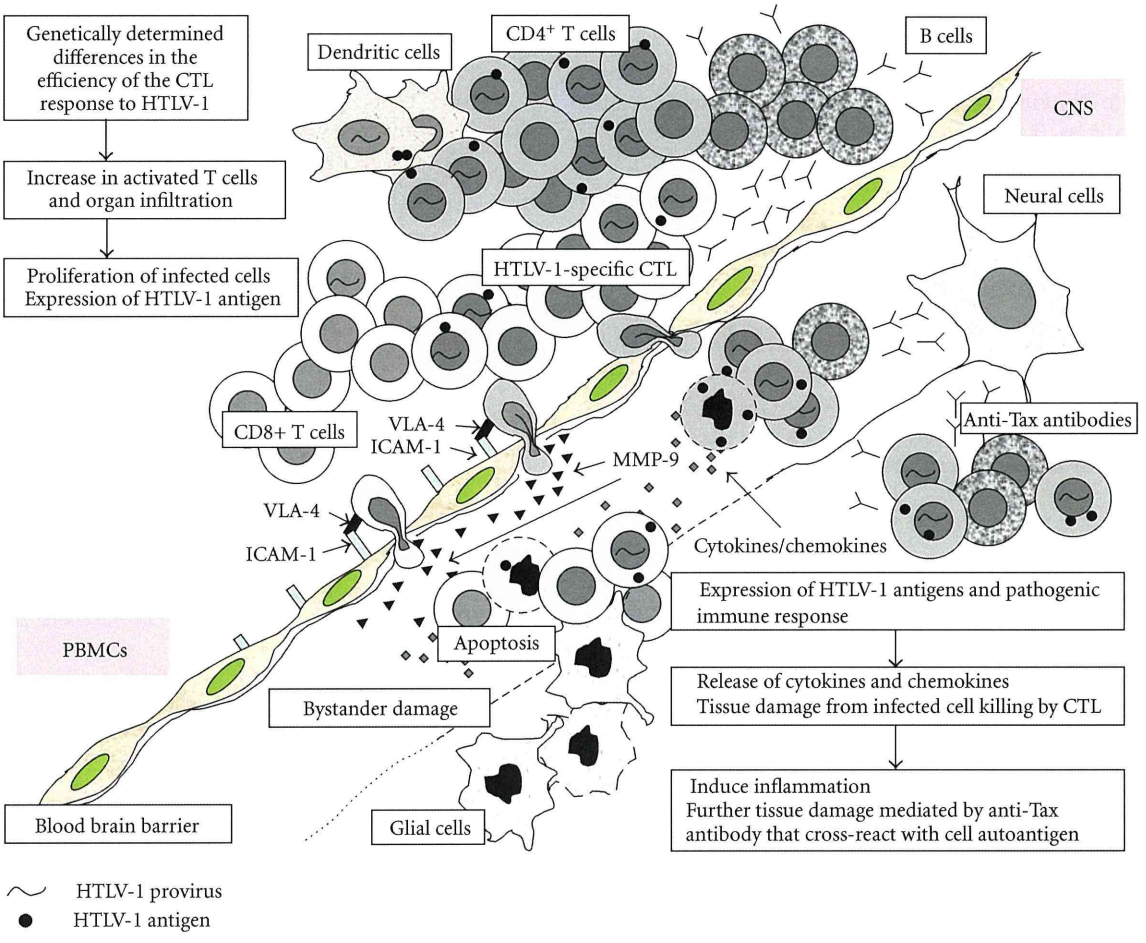

FIGURE 1: Hypothesis for the pathogenesis of human T-cell leukemia virus type-1 (HTLV-1) -associated myelopathy/tropical spastic paraparesis (HAM/TSP). Accumulating evidence suggests that the virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less 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. 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.

class II, CD86, and CD58 can block SLP [108]. Recently, it has been demonstrated that 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 [109]. In addition, other groups have obtained evidence that HTLV-1 transmission from DCs to T cells was mediated primarily by DC-SIGN [110], and DCs play a major part in generating and maintaining the Tax-specific $CD8^+$ T cells both *in vitro* and *in vivo* [111]. Moreover, using transgenic mouse models that permit conditional transient depletion of $CD11c^+$ DCs, and a chimeric HTLV-1 that carries the envelope gene from Moloney murine leukemia virus, Rahman et al. demonstrated the critical role of DCs in their ability to mount both innate and adaptive immune responses during early cell-free HTLV-1 infection [112, 113]. Since HTLV-1 can impair the differentiation of monocytes into DCs [114], the interaction of DCs with HTLV-1 plays a central part in the persistence and pathogenesis of HTLV-1.

5. Concluding Remarks

As shown in Figure 1, accumulating evidence suggests that the host immune response, especially the CTL response, plays a critical role in determining the risk of HAM/TSP. A less efficient CTL response against HTLV-1 may cause a higher PVL and higher antigen expression in infected individuals, which in turn lead to activation and expansion of antigen-specific T-cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression to HAM/TSP. Since HLA class 1 genotype determines only up to 50% of HAM/TSP risk in infected people [41], it is important to discover other factors that determine the efficiency of the CTL response

to HTLV-1 and the outcome of HTLV-1 infection. Studies of the HTLV-1 receptor and DCs are also critical in the development of vaccine approaches to elicit cellular immune responses to key viral proteins such as Tax and Env to ablate HTLV-1-infected T cells. Newer approaches using genetically engineered and/or humanized mouse models for HTLV-1 infection will help to develop effective treatment and prevention of HAM/TSP in the future.

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研究成果の刊行に関する一覧表

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雑誌

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Fan J, Ma G, Nosaka K, Tanabe J, Satou Y, Koito A, Wain-Hobson S, Vartanian JP, Matsuoka M	APOBEC3G generates nonsense mutations in human T-cell leukemia virus type 1 proviral genomes in vivo	J Virol	84	7278-7287	2010
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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.

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.

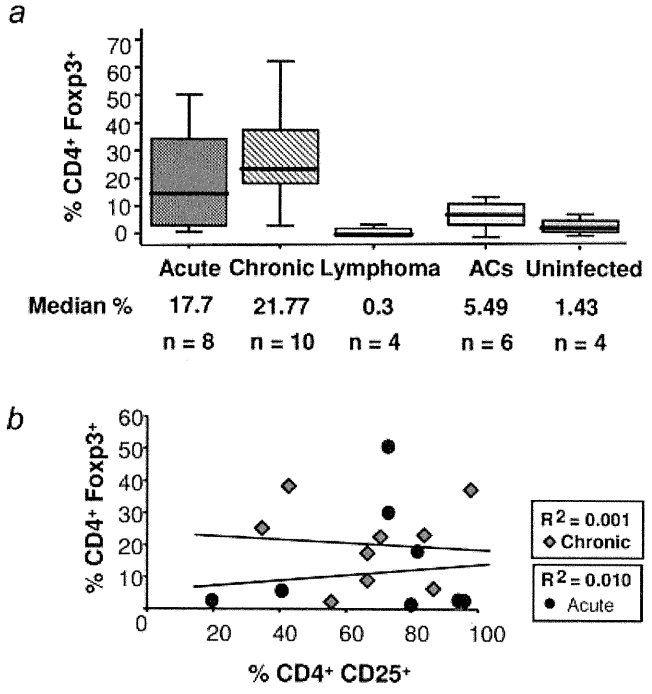


FIGURE 1 – FoxP3 and CD25 expression in patients with ATLL. (*a*) Spontaneous FoxP3 expression in $CD4^+$ cells after incubation *in vitro* for 18 hr. PBMCs were taken from patients with acute ATLL ($N = 8$), chronic ATLL ($N = 10$), lymphoma ($N = 4$), asymptomatic carriers (ACs, $N = 4$), uninfected 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.

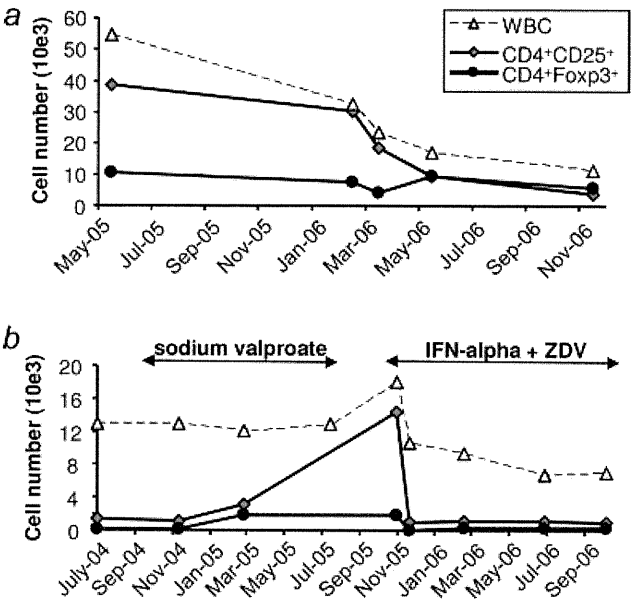


FIGURE 2 – Longitudinal follow-up of 2 patients with chronic ATLL. Each graph depicts the absolute numbers of white blood cells (WBC; white triangles), $CD4^+CD25^+$ cells and $CD4^+FoxP3^+$ cells. (*a*) In this patient, the chronic ATLL underwent spontaneous remission in March 2006. (*b*) A case of 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

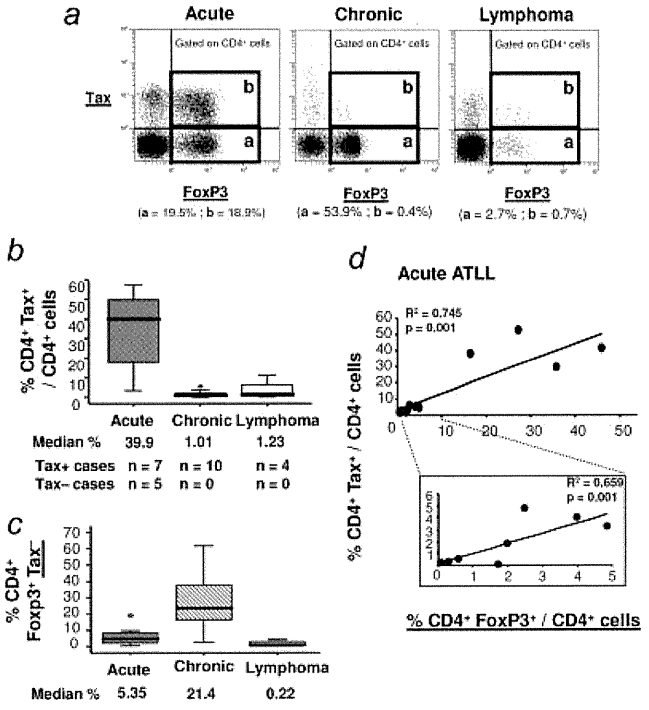


FIGURE 3 – FoxP3 and Tax expression in patients with ATLL. (*a*) Tax and FoxP3 expression in $CD4^+$ cells from representative patients with acute ATLL, chronic ATLL and lymphoma, after 18 hr incubation *in vitro*. Gate **a** represents the $CD4^+FoxP^+Tax^-$ population and gate **b** represents the $CD4^+FoxP3^+Tax^+$ 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 $CD4^+TCRVβn^+$ cells inhibit heterologous $CD4^+CD25^-$ T-cell proliferation

To test the functional capacity of the $TCRVβn^+$ 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 ($TCRVβn^+$-depleted) population (Fig. 6a). We then added the 3 respective cell populations ($CD4^+TCRVβn^+$; $CD4^+CD25^+$; $CD4^+TCRVβn^-CD25^-$) 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 $TCRVβn^+$ 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 $TCRVβn^+$ clonal population in each case caused little inhibition of proliferation of $CD4^+CD25^-$ cells similar to the degree of inhibition caused by the $CD4^+TCRVβn^-CD25^-$ cells.

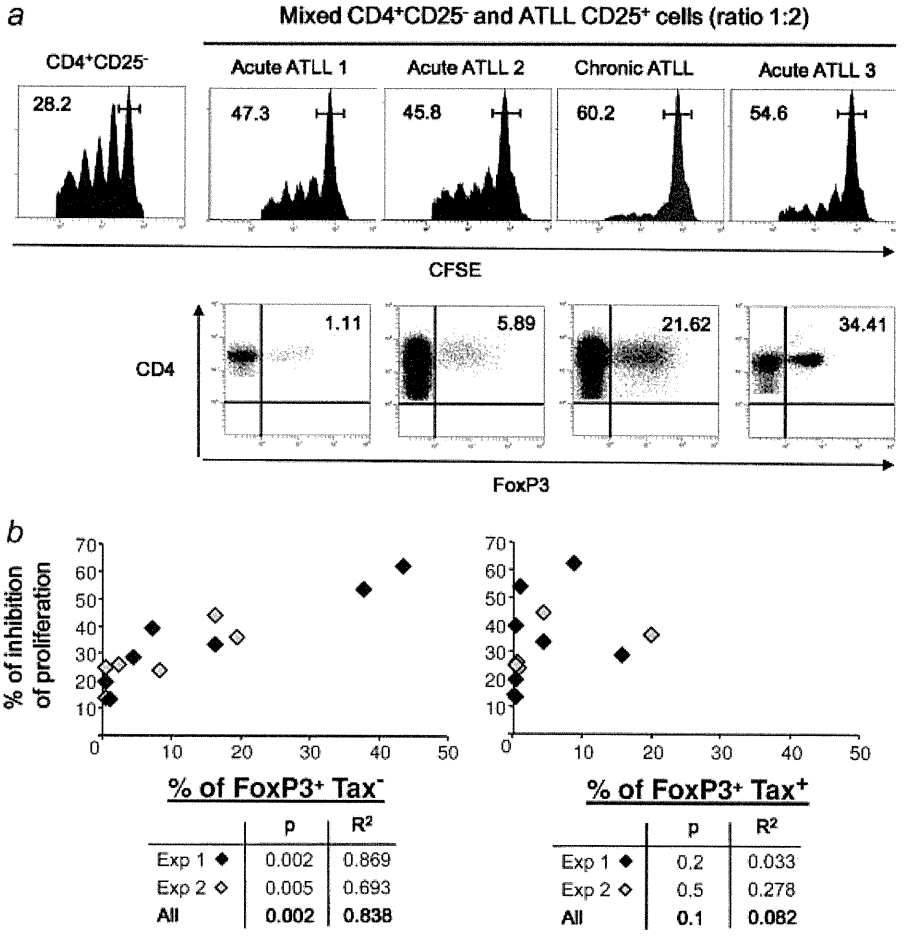
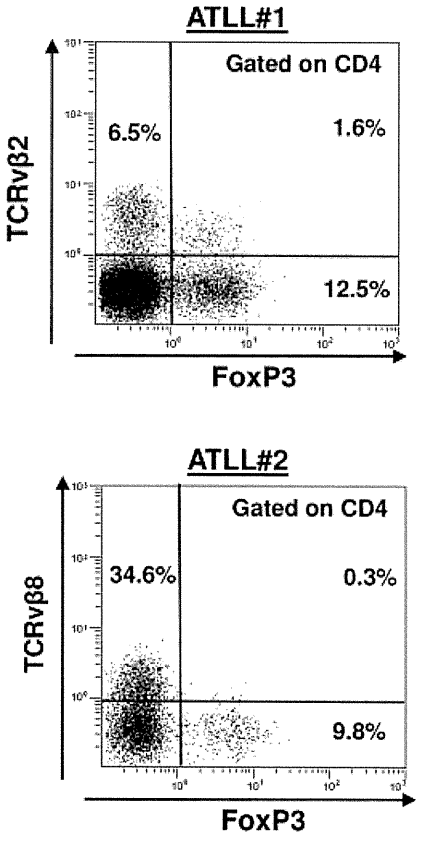


FIGURE 4 – Effect of ATLL cells and FoxP3 expression on proliferation of uninfected $CD4^+CD25^-$ cells. In each of two independent experiments, we tested the ability of $CD25^+$ ATLL cells to inhibit proliferation of $CD4^+CD25^-$ cells. In Experiment 1, effector ($CD25^+$) cells from 7 patients with 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.



$FoxP3^+$ 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 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.