

HAM/TSP patients, as evidenced by their overproduction of IFN- $\gamma$ , suggesting that HTLV-1 may intracellularly induce T cell plasticity from Treg to IFN- $\gamma$ <sup>+</sup> T cells. This review examines the recent research into the association between HTLV-1 and Treg cells that has greatly enhanced understanding of the pathogenic mechanisms underlying immune dysregulation in HTLV-1-associated neuroinflammatory disease.

**Keywords:** HTLV-1; HAM/TSP; ATL; CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cell; regulatory T cell; exFoxp3<sup>+</sup> cell; inflammation; immune-dysfunction

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## 1. Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus associated with chronic, persistent infection of human T cells. HTLV-1 infection is endemic in Japan, the Caribbean, and part of South America, Africa, the Middle East, and Melanesia [1]. Studies conducted in HTLV-1 endemic areas have demonstrated that HTLV-1 infection is associated with a variety of human diseases, including an aggressive mature T cell malignancy termed adult T-cell leukemia (ATL) [2], which is defined as neoplastic growth of HTLV-1-infected T cells. HTLV-1 is also associated with non-neoplastic inflammatory conditions such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4], uveitis [5], Sjögren syndrome [6], bronchoalveolitis, arthritis [7], and polymyositis [8], where high tissue concentrations of HTLV-1 infected T lymphocytes have been observed. Importantly, some patients have more than one of these HTLV-1-associated inflammatory conditions [9].

Although HTLV-1-associated disorders have been extensively studied, the exact mechanism by which HTLV-1 induces these inflammatory conditions is not completely understood. The proviral load of HTLV-1 may contribute to development of HTLV-1-associated inflammatory conditions, since the number of HTLV-1-infected T cells circulating in the peripheral blood is higher in patients with HAM/TSP than in asymptomatic HTLV-1-infected individuals [10,11], and is even higher in the cerebrospinal fluid of patients with HAM/TSP [12]. In HAM/TSP patients, the proviral load correlates with not only the percentage of activated CD4<sup>+</sup> T cells but also with that of HTLV-1-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) [11,13]. These HTLV-1-specific CTLs produce various cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , that may suppress viral replication and kill infected cells and/or promote bystander activation and killing of nearby resident cells in the central nervous system (CNS) [14–17]. In addition, increased viral expression, particularly of the transactivating viral gene encoding HTLV-1 Tax, has also been hypothesized to play a role in HTLV-1 disease progression [11,12]. Transgenic mice expressing HTLV-1 Tax develop an inflammatory arthropathy [18], and transgenic rats expressing HTLV-1 env-pX develop destructive arthropathy, Sjögren syndrome, vasculitis, and polymyositis [19]. These findings support the hypothesis that HTLV-1 *tax* is one of the exogenous retrovirus genes responsible for immune dysregulation.

HTLV-1 Tax is a transactivator/oncoprotein that has potent effects on infected T cells, including activation of nuclear factor(NF)- $\kappa$ B [20] with subsequent enhancement of cell activation and proliferation and expression of various cellular genes, such as IL-2 [21], the  $\alpha$ -chain of the IL-2 receptor (IL-2R $\alpha$ ) [22], IL-15 [23], and IL-15R $\alpha$  [24]. Such virus-induced intracellular activation may

directly contributes to T cell activation and the *ex vivo* T cell proliferation observed in patients with HAM/TSP [25]. These findings suggest that invasion by HTLV-1-infected T cells, together with viral gene expression and cellular-signaling mechanisms, trigger a strong virus-specific immune response and increased proinflammatory cytokine production, leading to CNS inflammation and autologous tissue damage. However, the precise mechanisms underlying the induction of immune activation by HTLV-1-infected T cells are not well understood.

## 2. HTLV-1 and Regulatory T Cells

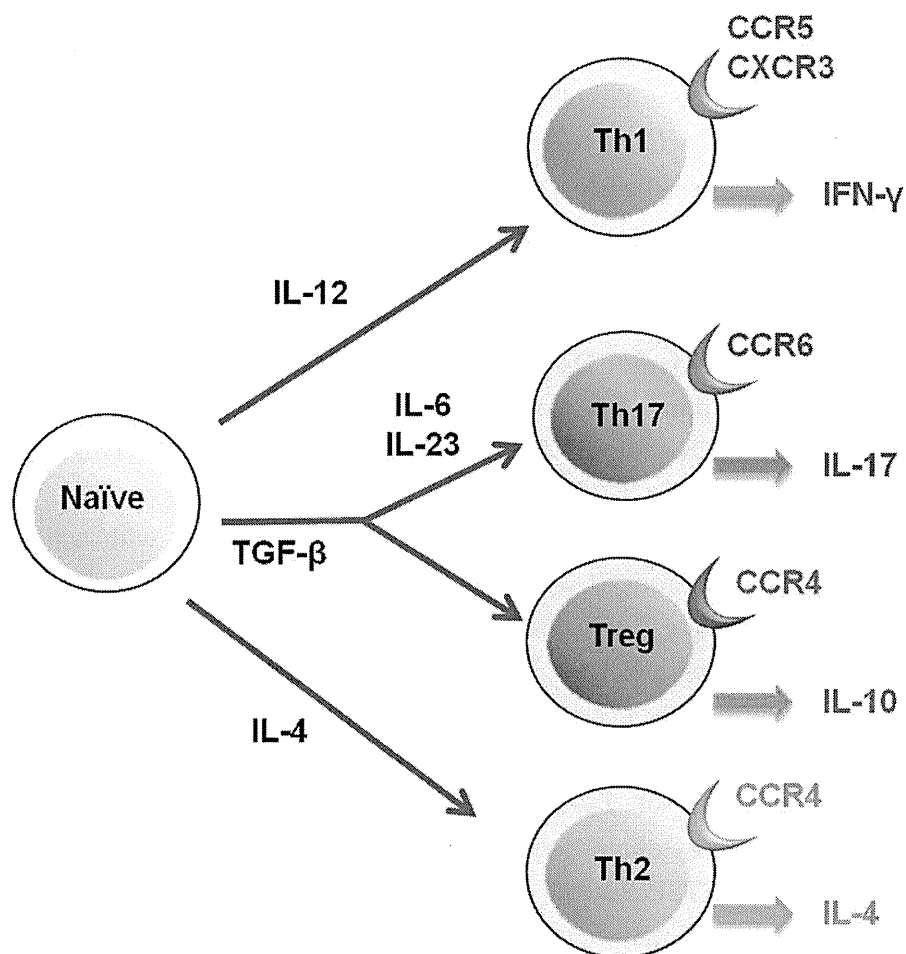
The recent discovery of regulatory T cells (Treg cells) has generated new opportunities for and increased interest in elucidating the above mentioned mechanisms. In healthy individuals, the Treg cells, a subset of CD4<sup>+</sup>CD25<sup>+</sup> T cells, play a key role in maintaining immune system homeostasis by suppressing the proliferation of and cytokine production by pathogenic T cells [26]. Although Treg cells are phenotypically similar to activated T cells, they can be identified *ex vivo* by their intracellular expression of the transcriptional regulator Foxp3 [27], which is critical in the development and functioning of Treg cells in both mice and humans. Significant reductions in Foxp3 expression and/or Treg cell function have been observed in patients with several types of human autoimmune diseases [28], suggesting that defects in Foxp3 expression and/or Treg functioning may precipitate loss of immunological tolerance. CD4<sup>+</sup>CD25<sup>+</sup> T cells are also the predominant viral reservoir in the peripheral blood of HTLV-1-infected individuals [29]. Recently, significant reductions in Foxp3 expression and Treg cell function have been observed in CD4<sup>+</sup>CD25<sup>+</sup> T cells from patients with HAM/TSP [30–34]. Furthermore, decreased expression levels of CTL antigen-4 (CTLA-4), a Treg-associated immune-suppressive molecule, and glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) have also been observed on the CD4<sup>+</sup>CD25<sup>+</sup> T cells of HAM/TSP patients [30,34]. Notably, overexpression of HTLV-1 Tax has been observed to reduce Foxp3 expression and inhibit the suppressive function of Treg cells *in vitro* [30]. Furthermore, because of a Tax-induced defect in TGF- $\beta$  signaling, Foxp3 expression was decreased and Treg functions were impaired in patients with HAM/TSP [35]. Recently, significantly decreased numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells were observed in transgenic mice expressing HTLV-1 Tax that develop an inflammatory arthropathy [36]. In addition, increased viral expression of the HTLV-1 bZIP factor (*HBZ*) gene encoding the minus strand of HTLV-1 has also been suggested to play a role in HTLV-1 disease progression [37], and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in *HBZ* transgenic mice were functionally impaired [38]. These findings indicate that HTLV-1-induced dysfunctioning of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells may be one of the mechanisms underlying the induction of immune activation by HTLV-1-infected T cells.

In contrast to the decreased expression of Foxp3 in CD4<sup>+</sup>CD25<sup>+</sup> T cells observed in HAM/TSP patients [30–34], most CD4<sup>+</sup>CD25<sup>+</sup> ATL cells have been shown to express Foxp3 in patients with ATL [39,40]. Therefore, it has been hypothesized that ATL cells may be derived from Treg cells [41]. Interestingly, some ATL cells exhibit immunosuppressive functions similar to those of Treg cells, which may contribute to clinically observed cellular immunodeficiency in ATL patients [41–43], although some of these ATL cells lose this regulatory function [44].

### 3. HTLV-1 and CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T Cells

Although HTLV-1 has been reported to infect a number of cell types both *in vitro* and *in vivo* [29,45–49], CD4<sup>+</sup> Th cells, which play a central role in adaptive immune responses, are the predominant viral reservoir in the peripheral blood [50]. To understand the effects of HTLV-1 infection on the functioning of CD4<sup>+</sup> Th cells, it is necessary to discover if, and if so which of the Th subpopulations is preferentially infected with HTLV-1. Based on their functions, patterns of cytokine secretion, and expression of specific transcription factors and chemokine receptors, CD4<sup>+</sup> Th cells, which are differentiated from naïve CD4<sup>+</sup> T cells, are classified into four major lineages: Th1, Th2, Th17, and Treg cells (Figure 1).

**Figure 1.** T cell subsets of CD4<sup>+</sup> T helper cells. Th cells are differentiated from naïve CD4<sup>+</sup> T cells into 4 major lineages: Th1, Th2, Th17, and T-regulatory (Treg) cells. Each Th subset exhibits characteristic functions, patterns of cytokine secretion, and expression of specific chemokine receptors.



The chemokine receptor CCR4 has recently been found to be expressed on HTLV-1-infected leukemia cells in ATL patients [51]. Because CCR4 is known to be selectively expressed on Treg and Th2 cells [51–53] (Figure 1) and because most ATL cells express high levels of Foxp3, it has been hypothesized that ATL cells may be derived from Treg cells [41]. Although it has been

demonstrated that  $CD4^+CD25^+$  T cells in HAM/TSP patients exhibit reduced Foxp3 expression and Treg suppression [30–33] and that HTLV-1-infected  $CD4^+$  T cells in HAM/TSP patients produce Th1 cytokines (IFN- $\gamma$ ) [16,30], it has also been observed that CCR4 selectively overexpresses on HTLV-1-infected T cells in HAM/TSP patients [54]. Furthermore, the majority of  $CD4^+CD25^+CCR4^+$  T cells have been found to be infected with HTLV-1 and this T cell subset has increased numbers in HAM/TSP patients [54]. Thus,  $CD4^+CD25^+CCR4^+$  T cells are a major reservoir of HTLV-1-infected T cells, which are increased in numbers in both HAM/TSP and ATL patients.

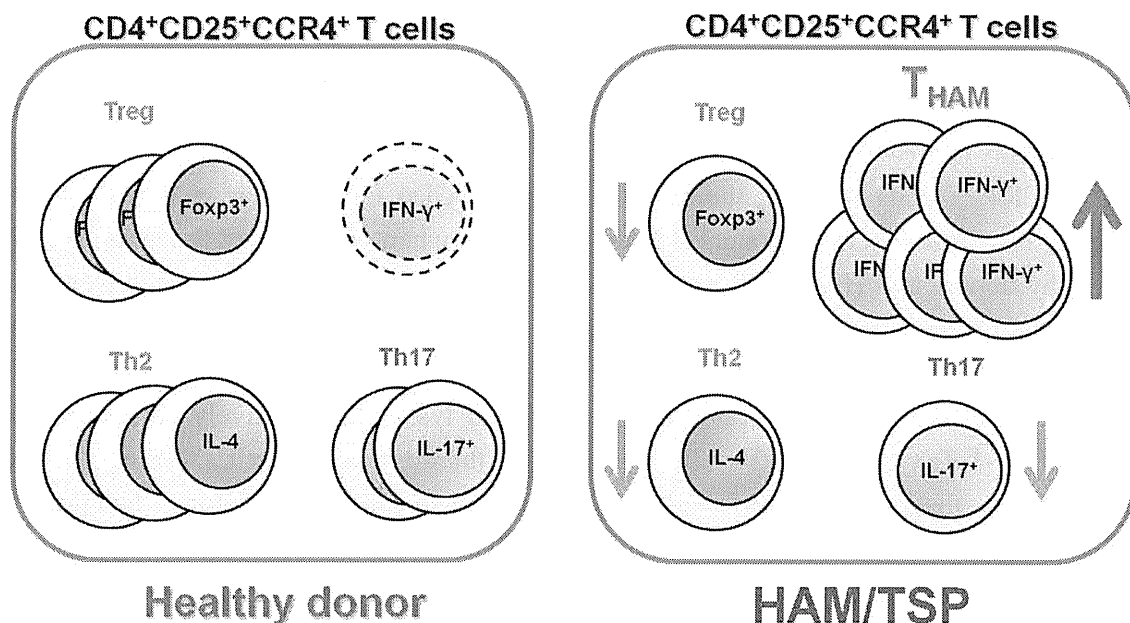
#### 4. HTLV-1 and Foxp3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T Cells

Although CCR4 is known to be selectively expressed on Treg and Th2 cells in healthy individuals, more detailed flow cytometric analysis of Foxp3 expression in  $CD4^+CD25^+CCR4^+$  T cells of HAM/TSP patients demonstrated that the frequency of the Foxp3<sup>-</sup> population was greatly increased in  $CD4^+CD25^+CCR4^+$  T cells [54]. Moreover, analysis of proinflammatory cytokine expression in this Foxp3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cell subset demonstrated that these cells uniquely produced multiple proinflammatory cytokines such as IL-2, IL-17, and few IFN- $\gamma$  in healthy individuals while Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells (Treg cells) did not. Furthermore, it was demonstrated that HAM/TSP patients had only few Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells that did not produce such cytokines [54]. The Foxp3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells in HAM/TSP were greater in number and overproduced IFN- $\gamma$  [54]. Further, the proportion of these IFN- $\gamma$ -producing Foxp3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells may have a functional consequence, since the presence of this subpopulation could be correlated with disease activity and severity of HAM/TSP *in vivo* [54]. Thus, in a  $CD4^+CD25^+CCR4^+$  T cell population that mainly consists of suppressive T cell subsets such as Treg and Th2 under healthy conditions, IFN- $\gamma$ -producing Foxp3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells, rarely encountered in healthy individuals, were increased in number and overproduced IFN- $\gamma$  in HAM/TSP patients (Figure 2). We therefore propose to call this IFN- $\gamma^+$ Foxp3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cell subset T<sub>HAM</sub> cells. Interestingly, increased numbers of Foxp3<sup>low</sup>CD4<sup>+</sup>CD25<sup>+</sup> memory T cells, which have cytokine secretion patterns similar to those of T<sub>HAM</sub> cells, have recently been observed in patients with active systemic lupus erythematosus (SLE) [55]. Therefore, it would be of interest to build on this finding by confirming whether this newly defined unique T cell subset, which has been observed in both HAM/TSP and SLE patients, is found in both these patient groups and can be functionally deregulated in other immunological diseases.

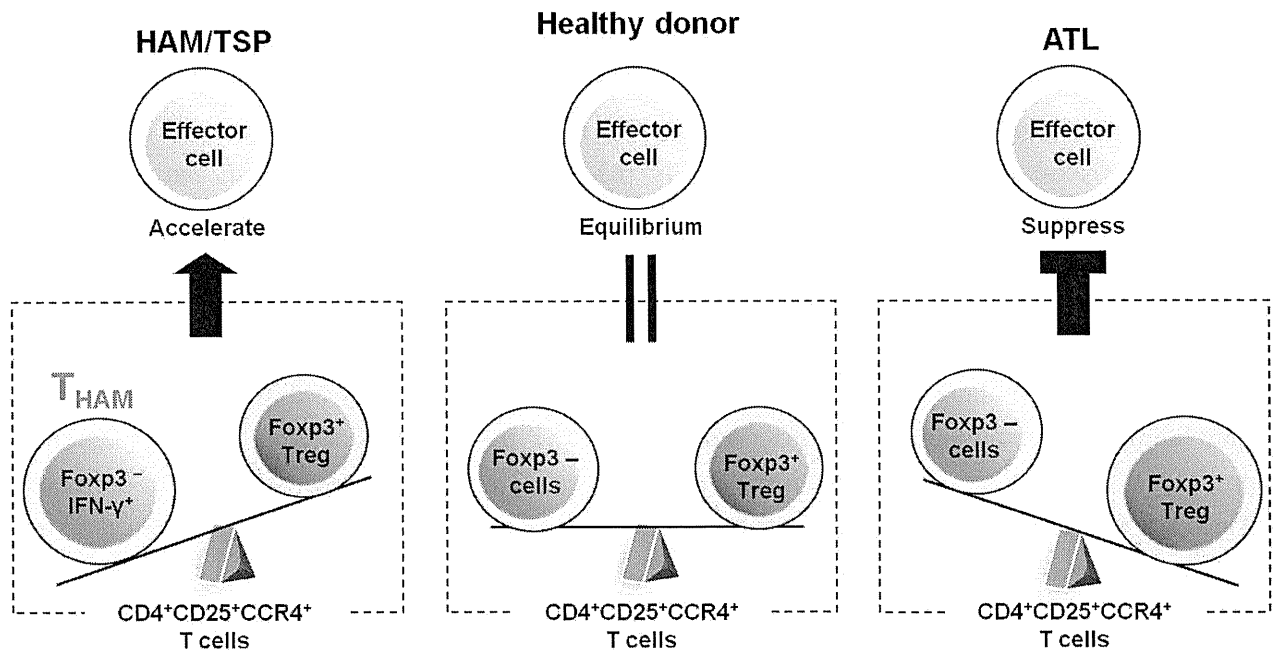
Although most  $CD4^+CD25^+CCR4^+$  T cells are infected with HTLV-1 in both HAM/TSP and ATL patients [54,56], the ratio of T<sub>HAM</sub> cells (CCR4<sup>+</sup>Foxp3<sup>-</sup> with IFN- $\gamma$  production) to Treg cells (CCR4<sup>+</sup>Foxp3<sup>+</sup> with no cytokine production) in the  $CD4^+CD25^+CCR4^+$  T cell subset has been found to be high in HAM/TSP patients but low in ATL patients [54]. This differential T<sub>HAM</sub>/Treg ratio in HTLV-1-infected T cells may be associated with the differential immune responses observed between HAM/TSP and ATL patients (Figure 3). ATL patients tend to have very low numbers of Tax-specific CD8<sup>+</sup> T cells in peripheral blood mononuclear cells (PBMCs) and to develop opportunistic infections [57,58], while HAM/TSP patients tend to have high numbers of Tax-specific CD8<sup>+</sup> CTLs [11,12,14,59]. As  $CD4^+CD25^+$  T cells with high levels of Foxp3 expression have been reported to have an immunosuppressive function in ATL patients [41–43], the increased number of

CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> leukemia T cells with Treg functions observed in ATL patients may contribute to their clinically observed cellular immunodeficiency. However, HAM/TSP patients show very high cellular and humoral immune responses, such as high proportions of Tax-specific CD8<sup>+</sup> T cells, as well as cytomegalovirus (CMV)-specific CD8<sup>+</sup> T cells in the PBMCs [14,33]; high antibody titer to HTLV-1 [9]; and increased production of proinflammatory cytokines, such as IL-12 and IFN- $\gamma$  [60]. It has been reported that CD4<sup>+</sup>CD25<sup>+</sup> T cells with low expression of Foxp3 [30] and HTLV-1 Tax-expressing Foxp3<sup>+</sup> Treg cells [61] extracted from HAM/TSP patients exhibit defective immunosuppressive functioning. Moreover, it has been demonstrated that HTLV-1-infected IFN- $\gamma$ -overproducing CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup>Foxp3<sup>-</sup> T cells (T<sub>HAM</sub> cells) increase in number in HAM/TSP patients, and their levels can be correlated with disease severity [54]. Thus, CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells with increased proinflammatory functioning, together with a defective Treg compartment [30–33,54], may overcome the regulatory effect of HTLV-1-uninfected Treg cells [61] and at least partly account for the heightened immune response observed in HAM/TSP patients. Collectively, these observations support the hypothesis that an imbalance in the T<sub>HAM</sub>/Treg ratio in HTLV-1-infected CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells is an important contributing factor in the immunological differences in host immune response observed between HAM/TSP and ATL patients (Figure 3).

**Figure 2.** Cellular components of CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells in healthy donors and HAM/TSP patients. In healthy donors, the CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cell population primarily consists of suppressive T cell subsets, such as Treg and Th2, whereas that of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients consists of an increased number of IFN- $\gamma$ -producing Foxp3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells (T<sub>HAM</sub> cells).



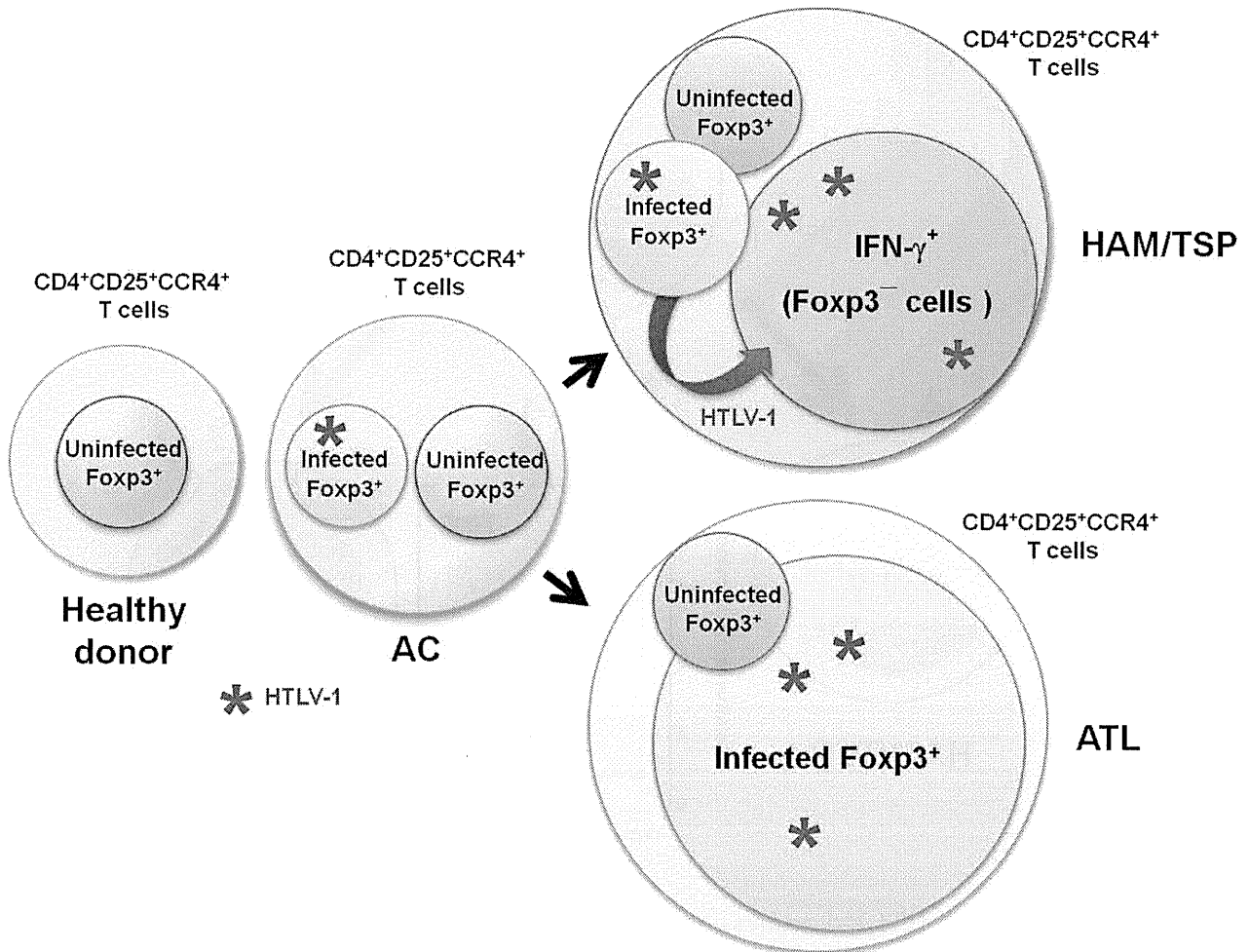
**Figure 3.** Differential immune responses and  $T_{HAM}/Treg$  ratios in  $CD4^+CD25^+CCR4^+$  T cells in HAM/TSP and adult T cell leukemia/lymphoma (ATL) patients.



### 5. Increased Numbers of $CD4^+FoXP3^+$ Cells in HAM/TSP Patients

Recently, it has been reported that the number of  $CD4^+FoXP3^+$  cells increases in HTLV-1-infected asymptomatic carriers, and is even higher in patients with HAM/TSP [61]. Although this report initially appears to conflict with the observations described above, it may not. In contrast to the decreased number of  $CD4^+$  T cells in patients with human immunodeficiency virus (HIV) infection, the number of HTLV-1 infected  $CD4^+$  T cells—most of which are  $CD4^+CD25^+CCR4^+$  T cells—in HAM/TSP patients is greatly increased. Therefore, although the percentage of  $FoXP3^+$  cells among the  $CD4^+CD25^+CCR4^+$  T cells is lower, the overall number of  $CD4^+FoXP3^+$  cells in HAM/TSP patients may be higher than that in healthy donors (Figure 4). Indeed, when we analyzed the number of  $FoXP3^+$  cells in healthy donors and HAM/TSP patients, we found it to be nearly equivalent between the two groups or slightly higher in HAM/TSP patients [54]. This difference (from slightly high to higher) would depend on the number of HTLV-1-infected  $CD4^+$  T cells in the samples tested. Importantly, Toulza *et al.* demonstrated that the rate of CTL-mediated lysis was negatively correlated with the number of HTLV-1-Tax<sup>-</sup> $CD4^+FoXP3^+$  cells, but not with the number of Tax<sup>+</sup> $CD4^+FoXP3^+$  cells [61], again suggesting that HTLV-1-infected Treg cells lose their regulatory function, while HTLV-1-uninfected Treg cells contribute substantially to immune control of HTLV-1 infection.

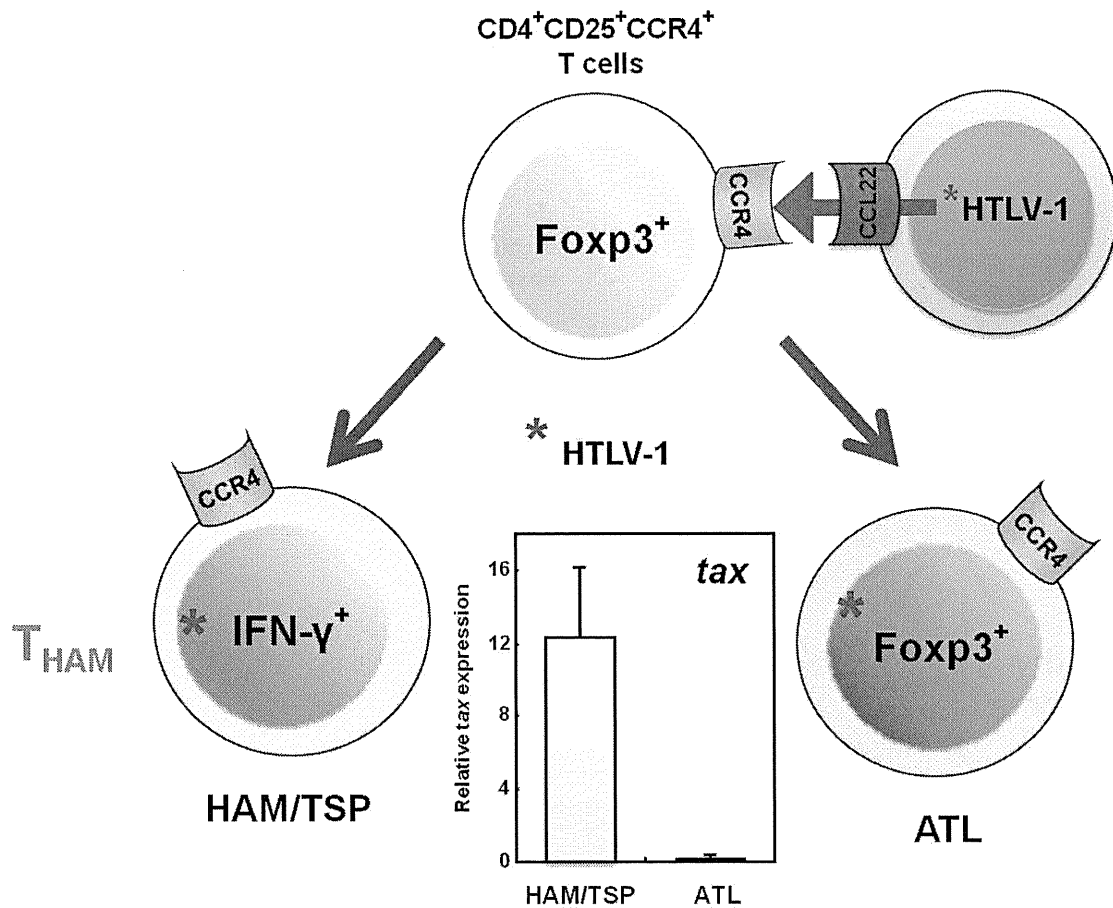
**Figure 4.** Scheme of proportion of each cellular component in  $CD4^+CD25^+CCR4^+$  T cells of healthy donors, asymptomatic carriers (AC), and patients with HAM/TSP or ATL. Although the proportion of  $Foxp3^+$  cells among the  $CD4^+CD25^+CCR4^+$  T cells is lower in HAM/TSP patients, the overall number of  $CD4^+Foxp3^+$  cells in HAM/TSP patients is higher than that in healthy donors. In ATL patients, the majority of  $CD4^+CD25^+CCR4^+$  T cells are  $Foxp3^+$  cells.



## 6. Does the $T_{HAM}$ Cell Population Include ex $Foxp3^+$ Cells?

According to Hieshima *et al.*'s recent delineation of the molecular mechanism underlying HTLV-1 tropism to  $CCR4^+CD4^+$  T cells [60], HTLV-1 Tax does not induce expression of CCR4, but Tax does induce expression of CCL22, which is the ligand for CCR4. Therefore, HTLV-1-infected T cells produce CCL22 through Tax and selectively interact with  $CCR4^+CD4^+$  T cells, resulting in preferential transmission of HTLV-1 to  $CCR4^+CD4^+$  T cells (Figure 5). In HTLV-1-seronegative healthy individuals,  $CD4^+CD25^+CCR4^+$  T cell populations primarily consist of suppressive T cell subsets, such as Treg and Th2 cells [61]. However, as described above, cells of this T cell subset become Th1-like cells that overproduce IFN- $\gamma$  in patients with HAM/TSP, while leukemogenesis develops and maintains the  $Foxp3^+$  Treg phenotype in ATL patients (Figure 5).

**Figure 5.** Differential fate of HTLV-1-infected CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells in HAM/TSP and ATL patients. After HTLV-1 infection, CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells in HAM/TSP patients, which are primarily Th2 and Treg cells before infection, become IFN-γ<sup>+</sup>Foxp3<sup>-</sup> T cells (T<sub>HAM</sub> cells) with high levels of intracellular HTLV-1 *tax* expression. In ATL patients, leukemogenesis develops and the Foxp3<sup>+</sup> Treg phenotype is maintained.

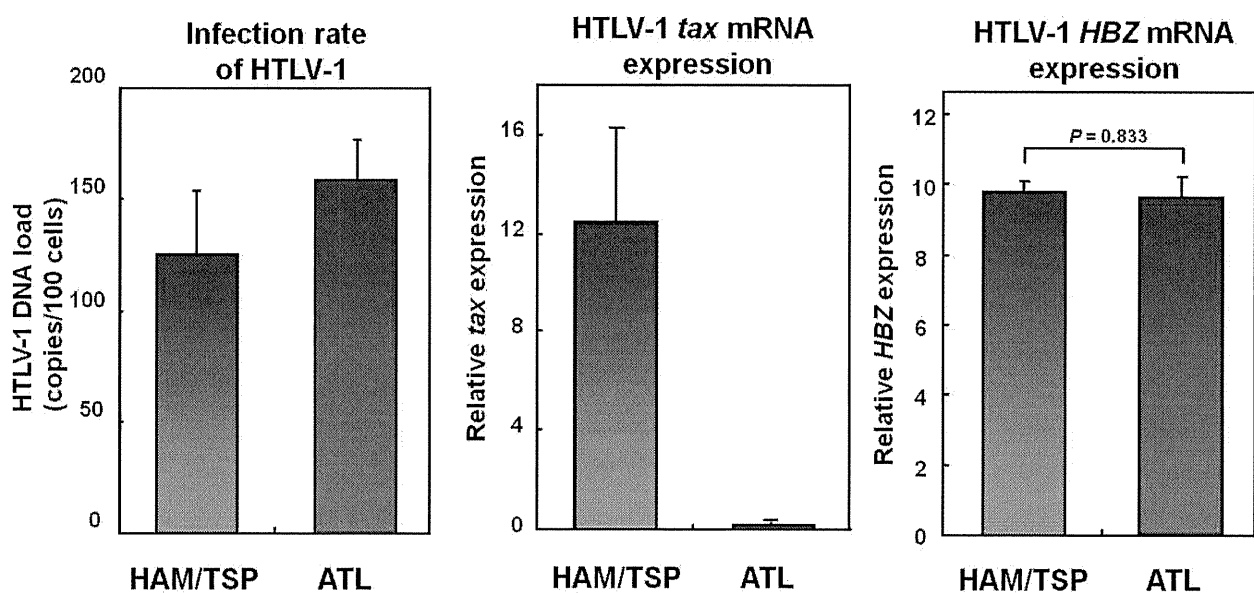


To determine whether HTLV-1 expression contributes to the differential fate of HTLV-1-infected CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells between HAM/TSP and ATL patients, differences in the HTLV-1 proviral load and the HTLV-1 *tax* mRNA and HTLV-1 *HBZ* mRNA expression of these populations were analyzed (Figure 6). Although HTLV-1 *tax* mRNA expression in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells was found to be significantly higher in HAM/TSP patients than in ATL patients, HTLV-1 proviral DNA loads and *HBZ* mRNA expression levels were found to be equivalent in the two groups [54] (Figure 6). This high HTLV-1 Tax expression in HAM/TSP CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells (Foxp3<sup>-</sup>) and low HTLV-1 Tax expression in ATL CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells (Foxp3<sup>+</sup>) suggests that intracellular HTLV-1 expression may act as a “switch” that directs T cell plasticity from Foxp3<sup>+</sup> Treg cells to IFN-γ<sup>+</sup>Foxp3<sup>-</sup> T cells. Indeed, a recent report highlighted that loss of Foxp3 in Treg cells and acquisition of IFN-γ may result in conversion of suppressor T cells into highly autoaggressive lymphocytes (exFoxp3<sup>+</sup> cells), which can contribute to the development of autoimmune conditions [62,63]. These findings support the hypothesis that HTLV-1 *tax* may be one of the exogenous retrovirus genes responsible for immune dysregulation through its interference in the equilibrium between inflammation and tolerance.



This hypothesis is currently being tested as a means of elucidating the precise molecular mechanisms by which HTLV-1 influences the fate and function of CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells, especially Foxp3<sup>+</sup> Treg cells. Further research investigating this hypothesis using animal models is required, as is further work to pathologically identify the exFoxp3<sup>+</sup> cells in the spinal cord lesions of HAM/TSP patients.

**Figure 6.** Increased HTLV-1 *tax* mRNA expression in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells in HAM/TSP patients. The HTLV-1 proviral load in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells from HAM/TSP and ATL patients was quantified by real-time PCR (left panel, n = 3). Expression levels of HTLV-1 *tax* mRNA (center panel, HAM/TSP: n = 4, ATL: n = 3) and *HBZ* mRNA (right panel, n = 5) in CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cells from HAM/TSP and ATL patients were quantified by real-time RT-PCR. Data are presented as mean ± standard error.



## 7. Mechanisms Underlying Increased HTLV-1 Tax Expression in HAM/TSP Patients

As described above, higher levels of HTLV-1 Tax expression have been observed in HAM/TSP patients [11], and a correlation between Tax expression and disease risk [64] has been identified. Both findings, together with experimental evidence [65] and theoretical justification [66] for selective proliferation of HTLV-1 expressing T cells *in vivo*, indicate that increased HTLV-1 provirus expression may play an important role in the pathogenesis of HAM/TSP. However, the molecular mechanisms underlying the increased levels of HTLV-1 provirus expression in HAM/TSP patients are not understood. Evidence continues to accumulate that the genomic integration site of HTLV-1 provirus affects the level of provirus expression. Continued accumulation of evidence is aided by the availability of the human genome sequence, which has enabled large-scale research into HTLV-1 integration sites. This research has demonstrated that the provirus integration sites of HTLV-1 *in vivo* are not randomly distributed within the human genome but rather associated with transcriptionally active regions [67,68]; that the frequent integration into these transcription units is associated with increased levels of provirus expression; and, importantly, that the increased number of integration sites in

transcription units is associated with HAM/TSP [68]. Future research should endeavor to elucidate the mechanisms underlying the immune dysregulation observed in HAM/TSP patients.

## 8. Conclusion

HTLV-1 initiates persistent infection of CD4<sup>+</sup> T cells and results in the development of HAM/TSP, a chronic neuroinflammatory disorder characterized by very high strong cellular and humoral immune responses. Because a higher viral load in HTLV-1-infected individuals increases the risk of HAM/TSP and is associated with high cellular and humoral immune responses, HTLV-1 infection-induced immune dysregulation may play an important role in the development and pathogenesis of this disease. The recent discovery of Treg cells has provided new opportunities for and generated increased interest in elucidating the mechanisms underlying the induction of immune activation by HTLV-1-infected T cells. Among the CD4<sup>+</sup> T helper cell populations that play a central role in adaptive immune responses, the CD4<sup>+</sup>CD25<sup>+</sup>CCR4<sup>+</sup> T cell population, which primarily consists of suppressive T cell subsets, such as the Treg and Th2 subsets, in healthy individuals, is the predominant viral reservoir of HTLV-1 in both ATL and HAM/TSP patients. Interestingly, cells of this T cell subset become Th1-like cells, overproducing IFN- $\gamma$  in HAM/TSP patients, while leukemogenesis develops and maintains the Foxp3<sup>+</sup> Treg phenotype in ATL patients. These results indicate that HTLV-1 may intracellularly induce T cell plasticity from Treg to IFN- $\gamma$ <sup>+</sup> T cells, which may contribute to the development of HAM/TSP. As such, these results support the hypothesis that HTLV-1 is one of the exogenous retrovirus genes responsible for immune dysregulation through its interference in the equilibrium maintained among host immune responses. Because the majority of immune disorders are of unknown etiology, the discovery of HTLV-1 and its association with inflammatory conditions has greatly enhanced our understanding of the pathogenic mechanisms underlying organ-specific immune disorders. Further investigation of the mechanism underlying HTLV-1 action in the immune system may result in identification of new molecular pathways that will further elucidate the basic mechanisms underlying immune-mediated disorders.

## Conflict of interest

The authors declare no conflicts of interest.

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## References and Notes

1. Birman, B.M.; Breen, E.C.; Stuver, S.; Cranston, B.; Martinez-Maza, O.; Falk, K.I.; Okayama, A.; Hanchard, B.; Mueller, N.; Hisada, M. Population differences in immune marker profiles associated with human T-lymphotropic virus type I infection in Japan and Jamaica. *Int. J. Cancer* **2009**, *124*, 614–621.
2. Uchiyama, T.; Yodoi, J.; Sagawa, K.; Takatsuki, K.; Uchino, H. Adult T-cell leukemia: Clinical and hematologic features of 16 cases. *Blood* **1977**, *50*, 481–492.
3. Gessain, A.; Barin, F.; Vernant, J.C.; Gout, O.; Maurs, L.; Calender, A.; de The, G. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* **1985**, *2*, 407–410.
4. Osame, M.; Usuku, K.; Izumo, S.; Ijichi, N.; Amitani, H.; Igata, A.; Matsumoto, M.; Tara, M. HTLV-I associated myelopathy, a new clinical entity. *Lancet* **1986**, *1*, 1031–1032.
5. Mochizuki, M.; Watanabe, T.; Yamaguchi, K.; Yoshimura, K.; Nakashima, S.; Shirao, M.; Araki, S.; Takatsuki, K.; Mori, S.; Miyata, N. Uveitis associated with human T-cell lymphotropic virus type I. *Am. J. Ophthalmol.* **1992**, *114*, 123–129.
6. Eguchi, K.; Matsuoka, N.; Ida, H.; Nakashima, M.; Sakai, M.; Sakito, S.; Kawakami, A.; Terada, K.; Shimada, H.; Kawabe, Y.; *et al.* Primary Sjogren's syndrome with antibodies to HTLV-I: Clinical and laboratory features. *Ann. Rheum. Dis.* **1992**, *51*, 769–776.
7. Nishioka, K.; Maruyama, I.; Sato, K.; Kitajima, I.; Nakajima, Y.; Osame, M. Chronic inflammatory arthropathy associated with HTLV-I. *Lancet* **1989**, *1*, 441.
8. Morgan, O.S.; Rodgers-Johnson, P.; Mora, C.; Char, G. HTLV-1 and polymyositis in Jamaica. *Lancet* **1989**, *2*, 1184–1187.
9. Nakagawa, M.; Izumo, S.; Ijichi, S.; Kubota, H.; Arimura, K.; Kawabata, M.; Osame, M. HTLV-I-associated myelopathy: Analysis of 213 patients based on clinical features and laboratory findings. *J. Neurovirol.* **1995**, *1*, 50–61.
10. Nagai, M.; Usuku, K.; Matsumoto, W.; Kodama, D.; Takenouchi, N.; Moritoyo, T.; Hashiguchi, S.; Ichinose, M.; Bangham, C.R.; Izumo, S.; *et al.* Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: High proviral load strongly predisposes to HAM/TSP. *J. Neurovirol.* **1998**, *4*, 586–593.
11. Yamano, Y.; Nagai, M.; Brennan, M.; Mora, C.A.; Soldan, S.S.; Tomaru, U.; Takenouchi, N.; Izumo, S.; Osame, M.; Jacobson, S. Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). *Blood* **2002**, *99*, 88–94.
12. Nagai, M.; Yamano, Y.; Brennan, M.B.; Mora, C.A.; Jacobson, S. Increased HTLV-I proviral load and preferential expansion of HTLV-I Tax-specific CD8+ T cells in cerebrospinal fluid from patients with HAM/TSP. *Ann. Neurol.* **2001**, *50*, 807–812.
13. Nagai, M.; Kubota, R.; Gretten, T.F.; Schneck, J.P.; Leist, T.P.; Jacobson, S. Increased activated human T cell lymphotropic virus type I (HTLV-I) Tax11-19-specific memory and effector CD8+ cells in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis: Correlation with HTLV-I provirus load. *J. Infect. Dis.* **2001**, *183*, 197–205.

14. Jacobson, S.; Shida, H.; McFarlin, D.E.; Fauci, A.S.; Koenig, S. Circulating CD8<sup>+</sup> cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. *Nature* **1990**, *348*, 245–248.
15. Kubota, R.; Kawanishi, T.; Matsubara, H.; Manns, A.; Jacobson, S. HTLV-I specific IFN-gamma<sup>+</sup> CD8<sup>+</sup> lymphocytes correlate with the proviral load in peripheral blood of infected individuals. *J. Neuroimmunol.* **2000**, *102*, 208–215.
16. Hanon, E.; Goon, P.; Taylor, G.P.; Hasegawa, H.; Tanaka, Y.; Weber, J.N.; Bangham, C.R. High production of interferon gamma but not interleukin-2 by human T-lymphotropic virus type I-infected peripheral blood mononuclear cells. *Blood* **2001**, *98*, 721–726.
17. Vine, A.M.; Heaps, A.G.; Kaftantzi, L.; Mosley, A.; Asquith, B.; Witkover, A.; Thompson, G.; Saito, M.; Goon, P.K.; Carr, L.; *et al.* The role of CTLs in persistent viral infection: Cytolytic gene expression in CD8<sup>+</sup> lymphocytes distinguishes between individuals with a high or low proviral load of human T cell lymphotropic virus type 1. *J. Immunol.* **2004**, *173*, 5121–5129.
18. Iwakura, Y.; Tosu, M.; Yoshida, E.; Takiguchi, M.; Sato, K.; Kitajima, I.; Nishioka, K.; Yamamoto, K.; Takeda, T.; Hatanaka, M.; *et al.* Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I. *Science* **1991**, *253*, 1026–1028.
19. Nakamaru, Y.; Ishizu, A.; Ikeda, H.; Sugaya, T.; Fugo, K.; Higuchi, M.; Yamazaki, H.; Yoshiki, T. Immunological hyperresponsiveness in HTLV-I LTR-env-pX transgenic rats: A prototype animal model for collagen vascular and HTLV-I-related inflammatory diseases. *Pathobiology* **2001**, *69*, 11–18.
20. Aono, H.; Fujisawa, K.; Hasunuma, T.; Marriott, S.J.; Nishioka, K. Extracellular human T cell leukemia virus type I tax protein stimulates the proliferation of human synovial cells. *Arthritis Rheum.* **1998**, *41*, 1995–2003.
21. Siekevitz, M.; Feinberg, M.B.; Holbrook, N.; Wong-Staal, F.; Greene, W.C. Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type I. *Proc. Natl. Acad. Sci. U. S. A.* **1987**, *84*, 5389–5393.
22. Cross, S.L.; Feinberg, M.B.; Wolf, J.B.; Holbrook, N.J.; Wong-Staal, F.; Leonard, W.J. Regulation of the human interleukin-2 receptor alpha chain promoter: Activation of a nonfunctional promoter by the transactivator gene of HTLV-I. *Cell* **1987**, *49*, 47–56.
23. Azimi, N.; Brown, K.; Bamford, R.N.; Tagaya, Y.; Siebenlist, U.; Waldmann, T.A. Human T cell lymphotropic virus type I Tax protein trans-activates interleukin 15 gene transcription through an NF-kappaB site. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 2452–2457.
24. Mariner, J.M.; Lantz, V.; Waldmann, T.A.; Azimi, N. Human T cell lymphotropic virus type I Tax activates IL-15R alpha gene expression through an NF-kappa B site. *J. Immunol.* **2001**, *166*, 2602–2609.
25. Waldmann, T.A. The biology of interleukin-2 and interleukin-15: Implications for cancer therapy and vaccine design. *Nat. Rev. Immunol.* **2006**, *6*, 595–601.
26. Sakaguchi, S.; Sakaguchi, N.; Asano, M.; Itoh, M.; Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **1995**, *155*, 1151–1164.

27. Hori, S.; Nomura, T.; Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **2003**, *299*, 1057–1061.
28. Sakaguchi, S.; Yamaguchi, T.; Nomura, T.; Ono, M. Regulatory T cells and immune tolerance. *Cell* **2008**, *133*, 775–787.
29. Yamano, Y.; Cohen, C.J.; Takenouchi, N.; Yao, K.; Tomaru, U.; Li, H.C.; Reiter, Y.; Jacobson, S. Increased expression of human T lymphocyte virus type I (HTLV-I) Tax11-19 peptide-human histocompatibility leukocyte antigen A\*201 complexes on CD4+ CD25+ T Cells detected by peptide-specific, major histocompatibility complex-restricted antibodies in patients with HTLV-I-associated neurologic disease. *J. Exp. Med.* **2004**, *199*, 1367–1377.
30. Yamano, Y.; Takenouchi, N.; Li, H.C.; Tomaru, U.; Yao, K.; Grant, C.W.; Maric, D.A.; Jacobson, S. Virus-induced dysfunction of CD4+CD25+ T cells in patients with HTLV-I-associated neuroimmunological disease. *J. Clin. Invest.* **2005**, *115*, 1361–1368.
31. Oh, U.; Grant, C.; Griffith, C.; Fugo, K.; Takenouchi, N.; Jacobson, S. Reduced Foxp3 protein expression is associated with inflammatory disease during human t lymphotropic virus type 1 Infection. *J. Infect. Dis.* **2006**, *193*, 1557–1566.
32. Michaelsson, J.; Barbosa, H.M.; Jordan, K.A.; Chapman, J.M.; Brunialti, M.K.; Neto, W.K.; Nukui, Y.; Sabino, E.C.; Chieia, M.A.; Oliveira, A.S.; *et al.* The frequency of CD127low expressing CD4+CD25high T regulatory cells is inversely correlated with human T lymphotropic virus type-1 (HTLV-1) proviral load in HTLV-1-infection and HTLV-1-associated myelopathy/tropical spastic paraparesis. *BMC Immunol.* **2008**, *9*, 41.
33. Hayashi, D.; Kubota, R.; Takenouchi, N.; Tanaka, Y.; Hirano, R.; Takashima, H.; Osame, M.; Izumo, S.; Arimura, K. Reduced Foxp3 expression with increased cytomegalovirus-specific CTL in HTLV-I-associated myelopathy. *J. Neuroimmunol.* **2008**, *200*, 115–124.
34. Ramirez, J.M.; Brembilla, B.N.; Sorg, O.; Chicheportiche, R.; Matthes, T.; Dayer, J.M.; Saurat, J.H.; Roosnek, E.; Chizzolini, C. Activation of the aryl hydrocarbon receptor reveals distinct requirements for IL-22 and IL-17 production by human T helper cells. *Eur. J. Immunol.* **2010**, *40*, 2450–2459.
35. Grant, C.; Oh, U.; Yao, K.; Yamano, Y.; Jacobson, S. Dysregulation of TGF-beta signaling and regulatory and effector T-cell function in virus-induced neuroinflammatory disease. *Blood* **2008**, *111*, 5601–5609.
36. Ohsugi, E.; KUmazaka, T. Low CD4/CD8 T-cell ratio associated with inflammatory arthropathy in human T-cell leukemia virus type I Tax transgenic mice. *PLoS ONE* **2011**, *6*, e18518.
37. Saito, M.; Matsuzaki, T.; Satou, Y.; Yasunaga, J.; Saito, K.; Arimura, K.; Matsuoka, M.; Ohara, Y. *In vivo* expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). *Retrovirology* **2009**, *6*, 19.
38. Satou, Y.; Yasunaga, J.; Zhao, T.; Yoshida, M.; Miyazato, P.; Takai, K.; Shimizu, K.; Ohshima, K.; Green, P.L.; Ohkura, N.; *et al.* HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation *in vivo*. *PLoS Pathog.* **2011**, *7*, e1001274.
39. Karube, K.; Ohshima, K.; Tsuchiya, T.; Yamaguchi, T.; Kawano, R.; Suzumiya, J.; Utsunomiya, A.; Harada, M.; Kikuchi, M. Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. *Br. J. Haematol.* **2004**, *126*, 81–84.

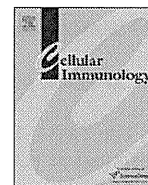
40. Roncador, G.; Garcia, J.F.; Maestre, L.; Lucas, E.; Menarguez, J.; Ohshima, K.; Nakamura, S.; Banham, A.H.; Piris, M.A. FOXP3, a selective marker for a subset of adult T-cell leukaemia/lymphoma. *Leukemia* **2005**, *19*, 2247–2253.
41. Kohno, T.; Yamada, Y.; Akamatsu, N.; Kamihira, S.; Imaizumi, Y.; Tomonaga, M.; Matsuyama, T. Possible origin of adult T-cell leukemia/lymphoma cells from human T lymphotropic virus type-1-infected regulatory T cells. *Cancer Sci.* **2005**, *96*, 527–533.
42. Chen, S.; Ishii, N.; Ine, S.; Ikeda, S.; Fujimura, T.; Ndhlovu, L.C.; Soroosh, P.; Tada, K.; Harigae, H.; Kameoka, J.; *et al.* Regulatory T cell-like activity of Foxp3+ adult T cell leukemia cells. *Int. Immunol.* **2006**, *18*, 269–277.
43. Matsubar, Y.; Hori, T.; Morita, R.; Sakaguchi, S.; Uchiyama, T. Delineation of immunoregulatory properties of adult T-cell leukemia cells. *Int. J. Hematol.* **2006**, *84*, 63–69.
44. Shimauchi, T.; Kabashima, K.; Tokura, Y. Adult T-cell leukemia/lymphoma cells from blood and skin tumors express cytotoxic T lymphocyte-associated antigen-4 and Foxp3 but lack suppressor activity toward autologous CD8+ T cells. *Cancer Sci.* **2008**, *99*, 98–106.
45. Koyanagi, Y.; Itoyama, Y.; Nakamura, N.; Takamatsu, K.; Kira, J.; Iwamasa, T.; Goto, I.; Yamamoto, N. *In vivo* infection of human T-cell leukemia virus type I in non-T cells. *Virology* **1993**, *196*, 25–33.
46. Nagai, M.; Brennan, M.B.; Sakai, J.A.; Mora, C.A.; Jacobson, S. CD8(+) T cells are an *in vivo* reservoir for human T-cell lymphotropic virus type I. *Blood* **2001**, *98*, 1858–1861.
47. Jones, K.S.; Petrow-Sadowski, C.; Huang, Y.K.; Bertolette, D.C.; Ruscetti, F.W. Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4(+) T cells. *Nat. Med.* **2008**, *14*, 429–436.
48. Enose-Akahata, Y.; Oh, U.; Grant, C.; Jacobson, S. Retrovirally induced CTL degranulation mediated by IL-15 expression and infection of mononuclear phagocytes in patients with HTLV-I-associated neurologic disease. *Blood* **2008**, *112*, 2400–2410.
49. Azakami, K.; Sato, T.; Araya, N.; Utsunomiya, A.; Kubota, R.; Suzuki, K.; Hasegawa, D.; Izumi, T.; Fujita, H.; Aratani, S.; *et al.* Severe loss of invariant NKT cells exhibiting anti-HTLV-1 activity in patients with HTLV-1-associated disorders. *Blood* **2009**, *114*, 3208–3215.
50. Richardson, J.H.; Edwards, A.J.; Cruickshank, J.K.; Rudge, P.; Dalgleish, A.G. *In vivo* cellular tropism of human T-cell leukemia virus type 1. *J. Virol.* **1990**, *64*, 5682–5687.
51. Yoshie, O.; Fujisawa, R.; Nakayama, T.; Harasawa, H.; Tago, H.; Izawa, D.; Hieshima, K.; Tatsumi, Y.; Matsushima, K.; Hasegawa, H.; *et al.* Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood* **2002**, *99*, 1505–1511.
52. Imai, T.; Nagira, M.; Takagi, S.; Kakizaki, M.; Nishimura, M.; Wang, J.; Gray, P.W.; Matsushima, K.; Yoshie, O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.* **1999**, *11*, 81–88.
53. Iellem, A.; Mariani, M.; Lang, R.; Recalde, H.; Panina-Bordignon, P.; Sinigaglia, F.; D'Ambrosio, D. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J. Exp. Med.* **2001**, *194*, 847–853.

54. Yamano, Y.; Araya, N.; Sato, T.; Utsunomiya, A.; Azakami, K.; Hasegawa, D.; Izumi, T.; Fujita, H.; Aratani, S.; Yagishita, N.; *et al.* Abnormally high levels of virus-infected IFN-gamma+ CCR4+ CD4+ CD25+ T cells in a retrovirus-associated neuroinflammatory disorder. *PLoS ONE* **2009**, *4*, e6517.
55. Miyara, M.; Yoshioka, Y.; Kitoh, A.; Shima, T.; Wing, K.; Niwa, A.; Parizot, C.; Taflin, C.; Heike, T.; Valeyre, D.; *et al.* Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* **2009**, *30*, 899–911.
56. Yoshie, O. Expression of CCR4 in adult T-cell leukemia. *Leuk. Lymphoma* **2005**, *46*, 185–190.
57. Kannagi, M. Immunologic control of human T-cell leukemia virus type I and adult T-cell leukemia. *Int. J. Hematol.* **2007**, *86*, 113–117.
58. Matsuoka, M. Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL). *Retrovirology* **2005**, *2*, 27.
59. Kubota, R.; Nagai, M.; Kawanishi, T.; Osame, M.; Jacobson, S. Increased HTLV type 1 tax specific CD8+ cells in HTLV type 1-associated myelopathy/tropical spastic paraparesis: Correlation with HTLV type 1 proviral load. *AIDS Res. Hum. Retroviruses* **2000**, *16*, 1705–1709.
60. Furuya, T.; Nakamura, T.; Fujimoto, T.; Nakane, S.; Kambara, C.; Shirabe, S.; Hamasaki, S.; Motomura, M.; Eguchi, K. Elevated levels of interleukin-12 and interferon-gamma in patients with human T lymphotropic virus type I-associated myelopathy. *J. Neuroimmunol.* **1999**, *95*, 185–189.
61. Toulza, F.; Heaps, A.; Tanaka, Y.; Taylor, G.P.; Bangham, C.R. High frequency of CD4+FoxP3+ cells in HTLV-1 infection: Inverse correlation with HTLV-1-specific CTL response. *Blood* **2008**, *111*, 5047–5053.
62. Zhou, X.; Bailey-Bucktrout, S.L.; Jeker, L.T.; Penaranda, C.; Martinez-Llordella, M.; Ashby, M.; Nakayama, M.; Rosenthal, W.; Bluestone, J.A. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells *in vivo*. *Nat. Immunol.* **2009**, *10*, 1000–1007.
63. Tsuji, M.; Komatsu, N.; Kawamoto, S.; Suzuki, K.; Kanagawa, O.; Honjo, T.; Hori, S.; Fagarasan, S. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* **2009**, *323*, 1488–1492.
64. Asquith, B.; Mosley, A.J.; Heaps, A.; Tanaka, Y.; Taylor, G.P.; McLean, A.R.; Bangham, C.R. Quantification of the virus-host interaction in human T lymphotropic virus I infection. *Retrovirology* **2005**, *2*, 75.
65. Asquith, B.; Zhang, Y.; Mosley, A.J.; de Lara, C.M.; Wallace, D.L.; Worth, A.; Kaftantzi, L.; Meekings, K.; Griffin, G.E.; Tanaka, Y.; *et al.* *In vivo* T lymphocyte dynamics in humans and the impact of human T-lymphotropic virus 1 infection. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 8035–8040.
66. Asquith, B.; Bangham, C.R. How does HTLV-I persist despite a strong cell-mediated immune response? *Trends Immunol.* **2008**, *29*, 4–11.
67. Derse, D.; Crise, B.; Li, Y.; Princler, G.; Lum, N.; Stewart, C.; McGrath, C.F.; Hughes, S.H.; Munroe, D.J.; Wu, X. Human T-cell leukemia virus type 1 integration target sites in the human genome: Comparison with those of other retroviruses. *J. Virol.* **2007**, *81*, 6731–6741.

68. Meekings, K.N.; Leipzig, J.; Bushman, F.D.; Taylor, G.P.; Bangham, C.R. HTLV-1 integration into transcriptionally active genomic regions is associated with proviral expression and with HAM/TSP. *PLoS Pathog.* **2008**, *4*, e1000027.

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## Advantage of higher-avidity CTL specific for Tax against human T-lymphotropic virus-1 infected cells and tumors

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### ABSTRACT

Strong CTL response can be observed and associated with the control of proviral load in human T-lymphotropic virus type 1 (HTLV-1) infection. However, there are few details with regard to how HTLV-1 specific CTLs work against HTLV-1 infected cells and adult T-cell leukemia cells (ATLs). In this study, using Tax-specific CTL lines with high- and low-functional avidity developed from HLA-A2-transgenic mice, we showed that higher avidity CTLs specific for Tax expressing larger numbers of TCRs and better binding strength to the antigen-HLA-A2 complex are much more efficient at eliminating HTLV-1 infected cells and, in particular, ATL tumor cells with the ability of recognizing a latent level of Tax product detected only with a real-time PCR. These findings suggest that such higher avidity CTLs specific for Tax in HTLV-1 could be responsible for preventing the development of HTLV-1 infection by detecting trace amount of antigens.

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### 1. Introduction

The human T-lymphotropic virus type 1 (HTLV-1) causes two distinct types of disease: a CD4<sup>+</sup> T cell malignancy known as adult T cell leukemia (ATL) [1,2] and a range of inflammatory disease, of which HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the best recognized and most widely studied [3,4]. In patients with HTLV-1 infection, the proviral load of HTLV-1 is usually stable over time [5]. However, the factors determining the set point of proviral load in each person remain to be elucidated. In particular, CTLs are active in individuals with low proviral load, in whom immunosurveillance could be more effective [6,7]. Several studies have reported that high-levels of HTLV-1-specific CTL activity can be observed in HAM/TSP patients and some asymptomatic HTLV-1 carriers, while ATL patients apparently lack HTLV-1-specific CTL activity, although it can be sporadically induced during the remission stages or after mitogenic stimulation with multiple in vitro antigenic stimulations of peripheral blood mononuclear cells [8,9]. One of the major target antigens by HTLV-1-specific CTLs in human is Tax protein [10,11], which is a

molecule responsible for T-cell immortalization [12,13]. CTLs induced in ATL patients in remission are able to lyse autologous tumor cells in vitro [14]. These observations suggest that HTLV-1-specific CTLs could play a critical role in host immunosurveillance against ATLs.

While the number of HTLV-1-specific CTLs elicited is unquestionably important [7], recent studies have identified an additional parameter, functional avidity, as critical in determining the efficiency of viral clearance [15–18]. T-cell avidity is a measure of the sensitivity of T cells recognizing a cognate antigen. High-avidity CTLs are those that can recognize antigen-presenting cells (APCs) bearing very low levels of peptide-major histocompatibility complex (MHC) antigen, whereas low-avidity CTLs require much more peptide-MHC antigen to be activated or to exert effector function [15,19–21].

In this study, in order to clarify whether Tax-specific CTLs with higher avidity are critical as a deterrent to control the proliferation of ATL and the expansion of HTLV-1 infection, we developed two CTL lines specific for Tax11-19 antigen having high- and low-avidity from HLA-A2 transgenic mice in vitro. Using these CTLs, we demonstrate not only that Tax product is a critical antigen but also in particular that the specific CTLs with higher avidity for Tax11-19 have a selective advantage on recognition of human ATLs and HTLV-1 infected cells compared with those with low avidity in vitro.

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## 2. Materials and methods

### 2.1. Synthetic peptides

The Tax11-19 peptide, LLFGYPVYV, was purchased from Asahi Technoglass (Chiba, Japan) and used as an HLA-A2-restricted CTL antigen [11].

### 2.2. Cells

C1RAAD cell line (HMYC1R transfected with HLA chimeric molecule containing  $\alpha 1$  and  $\alpha 2$  domains from human HLA-A2.1 and  $\alpha 3$  from mouse H-2D<sup>d</sup>) was described previously [22]. Cell lines were maintained in culture medium (CTM; 1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid (EHAA)) containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 4 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

HTLV-1-infected human ATL cell lines, KK-1 and KOB, were derived from the peripheral blood and ascites of ATL patients, respectively [23,24]. Human IL-2 dependent T cell line (HCT-4) was derived from the cerebrospinal fluid of a HAM/TSP patient [25]. KK-1, KOB, and HCT-4 were used as a target. Cells were maintained in CTM with 100 units/ml of recombinant human IL-2 (Imunace<sup>®</sup>35, Shionogi, Osaka, Japan).

### 2.3. Mice

Transgenic HHD-2 mice (gift from Dr. François Lemonnier, Institute Pasteur, Paris, France) were bred in our colony at the Institute of the Experimental Animals at St. Marianna University. HHD-2 mice are characterized by knock-out of the murine  $\beta_2$ -microglobulin gene, as well as murine H-2D<sup>b</sup>, transgenic expression of human HLA-A2.1 with a covalently-linked human  $\beta_2$ -microglobulin and a murine D<sup>b</sup>-derived  $\alpha 3$  domain to allow interaction with mouse CD8 [26]. All animal studies were approved by the Institute of Experimental animals at St. Marianna University.

### 2.4. Binding assay

Peptide binding to HLA-A2 molecules was measured using T2 mutant cell lines as described previously [27,28]. T2 cells ( $3 \times 10^5$ /well) were incubated overnight in 96-well plates with culture medium (a 1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid (EHAA) containing 2% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin) with 10  $\mu$ g/ml human  $\beta_2$ -microglobulin (Sigma-Aldrich, St. Louis, MO) and different peptide concentration. On the following day, cells were washed at  $190 \times g$  for 5 min twice with cold PBS containing 2% FBS and incubated for 30 min at 4 °C with anti-HLA-A2.1 BB7.2 mAb (1/100 dilution of hybridoma supernatant) and 5  $\mu$ g/ml FITC-labeled goat anti-mouse Ig (BD Pharmingen, San Diego, CA). Cells were washed twice after each incubation; subsequently, HLA-A2.1 expression was measured by flow cytometry (FACScan; BD Biosciences, Mountain View, CA). HLA-A2.1 expression was quantified as fluorescence index (FI) according to the formula: FI = ((geometric mean fluorescence with peptide – geometric mean fluorescence without peptide)/geometric mean fluorescence without peptide). FI<sub>0.5</sub> is the concentration required to give an FI of 0.5, meaning a 50% increase in HLA-A2 on the cell surface. Background fluorescence without BB7.2 was subtracted for each individual value. To compare the different peptides, FI<sub>0.5</sub> was calculated from the titration curve for each peptide. Each sample was tested in triplicate. Values were expressed as mean in triplicate.

### 2.5. CTL generation in HHD-2 transgenic mice

The method for generating antigenic peptide-specific CTL lines from HHD mice was described previously [28,29]. Mice aged more than 8 weeks were immunized subcutaneously in the base of the tail with 100  $\mu$ l of an emulsion containing 1:1 incomplete Freund's adjuvant (IFA), antigenic CTL peptide and cytokines (50 nmol Tax (11-19) peptide, 25 nmol HBV core 128–140 helper epitope, 3  $\mu$ g of rmlL-12 and 3  $\mu$ g of rmGM-CSF). Mice were boosted 2 weeks later, with the spleens removed 10–14 days after the boost. Immune spleen cells ( $2.5 \times 10^6$ /well) were stimulated in 24-well plates with autologous spleen cells ( $5 \times 10^6$ /well) pulsed for 30 min with 10  $\mu$ M Tax11-19 peptide for the development of low-avidity CTL lines (LCTL) or with 10 nM for high-avidity CTL lines (HCTL) in CTM supplemented with 10% T-stim<sup>®</sup> (Collaborative Biochemical Products, Bedford, MA). Following a minimum of four in vitro stimulations with the peptide-pulsed syngeneic spleen cells, two CTL lines were maintained by weekly restimulation with  $1 \times 10^6$  cells/well with  $4 \times 10^6$  peptide-pulsed mitomycin C-treated syngeneic spleen cells as feeders.

### 2.6. Cytotoxic assay

CTL activity was measured with <sup>51</sup>Cr-labeled target cells. Target cells ( $1 \times 10^6$ ) were pulsed in 100  $\mu$ l of 150  $\mu$ Ci <sup>51</sup>Cr for 1 h and were washed three times, with 5000 cells/well then added to 96-well round-bottom plates containing different peptide concentrations. Effector cells were introduced followed by additional incubation. Supernatants were then harvested and analyzed. The percentage of specific <sup>51</sup>Cr release was calculated as  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . Spontaneous release was determined from target cells that had been incubated in the absence of effector cells, while maximum release was determined in the presence of 2% TRITON<sup>®</sup> X-100 Detergent (CALBIOCHEM, La Jolla, CA). Each sample was tested in triplicate. Values were expressed as means  $\pm$  SEM of triplicates.

### 2.7. IFN- $\gamma$ ELISA assay

IFN- $\gamma$  in the culture supernatant harvested at 24 h was determined using an ELISA kit (R&D, Minneapolis, MN) according to the manufacturer's instructions. All samples were analyzed in triplicate. Values were expressed as means  $\pm$  SEM of triplicates.

### 2.8. TCR V $\beta$ screenings of CTLs

We assessed a V $\beta$  usage pattern between HCTL and LCTL using V $\beta$  TCR screening kit by a flow cytometry analysis (BD Bioscience Pharmingen, San Diego, CA).

### 2.9. Flow cytometry

We used a PE-Tax11-19/HLA-A\*0201 tetramer-LLFGYPVYV (Medical & Biological Laboratories, Nagoya, Japan) and PE-hamster anti-mouse CD3 $\epsilon$  Ab (145-2C11, BD Bioscience Pharmingen, San Diego, CA). Cells were centrifuged and washed twice with PBS containing 0.5% BSA, and then resuspended in 1% BSA/PBS. Cells were incubated 40 min at 4 °C with the antibody and then washed three times. The tetramer and anti CD3 $\epsilon$  Ab were titrated for staining simultaneously.

In order to compare the affinity of T cell receptor between HCTLs and LCTLs, indexes were calculated using the following two equations: ratio of geometric mean (RGM) = (geometric mean using tetramer or anti-CD3 $\epsilon$  Ab)/(geometric mean using control Ab). Each sample was tested in triplicate.

## 2.10. Western blotting

KK-1, KOB, and HCT-4 were lysed using standard lysis buffer (20 mM Tris-HCl, 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, and protease inhibitor cocktail (Roche, Mannheim, Germany)). Lysates were stored at -80 °C until use. Protein concentration was determined using the Bradford method (Bio-Rad protein assay reagent; Bio-Rad laboratories, Hercules, CA). Equal amounts (30 µg) of protein were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Following the transfer, membranes were blocked with Difco Skim milk (BD Bioscience, San Diego, CA) overnight at 4 °C. The working concentrations of the first Abs were 1 µg/ml for anti-Tax Ab (Lt-4) [30] and anti murine β-actin Ab (SIGMA, St. Louis, MO), and 1:10,000 for HRP-conjugated anti-mouse IgG Ab (SIGMA, St. Louis, MO). The membrane was washed, and was reacted with the appropriate second antibody. Finally, signals were visualized using the extended cavity laser (ECL) system (GE Healthcare Bio-sciences KK, Tokyo, Japan).

## 2.11. Real-time reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from cells using TRIzol<sup>®</sup> Reagent (Invitrogen, Carisbad, CA). First-stand cDNA was synthesized with random hexamers and reverse transcriptase (ReverTraAce; Toyobo, Japan) using 1 µg of total RNA in a reaction volume of 20 µl. Real-time PCR reactions were carried out using TaqMan<sup>®</sup> Universal Master Mix (Applied Biosystems, Carisbad, CA). ABI Prism 7500 SDS was programmed to an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers and probe for detecting the HTLV-1 Tax or GAPDH mRNA were used as described previously [31]. Relative quantification of mRNA was performed using the comparative threshold cycle method with GAPDH as an endogenous control. For each sample, target gene expression was normalized against the expression of GAPDH. To determine relative expression levels, the following formula was used: target gene expression =  $2^{-\text{Ct}(\text{target}) - \text{Ct}(\text{GAPDH})}$ . Each sample was tested in triplicate. Values were expressed as means ± SEM of triplicates.

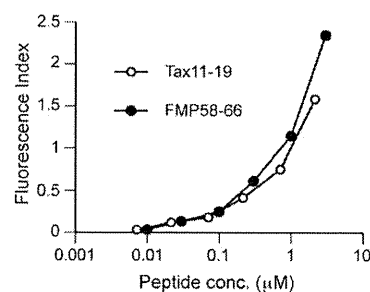
## 3. Results

### 3.1. Binding affinity of Tax11-19 for HLA-A2 molecule

Before attempting to develop Tax-specific CTL lines from HLA-A2 transgenic HHD mice, we evaluated the binding affinity of Tax11-19 peptide by T2 binding assay, which measures the cell surface stabilization of HLA-A2 molecules. Tax11-19 peptide displayed a binding capacity for the HLA-A2 molecule that was nearly equal to that of the positive control, the highly antigenic influenza virus matrix peptide (FMP58-66) [32] ( $\text{FI}_{0.5} = 0.329$  for Tax11-19,  $0.284 \mu\text{M}$  for FMP58-66) (Fig. 1). These data suggest that Tax11-19 would be a very strong antigenic peptide restricted to the HLA-A2 molecule.

### 3.2. Recognition of Tax11-19 peptide by CTL lines of different avidity

Based on the observation that Tax11-19 showed strong antigenicity inducing specific CTLs, we next attempted to develop low-avidity CTLs (LCTL) and high-avidity CTLs (HCTL) from HLA-A2 transgenic mice. HCTL were generated by weekly stimulation using low concentrations (10 nM) of the Tax peptide pulsed onto APCs, while LCTLs were also generated using 10 µM of the Tax peptide pulsed onto APCs. Using these different CTL lines, we examined Tax-specific CTLs-mediated cytotoxicity with Tax peptide titrated over a range of concentrations. The titration curve showed a



**Fig. 1.** Comparison of HLA-A2 binding curves between Tax11-19 and FMP58-66 peptide in T2-binding assay. The binding affinity of Tax11-19 for HLA-A2 molecule is almost as strong as that of FMP58-66 in influenza A virus.

0.5–1 log<sub>10</sub> difference in functional avidity measured as the peptide concentration necessary to produce 50% lysis (Fig. 2A). Similarly, we examined their properties in antigen-specific IFN-γ production from these CTL lines (Fig. 2B). With a 24 h assay, HCTLs showed more IFN-γ production than LCTLs even at lower concentration of Tax antigen. These data suggest that the two different CTL lines specific for Tax have different functional avidity.

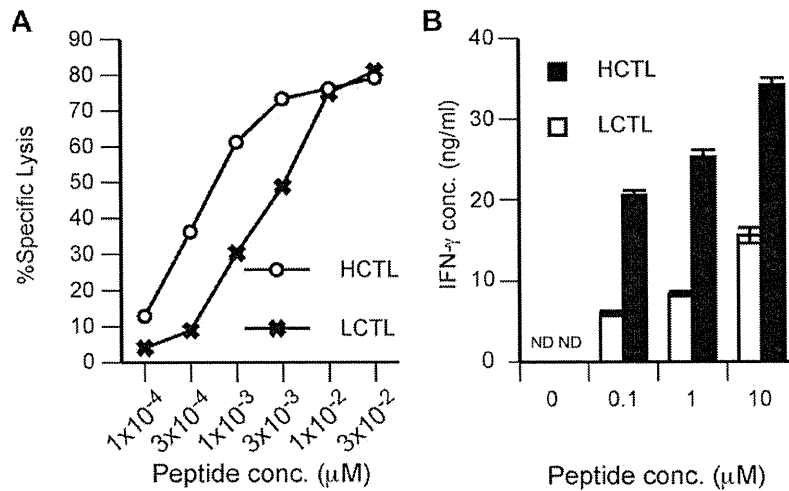
### 3.3. Different Vβ usage and binding ability to Tax-tetramer between high- and low-avidity CTLs

In order to confirm whether these CTLs with different avidity possessed different TCR structures, we assessed the difference in Vβ usage pattern between HCTLs and LCTLs using flow cytometric analysis (FCM). On FCM, antibodies available for screening were those for Vβ 2, 3, 4, 5, 6, 7, 8.1, 8.2, 8.3, 9, 10, 11, 12, 13, 14, and 17. On FCM, no Vβ were detected in LCTLs, while only Vβ5 was detected in HCTLs (Fig. 3A). The data suggested that the major TCR repertoire of HCTL is Vβ5, indicating that these two Tax-specific CTL lines have different TCR structures.

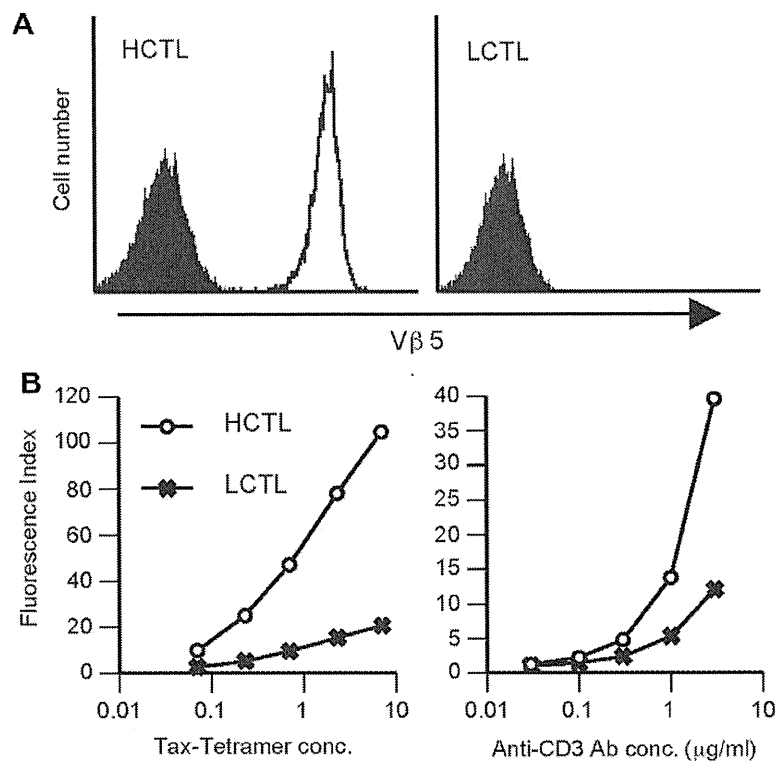
We next compared the binding affinity of TCR between HCTL and LCTL using Tax11-19/HLA-A2 tetramer-LLFGYPVYV and anti-CD3 Ab (Fig. 3B). On FCM with both Tax11-19-tetramer and anti-CD3 Ab titration, HCTLs showed a stronger fluorescence than LCTLs (Fig. 3B). On Tax11-19-tetramer assay, the ratio of fluorescence index (HCTL/LCTL) was ~5-fold at any titrated concentration, and it took 1.5 logs more tetramer to achieve the same level of staining. In the titration of anti-CD3 Ab, the ratio was ~3-fold and also it required about 3-fold more antibody to reach the same level of staining. These findings suggested that HCTLs not only have higher TCR affinity but also express greater numbers of TCR molecules on their surface when compared with LCTLs.

### 3.4. Recognition of human ATL targets by Tax-specific CTLs from HHD mice

We further examined whether these murine CTL lines with different functional avidity could induce cytotoxic activity against human ATL targets. We used the HTLV-1-infected human ATL cell lines, KK-1 (HLA-A2) and KOB (HLA-A30) as target cells derived from peripheral blood and ascites of ATL patients, respectively [23,24]. These murine CTL lines did not show strong cytotoxicity against human ATL lines as against murine targets with a 4 h assay, as it was previously reported that species specificity between murine CD8 and the α3 domain of human HLA-A2 may reduce the recognition ability by CTLs [33]. However, on a 12 h assay, cytotoxicity against human ATL was observed in an HLA-A2 restricted manner (Fig. 4A). HCTLs were especially more efficient at killing at low E/T ratios. Furthermore, on kinetics assay, HCTLs showed more efficient cytotoxicity against the human ATL target (KK-1) than LCTLs (Fig. 4B).



**Fig. 2.** Difference in functional avidity between HCTLs and LCTLs. (A) Recognition by the Tax11-19 peptide specific CTLs, HCTL and LCTL, of Tax11-19 antigenic peptide from 10<sup>-4</sup> to 10 μM when presented on C1RAAD target cells. The effector to target-cell (E/T) ratio was 20:1. Error bars were omitted because all SEMs were <3.5%. (B) Comparison of Tax11-19-specific IFN-γ production between HCTLs and LCTLs. A total of 200,000 CTL cells were cultured with 100,000 mytomycin-c treated C1RAAD cell with 0.1–10 μM Tax11-19 peptide. Culture supernatants at 24 h were assayed using IFN-γ ELISA kit according to the manufacturer's instructions. ND, not detected.



**Fig. 3.** TCR Vβ usage and expression level of TCR complex on Tax-specific CTLs with different functional avidity. (A) Comparison of Vβ usage pattern between HCTLs and LCTLs cytometry analysis (FCM). No Vβs among available anti-Vβ antibodies were detected in LCTL but only Vβ5 was detected in HCTL. (B) Comparison of binding curves for human Tax11-19-tetramer and anti-CD3ε Ab between HCTLs and LCTLs. HCTLs consistently showed a stronger fluorescence index than LCTLs; for Tax11-19-tetramer, the ratio of fluorescence index (LCTL/HCTL) was ~5-fold, and for anti-CD3ε Ab, it was ~3-fold.

### 3.5. Recognition of HTLV-1 infected human T cells by Tax-specific CTL from HHD mice

Next, in order to examine a comparison of the cytotoxicity against HTLV-1 infected non-tumor cells, we used HTLV-1 infected human T cells (HCT-4) derived from a patient with HAM/TSP [25].

On a 12 h lytic assay, HCTLs showed more efficient cytotoxicity against the HTLV-1 infected human T cells while LCTLs were not able to kill the targets under the these experimental conditions (Fig. 5A). At no time point was there detectable killing by LCTLs (Fig. 5B). These findings suggested that the superior recognition ability by the CTLs with higher functional avidity may have a more