3. Th1 or Th2 Activation Status in the Increased Transmigrating Activity of HTLV-I-Infected CD4$^+$ T Cells in HAM/TSP Patients

Helper T cells are generally divided into two distinct populations, Th1 and Th2, based on their cytokine-production profiles [45, 46]. The former produce, among others, interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin, while the latter produce interleukin-4, interleukin-5, interleukin-6 and interleukin-10 (IL-4, IL-5, IL-6 and IL-10). Although interleukin-12 (IL-12) is secreted, as both the biologically active form (p70 heterodimer) and the biologically inactive form (p40 chains) [47], mainly from antigen-presenting cells, such as macrophages and dendritic cells, IL-12 induces IFN-γ production from Th1 cells [48]. Thus, IL-12 and IFN-γ are very important cytokines that regulate the differentiation to the Th1 type and induce cell-mediated immunity [45]. On the other hand, the differentiation to the Th2 type that induces humoral immunity is regulated by IL-4 and IL-10 [49, 50]. Of these cytokines, IL-4 is very important for the differentiation to the Th2 type [46]. The importance of an imbalance between Th1 and Th2 in the pathogenesis of autoimmune or inflammatory diseases has been reported [49-51].

We have previously reported simultaneous up-regulation of mRNA expression of inflammatory cytokines, such as IFN-γ, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-1α in the peripheral blood lymphocytes of HAM/TSP patients [52]. Subsequently, we demonstrated that the spontaneous production of inflammatory cytokines, such as IFN-γ, TNF-α, and GM-CSF, but not IL-4, increased simultaneously in cultured the peripheral blood CD4$^+$, but not CD8$^+$, T cells [53]. These findings suggested that the Th1 cell population rather than the Th2 cell population predominates in the peripheral blood CD4$^+$ T cells of HAM/TSP patients. Therefore, to clarify the immunological balance in HAM/TSP patients, we measured IL-12 and IFN-γ which play an important role in the Th1 state, and IL-4 and IL-10 as Th2 cytokine, in both the sera and cerebrospinal fluid (CSF) and compared the results to those for the controls including anti-HTLV-I-seropositive carriers [54].

As shown in figure 7a, serum levels of total IL-12 in 22 HAM/TSP patients (mean ± SE; 128.68 ± 12.21 pg/mL) were significantly higher than in 22 controls including 9 anti-HTLV-I-seropositive carriers (mean ± SE; 72.36 ± 8.80 pg/mL). In addition, serum p70 heterodimer, which is a biologically active form of IL-12, was detected in 9 and 2 of 22 HAM/TSP patients and 22 controls, respectively. Serum levels of p70 heterodimer in HAM/TSP patients (mean ± SE; 3.20 ± 0.98 pg/mL) were also significantly higher than in controls (mean ± SE; 0.36 ± 0.25 pg/mL) (figure 7b). However, CSF levels of total IL-12 in 15 HAM/TSP patients were the same as in 13 controls, including 7 anti-HTLV-I-seropositive carriers (mean ± SE; 16.53 ± 4.41 and 9.46 ± 3.92 pg/mL, respectively) (figure 7c). In addition, CSF p70 heterodimer was detected in only one of 15 HAM/TSP patients and not detected in 13 controls (data not shown). On the other hand, serum IFN-γ levels in 22 HAM/TSP patients (mean ± SE; 0.33 ± 0.09 IU/mL) were significantly higher than in 22 controls, including 9 anti-HTLV-I-seropositive carriers (mean ± SE; 0.20 ± 0.10 IU/mL) (figure 8a). Finally, as shown in figure 8b, CSF IFN-γ levels were also significantly higher in 15 HAM/TSP patients (mean ± SE; 0.40 ± 0.07

IU/mL) than in 13 controls, including 7 anti-HTLV-I-seropositive carriers (mean ± SE; 0.15 ± 0.04 IU/mL).

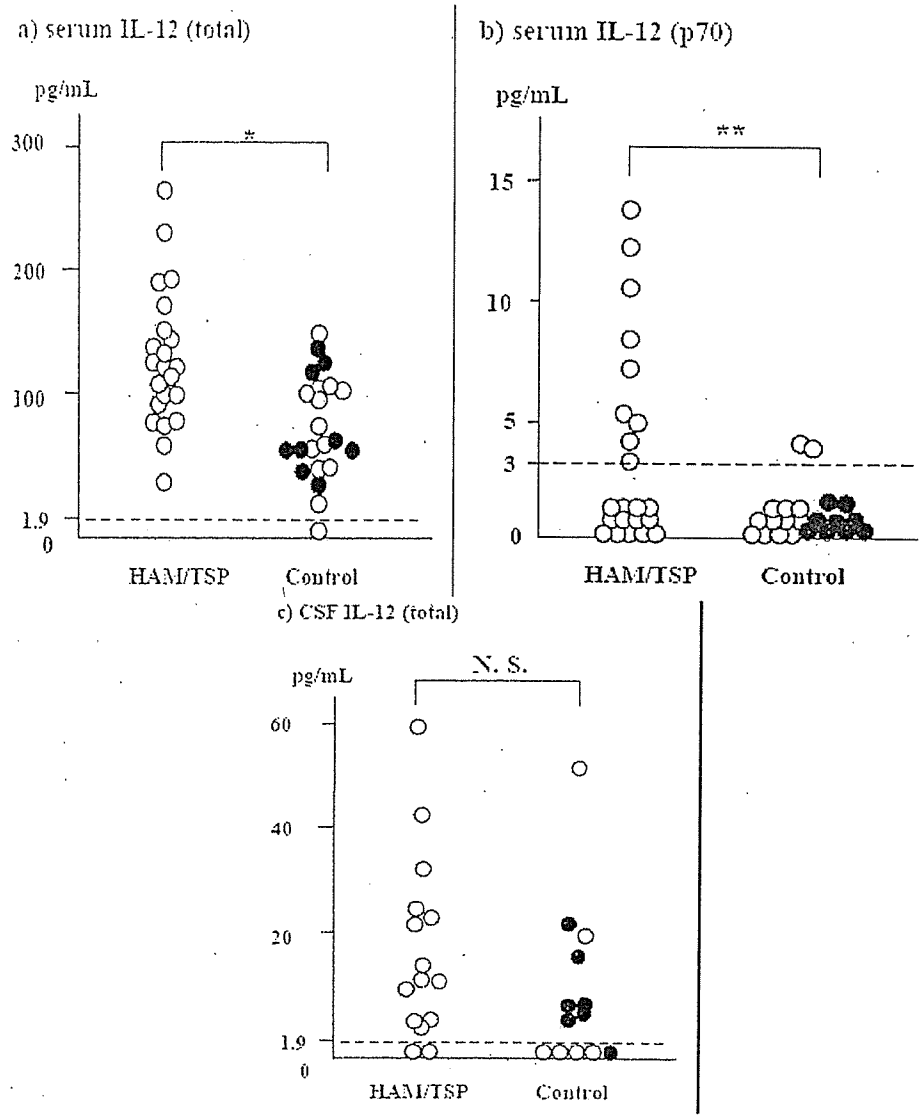


Figure 7. Serum levels of total IL-12 (p70 heterodimer plus p40 chains) and IL-12 (p70 heterodimer) and cerebrospinal fluid (CSF) levels of total IL-12. a) Serum total IL-12: serum levels of total IL-12 in HAM/TSP patients were higher than in the controls (mean ± SE; 128.68 ± 12.21 and 72.36 ± 8.80 pg/mL) (*$p = 0.0011$). b) Serum p70 heterodimer: serum levels of p70 heterodimer in HAM/TSP patients were higher than in the controls (mean ± SE; 3.20 ± 0.98 and 0.36 ± 0.25 pg/mL) (**$p = 0.0095$). c) CSF total IL-12: CSF levels of total IL-12 of HAM/TSP patients were the same as those of the controls (mean ± SE; 16.53 ± 4.41 and 9.46 ± 3.92 pg/mL) (N.S.: not significant). Minimal measurable levels (indicated by dotted lines): 1.9 pg/mL for total IL-12, 3.0 pg/mL for p70 heterodimer. HAM: HAM/TSP patients (serum: n=22; CSF: n=15), Controls: Anti-HTLV-I-seropositive carriers (closed circle) (serum: n=9; CSF: n=7) and anti-HTLV-I-seronegative controls (serum: n=13; CSF: n=6). Mann-Whitney U-test was used for statistical analysis. Quotation from Ref. 54.

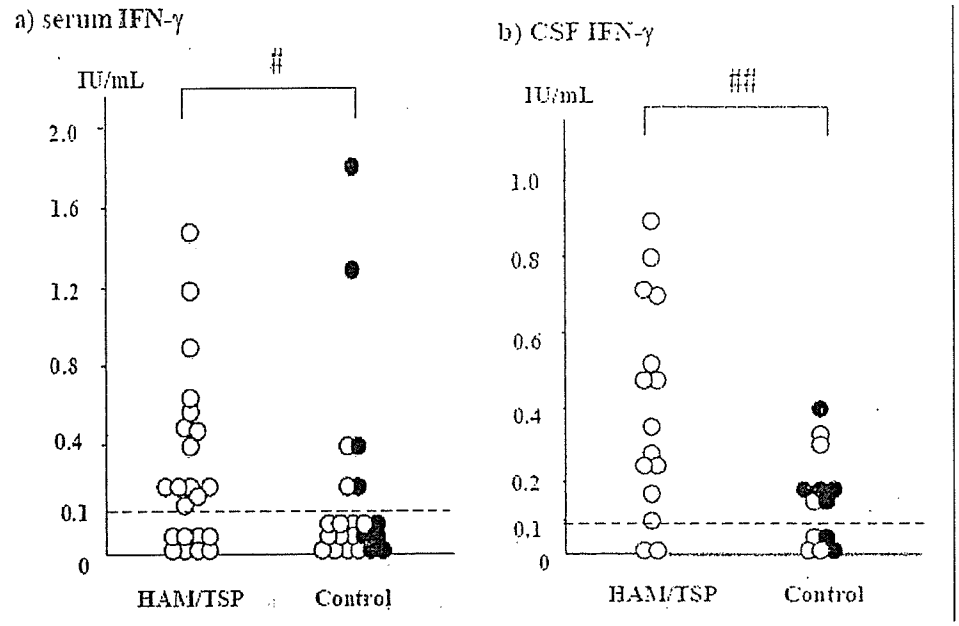


Figure 8. Serum and CSF levels of IFN-γ. a) Serum IFN-γ: serum levels of IFN-γ of HAM/TSP patients were higher than in the controls (mean ± SE; 0.33 ± 0.09 and 0.20 ± 0.10 IU/mL) (#p = 0.0035). b) CSF IFN-γ: CSF levels of IFN-γ of HAM/TSP patients were also higher than in the controls (mean ± SE; 0.40 ± 0.07 and 0.15 ± 0.04 IU/mL) (##p = 0.015). Minimal measurable levels (indicated by dotted lines): 0.1 IU/mL for IFN-γ. HAM: HAM/TSP patients (serum: n=22; CSF: n=15), Controls: Anti-HTLV-I-seropositive carriers (closed circle) (serum: n=9; CSF: n=7) and anti-HTLV-I-seronegative controls (serum: n=13; CSF: n=6). Mann-Whitney's U-test was used for statistical analysis. Quotation from Ref. 54.

Next, we measured IL-4 and IL-10 as Th2 cytokines. Serum IL-4 was present in only 4 of 22 HAM/TSP patients and in 3 of 22 controls. Serum IL-4 levels in HAM/TSP patients (mean ± SE; 1.55 ± 0.73 pg/mL) were the same as in controls (mean ± SE; 2.22 ± 1.27 pg/mL). In addition, although serum IL-10 was detected in 7 of 22 HAM/TSP patients and in 10 of 22 controls, there were no significant differences in the levels between both groups (mean ± SE; 3.28 ± 1.52 pg/ml, mean ± SE; 3.06 ± 0.90 pg/mL, respectively). On the other hand, although CSF IL-4 was present in only 4 anti-HTLV-I-seropositive carriers of 13 controls, including 7 anti-HTLV-I-seropositive carriers (mean ± SE; 3.00 ± 1.32 pg/mL), CSF IL-4 was not detected in 15 HAM/TSP patients (p = 0.023). CSF IL-10 was present in 5 of 15 HAM/TSP patients and in 7 or 13 controls, including 7 anti-HTLV-I-seropositive carriers. The CSF IL-10 levels in HAM/TSP patients (mean ± SE; 7.20 ± 3.96 pg/mL) was the same as in controls (mean ± SE; 5.39 ± 2.23 pg/mL).

These findings indicate that the immunological balance of helper T cells between Th1 and Th2 is toward Th1 in both the peripheral blood and the central nervous system (CNS) in HAM/TSP patients. Increased serum but not CSF levels of IL-12 suggest that differentiation of the helper T cells population to Th1 is induced outside of the CNS in HAM/TSP patients and these Th1 cells migrate to the CNS. In addition, in considering that IL-4 in the CSF was not detected in any of HAM/TSP patients although all 4 of the controls in whom IL-4 in the CSF was detected were anti-HTLV-I-seropositive carriers, these findings are a strong indication that

the balance of the helper T cells which function in the CNS of HAM/TSP patients tends toward Th1, rather than Th2.

How IL-12 production is up-regualted in HAM/TSP patients is unclear. The increased CD40 ligand expression on activated $CD4^+$ T cells can induce increased IL-12 production in monocytes [55]. In addition, the combination of CD40 ligand and INF-γ can generate large amounts of IL-12 in some population of human monocyte-derived dendritic cells [56]. However, as far as we determined by comparing the percentage of CD40 ligand-positive cells in the peripheral blood $CD4^+$ T cells of HAM/TSP patients and controls, there was no significant difference between the two groups [57]. Therefore, although elevated serum IL-12 levels in HAM/TSP patients might be based on different mechanisms from CD40/CD40 ligand interaction, it is conceivable that it is strongly involved in Th1 activation or maturation in HAM/TSP patients through the IL-12/IL-12 receptor axis.

In differentiation of naive T cells into Th1 or Th2 helper T cells, cytokine signaling plays an important role. IL-12 receptor/signal transducers and activators of transcription 4 (IL-12R/STAT4) and IL-4R/STAT6 signaling are involved in Th1 and Th2 differentiation, respectively [58]. In addition, T-bet, which is a member of the T-box family, has been cloned as a Th1-specific transcription factor [59]. Therefore, both IL-12R/STAT4 signaling and T-bet work cooperatively in Th1 differentiation, and IL-4R/STAT6 signaling induces GATA-3 activation, one of the Th2-specific transcription factors in Th2 differentiation [60]. Recently, the importance of negative regulation of cytokine signal transduction in the differentiation and homeostasis of the immune system has become somewhat clearer [61]. The suppressor of cytokine signaling (SOCS) family is a representative of the negative regulators which act through a feedback mechanism or an inhibitory signal to the Janus kinases (JAK)/STAT systems in cytokine signaling [62]. In this system, SOCS1 is induced by IFN-γ receptor (IFN-γR)/STAT1 signaling and inhibits IL-4R/STAT6 signaling concomitant with the negative feedback to its own JAK/STAT1 signaling [61, 63]. Although SOCS3 is induced by a number of humoral factors, such as IL-2, IL-6, IL-10, growth hormone, prolactin and leptin, with simultaneous negative feed back to their own JAK/STATs [64], it was reported that SOCS3 also inhibits IL-12R/STAT4 signaling [65]. These findings suggest that up-regulation of SOCS1 expression, based on activation of IFN-γ signaling, inhibits Th2 differentiation by blocking the IL-4R/STAT6 signaling pathway, and that down-regulation of SOCS3 expression leads to Th1 differentiation. Indeed, it has been reported that Th1 cells express significantly higher levels of SOCS1 than SOCS3 and expression levels of each SOCS protein are reversed in Th2 cells, indicating that either SOCS1 or SOCS3 protein is a marker for Th1 cells or Th2 cells, respectively [65, 66]. To clarify the Th1 activation state in HAM/TSP patients from the standpoint of cytokine signaling, we analyzed mRNA expression of T-bet, GATA-3, IL-12Rβ2, SOCS1 and SOCS3 in the peripheral blood mononuclear cells (PBMC) of HAM/TSP patients, as compared to either anti-HTLV-I-seropositive carriers or anti-HTLV-I-seronegative controls, by quantitative real-time RT-PCR [67].

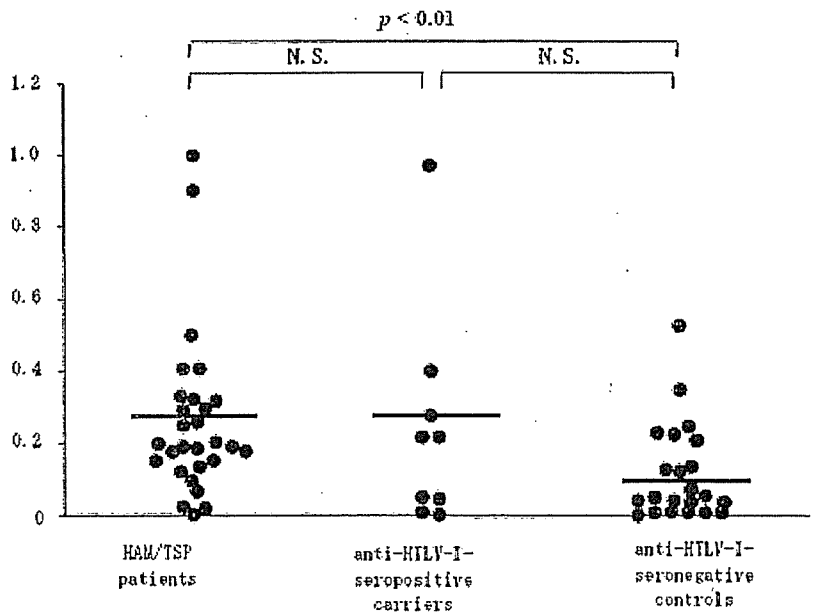
After each mRNA expression was evaluated by normalization to β2-microglobulin (β2m), the value of each mRNA expression was determined (table 1). As shown in figure 9a and 9b, mRNA expression of both T-bet and GATA-3 was significantly increased in PBMC of 28 HAM/TSP patients, compared to 22 anti-HTLV-I-seronegative controls. However, there was no significant difference between HAM/TSP patients and anti-HTLV-I-seropositive carriers

(figure 9a and 9b). In addition, mRNA expression of IL-12Rβ2 was significantly higher in HAM/TSP patients than in anti-HTLV-I-seronegative controls (figure 10). Although mRNA expression of T-bet, GATA-3 and IL-12Rβ2 seemed to be higher in anti-HTLV-I-seropositive carriers without HAM/TSP than in anti-HTLV-I-seronegative controls, these differences were not statistically significant. In addition, no significant differences in mRNA expression were found among HTLV-I-infected individuals with or without HAM/TSP.

Table 1. mRNA expression of cytokine signaling molecules.

	HAM/TSP patients	anti-HTLV-I-seropositive carriers without HAM/TSP	anti-HTLV-I seronegative controls
T-bet	0.260 ± 0.043	0.241 ± 0.100	0.118 ± 0.028
GATA-3	0.167 ± 0.040	0.115 ± 0.051	0.022 ± 0.005
IL-12Rβ2	0.321 ± 0.126	0.288 ± 0.104	0.114 ± 0.023
SOCS1	0.338 ± 0.062	0.147 ± 0.038	0.035 ± 0.007
SOCS3	4.860 ± 0.818	6.714 ± 1.467	13.234 ± 1.385

After each mRNA expression was evaluated by normalization to β2m, the value of each cytokine; signaling molecule mRNA expression was presented as mean ± SE. Quotation from Ref. 67.



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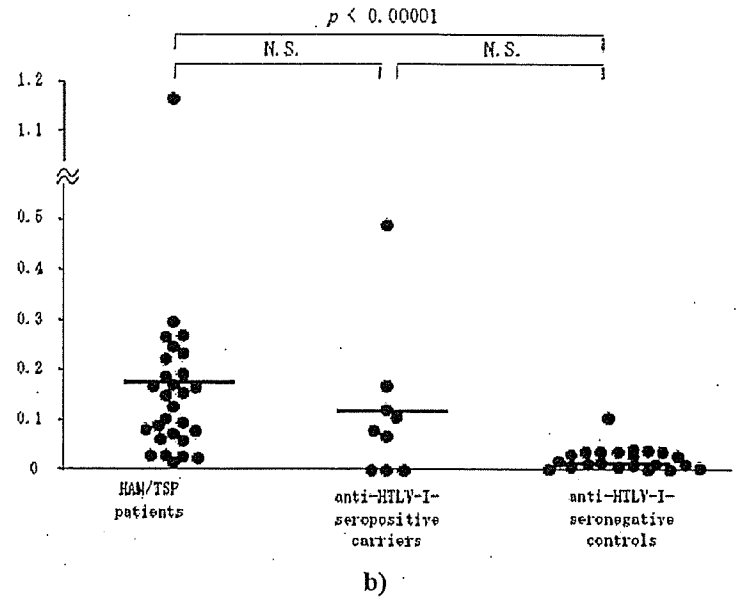


Figure 9. Comparison of mRNA expression of T-bet and GATA-3 in PBMC. mRNA expression of both T-bet (a) and GATA-3 (b) in PBMC was significantly increased in HAM/TSP patients compared to anti-HTLV-I-seronegative controls. Although mRNA expression of T-bet and GATA-3 appeared to be higher in anti-HTLV-I-seropositive carriers than in anti-HTLV-I-seronegative controls, this difference was not statistically significant. Mann-Whitney's U-test was used for statistical analysis. Quotation from Ref. 67.

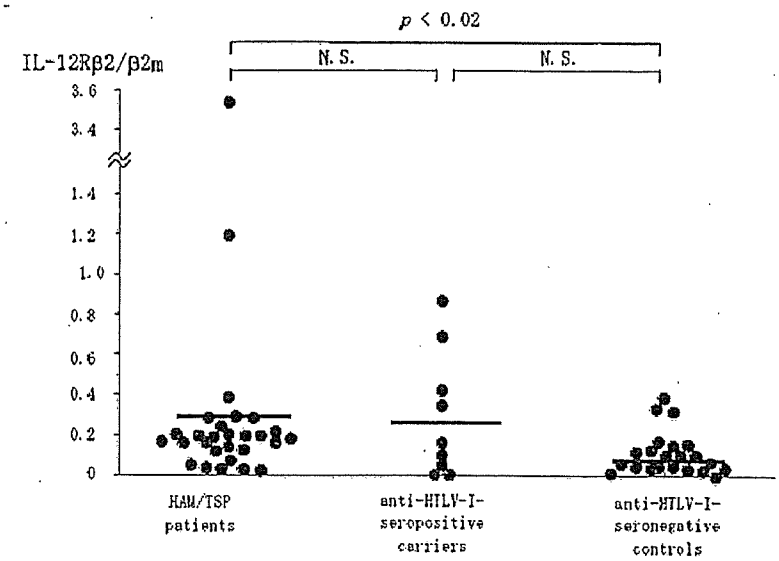
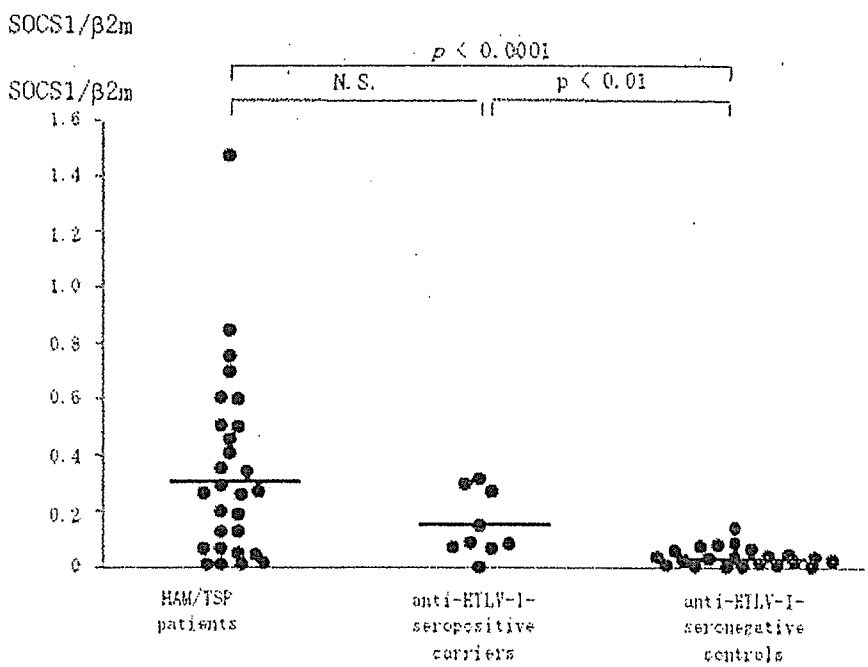


Figure 10. Comparison of mRNA expression of IL-12Rβ2 in PBMC. mRNA expression of IL-12Rβ2 in PBMC was significantly higher in HAM/TSP patients than in anti-HTLV-I-seronegative controls. Although mRNA expression of IL-12Rβ2 seemed to be higher in anti-HTLV-I-seropositive carriers than in anti-HTLV-I-

seronegative controls, this difference was not statistically significant. Mann-Whitney's U-test was used for statistical analysis. Quotation from Ref. 67.

mRNA expression of SOCS1 was significantly increased in HAM/TSP patients and in anti-HTLV-I-seropositive carriers without HAM/TSP, compared to anti-HTLV-I-seronegative controls (figure 11a). Conversely, mRNA expression of SOCS3 was significantly decreased in both HAM/TSP patients and anti-HTLV-I-seropositive carriers, compared to anti-HTLV-I-seronegative controls (figure 11b). However, no significant difference in mRNA expression of SOCS3 was found between HAM/TSP patients and anti-HTLV-I-seropositive carriers.

In both HAM/TSP patients and anti-HTLV-I-seropositive carriers, increased mRNA expression of SOCS1 indicated up-regulation of IFN-γ signaling, and decreased mRNA expression of SOCS3 indicated down-regulation of IL-4 signaling. However, GATA-3 mRNA expression seemed to also be up-regulated in both HAM/TSP patients and anti-HTLV-I-seropositive carriers. Therefore, we analyzed the correlation between T-bet and GATA-3 mRNA expression in both HAM/TSP patients and anti-HTLV-I-seropositive carriers. As shown in figure 12, mRNA expression of both transcription factors showed moderately positive and strongly positive correlation in HAM/TSP patients (rs = 0.393; p = 0.041) and in anti-HTLV-I-seropositive carriers (rs = 0.754; p = 0.033), respectively. There were no correlation between either T-bet or SOCS1 and HTLV-I tax mRNA expression in both HAM/TSP patients and anti-HTLV-I-seropositive carriers (data not shown). However, as shown in figure 13, there was a moderately positive correlation between IL-12Rβ2 and HTLV-I tax mRNA expression in HAM/TSP patients (rs = 0.380; p = 0.048) but not in anti-HTLV-I-seropositive carriers (rs = 0.197; p = 0.367).



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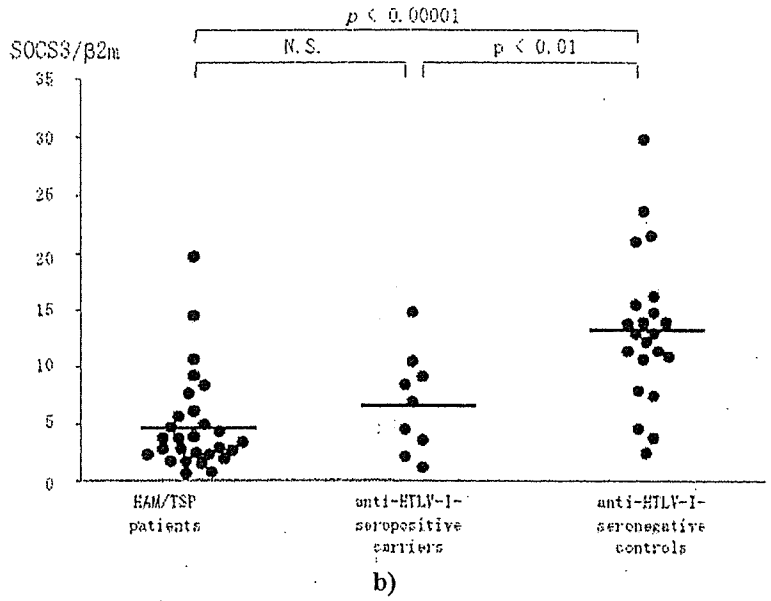


Figure 11. Comparison of mRNA expression of SOCS1 and SOCS3 in PBMC. mRNA expression of SOCS1 in PBMC (a) was significantly higher in both HAM/TSP patients and anti-HTLV-I-seropositive carriers than in anti-HTLV-I-seronegative controls. Although mRNA expression of SOCS1 seemed to be higher in HAM/TSP patients than in anti-HTLV-I-seropositive carriers, this difference was not statistically significant. Conversely, mRNA expression of SOCS3 in PBMC (b) was significantly decreased in both HAM/TSP patients and anti-HTLV-I-seropositive carriers, compared to anti-HTLV-I-seronegative controls. There was no significant difference in mRNA expression of SOCS3 between HAM/TSP patients and anti-HTLV-I-seropositive carriers. Mann-Whitney's U-test was used for statistical analysis. Quotation from Ref. 67.

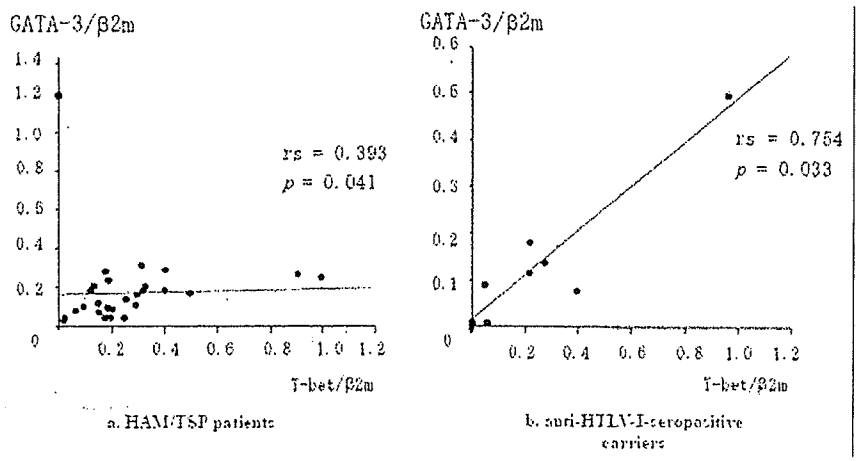


Figure 12. Correlation between T-bet and GATA-3 mRNA expression among HAM/TSP patients and anti-HTLV-I-seropositive carriers. mRNA expression of both transcription factors showed moderately positive correlation in HAM/TSP patients and strongly positive correlation in anti-HTLV-I-seropositive carriers, respectively. Nonparametric Spearman's rank correlation test was used for statistical analysis. a) HAM/TSP

patients (rs = 0.393, p = 0.041); b) anti-HTLV-I-seropositive carriers (rs = 0.754, p = 0.033). Quotation from Ref. 67.

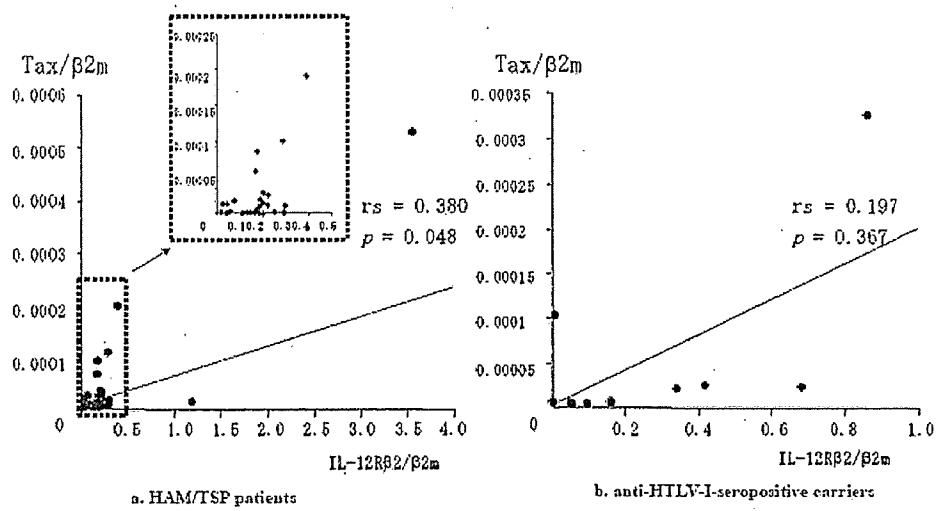


Figure 13. Correlation between IL-12Rβ2 and HTLV-I tax mRNA expression among HAM/TSP patients and anti-HTLV-I-seropositive carriers. There was a moderately positive correlation in only HAM/TSP patients, but not in anti-HTLV-I-seropositive carriers. Nonparametric Spearman's rank correlation test was used for statistical analysis. a) HAM/TSP patients (rs = 0.380, p = 0.048); b) anti-HTLV-I seropositive carriers (rs = 0.197, p = 0.367). Quotation from Ref. 67.

In this study, we showed the increased mRNA expression of T-bet, which is a master switch in Th1 differentiation [68, 69], IL-12Rβ2 and SOCS1, and the decreased mRNA expression of SOCS3 in PBMC of HAM/TSP patients, compared to them of anti-HTLV-I-seronegative controls, strongly indicating the up-regulated Th1 related cytokine signaling with the down-regulated Th2 related cytokine signaling in HAM/TSP patients. However, significant differences were not detected in mRNA expression of the signaling molecules analyzed in this study between HAM/TSP patients and anti-HTLV-I-seropositive carriers. Moreover, the pattern of SOCS1 and SOCS3 mRNA expression indicated that the status of anti-HTLV-I-seropositive carriers was similar to that of HAM/TSP patients. But the up-regulated Th1-cytokine related signaling with the down-regulated Th2-cytokine related signaling seems to be more obvious in HAM/TSP patients than in anti-HTLV-I-seropositive carriers.

In addition, unexpectedly, GATA-3 mRNA expression was also increased in PBMC of HAM/TSP patients, as compared to anti-HTLV-I-seronegative controls. With regard to these findings, there was some correlation between T-bet and GATA-3 mRNA expression among HTLV-I-infected individuals with and without HAM/TSP and a stronger correlation between both transcription factors in anti-HTLV-I-seropositive carriers than in HAM/TSP patients, suggesting that Th1/Th2 balance might be under stronger compensatory control in the expression levels of transcription factors in anti-HTLV-I-seropositive carriers than in HAM/TSP patients.

IL-12Rβ2 , which is located up-stream of STAT4 signaling and plays a very important role in Th1 differentiation [68], is induced by IL-12 itself through STAT4 signaling [70]. Therefore, the increased IL-12Rβ2 mRNA expression in HAM/TSP patients is consistent with the

elevated serum levels of IL-12 in HAM/TSP patients. However, there were no significant differences in mRNA expression of IL-12Rβ2 between HAM/TSP patients and anti-HTLV-I-seropositive carriers. Interestingly, though, there was a moderately positive correlation between IL-12Rβ2 and HTLV-I tax mRNA expression in HAM/TSP patients but not anti-HTLV-I-seropositive carriers. As IL-12Rβ2 is a most reliable marker of Th1 cells, our data suggest that HTLV-I-infected Th1 cells are increased in PBMC of HAM/TSP patients although HTLV-I tax might be cooperatively involved in IL-12R/STAT4 signaling.

It has been reported that intracellular IFN-γ^+/IL-4^+ cell ratio in the peripheral blood $CD4^+$ T cells is increased in HAM/TSP patients [71]. More recently, it has been demonstrated that IFN-γ producing cells in the population of HTLV-I tax-expressing cells are increased in HAM/TSP patients, compared to anti-HTLV-I-seropositive carriers [72]. Considering these findings together with our data presented in this section, the immunological status in HAM/TSP patients is under the up-regulated Th1 and the down-regulated Th2 state systemically, and it is conceivable that Th1 activation in HAM/TSP patients is based on the increase of HTLV-I-infected Th1 cells. However, if referring to only the Th1 activation state, it cannot be distinguished clearly between HAM/TSP patients and anti-HTLV-I-seropositive carriers because of the similar expression pattern of SOCS1/SOCS3 mRNA, as mentioned above. Therefore, the most important prerequisite for the development of HAM/TSP from the HTLV-I asymptomatic carrier state must be based on the exaggerated transmigration activity of HTLV-I-infected Th1 cells mediated by the increased activities of several kinds of molecules, such as MMP and AP-N, to the tissues, in addition to Th1 activation.

4. Do the Cells that Have the Potential to Transmigrate into the Tissues Really Have the Characteristics of Th1 Cells in HAM/TSP Patients?

We suggested that the immunological status of HAM/TSP patients is under systemic Th1 activation, based on an increased number of HTLV-I-infected Th1 cells. Then, how does this situation trigger the pathological process in the spinal cords of HAM/TSP patients? In other words, do the cells that have the potential to transmigrate into the tissues really have the characteristics of Th1 cells? We previously reported that the production of IFN-γ, TNF-α and GM-CSF by EC-adherent T cells in HAM/TSP patients was significantly increased, as compared to EC-non-adherent T cells. Moreover, the production of these Th1-related cytokines in each EC-adherent T cell population was significantly increased in HAM/TSP patients compared to anti-HTLV-I-seronegative controls, suggesting that the T cells which have the potential to transmigrate into tissues have increased activity of Th1 cytokine production [73].

When T cells transmigrate into the tissues through vascular EC, the rolling phenomenon is the initial step for the initial attachment of T cells [19]. In this step, selectin and its ligands, which are expressed on vascular EC and T cells, respectively, play an important role [74]. Of these, sialyl Lewis^x antigen (sLe^x) is a ligand for both E- and P-selectin [75]. Therefore, it is suggested that T cells expressing sLe^x have the potential to transmigrate into the tissues. In addition, it was reported that Th1 cells, but not Th2 cells, can bind to E- and P-selectin, indicating that each ligand, sLe^x, for each selectin is a phenotypic markers of Th1 cells [76].

We used KM 93 monoclonal antibody, which recognizes sLe^x [77], and compared the frequency of sLe^{x+} cells, together with cytokine (IFN-γ and IL-4) production, in the peripheral blood CD4$^+$ T cells between HAM/TSP patients and controls, including anti-HTLV-I-seropositive carriers. In addition, we compared the HTLV-I proviral load between the sLe^{x+} and sLe^{x-} cell population in the peripheral blood CD4$^+$ T cells of HAM/TSP patients [78].

As shown in figure 14, the percentage of sLe^{x+} cells in the peripheral blood CD4$^+$ T cells in HAM/TSP patients (29.7 ± 4.6 %) was significantly higher than in controls, including anti-HTLV-I-seropositive carriers (13.9 ± 1.6 %). Next, either sLe^{x+} or sLe^{x-} cells, separated by the panning method, were cultured for 48 hrs, and cytokines in the culture supernatants were measured. A significantly higher IFN-γ production by sLex$^+$ cells in the peripheral blood CD4$^+$ T cells was noted in HAM/TSP patients (24.1 ± 8.0 IU/mL) compared to controls including anti-HTLV-I-seropositive carriers (1.2 ± 0.9 IU/mL) (figure 15a). However, there was no significant difference in IL-4 production by this cell population between HAM/TSP patients and controls (1.1 ± 1.1 vs. 1.9 ± 1.3 pg/mL, respectively) (data not shown). In addition, IFN-γ production in the sLex$^+$ cell population (24.1 ± 8.0 IU/mL) was significantly higher than in the sLex$^-$ cell population (2.6 ± 0.9 IU/mL) of the peripheral blood CD4$^+$ T cells of HAM/TSP patients (figure 15b). Conversely, IL-4 production in the sLex$^+$ cell population was significantly lower than in the sLex$^-$ cell population of the peripheral blood CD4$^+$ T cells of HAM/TSP patients (1.1 ± 1.1 vs. 11.5 ± 4.7 pg/mL, respectively, p = 0.0431) (data not shown). However, there was no significant difference in IFN-γ and IL-4 production between sLex$^+$ and sLex$^-$ cell populations in the peripheral blood CD4$^+$ T cells of controls (IFN-γ; 1.2 ± 0.9 vs. 1.0 ± 0.8 IU/mL, IL-4; 1.9 ± 1.3 vs. 6.5 ± 3.7 pg/mL, respectively) (data not shown).

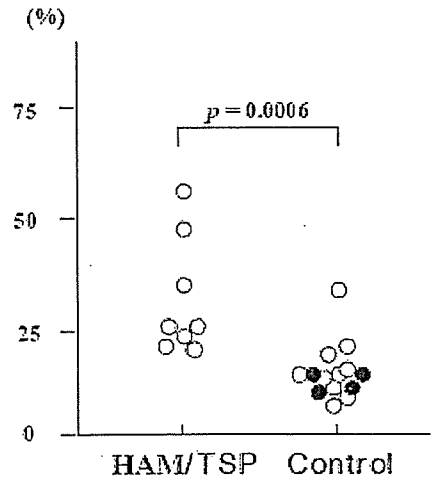


Figure 14. Comparison of the frequency of sLe^{x+} cells in peripheral blood CD4$^+$ T cells between HAM/TSP patients and controls. The percentage of sLe^{x+} cells in peripheral blood CD4$^+$ T cells was significantly higher in HAM/TSP patients (29.7 ± 4.6%) than in controls (13.9 ± 1.6%) (p = 0.0006). HAM: HAM/TSP patients (n=8); Control: anti-HTLV-I seropositive carriers (closed circles) (n=4) and anti-HTLV-I-seronegative controls (n=10). Mann-Whitney's U-test was used for statistical analysis. Quotation from Ref. 78.

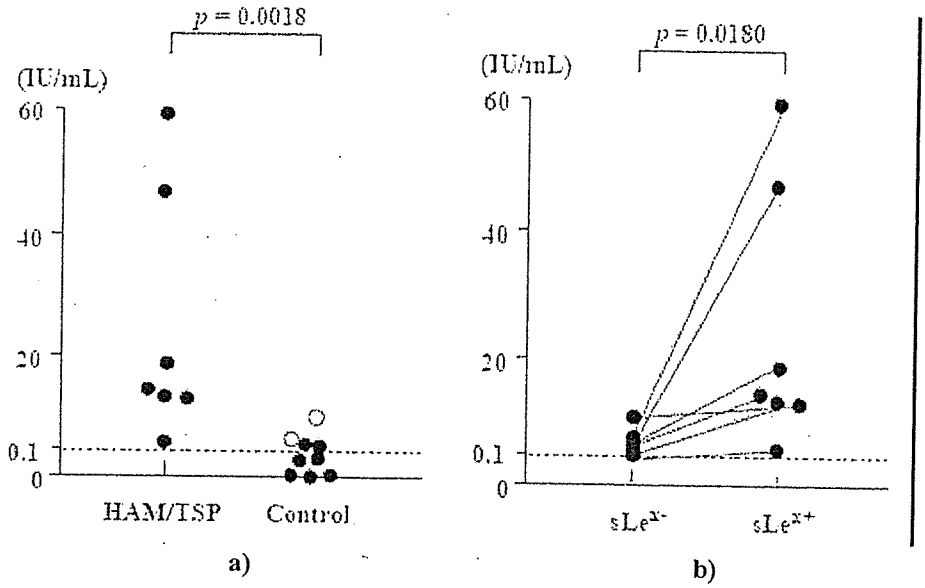


Figure 15. Comparison of IFN-γ production by the sLe^{x+} and sLe^{x-} cell populations in peripheral blood $CD4^+$ T cells between HAM/TSP patients and controls. a) IFN-γ production by sLe^{x+} cell population in peripheral blood $CD4^+$ T cells in HAM/TSP patients (24.1 ± 8.0 IU/mL) was significantly higher than in controls (1.2 ± 0.9 IU/mL, p=0.0018). Mann-Whitney's U-test was used for statistical analysis. b) IFN-γ production by the sLe^{x+} cell population (24.1 ± 8.0 IU/mL) was significantly higher than by the sLe^{x-} cell population in peripheral blood $CD4^+$ T cells of HAM/TSP patients (2.6 ± 0.9 IU/mL, p=0.0180). Wilcoxon single-rank test was used for statistical analysis. HAM: HAM/TSP patients (n=7); Control: anti-HTLV-I-seropositive carriers (n=2) (open circles) and anti-HTLV-I-seronegative controls (n=7); sLe^{x+}: sLe^{x+} cell population; sLe^{x-}: sLe^{x-} cell population. Minimal measurable level: 0.1 IU/mL. Quotation from Ref. 78.

In comparing the HTLV-I proviral load, quatitated by competitive PCR methods, we found the HTLV-I proviral load in the $sLex^+$ cell population to be two- to eight-fold higher than in the $sLex^-$ cell population in the peripheral blood $CD4^+$ T cells of all eight HAM/TSP patients (figure 16). However, HTLV-I proviral load in the $sLex^+$ cell population was almost same in the sLex- cell population in the peripheral blood $CD4^+$ T cells of an anti-HTLV-I-seropositive carrier, although the HTLV-I proviral load copy numbers were very low in this case (figure 16).

Our data, clearly demonstrating that the peripheral blood $CD4^+$ T cells expressing sLe^x were increased in HAM/TSP patients, may be related to the following findings. Firstly, it might be based on the high HTLV-I proviral load itself [3, 4], which is the most characteristic virological abnormality in the peripheral blood of HAM/TSP patients. It has been reported that the HTLV-I tax protein transactivates fucosyltransferase VII, which is a key element in regulating the synthesis of E- and P-selectin ligands in lymphocytes [79-81]. Therefore, the higher the numbers of HTLV-I-infected cells are, the higher the numbers of sLe^{x+} cells would be. This fact was also supported by our findings that HTLV-I proviral load in the sLe^{x+} cell population was significantly higher than in the sLe^{x-} cell population. Secondly, although serum IL-12 levels are increased in HAM/TSP patients, it is known that IL-12 up-regulates the activity and expression of selectin ligand via up-regulation of fucosyltransferase VII expression [82]. Thus, the increased number of sLe^{x+} cells in the peripheral blood $CD4^+$ T cells of

HAM/TSP patients might be mediated by high HTLV-I proviral loads and by increased serum IL-12.

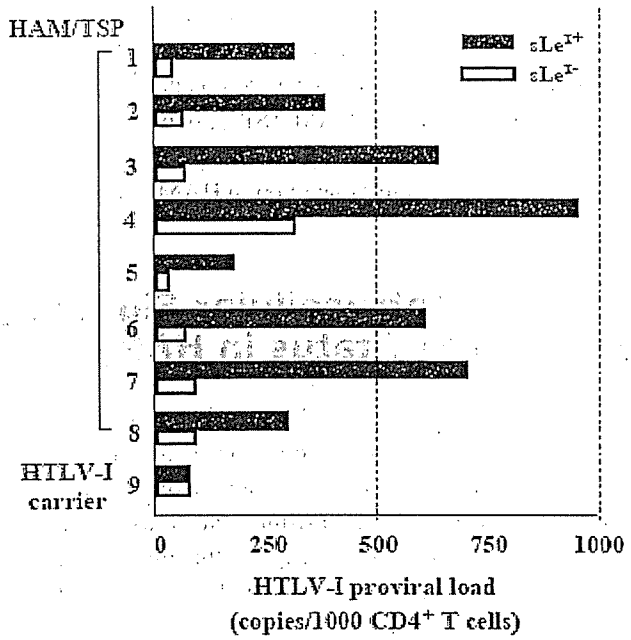


Figure 16. Comparison of HTLV-I proviral load in sLe^{x+} and sLe^{x-} cell populations in peripheral blood CD4$^+$ T cells of HAM/TSP patients. Quantitative analysis of HTLV-I proviral DNA in the sLe^{x+} and sLe^{x-} cell populations in peripheral blood CD4$^+$ T cells. HTLV-1 proviral DNA quantitated in the sLe^{x+} cell population was higher than in the sLe^{x-} cell population in all eight HAM/TSP patients (lane 1 – 8). However, HTLV-I proviral DNA quantitated in both populations was almost the same in an anti-HTLV-I-seropositive carrier (lane 9). sLe^{x+} : sLe^{x+} cell population; sLe^{x-} : sLe^{x-} cell population. Quotation from Ref. 78.

When we compared the potential of IFN-γ production in the sLe^{x+} cell population in the peripheral blood CD4$^+$ T cells between HAM/TSP patients and controls, HAM/TSP patients had significantly increased potential as compared to controls, suggesting that Th1 cells of HAM/TSP patients are obviously also under the activated status of Th1 function. Furthermore, when we compared the activity of both IFN-γ and IL-4 production between sLe^{x+} and sLe^{x-} cell populations of the peripheral blood CD4$^+$ T cells of HAM/TSP patients, IFN-γ production was significantly higher in the former than in the latter population, but vice versa for IL-4 production. However, there was no significant difference in the production of both cytokines between sLe^{x+} and sLe^{x-} cell populations of the peripheral blood CD4$^+$ T cells in controls. These findings strongly suggest that the cells having the potential to transmigrate into the tissues have activated Th1 function and this cell population is increased in the peripheral blood CD4$^+$ T cells of HAM/TSP patients.

So, what is the relationship between Th1 function and HTLV-I infection in the peripheral blood CD4$^+$ T cells of HAM/TSP patients? When we compared the HTLV-I proviral load between between sLe^{x+} and sLe^{x-} cell populations of the peripheral blood CD4$^+$ T cells of

HAM/TSP patients, HTLV-I proviral load in the sLe^{x+} cell population was two- to eight-fold higher than in the sLe^{x-} cell population, indicating that HTLV-I-infected $CD4^+$ T cells are concentrated in the cell population having the potential to transmigrate into the tissues.

Overall, our data indicated that the cells having the potential to transmigrate into the tissues has the characteristics of Th1 cells and are constituted of much numbers of HTLV-I-infected cells compared to the cells not having the potentials to transmigrate into the tissues in the peripheral blood $CD4^+$ T cells of HAM/TSP patients. Therefore, these findings strongly suggested that HTLV-I-infected Th1 cells play a very important role as the first trigger in the development of chronic spinal cord inflammation in HAM/TSP.

5. What Type of Intracellular Signaling Molecule is Involved in Th1 Status in HAM/TSP Patients?

Even if sLe^{x+} antigen is a ligand for the recruitment of Th1 cells into tissues [76, 81], HTLV-I tax protein can up-regulate the expression of this antigen on HTLV-I-infected cells [79] as mentioned above, indicating that the characteristics of Th1 cells are not explainable by only the expression of this ligand in HTLV-I-infected status. Indeed, leukemia cells in patients with ATL and the related cell lines strongly express this ligand [79, 83]. In addition, the pattern of chemokine receptor expression suggests that ATL cells originate from Th2 or regulatory T cells [84, 85]. Although IFN-γ expression, a representative Th1 cytokine, is up-regulated with high proviral load in HAM/TSP patients, it is still not clear which intracellular signaling induces such a status. Thus, what type of signaling molecule is involved in Th1 activation in HTLV-I-infected cells from HAM/TSP patients? And how is the signaling molecule related to the high HTLV-I proviral load in the peripheral blood in HAM/TSP patients?

Numerous signaling molecules are involved in the regulation of expression of IFN-γ [46, 58]. One of these is the p38 mitogen-activated protein kinase (p38 MAPK), which is involved in Th1 differentiation with IFN-γ induction through the downstream target, activation transcription factor (ATF)-2 [86-88]. The p38 MAPK, which is phosphorylated by activated MAPK kinase, phosphorylates ATF-2 in turn and the phosphorylated ATF-2 induces transcription of the gene encoding IFN-γ. In addition, the importance of p38 MAPK signaling pathway in the Th1 differentiation, also in T cell receptor (TCR) or STAT-4 -independent manner, has been reported [89, 90].

On the other hand, HTLV-I tax protein, which is the gene product of pX region in the 3' terminal region of HTLV-I genome, plays an important role in transcription of HTLV-I proviral genome from HTLV-I long terminal repeat (LTR) [1]. However, it is well known that tax binds HTLV-I LTR not directly but through binding of the cAMP response element binding protein and ATF-1 or ATF-2 (CREB/ATF family) [91, 92], implying that the efficient recruitment of CREB and ATF to LTR of HTLV-I provirus is necessary for efficient HTLV-I replication. ATF-2 is the downstream target of p38 MAPK, MAPKAP kinase-2 [93] as mentioned above.

If p38 MAPK signaling is activated in HTLV-I-infected T cells of HAM/TSP patients, such status might be linked to both up-regulated spontaneous IFN-γ expression and high HTLV-I proviral load in HAM/TSP patients. We first analyzed activated (phosphorylated)-p38 MAPK expression with regard to both spontaneous IFN-γ production and HTLV-I p19 antigen

expression as indicators of HTLV-I expression in IL-2-dependent HTLV-I-infected T cell lines derived from HAM/TSP patients, compared with cells derived from ATL patients. Second, we analyzed the suppressive effect of a pyrinidyl imidazole inhibitor, SB203580, which is a p38 MAPK specific inhibitor [94], on both spontaneous IFN-γ production and HTLV-I p19 antigen expression in HTLV-I-infected T cell lines and the peripheral blood CD4$^+$ T cells derived from HAM/TSP patients [95].

As shown in table 2, both IFN-γ and HTLV-I 19 antigen levels were significantly higher in two HTLV-I-infected T cell lines from HAM/TSP patients (HCT-1 and HCT-4) [96] than in three HTLV-I-infected T cell lines from ATL patients (KK-1, KOB, and SO-4) [97]. Based on this finding, we next analyzed the expression of phosphorylated-p38 MAPK in each cell line, using Western blot. As shown in figure 17, the expression of phosphorylated-p38 MAPK was significantly increased in both HCT-1 and HCT-4. On the other hand, although the expression of phosphorylated-p38 MAPK was faintly detected in SO-4, it was not detected in both KK-1 and KOB (figure 17). These results suggest that high IFN-γ and HTLV-I p19 antigen expression in HTLV-I-infected T cells from HAM/TSP patients depend on activation of the p38 MAPK signaling pathway.

Table 2. Levels of IFN-γ and HTLV-I p19 antigen in culture supernatants of HTLV-I-infected T-cell lines

Cell lines	IFN-γ (IU/mL)	HTLV-I p19 (pg/mL)
HAM		
HCT-1	65.30	16,450.0
HCT-4	148.80	43,485.0
ATL		
KK-1	0.79	409.0
KOB	0.66	1,456.0
SO-4	0.77	< 25.0

Levels of IFN-γ and HTLV-I p19 antigen were measured by ELISA in culture supernatant of each cell line, cultured at 1 x 10^5 cells/mL in the presence of 100 units/mL of IL-2 for 48 hrs. The minimum detectable level of IFN-γ was 0.03 IU/mL and that of HTLV-I p19 antigen was 25.0 pg/mL. Quotation from Ref. 95.

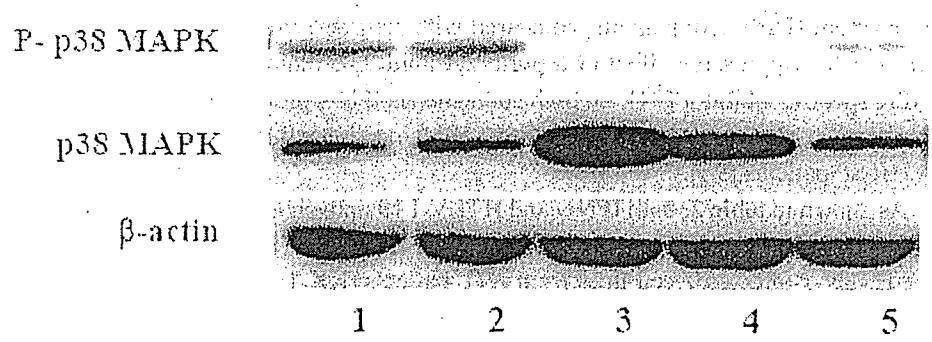
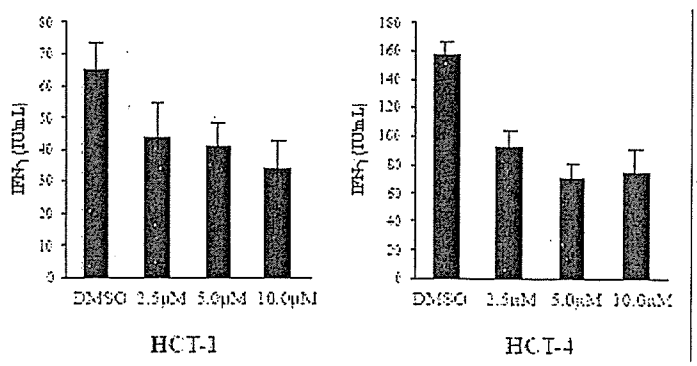
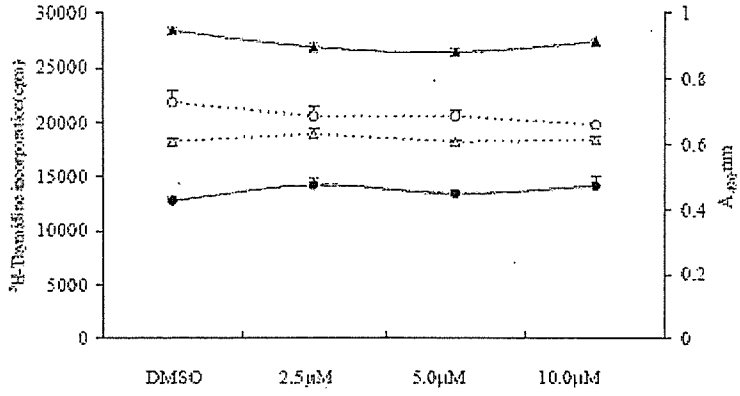


Figure 17. Western blot analysis of p38 MAPK expression in HTLV-I-infected T cell lines. Lanes 1 and 2: HTLV-I-infected T cell lines (HCT-1 and HCT-4) derived from HAM/TSP patients, respectively. Lanes 3, 4, and 5: HTLV-I-infected T cell lines (KK-1, KOB, and SO-4) derived from ATL patients, respectively. P-p38 MAPK: phosphorylated-p38 MAPK; β-actin: internal control. Quotation from Ref. 95.

To confirm whether this p38 MAPK signaling pathway is functionally activated for IFN-γ induction in HCT-1 and HCT-4, we analyzed the effect of a p38 MAPK specific inhibitor, SB203580, on spontaneous IFN-γ production by both cell lines. As shown in figure 18a, SB203580 suppressed dose-dependently, by up to about 50%, IFN-γ production by both cell lines. To determine whether the SB203580-induced suppression of IFN-γ production was dependent on inhibition of cell proliferation, we checked the changes in cell proliferation of both HCT-1 and HCT-4 treated with SB203580. However, as shown in figure 18b, SB203580 did not affect cell proliferation. In addition, the MTS assay revealed that this treatment also did not affect cell viability (figure 18b). These results indicate that p38 MAPK signaling is functionally activated for IFN-γ induction in both cell lines derived from HAM/TSP patients and the suppression of spontaneous IFN-γ production by treatment with SB203580 was based on inhibition of p38 MAPK signaling pathway in IFN-γ induction, but not suppression of cell proliferation.



a)



b)

Figure 18. Effects of SB203580 on HTLV-I-infected T cell lines derived from HAM/TSP patients (HCT-1 and HCT-4). a) SB203580 suppresses spontaneous IFN-γ production by HCT-1 and HCT-4. HCT-1 or HCT-4 cells were cultured at a density of 1 x 10^5 cells/mL in the presence of DMSO only or different concentrations (2.5, 5.0 and 10.0 μM) of SB203580 with 100 units/mL of IL-2 for 48 hrs, and then the culture supernatants were collected. Levels of IFN-γ in culture supernatants were measured by ELISA. Data are expressed as mean±SEM of triplicate cultures. b) Cell proliferation and cell viability assays. HCT-1 or HCT-4 cells were cultured in the presence of DMSO only or different concentrations of SB203580 with 100 units/mL of IL-2 in 96-well plates. For cell proliferation assay, cells were pulsed for the last 12 hrs of a 48-hr incubation with [^{3}H]TdR. Data are mean±SEM of triplicate cultures. HCT-1: ● - ●, HCT-4: ○···○. For cell viability, the MTS assay was performed according to the manufacturer's instructions. Data represent OD titer at wavelength of 490 nm. Data are mean±SEM of triplicate cultures. HCT-1: ▲ - ▲; HCT-4: △...△. Quotation from Ref. 95.

Then, is p38 MAPK signaling also involved in spontaneous IFN-γ production by peripheral blood CD4$^+$ T cells in HAM/TSP patients? To confirm this, we treated cells with SB203580 and evaluated the suppressive effect on IFN-γ production. The suppression ratio of IFN-γ production (SR) was determined as follows: SR = (IFN-γ level in culture supernatant of cultured CD4$^+$ T cells in the presence of DMSO only - IFN-γ level in supernatant of cultured CD4$^+$ T cells in the presence of 10 μM SB203580) / (IFN-γ level in supernatant of cultured CD4$^+$ T cells in the presence of DMSO only). As shown in figure 19, SB203580 induced about 24-79% suppression of IFN-γ production by peripheral blood CD4$^+$ T cells of all 6 HAM/TSP patients (figure 19, p=0.0156). However, SB203580 produced only about 20% suppression of IFN-γ production by peripheral blood CD4$^+$ T cells of 7 controls, including 3 anti-HTLV-I-seropositive carriers (figure 19). As in HTLV-I-infected T-cell lines, SB203580 did not affect the viability of peripheral blood CD4$^+$ T cells (data not shown). These findings suggest that the p38 MAPK signaling pathway is also preferentially involved in spontaneous IFN-γ production by peripheral blood CD4$^+$ T cells in HAM/TSP patients.

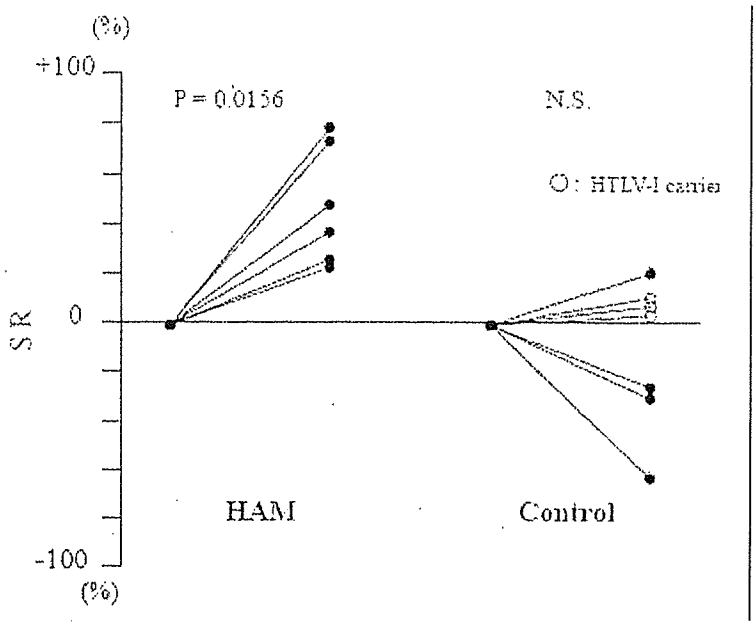


Figure 19. SB203580 suppresses IFN-γ production by peripheral blood $CD4^+$ T cells. Peripheral blood $CD4^+$ T cells were cultured at a densisty of 1 x 10^6 cells/mL in the presence of DMSO only or 10 μM SB203580 for 72 hrs, and the culture supernatants were collected. IFN-γ levels in each culture supernatant were measured by ELISA. The suppression ratio of IFN-γ production (SR) was determined as follows: SR = [(IFN-γ level in culture supernatant of cultured $CD4^+$ T cells in the presence of DMSO only - IFN-γ level in supernatant of cultured $CD4^+$ T cells in the presence of 10 μM SB203580) / (IFN-γ level in supernatant of cultured $CD4^+$ T cells in the presence of DMSO only)]. The sign test was used for statistical analysis. Open circle: HTLV-I-seropositive carriers; N.S.: not significant. Quotation from Ref. 95.

Next, we analyzed the involvement of p38 MAPK signaling in HTLV-I expression. Treatment of HCT-1 and HCT-4 with SB203580 suppressed HTLV-1 p19 antigen expression by about 22% and 10%, respectively (figure 20a). However, the same treatment did not reduce HTLV-I p19 antigen expression in the HTLV-I-transformed T-cell line, MT-2 (figure 20a), which produce low amounts of IFN-γ without the activation of p38 MAPK (data not shown). In addition, SB203580 produced about 24-66% reduction of HTLV-I p19 antigen expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients (figure 20b). These findings suggest that the p38 MAPK signaling pathway is also involved in HTLV-I expression in HTLV-I-infected cells from HAM/TSP patients.

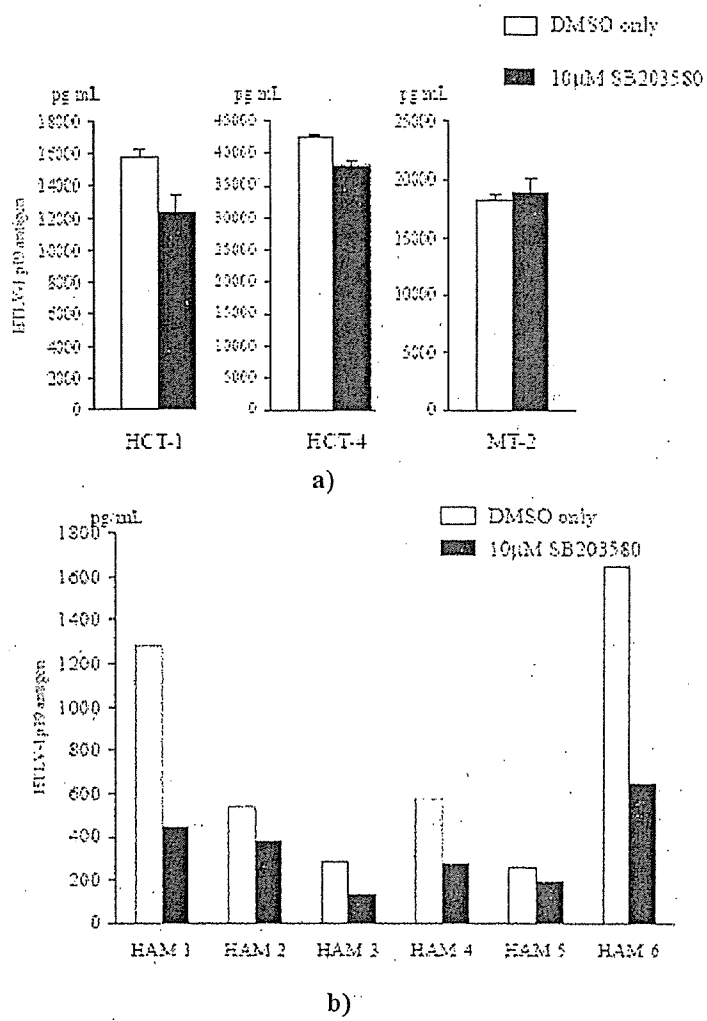


Figure 20. SB203580 reduces HTLV-I p19 antigen expression in HCT-1, HCT-4 and peripheral blood CD4$^+$ T cells of HAM/TSP patients. a) HCT-1 or HCT-4 cells were cultured at concentration of 1 x 10^5 cells/mL in the presence of DMSO only or 10 μM SB203580 with 100 units/mL of IL-2 for 48 hrs. As control, MT-2 cells were cultured without IL-2 for 48 hrs in the same condition. The culture supernatants were collected, and the levels of HTLV-I p19 antigen were measured by ELISA. Data are mean±SEM of triplicate cultures. Open bars: DMSO only; solid bars: 10 μM SB203580. b) Peripheral blood CD4$^+$ T cells of HAM/TSP patients were cultured at 1 x 10^6 cells/mL in the presence of DMSO only or 10 μM SB203580 for 72 hrs. The culture supernatants were collected, and the levels of HTLV-I p19 antigen in the culture supernatant were measured by ELISA. Open bars: DMSO only; solid bars: 10 μM SB203580. Quotation from Ref. 95.

In this study, we clearly demonstrated the involvement of p38 MAPK signaling for IFN-γ expression in HAM/TSP patients. Our data, such as the analysis of the relationship between activated p38 MAPK and IFN-γ expression in three cell lines derived from ATL patients, also support the hypothesis that ATL cells originate from Th2 cells [84, 85]. Although it is well known that IFN-γ expression is regulated by various signaling molecules, we focused on the involvement of the p38 MAPK signaling pathway for IFN-γ expression in HAM/TSP patients because it plays an important role in IFN-γ induction in TCR-independent condition [89],

which is consistent with up-regulated spontaneous IFN-γ expression in the peripheral blood $CD4^+$ T cells of HAM/TSP patients. Although other signaling pathways, such as nuclear factor kappa B (NF-κB) and c-JUN N-terminal kinase or IFN-γ promoter activation by HTLV-I tax itself [58, 87, 98] might also contribute to IFN-γ induction, activation of the p38 MAPK signaling pathway seems to be most important. While it is not clear whether increased IFN-γ production by peripheral blood $CD4^+$ T cells of HAM/TSP patients is derived from HTLV-I-infected cells only, TCR-independent IFN-γ induction also occurs over a relatively long period compared with TCR-dependent IFN-γ induction [89]. Therefore, activation of p38 MAPK signaling and the related up-regulation of IFN-γ expression seem consistent with the clinical course of HAM/TSP with chronic inflammatory status, such as slow progression.

One of the downstream targets of p38 MAPK is ATF-2, which binds HTLV-I LTR in concert with CREB and HTLV-I tax and enables efficient replication of HTLV-I, as mentioned above. Therefore, there was the possibility that activation of p38 MAPK signaling itself links to up-regulation of HTLV-I expression. Indeed, the interruption from p38 MAPK to ATF-2 signaling by SB203580 treatment induced the down-regulation of HTLV-I p19 antigen production from HTLV-I-infected T cell lines of HAM/TSP patients. In addition, very interestingly, HTLV-I p19 antigen expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients was also significantly suppressed by SB203580 treatment. These findings suggest that activation of the p38 MAPK signaling pathway plays a crucial role in HTLV-I expression and leads to high HTLV-I proviral load in the peripheral blood of HAM/TSP patients.

In this section, we have demonstrated that activation of the p38 MAPK signaling pathway is involved in the up-regulation of IFN-γ expression in HAM/TSP patients with high HTLV-I proviral load, suggesting that this signaling pathway functions as the one of the causes of Th1 activation based on high HTLV-I proviral load in the peripheral blood of HAM/TSP patients. Although it is not clear how p38 MAPK signaling is activated in HAM/TSP patients, perpetuation of activation of the p38 MAPK signaling pathway in the HTLV-I-infected state might strongly contribute to the development of HAM/TSP. Therefore, p38 MAPK might be a potential target in the treatment of HAM/TSP.

6. Which Kind of Status is Involved in the Long-Standing Perpetuation of a Chronic Inflammatory State in the Spinal Cords of HAM/TSP Patients?

As mentioned above, the increase of activated HTLV-I-infected Th1 cells have the potential to trigger the first step of the pathogenetic process in the spinal cords of HAM/TSP patients. However, HAM/TSP manifests as a chronic progressive disease, indicating that the pathological process involves long-standing perpetuation of a chronic inflammatory state in the spinal cords. Therefore, the establishment of the pathological process in the spinal cords of HAM/TSP patients is not explained by only the up-regulated transmigration activity of activated HTLV-I-infected Th1 cells. Thus, the peripheral blood $CD4^+$ T cells of HAM/TSP patients must settle locally over a long period, after transmigration into the spinal cords, for the establishment of the initial pathological process.