Methods

Patient samples

Blood samples were obtained from twelve patients with HAM/TSP (HAM#1-12), six HTLV-I-seropositive asymptomatic carriers (AC#1-6), and ten HTLV-I-seronegative healthy donors (ND#1-10). Diagnosis of HAM/TSP was based on WHO diagnostic criteria. Three patients with HAM/TSP were HLA-A*201$^+$. PBMCs were isolated by Ficoll-Hypaque (Lonza Walkersville, Walkersville, MD) centrifugation. The PBMCs obtained from HTLV-I-infected patients or ND were cryopreserved in liquid nitrogen until use. Informed consent was obtained from each subject. The study was reviewed and approved by the National Institute of Neurological Disorders and Stroke (NINDS) Institutional Review Board. Informed consent was obtained in accordance with the Declaration of Helsinki.

Antibodies and reagents

For flow cytometry, antibodies for human CD3, CD4, CD8, CD14, CD16, CD28, CD49d, CD107a, IFN-γ, TNF-α, and HLA-DR (all from BD Biosciences, San Jose, CA), CX_3CR1 (Medical and Biological laboratories, Nagoya, Japan), HLA-ABC (AbD Serotec, Oxford, UK), and anti-Tax monoclonal antibody (Lt-4) were used. For immunohistochemistry, rabbit polyclonal anti-human CX_3CR1 (abcam, Cambridge, MA) was used as primary antibody. Minocycline was purchased from Sigma (St. Louis, MO).

Cell culture

PBMCs of NDs or patients with HAM/TSP were suspended at 1 × 10^6 cells/mL in RPMI media (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine), and cultured for 24 hours either with or without minocycline in 24 well plate in 5% CO_2 incubator at 37°C. The culture supernatants were collected, centrifuged at 2000 g for 10 min to remove cellular debris and stored at -80°C until use. The cultured cells were collected for immunofluorescence staining or stored at -80°C until use. For immunofluorescence staining of MHC class I on MPs, PBMCs were collected after culture for 5 hours or 18 hours.

To examine Tax expression in CD4$^+$ T cells cocultured with or without CD14$^+$ cells, CD4$^+$CD25$^+$ T cells or CD4$^+$CD25$^-$ T cells and CD14$^+$ cells were magnetically isolated from PBMCs of HTLV-I-infected patients by CD4$^+$CD25$^+$ Regulatory T cell Isolation Kit and CD14 MicroBeads (both from Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according to the manufacture's instruction, and 2 × 10^5 cells of each CD4$^+$ T cells were cocultured with or without the same amount of autologous CD14$^+$ cells for 18 hours in 48 well plate in 5% CO_2 incubator at 37°C.

ELISA

IL-1β was detected in the PBMC culture supernatants of NDs and patients with HAM/TSP using Human IL-1β Quantikine ELISA (R & D systems), according to the manufacturer's instructions

CD107a mobilization assay

CD107a mobilization assay was performed as previously described [19]. To detect spontaneous degranulation and IFN-γ expression in CD8$^+$ T cells, PBMCs of patients with HAM/TSP were cultured for 24 hours. To detect Tax11-19 specific responses, PBMCs were stimulated with an appropriate concentration of HTLV-I Tax11-19 LLFGYPVYV and 1 μg/mL each of CD28 and CD49d for 5 hours. In treatment of minocycline, appropriate minocycline was added into the culture. Conjugated CD107a antibody, 0.7 μL/mL of GoldiStop™ (BD Biosciences), and 1 μg/mL of brefeldin A (Sigma) were added into the culture for 5 hours before the time point for detection.

Flow cytometry

For analysis of peripheral blood monocyte populations, patients' PBMCs were stained with CD3, CD4, CD8, CD14, CD16, HLA-DR and CX_3CR1. Expression of CD107a, IFN-γ, TNF-α and intracellular Tax in the cultured or uncultured PBMCs was examined by flow cytometoric analysis. First, PBMCs were surface-stained with specific antibodies. After fixation and permeabilization with Fixation/Permeabilization solution (BD Biosciences) according to the manufacturer's instructions, the cells were intracellularly stained with anti-IFN-γ, anti-TNF-α or anti-Tax for each experiment. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) or LSR II (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Lymphoproliferation assay

Lymphoproliferation assay was performed as previously described [55]. PBMCs were suspended in RPMI medium supplemented with 5% human AB serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine, and plated in triplicate on a round bottom 96-well plate at a concentration of 3 × 10^5 cells/well with or without minocycline. The cells were cultured in 5% CO_2 incubator at 37°C, and pulsed after 3 to 5 days of culture for 4 h with 1 μCi [^{3}H] thymidine. The average cpm from each of the wells was plotted.

Immunohistochemistry

Spinal cord tissues from a patient with HAM/TSP were fixed with buffered formalin and embedded in paraffin wax. Microtome sections were cut 10 μm thick. Sections

were deparaffinized with xylene, rehydrated and immersed in Target Retrieval Solution, pH6.0, (Dako, Carpinteria, CA) at 121°C for 10 min. After blocking of endogenous peroxide with 3% hydrogen peroxide for 10 min, the sections were incubated with a rabbit anti-CX_3CR1 antibody (1 μg/ml) for one hour at room temperature. Reactivity was visualized with diaminobenzidine (DAB) using Envision™+system (Dako), followed by counterstaining with hematoxylin. The stained sections were visualized with Zeiss 200M Axiovert inverted microscope (Carl Zeiss MicroImaging Inc, Thornwood, NY). The image data of each section were created using Volocity imaging analysis software (Improvision, Waltham, MA).

Statistical analysis

Mann-Whitney test and Wilcoxon matched-pairs signed rank test were used for comparison between groups. Simple linear regression analysis was used for explaining a relationship between groups, respectively. All statistical analysis was performed using Prism (GraphPad software).

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Authors' contributions

YE-A designed the research, performed most of the experiments, analyzed results, made the figures and wrote the manuscript; EM analyzed immunohistochemical image, analyzed results, made the figures and wrote the manuscript; UO coordinated clinical work, analyzed results and wrote the manuscript; YT contributed reagents for analysis. SJ designed the research, analyzed results and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Activation of mTOR by human T-cell leukemia virus type 1 Tax is important for the transformation of mouse T cells to interleukin-2-independent growth

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Human T-cell leukemia virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia, and it immortalizes and transforms human T cells in both an interleukin (IL)-2-dependent and -independent manner. HTLV-1 encodes Tax, which plays crucial roles in HTLV-1-mediated immortalization and transformation of human T cells. A previous study showed that Tax can transform a mouse T-cell line, CTLL-2, from having IL-2-dependent growth to IL-2-independent growth. Given that the Akt/mTOR pathway is essential for IL-2-induced cell growth in T cells, we examined whether the Akt/mTOR pathway is involved in Tax-induced transformation to IL-2-independent growth. The stable and transient expression of Tax in CTLL-2 induced the phosphorylation of p70S6 kinase and ribosomal protein S6, downstream targets of the mTOR kinase, whereas that of Akt was only minimally induced. Studies with Tax mutants indicated that the activation of mTOR by Tax was correlated with the transformation of CTLL-2 cells to IL-2-independent growth. Rapamycin, an inhibitor of mTOR kinase, reduced the growth of Tax-transformed CTLL-2 cells. Moreover, the transduction of a constitutively active form of Akt in the CTLL-2 cells also induced IL-2-independent growth. Like CTLL-2/Tax, constitutive phosphorylation of p70S6 kinase was detected in the absence of IL-2 in all of the HTLV-1-infected human T-cell lines. These results suggest that Tax activates the mTOR pathway in T cells, and that this activation plays a crucial role in the growth of HTLV-1-infected T cells when a limited amount of IL-2 is available. (*Cancer Sci* 2012; 103: 369–374)

Human T-cell leukemia virus type 1 (HTLV-1) is an etiologic agent of adult T-cell leukemia (ATL),[1–4] which is characterized by monoclonal proliferation of HTLV-1-infected $CD4^+$ T cells. HTLV-1 is mainly transmitted from a mother to an infant through breast milk, but only 3–5% of infections develop ATL, with an average latency of 40–60 years.[4] HTLV-1 immortalizes $CD4^+$ T cells in an interleukin (IL)-2-dependent manner, although some cells acquire IL-2-independent growth properties.[5,6] Understanding how HTLV-1 immortalizes and transforms $CD4^+$ T cells is an important step to elucidate the molecular mechanism underlying the leukemogenesis.

In addition to its structural genes, HTLV-1 encodes several non-structural genes.[7] Of these, *tax1* is essential for the immortalization of $CD4^+$ T cells by HTLV-1.[8] For instance, recombinant HTLV-1 defective for the *tax1* gene does not immortalize human T cells and did not establish persistent infection in an animal model.[8] Moreover, Tax1 by itself immortalizes human $CD4^+$ T cells in an IL-2-dependent manner *in vitro*.[9,10] Therefore, Tax1 plays crucial roles in the IL-2-dependent immortalization of human T cells by HTLV-1.

HTLV-2, a close relative of HTLV-1, is not associated with ATL or any other types of leukemia.[11,12] HTLV-2 also encodes a transforming protein, Tax2, which has approximately 75% amino acid similarity to Tax1, and shares many functions, such as activation of NF-κB, CREB, and AP-1. Intriguingly, Tax1 has one dominant activity over Tax2; it converts a mouse T-cell line, CTLL-2, from IL-2-dependent growth to IL-2-independent growth much more efficiently than Tax2.[13,14] These results suggest that the activity of Tax1 for inducing the IL-2-independent growth of cells is associated with the HTLV-1-specific pathogenesis.

The Akt/mTOR kinase pathway is activated by IL-2 and transmits cell survival signals in T cells.[15,16] Moreover, aberrant activation of the Akt/mTOR pathway is frequently observed in leukemia and lymphoma.[17,18] Our data indicate that Tax1 activates the mTOR kinase in CTLL-2 cells and that this activation is important for the Tax1-induced transformation of CTLL-2 cells to IL-2-independent growth.

Materials and Methods

Cells and culture conditions. The cell lines used in this study have all been described previously.[19,20] SLB-1, MT-4, and HUT102 are HTLV-1-infected human T-cell lines. HUT78 and Jurkat are HTLV-1-negative human T-cell lines. These human T-cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL) (RPMI/10%FBS). Kit225 is an IL-2-dependent HTLV-1-negative human T-cell line originated from a patient with T-cell chronic lymphocytic leukemia,[21] and the cells were cultured in RPMI/10%FBS containing 0.5 nM IL-2 (RPMI/10%FBS/IL-2). ILT-Koy and ILT-Oot are IL-2-dependent HTLV-1-infected human T-cell lines, and they were cultured in RPMI/20%FBS/IL-2.[19] CTLL-2 is a mouse T-cell line that grows in an IL-2-dependent manner, and the cells were cultured in RPMI/10%FBS/IL-2 supplemented with 55 μM 2-mercaptoethanol (2-ME) (RPMI/10%FBS/IL-2/2-ME). CTLL-2 cells stably expressing Tax1 were cultured in RPMI/10%FBS/IL-2/2-ME containing 2 μg/mL puromycin.[14] Tax1-transformed IL-2-independent CTLL-2 cells were cultured in RPMI/10%FBS/2-ME. CTLL-2 cells stably expressing hAkt1mΔPH (CTLL-2/hAkt1mΔPH) were cultured in RPMI/10%FBS/IL-2/2-ME containing 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA, USA). Human embryonic kidney 293T cells were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL).

Plasmids. pHβPr-1-neo-Tax1, pHβPr-1-neo-TaxM22, and pHβPr-1-neo-Tax703 were expression plasmids encoding Tax1, TaxM22, and Tax703, respectively.[19,22] CS-EF-Tax1 is a lentiviral vector expressing Tax1.[23] To construct the expression

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vectors for Tax1 mutant genes, the TaxM22 and Tax703 cDNA fragments were amplified by PCR from the pHβPr-1-neo-TaxM22 and pHβPr-1-neo-Tax703 plasmids, respectively, and the fragments were inserted into the Gateway entry vector pENTR/D-TOPO (Invitrogen). These *tax* genes in the entry vectors were then transferred to the lentiviral vector CSII-EF-RfA (provided by Dr. H. Miyoshi, Riken Tsukuba Institute, Tsukuba, Japan) by an LR recombination reaction using LR clonase (Invitrogen), and they were designated CS-EF-TaxM22 and CS-EF-Tax703. The κB-Luc and WT-Luc plasmids are luciferase reporters regulated by the κB element of the IL-2 receptor α-chain gene and the Tax-inducible 21-bp sequence in the HTLV-1 long-terminal repeat, respectively.[13] The pGK/β-gal plasmid expresses β-galactosidase under the control of the phosphoglycerate kinase promoter and was used to normalize the transfection efficiency. The retroviral expression vector, pQCXIN-hAkt1mΔPH, was constructed by inserting the hAkt1mΔPH fragment from the pCS2-hAkt1mΔPH plasmid into the *Bam*HI site of pQCXIN (Clontech, Palo Alto, CA, USA). The pCS2-hAkt1mΔPH plasmid encoding a constitutively active human Akt1 (hAkt1mΔPH) was provided by Dr. Y. Gotoh, Institute of Molecular and Cellular Biosciences, University of Tokyo (Tokyo, Japan).[24]

Generation of lentiviruses and retroviruses. Recombinant lentiviruses were generated by transfecting pCAG-HIVgp, pCMV-VSV-G-RSV-Rev (provided by Dr. H. Miyoshi, Riken Tsukuba Institute) and the respective lentiviral vectors encoding GFP, Tax1, Tax703, and TaxM22 into 293T cells using FuGENE HD (Roche, Indianapolis, IN, USA). At 72 h after the transfection, the culture supernatants were collected, and the viral titers were measured by a Lenti-X qRT-PCR Titration Kit (Clontech). They were then infected into CTLL-2 cells (4×10^5) in 2 mL RPMI/10%FBS/IL-2/2-ME containing 8 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). Recombinant retroviruses were generated by transfecting pGag-pol-IRES-bs^r (a gift from Dr. T. Kitamura, Institute of Medical Science, University of Tokyo),[25] pMD.G, an expression vector for VSV-G, and the retroviral vector plasmid encoding hAktmΔPH into 293T cells using FuGENE 6 (Roche). At 72 h after transfection, the culture supernatant was collected and used to infect the CTLL-2 cells (4×10^5) in a final volume of 2 mL RPMI/10%FBS/IL-2/2-ME containing 8 μg/mL polybrene. At 48 h post-infection, the cells infected with lentiviruses or retroviruses were cultured in RPMI/10%FBS/IL-2/2-ME supplemented with 0.5 mg/mL G418 for more than 7 days, and the expression of hAkt1mΔPH in the established cells was measured by Western blot analysis.

Western blot analysis. The cell lysates were prepared by treating the cells with 1× SDS sample buffer (2% SDS, 62.5 mM Tris–HCl [pH 6.8], 10% glycerol, 0.01% bromophenol blue, 50 mM DTT). The protein concentrations of the cell lysates were measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysates (15–20 μg) were size-separated by SDS-PAGE, and electronically transferred onto a PVDF membrane. The membrane was incubated with 5% skim milk/TBS-T (20 mM Tris–HCl [pH7.6], 150 mM NaCl, 0.1 % Tween 20) at room temperature for 1 h, and further incubated with the primary antibodies. After washing with TBS-T, the membranes were incubated with an anti-rabbit or anti-mouse antibody or Protein A conjugated with HRP. The proteins recognized by the antibodies on the membrane were visualized using the ECL Western Blotting Detection System (GE Healthcare Bioscience, Little Chalfont, UK). The primary antibodies used in this study were an rabbit anti-Akt mAb, rabbit anti-phospho-Akt mAb, rabbit anti-p70 ribosomal S6 kinase (p70S6K) antibody, rabbit anti-phospho-p70S6K antibody, rabbit anti-ribosomal protein S6 (RPS6) antibody, rabbit anti-phospho-RPS6 antibody (Cell Signaling Technology, Beverly, MA, USA), a mouse anti-PTEN mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal anti-Tubulin antibody (Calbiochem, La Jolla, CA, USA), and a mouse anti-Tax mAb (Taxy7).[26]

Cell growth assay. CTLL-2 cells expressing Tax1 or parental CTLL-2 cells were cultured at 1×10^5 cells/mL in 1 mL RPMI/10%FBS/2-ME together with either 100 nM rapamycin or its solvent (DMSO) on a 24-well plate. CTLL-2/hAkt1mΔPH cells were washed once with RPMI 1640 medium, and were cultured at 2×10^5 cells/mL in 1 mL RPMI/10%FBS/2ME in the presence of either 100 nM rapamycin or its solvent (DMSO) on a 24-well plate. The number of viable cells was counted by a Trypan blue dye exclusion method using light microscopy. CTLL-2/hAkt1mΔPH cells were also cultured in 96-well plates at a cell density of 1000, 3000, or 9000 cells/well for 3 weeks without IL-2. The number of wells containing outgrowing cells was counted under a light microscope.

Luciferase assay. CTLL-2 cells (2.5×10^6) were transfected with pHβPr-1-neo-Tax1, pHβPr-1-neo-Tax703, or pHβPr-1-neo-TaxM22, together with κB-Luc or WT-Luc (CRE-Luc), and pGK/β-gal by using the DEAE dextran method as described previously.[19] At 48 h after transfection, the cell lysates were prepared, and the luciferase and β-galactosidase activities in the lysates were measured using the Luciferase Assay System (Promega, Fitchburg, WI, USA) and Galacto-Light System (Applied Biosystems, Foster City, CA, USA), respectively.

Results

Tax1 induces phosphorylation of S6K and RPS6 in a T-cell line. CTLL-2 is a mouse T-cell line, the growth of which is dependent on IL-2. We previously showed that HTLV-1 Tax1 transforms CTLL-2 cells to induce their IL-2-independent growth.[19] IL-2-induced growth in normal T cells requires activation of the Akt/mTOR pathway, so we examined the involvement of Akt/mTOR in Tax1-induced IL-2-independent cell growth of CTLL-2 cells. We previously established stable Tax1-expressing cells in the presence of IL-2 by transfecting them with a Tax1 expression plasmid.[14] Western blot analysis confirmed that two Tax1-transduced cell lines (Tax1-14, Tax1-18), but not the vector-only transduced cells, expressed the Tax1 protein (Fig. 1). These cells were then cultured with and without IL-2 for 18 h, and the status of the Akt/mTOR pathway was characterized by Western blot analysis. After IL-2 deprivation, two Tax1-transduced cell lines showed increased phosphorylation of S6K and RPS6, two well-known downstream targets of mTOR kinase, relative to the control cells, whereas they showed an equivalent level of phosphorylated Akt (Fig. 1). The CTLL-2/Tax1-14 cells showed stronger phosphorylation of S6K and RPS6 than CTLL-2/Tax1-18 cells, which correlated with the high Tax1 expression. Unlike Tax1, IL-2 induced the phosphorylation of Akt in CTLL-2 cells, indicating that Akt is able to be activated in CTLL-2 cells. To confirm the activity of Tax1 with regard to S6K and RPS6, it was transiently transduced into CTLL-2 cells by a lentiviral vector, and the cells were cultured in the absence of IL-2 for 18 h. The transient expression of Tax1 also induced the phosphorylation of S6K and RPS6, but not that of Akt (Fig. 2). Therefore, these results indicate that Tax1 induces the phosphorylation of S6K and RPS6 in CTLL-2 cells.

To obtain information about how Tax1 induces the phosphorylation of S6K and RPS6, we used two Tax1 mutants, Tax703 and TaxM22.[27,28] These *tax1* expression plasmids, together with luciferase reporter plasmids under the control of either NF-κB or CREB, were transfected into a mouse T-cell line (CTLL-2) using the DEAE–dextran method. At 48 h after transfection, the cell lysates were prepared, and the luciferase activities of the cells were measured. Tax703 efficiently activated NF-κB-dependent transcription, but the activity level of CREB was only half of that induced by the wild-type Tax.

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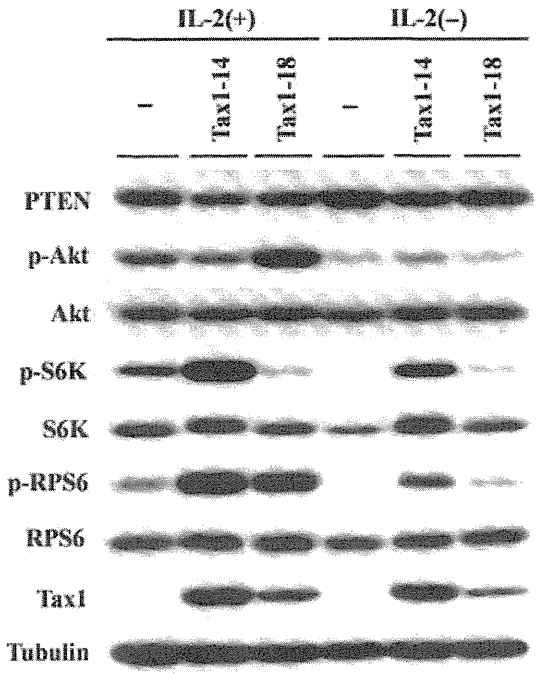

Fig. 1. Augmented phosphorylation of S6K and RPS6 is present in CTLL-2 mouse T cells expressing Tax1. CTLL-2 cells stably expressing Tax1 (Tax1-14, Tax1-18), maintained in the presence of 0.5 nM IL-2, were cultured with (+) or without (–) IL-2 for 18 h. Lysates prepared from the cells were then subjected to Western blot analysis with the indicated antibodies. The antibodies used were anti-Akt, anti-phospho-Akt, anti-p70S6K, anti-phospho-p70S6K, anti-RPS6, anti-phospho-RPS6, and anti-PTEN, anti-tubulin, and an anti-Tax mAb (Taxy7).

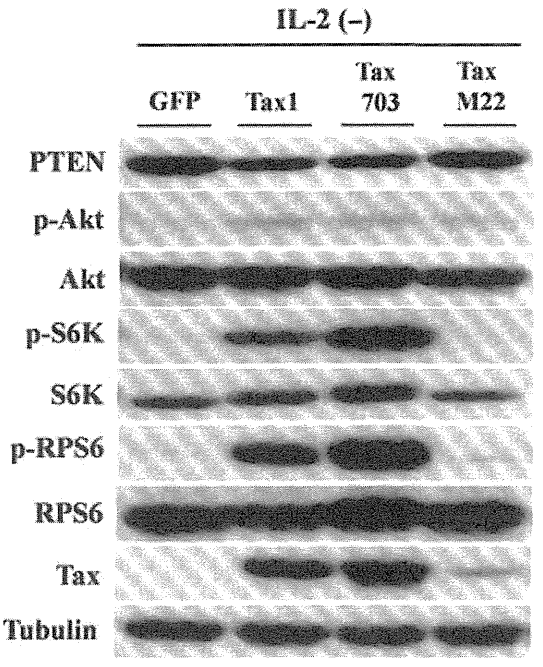

Fig. 2. Tax1 induces the phosphorylation of S6K and RPS6 in CTLL-2 mouse T cells. CTLL-2 cells (4×10^5) were infected with lentiviruses encoding Tax1, Tax703, or TaxM22. At 48 h after infection, the cells were washed with RPMI-1640 medium, and cultured without (–) IL-2 for 18 h. Lysates prepared from the cells were analyzed by Western blot analysis using the indicated antibodies.

TaxM22 only minimally activated NF-κB, and activated CREB more than did Tax703 (Fig. 3). Like Tax1, transduction of Tax703 into CTLL-2 cells induced the phosphorylation of S6K and RPS6, but not Akt. However, TaxM22 in the CTLL-2 cells did not induce any phosphorylation of S6K, RPS6, or Akt, although its inability to induce the phosphorylation of S6K and RPS6 may be due to the low protein expression in CTLL-2 cells. Collectively, these results suggest that the Tax1 functions associated with NF-κB activation play a major role in the phosphorylation of S6K and RPS6. We also noticed that stable, as well as transient, expression of Tax1 or Tax703 in CTLL-2 cells reduced the expression of PTEN, which is the main phosphatase of phosphorylated Akt. This is consistent with the results of a previous study indicating that Tax1 downregulates the expression of PTEN through the NF-κB pathway.[29]

mTOR plays a role in the IL-2-independent growth of CTLL-2/Tax1 cells. The above data suggested that Tax1 stimulates mTOR activity to phosphorylate S6K and RPS6 in CTLL-2 cells. To examine whether mTOR is involved in the Tax1-induced growth stimulation of CTLL-2 cells, Tax1-transformed IL-2-independent CTLL-2 cells were cultured in the presence of rapamycin, an mTOR kinase inhibitor,[30] and the number of viable cells was counted by light microscopy. Rapamycin inhibited the growth of Tax1-transformed CTLL-2 cells (Fig. 4A), which was associated with the decreased phosphorylation of RPS6 (Fig. 4B). Rapamycin also reduced the IL-2-induced growth of CTLL-2 cells (Fig. 4A), which was also associated with reduced phosphorylation of RPS6. These results suggest that mTOR activation plays a role in the Tax1-induced IL-2-independent growth of CTLL-2 cells.

Constitutively active Akt1 induces IL-2-independent growth of CTLL-2 cells. To further clarify the role of the Akt/mTOR pathway in the IL-2-independent growth of CTLL-2 cells, we established CTLL-2 cells expressing constitutively active Akt1 (hAkt1mΔPH) by retrovirus-mediated gene transfer in the presence of IL-2. Western blot analysis detected hAkt1mΔPH in the CTLL-2 cells (Fig. 5A). Next, the established cells were cultured without IL-2 for the indicated

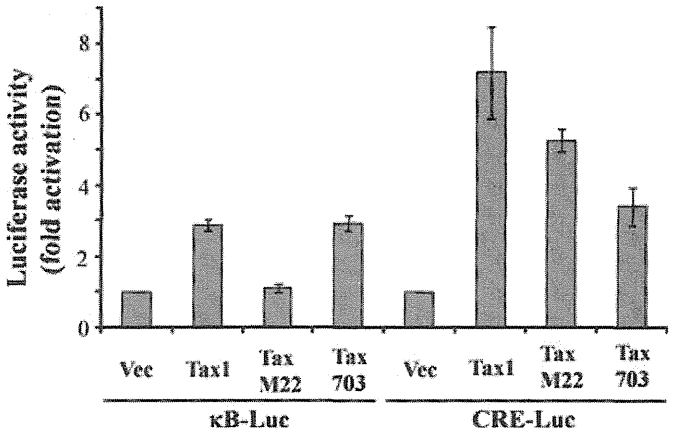

Fig. 3. CTLL-2 mouse T cells were transfected with pHβPr-1-neo-Tax1, pHβPr-1-neo-Tax703, or pHβPr-1-neo-TaxM22, together with κB-Luc or WT-Luc (CRE-Luc), and pGK/β-gal using the DEAE–dextran method. At 48 h after transfection, the cell lysates were prepared, and the luciferase and β-galactosidase activities in the lysates were determined using a luminometer. The activity of luciferase was normalized to that of β-galactosidase, and the fold activation was calculated as the ratio to that of the control transfection with the pHβPr-1-neo plasmid. The data shown are the averages of triplicate scores with standard deviations.

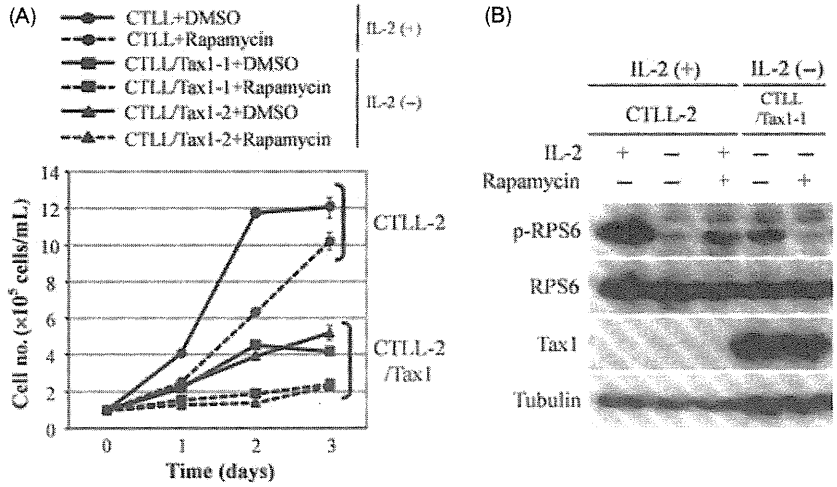

Fig. 4. Rapamycin suppresses the growth of Tax1-transformed CTLL-2 mouse T cells. (A) CTLL-2 cells and Tax1-transformed IL-2-independent CTLL-2 cells (Tax1-1 and Tax1-2, respectively) were cultured in the presence or absence of 100 nM rapamycin. The number of viable cells was counted by a Trypan blue dye exclusion method using a light microscope. (B) CTLL-2, Tax1-transformed IL-2-independent CTLL-2 cells (Tax1-1) were cultured in the presence or absence of 100 nM rapamycin. After 24 h in culture, cell lysates were prepared and subjected to Western blot analysis using the indicated antibodies. RPS6, ribosomal protein S6.

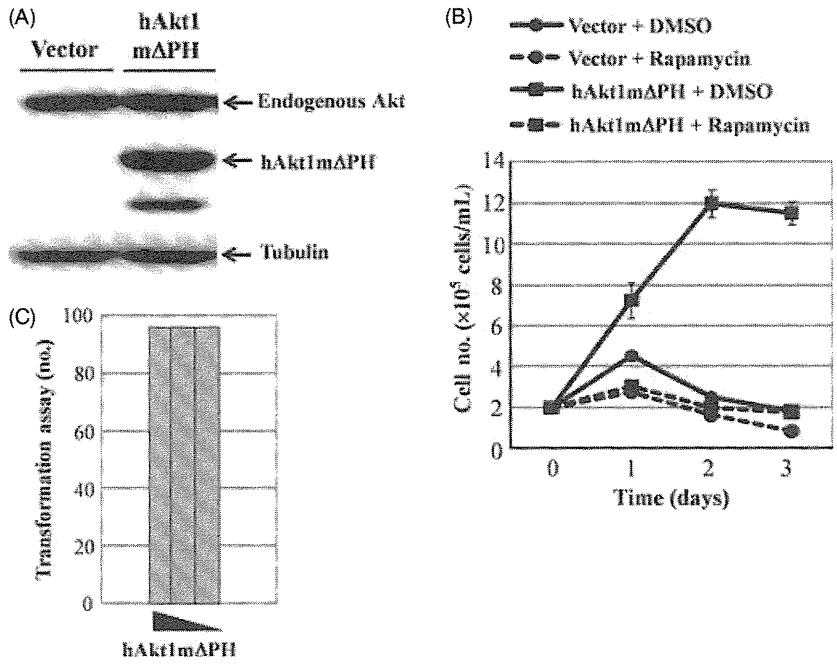

Fig. 5. Constitutively active Akt1 transforms CTLL-2 mouse T cells from IL-2-dependent growth to independent growth. (A) CTLL-2 cells were infected with retroviruses encoding hAktmΔPH, and the cells were cultured in medium containing IL-2 and G418 for 7 days. Cell lysates were prepared, and the expression of the hAktmΔPH protein was measured by Western blot analysis using an anti-Akt antibody. (B) CTLL-2 cells stably expressing hAkt1mΔPH were washed with RPMI-1640 medium, suspended in RPMI/10%FBS/2-ME in the presence or absence of 100 nM rapamycin, and seeded at 2×10^5 cells/mL in a 24-well plate. The number of viable cells was counted by a Trypan blue dye exclusion method using a light microscope. (C) CTLL-2 cells stably expressing hAkt1mΔPH were washed twice with PBS, and cultured without IL-2 at a cell density of 1000, 3000, or 9000 cells/well in 96-well plates for 3 weeks. The number of wells containing outgrowing cells was counted under a light microscope.

number of days, and the number of viable cells was counted using a Trypan blue exclusion method under a light microscope. Unlike the parental CTLL-2 cells, the cells expressing constitutively active Akt1 continuously grew in the absence of IL-2 (Fig. 5B). Moreover, the cell growth induced by constitutively active Akt1 was efficiently inhibited by rapamycin.

After obtaining these results, the CTLL-2/ hAkt1mΔPH cells were next cultured in the absence of IL-2 at a cell density of 1000, 3000, or 9000 cells/well in 96-well plates. After 3 weeks in culture, all the wells contained outgrowing cells (Fig. 5C). These results indicated that the activation of the Akt/mTOR pathway is sufficient to induce the IL-2-independent growth of CTLL-2 cells.

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Akt/mTOR pathway activated in HTLV-1-infected T-cell lines. There are two types of HTLV-1-infected human T-cell lines, one shows IL-2-dependent cell growth and the other shows IL-2-independent growth. For our subsequent study, we used two types of HTLV-1-negative human T-cell lines, IL-2-dependent (Kit225) and IL-2-independent (HUT78) cells. Jurkat cells are an HTLV-1-negative human T-cell line that has constitutively active Akt as a result of the deletion of the *PTEN* gene[31] (Fig. 6). The status of the Akt/mTOR pathway in these cell lines was characterized by Western blot analysis (Fig. 6). The phosphorylated forms of Akt, S6K, and RPS6 were higher in all the HTLV-1-infected human T-cell lines, except for MT-4 cells, than those in HUT78 or Kit225 cells in the absence of IL-2. These results indicated that the Akt/mTOR pathway is activated in most HTLV-1-infected human T-cell lines. MT-4 cells showed increased phosphorylation of S6K relative to the control cells, but no differences in the phosphorylation of RPS6 or Akt.

Discussion

After treatment with IL-2, the mTOR kinase is activated in T cells, which then induces the phosphorylation of its downstream targets, S6K and RPS6, the activation of which is essential for IL-2-induced cell growth.[32] In this study, we found that Tax1 induces the phosphorylation of S6K and RPS6 in the CTLL-2 T-cell line (Figs 1,2), and that the inhibition of mTOR by rapamycin reduced the growth of Tax1-transformed IL-2-independent CTLL-2 cells (Fig. 4A). Moreover, a constitutively active form of Akt1 transformed the CTLL-2 cells from having IL-2-dependent growth to have IL-2-independent growth (Fig. 5). Taken together, these results suggest that Tax1-mediated activation of mTOR kinase in CTLL-2 cells is crucial for their IL-2-independent growth.

Two previous studies showed that Tax1 activates Akt in a fibroblast cell line and T cells.[29,33] For instance, transient expression of Tax1 in primary human T cells purified from PBMC induced the phosphorylation of Akt through the activation of phosphatidylinositol 3-kinase (PI3K).[29] Although it is unclear why we could not detect the Tax1-induced phosphorylation of Akt in CTLL-2 cells, this might be due to the distinct experimental conditions used in the two studies, including the differences in cell types (purified primary human T cells versus an IL-2-dependent T-cell line). Therefore, the present study could not clarify whether the activation of mTOR by Tax1 is mediated by Akt or not.

Tax703 activated mTOR in CTLL-2 cells (Fig. 2). A previous study showed that Tax703 induced the IL-2-independent growth of CTLL-2 cells.[19] Taken together, these results support our hypothesis that mTOR activation by Tax1 is involved in the induction of IL-2-independent growth of CTLL-2 cells. It should be noted that Tax703 activates or represses several signaling pathways other than NF-κB-dependent transcriptional activation, such as transcriptional activation through E2F.[34] Therefore, the present data does not necessarily mean that mTOR is activated by Tax1 through NF-κB-inducible genes. As a result, a further analysis is required to elucidate how Tax1 activates mTOR.

All but one (MT-4) of the HTLV-1-infected T-cell lines that were examined possessed augmented phosphorylation of S6K and RPS6 relative to that in uninfected cells (Fig. 6). A previous study showed that rapamycin inhibits the growth of IL-2-independent HTLV-1-infected T-cell lines.[35] Taken together, the results of the present and previous studies suggest that Tax1 plays a role in mTOR activation in HTLV-1-infected T-cell lines, thereby mediating their IL-2-independent growth. Of the various HTLV-1-positive cell lines that were examined, MT-4 showed increased phosphorylation of S6K, but not of RPS6 nor Akt, relative to HTLV-1-negative cells. Therefore, S6K might be activated in MT-4 cells through an mTOR-independent mechanism.

The phosphorylation levels of S6K and RPS6 in IL-2-dependent HTLV-1-infected cells in the absence of IL-2 were closely equivalent to those of the IL-2-independent HTLV-1-infected cells (Fig. 6). These results suggest that mTOR activation is not sufficient for the IL-2-independent growth of HTLV-1-infected cells, and event(s) other than mTOR activation are required for their IL-2-independent growth. In this respect, some other viral protein different from Tax and/or the aberrant activation of cellular factor(s) is likely to be involved in the IL-2-independent growth of HTLV-1-infected T-cells, as Tax immortalizes human T cells only in an IL-2-dependent manner.

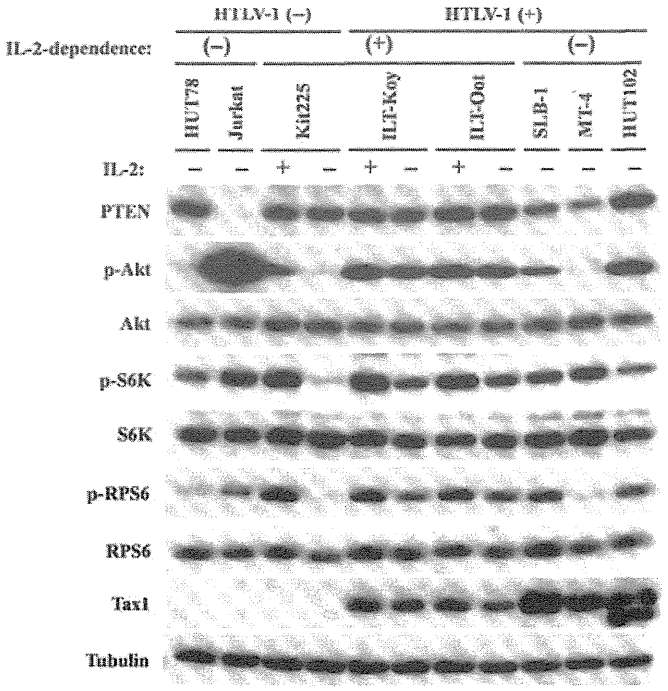

Fig. 6. Status of the Akt/mTOR pathway in HTLV-1 negative and positive human T-cell lines. IL-2-dependent human T-cell lines (Kit225, ILT-Koy, and ILT-Oot) were washed twice with PBS, and cultured in 5 mL of RPMI/10%FBS without IL-2 for 18 h. Cell lysates were prepared from these cells, as well as the indicated IL-2-independent HTLV-1 negative human T-cell lines (HUT78, Jurkat, and Kit225), and HTLV-1 positive human T-cell lines (SLB-1, MT-4, and HUT102), and their protein expression was assessed by Western blot analysis using the indicated antibodies. IL-2 dependence (+) indicates IL-2-dependent growth properties of the respective cells. HTLV-1(+), HTLV-1-infected cell lines.

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Disclosure Statement

The authors have no conflict of interest.

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Review Article

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

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

1. Introduction

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

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

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

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

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

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

3. Risk Factors for HAM/TSP

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

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

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

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

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

3.2. HTLV-1 Genotype and Genomic Integration Site. Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 *tax* gene variation and the risk of HAM/TSP [46]. The *tax* subgroup A, which belongs to cosmopolitan subtype A, was more frequently observed in HAM/TSP patients, and this association was independent of the protective effect of the HLA allele HLA-A*02. HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, *tax* subgroup B which belongs to cosmopolitan subtype B, but not against *tax* subgroup A in the Japanese population [46]. Interestingly, HLA-A*02 appeared not to give protection against infection with cosmopolitan subtype A in a population in Iran [40]. Moreover, the Iranian HTLV-1 strain has a Rex protein that is 20 amino acids longer than that of the Japanese strain that belongs to cosmopolitan subtype B. Experiments are now underway to compare the functions of these Rex proteins.

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

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

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

4. Immune Response to HTLV-1

4.1. Innate Immune Response

4.1.1. Natural Killer (NK) Cells. Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of NK cells (especially the $CD3^+CD16^+$ subset) than ACs although the results were not normalized with respect to PVL [51]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, a synthetic tetramer of HLA-E with the HLA-G signal sequence peptide was used to identify NK cells in HAM/TSP patients [52]. The results showed a significantly lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than ACs, and as in the earlier studies [51], this reduction in frequency was particularly notable in the $CD3^+$ cells, whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^-$ cells between patients with HAM/TSP and ACs [52]. Recent data also suggest that the frequency of invariant NKT (iNKT) cells in the peripheral blood of HAM/TSP patients is significantly decreased when compared with healthy subjects and/or ACs [53, 54]. These findings indicate that the activity of the NK or NKT cell response was associated with the absence of HAM/TSP. Interestingly, a previous uncontrolled preliminary trial of treatment of HAM/TSP with fermented milk containing viable *Lactobacillus casei* strain Shirota resulted in a significant increase in NK cell activity, with improvements in clinical symptoms [55]. Thus, circulating NK and NKT cells might also play an important role in the disease progression and the pathogenesis of HAM/TSP. Recently, it has been reported that in addition to the previously described $CD8^+$ T-cell spontaneous proliferation [56], $CD56^+$ NK cells also spontaneously proliferated *in vitro*, and spontaneous NK cell proliferation positively correlated with HTLV-1 PVL but not with the presence of HAM/TSP [57]. A hallmark of HTLV-1 infection is the *in vitro* proliferation of PBMCs when cultured in the absence of exogenous antigen or mitogen, referred to as spontaneous lymphocyte proliferation (SLP), and in HAM/TSP patients, the levels of SLP reflect the severity of the disease [58, 59]. Most of the high SLP observed in PBMCs from HAM/TSP patients is likely to be explained by a greater spontaneous expression of the provirus and consequently a greater proliferation of responding $CD8^+$ T cells in culture [56]. The greater proviral expression may be partly attributable to the impaired function and decreased number of NK cells in HAM/TSP patients. Although further

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

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

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

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

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

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

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

4.4. CD4$^+$ Helper T-Cell Response to HTLV-1. Antiviral CD4$^+$ T-cell responses are of central importance in driving B-cell and CD8$^+$ T-cell responses *in vivo*. The most common HTLV-1 antigen recognized by CD4$^+$ T-cells is the Env protein [87, 88], in contrast with the immunodominance of Tax in the CD8$^+$ T-cell response [89–91]. At a similar PVL, patients with HAM/TSP had significantly increased frequency of virus-specific CD4$^+$ T cells compared to ACs [88, 92]. The antiviral T-helper (Th)1 phenotype is also dominant among HTLV-1-specific CD4$^+$ T cells in both ACs and patients with HAM/TSP [93], and there is a higher frequency of IFN-γ, TNF-α, and IL-2 production by CD4$^+$ T cells in patients with HAM/TSP compared to AC of a similar PVL [93, 94]. A role for CD4$^+$ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts the immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in Southern Japan [37, 41] and Northeastern Iran [40]. Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380-394 peptide was used to analyze Env-specific CD4$^+$ T cells directly *ex vivo* [92]. The results showed that the frequency of tetramer$^+$CD4$^+$ T cells was significantly higher in HAM/TSP patients than ACs with similar PVL. Furthermore, direct ex vivo analysis of tetramer$^+$CD4$^+$ T cells from two unrelated DRB1*0101-positive HAM/TSP patients indicated that certain T-cell receptors (TCRs) Vβs were utilized and antigen-specific amino acid motifs were identified in complementarity determining region (CDR) 3 from both patients. These results suggest that the observed increase in virus-specific CD4$^+$ T cells in HAM/TSP patients, which may contribute to CD4$^+$ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected CD4$^+$ T cells but was the result of *in vivo* selection by specific MHC-peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 tetramer$^+$CD8$^+$ T cells [95] and muscle-infiltrating cells from HAM/TSP patients and HTLV-1-infected polymyositis patients [96].

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

4.6. Dendritic Cells (DCs). Dendritic cells are antigen-presenting cells which play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from HAM/TSP patients were infected with HTLV-1 [106], and the development of HAM/TSP is associated with rapid maturation of DCs [107]. As already mentioned, one of the hallmarks of HTLV-1 infection is the spontaneous lymphocyte proliferation (SLP). Interestingly, depletion of DCs from the HAM/TSP patient's PBMCs abolished SLP, whereas supplementing DCs restores proliferation [106]; supplementing B cells or macrophages had no effect. A DC-dependent mechanism of SLP was further supported by data showing that antibodies to MHC

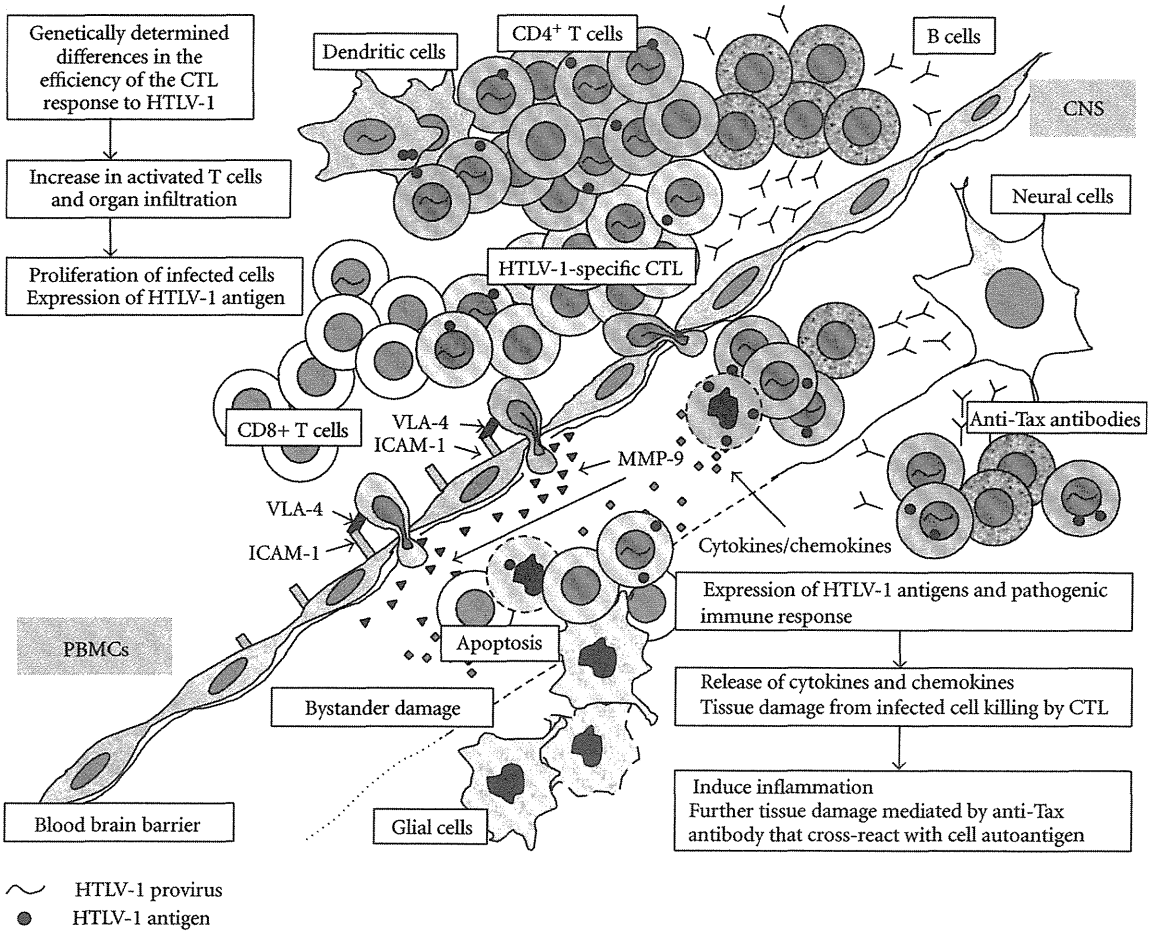

FIGURE 1: Hypothesis for the pathogenesis of human T-cell leukemia virus type-1 (HTLV-1) -associated myelopathy/tropical spastic paraparesis (HAM/TSP). Accumulating evidence suggests that the virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression in infected individuals, which lead to activation and expansion of antigen-specific T-cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development. It is also possible that the immunoglobulin G specific to HTLV-1-Tax, which cross-react with heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1), is associated with subsequent inflammation following initial tissue damage.

class II, CD86, and CD58 can block SLP [108]. Recently, it has been demonstrated that both myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1, and HTLV-1-infected DCs can rapidly transfer virus to autologous primary $CD4^+$ T cells [109]. In addition, other groups have obtained evidence that HTLV-1 transmission from DCs to T cells was mediated primarily by DC-SIGN [110], and DCs play a major part in generating and maintaining the Tax-specific $CD8^+$ T cells both *in vitro* and *in vivo* [111]. Moreover, using transgenic mouse models that permit conditional transient depletion of $CD11c^+$ DCs, and a chimeric HTLV-1 that carries the envelope gene from Moloney murine leukemia virus, Rahman et al. demonstrated the critical role of DCs in their ability to mount both innate and adaptive immune responses during early cell-free HTLV-1 infection [112, 113]. Since HTLV-1 can impair the differentiation of monocytes into DCs [114], the interaction of DCs with HTLV-1 plays a central part in the persistence and pathogenesis of HTLV-1.

5. Concluding Remarks

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

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

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