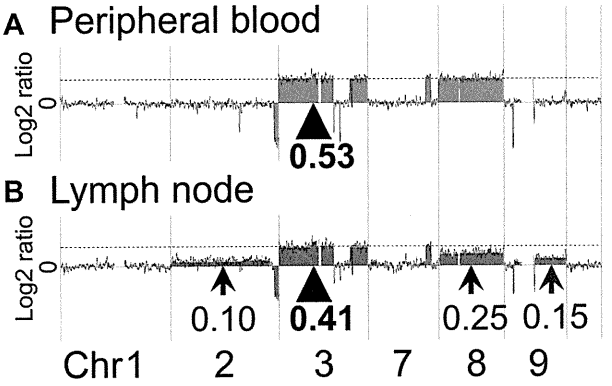


Figure 1. Representative profiles of the PB and LN samples of case 1. Array CGH results for case 1 are shown. (A) In the PB sample of case 1, regions of gain were detected. The log2 ratio of chromosome 3 was 0.53 (arrowhead). The log2 ratios of chromosomes 7 and 8 were the same as for chromosome 3 (dotted line). (B) In the LN sample of case 1, a log2 ratio imbalance was found. Log2 ratios among chromosomes 2, 3, 7, 8, and 9 differed. The log2 ratios of chromosome 3 and 7 were 0.41 (arrowhead and dotted line). Arrows show different log2 ratios: chromosome 2 = 0.10, chromosome 8 = 0.25, and chromosome 9 = 0.15.

57 years (range, 32-74 years). Detailed patient information is provided in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Four cell lines, SP-49,[9] HANK1,[10] ATN-1,[11] and Jurkat,[12] were also analyzed. SP-49 is a mantle cell lymphoma cell line, HANK1 is a natural killer/T-cell lymphoma line, ATN-1 is an ATLL cell line, and Jurkat is a T-cell lymphoblast–like cell line.

Peripheral blood samples were obtained from the blood of 8 healthy male donors. PBMCs were isolated by Ficoll-Paque PLUS centrifugation (GE Healthcare).

DNA extraction

$CD4^+$ cells in PB samples were purified using a magnetic-activated cell-sorting protocol (Miltenyi Biotec). High–molecular-weight DNA was extracted from $CD4^+$ cells, frozen LNs, and from the SP-49, HANK1, ATN-1, and Jurkat cell lines using standard proteinase K treatment and phenol-chloroform extraction.[13] Normal DNA was obtained from PBMC samples of 8 healthy male donors.

Oligo-array CGH

Characterization of the genomic aberrations was performed using Agilent 44K Whole Human Genome CGH arrays (Agilent Technologies) containing 44 000 probes. Procedures for DNA digestion, labeling, hybridization, scanning, and data analyses were performed according to the manufacturer's protocol (Agilent Technologies).

CGH data analysis

CGH data were extracted from scanned images using Feature Extraction software (version 10.3; Agilent Technologies). Raw data were transferred to the Genomic Workbench v5.0 software (Agilent Technologies) for further analysis. We defined gains and losses over a continuous 15-probe dataset as a linear log2 ratio average of ≥ 0.05 or ≤ -0.05, respectively, and microdeletion for a range of 3-15 probes as a linear log2 ratio average of ≤ -0.4. A detailed explanation of the log2 ratio is available in the supplemental data. The array CGH data have been deposited in ArrayExpress under the accession number E-MEXP-3042.

Southern blot analysis of HTLV-1 integration and TCRγ rearrangement

Integration of the HTLV-1 provirus genome and TCRγ rearrangement were assayed as described previously.[5,14] In brief, DNA samples (5 μg) of LNs were digested with restriction enzymes (PstI) and electrophoresed through 0.7% agarose gels. The DNA was then transferred onto a Hybond N^+ membrane (Amersham Pharmacia Biotech) and hybridized to randomly primed ^{32}P-labeled DNA probes specific for the HTLV-1 and TCRγ genes. Blots were then washed at the appropriate stringency and visualized by autoradiography. The HTLV-1 probe comprised a 1.0-kb fragment of the pX region, which was PCR amplified using the primers 5′-ccacttcccagggtttggacag-3′ and 5′-tctgcctctttttcgttaaaaagtagagaaatggg-3′, and the TCRγ probe comprised a 0.6-kb fragment of Jγ2.1.[14]

Results

Oligo-array CGH analysis against paired samples obtained from the PB and LNs

In all of the 13 acute-type ATLL cases, genomic aberrations were detected by oligo-array CGH. Representative profiles of the paired samples obtained from the PB and LNs in cases 1 and 2 are shown in Figure 1A and B and Figure 2A and B, respectively.

In the PB sample of case 1, genomic aberration regions showed a constant log2 ratio. Regions of gain were detected on chromosomes 3, 7, and 8. The log2 ratios corresponding to these regions were 0.53, suggesting that there was no imbalance (Figure 1A arrowhead). On the other hand, imbalance of the log2 ratio among chromosomes was found for the LN sample of case 1. Genomic aberrations of the case 1 LN sample were similar to

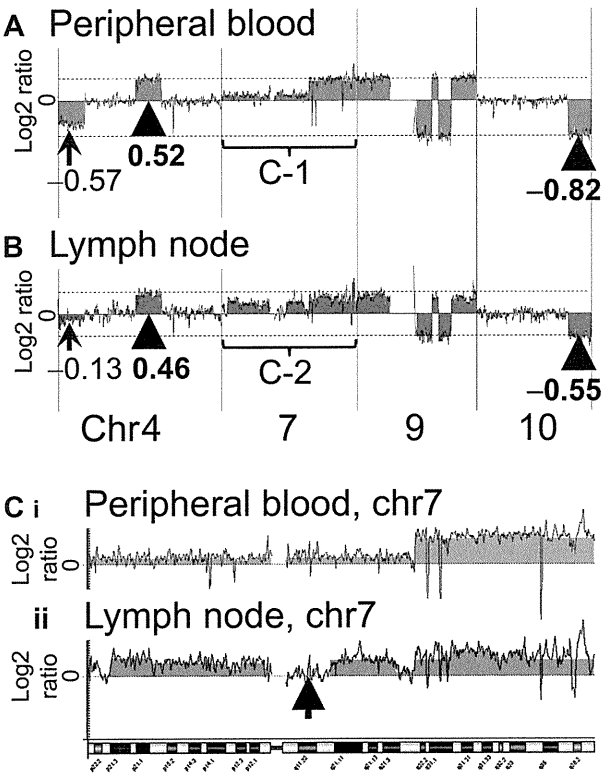


Figure 2. Representative profiles of the PB and LN samples of case 2. The results for case 2 were more complex than those for case 1. In both the PB and the LN samples of case 2, a log2 ratio imbalance was found. (A) In the PB sample, the arrowhead and dotted line indicate the majority of log2 ratios of gain and loss regions. Log2 ratios of the majority of loss regions were −0.82. The log2 ratio of chromosome 4 was −0.57. (B) In the LN sample, the arrowhead and dotted line indicate the majority of log2 ratios of gain and loss regions. Log2 ratios of the majority of loss regions were −0.55. The log2 ratio of chromosome 4 was −0.13. Chromosome 7 regions of PB and LN samples are magnified as Ci and Cii, respectively. (C) Chromosome 7 of the case 2 PB sample shows complex aberrations (i). This result also indicates a log2 ratio imbalance. Chromosome 7 of the case 2 LN sample shows more complex aberrations (ii). An arrow indicates a region (7q11.21-11q.23) without genomic aberration. Ci and Cii suggest that the genomic profiles of the PB and LN samples differ.

Table 1. Array CGH results of paired samples of acute-type ATLL

Case no.	Genome aberrations	Log2 imbalance		Genomic profiles of PB and LN	Common aberration regions between PB and LN	ATLL clones
		PB	LN			
1	+	−	+	different	+	Multiple subclones
2	+	+	+	different	+	Multiple subclones
3	+	−	−	same	+	Monoclone
4	+	+	+	different	+	Multiple subclones
5	+	+	−	different	+	Multiple subclones
6	+	−	−	same	+	Monoclone
7	+	+	+	different	+	Multiple subclones
8	+	−	+	different	+	Multiple subclones
9	+	−	+	different	+	Multiple subclones
10	+	−	+	different	+	Multiple subclones
11	+	−	−	same	+	Monoclone
12	+	−	−	same	+	Monoclone
13	+	+	+	different	+	Multiple subclones
Total	13 (100%)	5 (38.4%)	8 (61.5%)	9 (69.2%)	13 (100%)	9 (69.2%)

+ indicates present; and −, absent.

that of the PB sample. However, the log2 ratios among chromosomes 2, 3, 7, 8, and 9 differed as follows. Regions of gain were detected on chromosomes 2, 3, 7, 8, and 9, as shown by the log2 ratios: chromosome 2 = 0.10, chromosomes 3 and 7 = 0.41, chromosome 8 = 0.25, and chromosome 9 = 0.15 (Figure 1B arrowhead and arrows). The log2 ratio of chromosome 8 was lower than that of chromosomes 3 and 7. Gains of chromosomes 2 and 9 were detected in the LN sample, but not in the PB sample. These results indicated that a log2 ratio imbalance occurred in the LN sample.

Case 2 had a log2 ratio imbalance in both the PB and LN samples (Figure 2A). The genomic aberrations of the case 2 PB sample differed from those of the LN sample, as was also found with case 1. In the case 2 PB sample, regions of loss were detected on chromosomes 4, 9, and 10, as shown by the log2 ratios: chromosome 4 = −0.57 (Figure 2A arrow) and chromosomes 9 and 10 = −0.82 (Figure 2A arrowhead and dotted line). In the case 2 LN sample, regions of loss were also detected on chromosomes 4, 9, and 10, as shown by the log2 ratios: chromosome 4 = −0.13 (Figure 2B arrow) and, chromosomes 9 and 10 = −0.55 (Figure 2B arrowhead and dotted line). These data indicated that both samples had a log2 ratio imbalance. Complex genome aberrations were found for chromosome 7 in the paired samples of case 2. Consecutive gain regions were found in the whole of chromosome 7 of the PB sample (Figure 2Ci, and a region (7q11.21-11q.23) without genomic aberrations was found in chromosome 7 of the LN sample (Figure 2Cii arrow).

A log2 ratio imbalance among chromosomes was present in many other samples of acute-type ATLL, as summarized in Table 1.

Confirmation of log2 ratio imbalance among chromosomes

A log2 ratio imbalance among chromosomes was found in many ATLL clinical samples. We expected that a log2 ratio imbalance would indicate the presence of clones with different genomic aberrations. Therefore, we prepared 2 cell lines, SP-49 and HANK1, which possess different genomic aberrations. The genomic DNA of SP-49 was mixed with that of HANK1. We then conducted oligo-array CGH using the mixed-genomic DNA samples at various ratios.

Array CGH analysis of the SP-49 genome showed some genomic aberration regions, which were consistent with the G-band result that had been reported.[9] Log2 ratios of all 1-copy gain regions were 0.55, and log2 ratios of all 1-copy loss regions were −0.80. Imbalance of the log2 ratio among the chromosomes was not found. The same was true for HANK1, in which genomic aberration regions were consistent with the G-band result that had been reported and an imbalance of the log2 ratio among the chromosomes was not found.[10]

A representative array CGH result using a mixed-DNA sample at a ratio of 7:3 (SP-49:HANK1) is shown in Figure 3. The results showed an imbalance of the log2 ratio among chromosomes. It was possible to reproduce the log2 ratio imbalance. For example, the log2 ratios of chromosomes 2p14-pter, 2q14.3-qter, and 7p were 0.55, 0.15, and 0.46, respectively. These log2 ratios clearly differed. Furthermore, additional regions with different log2 ratios were found.

These results indicated that some of the clones present in the sample that had different genome profiles caused a log2 ratio imbalance in the array CGH result. The log2 ratio did not differ in chromosome 2p, which had a copy region identical to both SP-49 and HANK1.

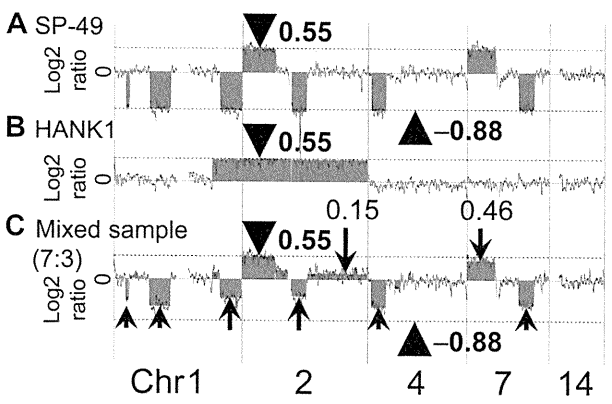


Figure 3. Confirmation of log2 ratio imbalance among chromosomes. The manner in which the log2 ratio imbalance occurred was confirmed. (A) SP-49 showed no imbalance. Log2 ratios of gain regions were 0.55 (arrowhead and dotted line). Log2 ratios of loss regions were −0.88 (arrowhead and dotted line). (B) HANK1 showed no imbalance. Log2 ratios of gain regions were 0.55 (arrowhead and dotted line). (C) Mixed-genomic DNA at a ratio of 7:3 reproduced the log2 ratio imbalance. The log2 ratio of chromosome 2p14-pter of the mixed DNA sample was 0.55 (arrowhead). Chromosome 2p had a copy region identical to both SP-49 and HANK1. Arrows indicate the log2 ratio imbalance.

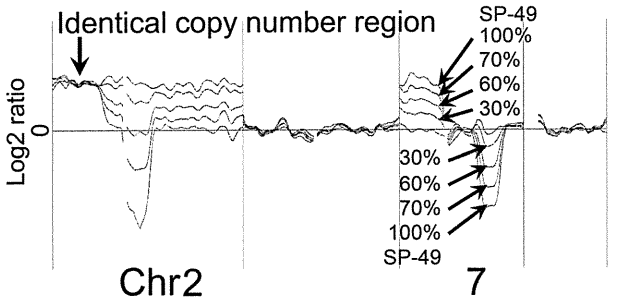


Figure 4. Log2 ratio reflects the ratio of tumor. The genome profiles of mixed-DNA samples comprising various ratios were superimposed. Gain was detected in chromosome 7 of all mixed samples, as shown by the log2 ratio: SP-49 = 0.55; 100%, 7:3 = 0.46; 70%, 6:4 = 0.32; 60%, 3:7 = 0.20; 30%. Loss was also detected in chromosome 7 of all mixed samples as shown by the log2 ratio: Sp-49 = −0.88; 100%, 7:3 = −0.62; 70%, 6:4 = −0.39; 60%, 3:7 = −0.14; 30%. Chromosome 2p had a copy region identical to both SP-49 and HANK1. The log2 ratios never changed in these regions.

Log2 ratios reflect the ratio of tumor

The genome profiles of mixed-DNA samples comprising various ratios were superimposed (Figure 4). The ratios of SP-49 to HANK1 were 7:3, 6:4, and 3:7. These results clearly revealed that the log2 ratio reflected the ratio of the tumor. When tumors included in a sample had identical genomic aberration regions, the log2 ratio never changed in these regions.

Southern blot analysis of HTLV-1 integration and TCRγ rearrangement

HTLV-1 integration

HTLV-1 integration was examined using Southern blot analysis, and the results showed HTLV-1 integration in all of the 11 cases examined. Eight of the 11 cases examined comprised a mono-integration band, whereas the others showed multi-integration bands. (Figure 5A)

TCRγ rearrangement

Southern blot analysis of TCR Jγ rearrangement was also conducted and evaluated as described previously by Moreau et al.[14] The results indicated that all samples were monoclonal (Figure 5B). Five of the 11 cases examined had a 6.8-kb rearrangement band, and 2 had a 2.9-kb rearrangement band. The others showed loss of germinal bands. In case 2, one allele of TCRγ was rearranged, because the germinal band of 8.0 kb was weaker than that of 4.9 kb. Case 7 lost all germinal bands, such as ATN-1, which is an ATLL cell line. This result indicated that both alleles of TCRγ were rearranged at Jγ2.3, because no deletion was found in case 7 by array CGH. Given that 3 or more TCR rearrangement bands were not found, no cases showed definite multi-clonality in tumor cells. These results indicated that the acute-type ATLL examined represented a monoclonal tumor comprising TCR rearrangements and with some possessing multiple integrations of HTLV-1.

Appearance of LN subclones before PB subclones

Array CGH analysis revealed that PB samples from 5 of 13 cases had homozygous loss regions that were not found in the corresponding LN samples of each case. In case 2, 1p12-1p13.1 of the PB sample was seen to represent homozygous loss, unlike the case with the LN sample (Figure 6). However, log2 ratios of same region in the LN sample seemed to be slightly lower than those of neighboring regions. This raised the possibility that a minor

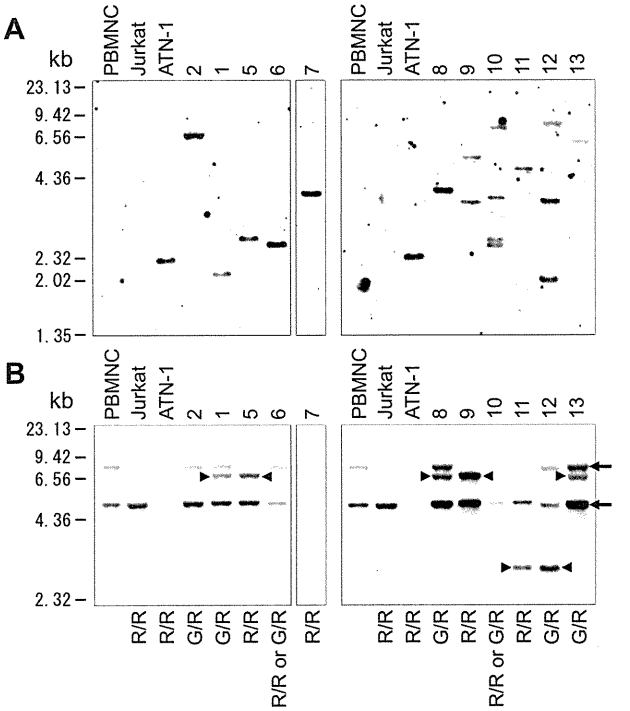


Figure 5. Southern blot analysis. (A) Southern blot analysis of HTLV-1 integration in 11 of 13 cases. (B) Southern blot analysis of TCRγ rearrangement. Arrows indicate the 8.00- and 4.9-kb germline bands. Arrowheads indicate the 6.8- and 2.9-kb rearrangement bands. G indicates a germinal allele; R, rearrangement allele; G/R, rearrangement of one allele; R/R, rearrangement of both alleles.

subclone was present. The PB samples from cases 1, 4, 8, and 10 also had homozygous loss regions that were not clearly found in the corresponding LN samples (Table 2).

No cases had a homozygous loss region in the LN samples when the PB samples had a heterozygous loss in the same regions. Array CGH and Southern blotting results indicated that multiple subclones had developed from one clone. Therefore, when 2 clones were found in a patient, the clone with homozygous loss must have developed from the clone with heterozygous loss. The homozygous loss analysis revealed that in about 40% of ATLL patients, subclones that had appeared in the PB were derived from LN subclones.

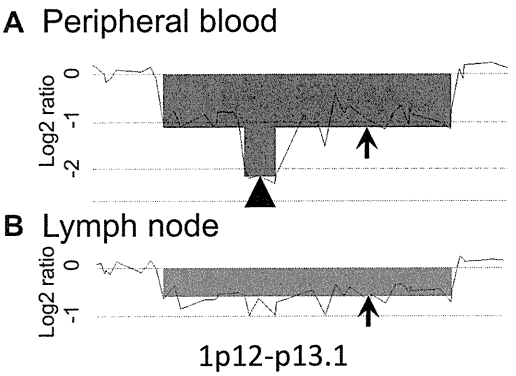


Figure 6. Homozygous loss region analysis. A representative homozygous loss region of case 2 is shown (1p12-p13.1). The total scale of the figure is approximately 2 Mb. The arrowhead indicates a homozygous loss region; arrows indicate heterozygous loss regions. Homozygous loss was found only in the PB sample. The log2 ratio of this region in the LN sample was slightly lower than that in the neighboring regions, suggesting the possibility that a minor subclone may exist in the LNs.

Table 2. PB samples of 5 of 13 cases only had homozygous loss regions that were not found in the LN samples

Case no.	Homozygous loss only in PB	Homozygous loss only in LN	Locus	Gene
1	+	–	3q22.3	*PCCB, STAG1*
2	+	–	1p12-p13.1	*IGSF3*
3	–	–		
4	+	–	6p22.3	*ATXN1*
5	–	–		
6	–	–		
7	–	–		
8	+	–	4q31.21	*INPP4B*
9	–	–		
10	+	–	9q31.2	*KLF4*
11	–	–		
12	–	–		
13	–	–		
Total	5	0		

+ indicates present; and –, absent.

Selected subclone of LNs in the PB

Tumor cells in the PB samples of some cases (eg, cases 1 and 9) appeared to have been selected from multiple subclones. In these cases, a log2 ratio imbalance was not found in the PB sample but was found in the LN sample. This indicated that PB samples were monoclonal and that the LN samples contained multiple subclones. Both samples from each case had common aberrations, and the LN samples had aberrations that were not found in the PB samples. These results may indicate that the LNs contain multiple subclones with different genomic aberrations, and that one of these subclones then appears in the PB (Figure 7).

Discussion

The imbalance and differing genomic profiles of PB and LN samples indicate that acute-type ATLL comprises multiple subclones

In this study, we revealed the presence of a log2 ratio imbalance among chromosomes of LN samples in many patients with acute-type ATLL. Most of the genomic profiles were found to differ from those of the PB samples. Although monoclonal proliferation of acute-type ATLL is referred to in the World Health Organization classification,[15] these data clearly show that acute-type ATLL contains multiple subclones that originate as a result of clonal evolution in ATLL patients.

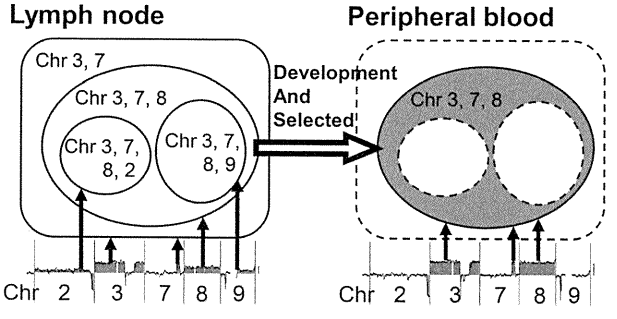


Figure 7. Selected subclone from the LN in the PB. Shown is a schematic representation of a selected subclone from the LN sample in the PB of case 1. In the LN sample of case 1, at least 4 subclones exist: a subclone with chromosome 3 and 7 aberrations; a subclone with additional chromosome 8 aberrations; a subclone with chromosome 3, 7, 8, and 2 aberrations; and a subclone with chromosome 3, 7, 8, and 9 aberrations. Among these subclones, a subclone with chromosome 3, 7, and 8 aberrations appeared in the PB sample.

Shinawi et al[16] reported a case of pediatric AML in which 2 clones with different chromosome aberrations showed a log2 ratio imbalance as detected by array CGH. We were able to reproduce a log2 ratio imbalance among chromosomes by mixing different ratios of DNA prepared from 2 different cell lines. The log2 ratio reflected the ratio of tumor clones. Based on these data, we analyzed the acute-type ATLL data and identified that a log2 ratio imbalance indicated the presence of multiple subclones in a sample. Minority clones with low log2 ratios could be found in this experiment by taking advantage of the high sensitivity associated with the use of array CGH. As a result, the presence of multiple subclones was unambiguously determined.

Cases showing different genomic profiles between PB and LN samples reached as high as 69%. We reported previously that paired samples obtained from different sites had different chromosomal aberrations in some cases.[17] We also reported that sequential samples at chronic and crisis or acute onset and relapse in each case showed different chromosome aberrations or integrations as determined by chromosomal CGH or Southern blot analysis.[17] Similar clonal change has been reported previously in some cases of B-cell lymphoma.[18] Although analysis of sequential samples is important when examining the stability of multiple subclones, it is difficult to acquire sequential samples from acute-type ATLL patients because these patients require immediate chemotherapy. However, chronic-type ATLL can be treated with "watchful waiting," so the clonal stability of ATLL may be explored in these patients.

Our data indicate that acute-type ATLL comprises multiple subclones with differing genomic aberrations. Several morphologic variants of ATLL have been described,[15] and the presence of a mixture of cells of different sizes has been reported. However, the histological type does not correspond to the clinical subtype.[19] Therefore, it is reasonable to postulate that the histological type does not always reflect the clinical features because the tumor subclones may differ at various sites.

HTLV-1 integration and TCRγ rearrangement determined by Southern blotting

We focused on the cell origin of the multiple subclones in each patient. Southern blot analysis revealed a monoclonal band of HTLV-1 integration or monoclonal rearrangement of TCRγ in all samples examined. These data indicated that the ATLL clones in each case had a common tumor cell origin. ATLL research and treatment utilize the Shimoyama classification. Acute-type ATLL represents one subtype in the classification, and is considered to be a monoclonal tumor. Our data are also consistent with this classification. However, it is possible that multiple subclones in the LNs possess a diversity that may account for the variable clinical manifestations and drug resistance that can occur during the treatment of ATLL.

Selection of leukemic clone and diversity in LNs

Array CGH suggested that the subclones in the PB and LNs differed even though they are derived from an identical monoclonal tumor cell, as determined by in Southern blot analysis. Given that the clones are derived from one clone, theoretically the clone with heterozygous loss is never derived from a cell with homozygous loss. Homozygous loss regions were only present in the PB samples examined at a frequency of 38% (5 of 13 cases examined). None of the 5 samples showed homozygous loss

regions found in the LN samples, indicating that in these cases, subclones present in the LNs were not derived from those in the PB. These results suggested that the selected subclones appeared in the PB after subclones developed in the LNs. However, it remains to be determined how these clones in the PB become stable during the course of disease. It is also important to determine whether the tumor cells in the PB can proliferate at the level of tumor cells in the LNs.

In conclusion, the results of the present study showed that there are multiple subclones in acute-type ATLL, all of which possess a common TCR rearrangement and the genomic profiles of which often differ between the PB and LNs. Cases were identified in which a selected subclone from multiple subclones in the LN samples was also identified in the PB samples. ATLL was clinically classified into 4 subtypes by Shimoyama. However, the specific genes that characterize acute-type ATLL have not been identified. Our results reveal that acute-type ATLL is a genetically heterogeneous neoplasm and that clonal evolution of ATLL takes place in the LNs.

Acknowledgments

We thank Dr Koichi Ohshima for diagnoses; Dr Shinobu Tsuzuki, Dr Kennosuke Karube, and Dr Keiichiro Honma for discussions and encouragement throughout this study; and Kyoko Hirano for outstanding technical assistance.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and from the Japan Society for the Promotion of Science.

Authorship

Contribution: A. Umino performed the experiments, analyzed data, and wrote the paper; M.N. analyzed data and performed experiments; A. Utsunomiya provided advice, discussed clinical data, and treated patients; K.T. provided advice, discussed clinical data, treated patients, and wrote the paper; N.T. analyzed and discussed clinical data; N.K. discussed clinical data; and M.S. supervised the research, discussed clinical data, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Masao Seto, MD, PhD, Division of Molecular Medicine, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681, Japan; e-mail: mseto@aichi-cc.jp.

References

1. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87). *Br J Haematol.* 1991;79(3):428-437.
2. Raimondi SC, Behm FG, Roberson PK, et al. Cytogenetics of childhood T-cell leukemia. *Blood.* 1988;72(5):1560-1566.
3. Shimoyama M, Abe T, Miyamoto K, et al. Chromosome aberrations and clinical features of adult T cell leukemia-lymphoma not associated with human T cell leukemia virus type I. *Blood.* 1987; 69(4):984-989.
4. Maciejewski JP, Tiu RV, O'Keefe C. Application of array-based whole genome scanning technologies as a cytogenetic tool in haematological malignancies. *Br J Haematol.* 2009;146(5):479-488.
5. Oshiro A, Tagawa H, Ohshima K, et al. Identification of subtype-specific genomic alterations in aggressive adult T-cell leukemia/lymphoma. *Blood.* 2006;107(11):4500-4507.
6. Tsukasaki K, Tsushima H, Yamamura M, et al. Integration patterns of HTLV-I provirus in relation to the clinical course of ATL: frequent clonal change at crisis from indolent disease. *Blood.* 1997;89(3):948-956.
7. Etoh K, Tamiya S, Yamaguchi K, et al. Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells in vivo. *Cancer Res.* 1997; 57(21):4862-4867.
8. Okamoto T, Ohno Y, Tsugane S, et al. Multi-step carcinogenesis model for adult T-cell leukemia. *Jpn J Cancer Res.* 1989;80(3):191-195.
9. Daibata M, Takasaki M, Hirose S, et al. Establishment of a new human B cell line carrying t(11;14) chromosome abnormality. *Jpn J Cancer Res.* 1987;78(11):1182-1185.
10. Kagami Y, Nakamura S, Suzuki R, et al. Establishment of an IL-2-dependent cell line derived from 'nasal-type' NK/T-cell lymphoma of CD2+, sCD3-, CD3epsilon+, CD56+ phenotype and associated with the Epstein-Barr virus. *Br J Haematol.* 1998;103(3):669-677.
11. Naoe T, Akao Y, Yamada K, et al. Cytogenetic characterization of a T-cell line, ATN-1, derived from adult T-cell leukemia cells. *Cancer Genet Cytogenet.* 1988;34(1):77-88.
12. Gillis S, Watson J. Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2-producing human leukemia T cell line. *J Exp Med.* 1980;152(6):1709-1719.
13. Seto M, Yamamoto K, Iida S, et al. Gene rearrangement and overexpression of PRAD1 in lymphoid malignancy with t(11;14)(q13;q32) translocation. *Oncogene.* 1992;7(7):1401-1406.
14. Moreau EJ, Langerak AW, van Gastel-Mol EJ, et al. Easy detection of all T cell receptor gamma (TCRG) gene rearrangements by Southern blot analysis: recommendations for optimal results. *Leukemia.* 1999;13(10):1620-1626.
15. Ohshima K, Jaffe ES, Kikuchi M. Adult T-cell leukaemia/lymphoma. In: The National Agency for Research on Cancer, Swerdlow S, Campo E, Lee Harris N, et al, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon: World Health Organization; 2008: 281-284.
16. Shinawi M, Erez A, Shardy DL, et al. Syndromic thrombocytopenia and predisposition to acute myelogenous leukemia caused by constitutional microdeletions on chromosome 21q. *Blood.* 2008;112(4):1042-1047.
17. Tsukasaki K, Krebs J, Nagai K, et al. Comparative genomic hybridization analysis in adult T-cell leukemia/lymphoma: correlation with clinical course. *Blood.* 2001;97(12):3875-3881.
18. Siegelman MH, Cleary ML, Warnke R, Sklar J. Frequent biclonality and Ig gene alterations among B cell lymphomas that show multiple histologic forms. *J Exp Med.* 1985;161(4):850-863.
19. Takeshita M, Akamatsu M, Ohshima K, et al. CD30 (Ki-1) expression in adult T-cell leukaemia/lymphoma is associated with distinctive immunohistological and clinical characteristics. *Histopathology.* 1995;26(6):539-546.

Viruses **2011**, *3*, 1532-1548; doi:10.3390/v3091532

OPEN ACCESS

viruses

ISSN 1999-4915

www.mdpi.com/journal/viruses

Review

Human T-Lymphotropic Virus Type 1 (HTLV-1) and Regulatory T Cells in HTLV-1-Associated Neuroinflammatory Disease

Natsumi Araya [1], Tomoo Sato [1], Naoko Yagishita [1], Hitoshi Ando [1], Atae Utsunomiya [2], Steven Jacobson [3] and Yoshihisa Yamano [1,*]

[1] Department of Rare Diseases Research, Institute of Medical Science, School of Medicine, St. Marianna University, Kawasaki 216-8511, Japan; E-Mails: araya@marianna-u.ac.jp (N.A.); tomoo@marianna-u.ac.jp (T.S.); yagi@marianna-u.ac.jp (N.Y.); hando@marianna-u.ac.jp (H.A.)

[2] Department of Hematology, Imamura Bun-in Hospital, Kagoshima 890-0064, Japan; E-Mail: autsunomiya@jiaikai.jp

[3] Viral Immunology Section, Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA; E-Mail: jacobsons@ninds.nih.gov

* Author to whom correspondence should be addressed; E-Mail: yyamano@marianna-u.ac.jp; Tel.: +81-44-977-8111; Fax: +81-44-977-9772.

Received: 2 June 2011; in revised form: 13 August 2011 / Accepted: 16 August 2011 / Published: 25 August 2011

Abstract: Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that is the causative agent of adult T cell leukemia/lymphoma (ATL) and associated with multiorgan inflammatory disorders, including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and uveitis. HTLV-1-infected T cells have been hypothesized to contribute to the development of these disorders, although the precise mechanisms are not well understood. HTLV-1 primarily infects $CD4^+$ T helper (Th) cells that play a central role in adaptive immune responses. Based on their functions, patterns of cytokine secretion, and expression of specific transcription factors and chemokine receptors, Th cells that are differentiated from naïve $CD4^+$ T cells are classified into four major lineages: Th1, Th2, Th17, and T regulatory (Treg) cells. The $CD4^+CD25^+CCR4^+$ T cell population, which consists primarily of suppressive T cell subsets, such as the Treg and Th2 subsets in healthy individuals, is the predominant viral reservoir of HTLV-1 in both ATL and HAM/TSP patients. Interestingly, $CD4^+CD25^+CCR4^+$ T cells become Th1-like cells in

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

Keywords: HTLV-1; HAM/TSP; ATL; $CD4^+CD25^+CCR4^+$ T cell; regulatory T cell; ex$Foxp3^+$ cell; inflammation; immune-dysfunction

1. Introduction

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

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

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

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

2. HTLV-1 and Regulatory T Cells

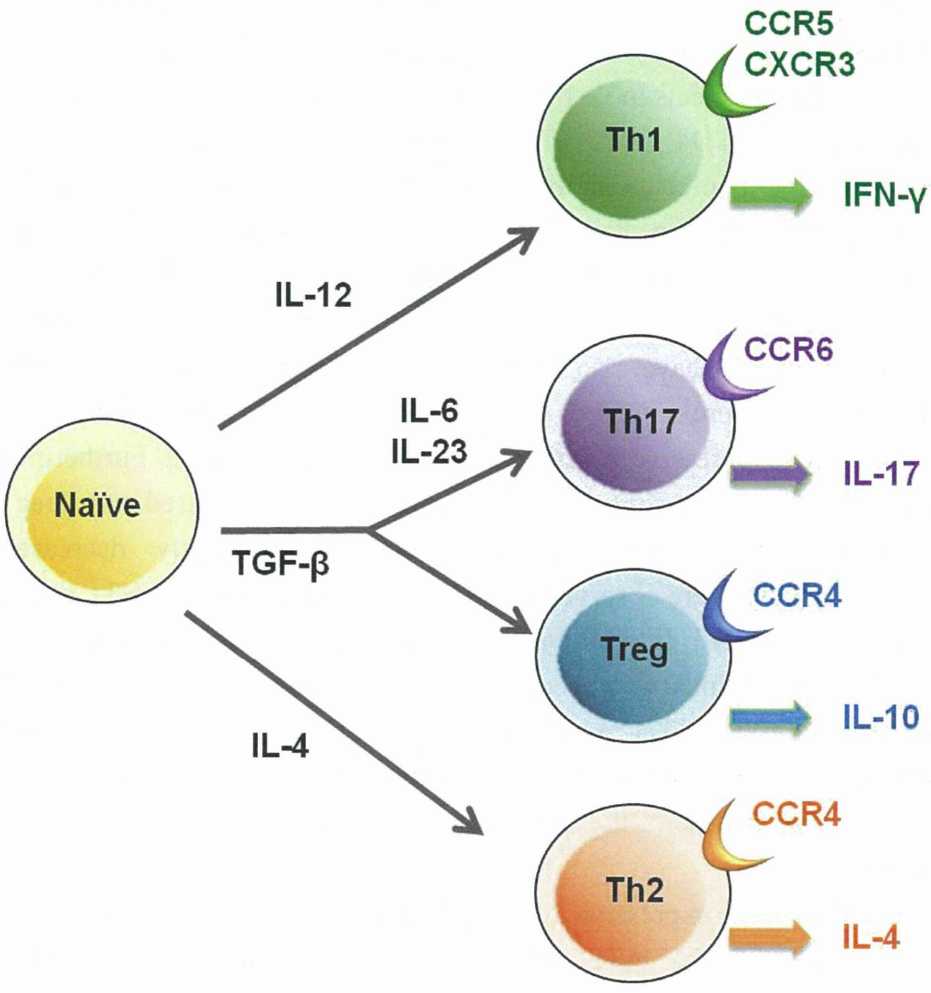
The recent discovery of regulatory T cells (Treg cells) has generated new opportunities for and increased interest in elucidating the above mentioned mechanisms. In healthy individuals, the Treg cells, a subset of $CD4^+CD25^+$ T cells, play a key role in maintaining immune system homeostasis by suppressing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

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

3. HTLV-1 and $CD4^+CD25^+CCR4^+$ T Cells

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

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



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

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

4. HTLV-1 and $Foxp3^-CD4^+CD25^+CCR4^+$ T Cells

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

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

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

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

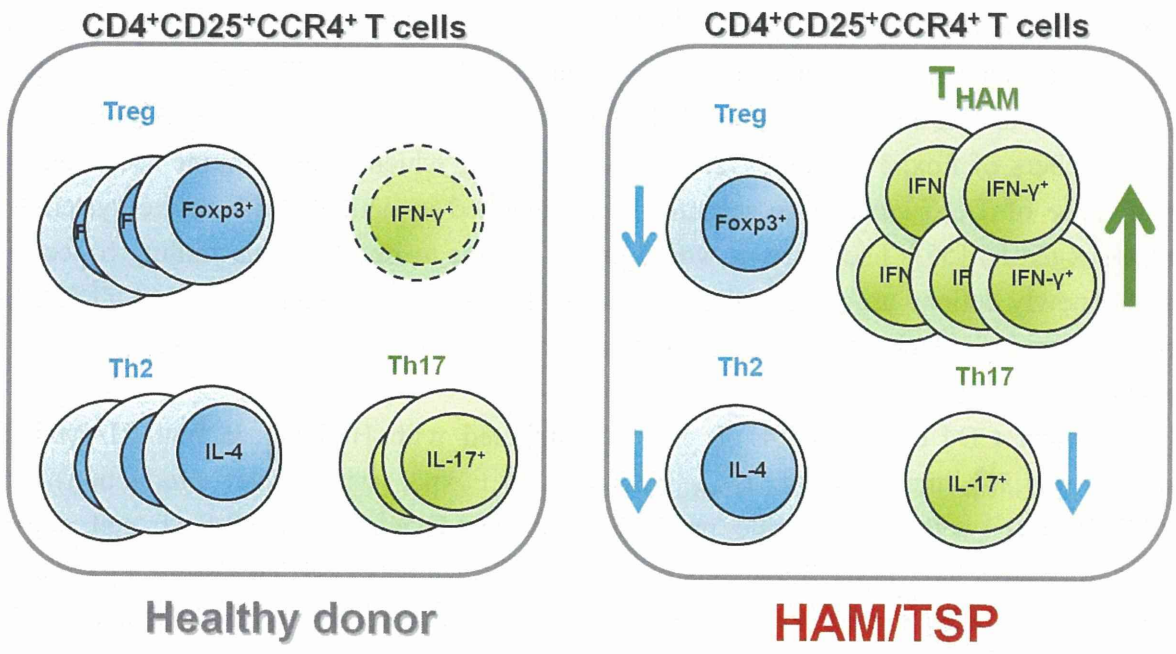
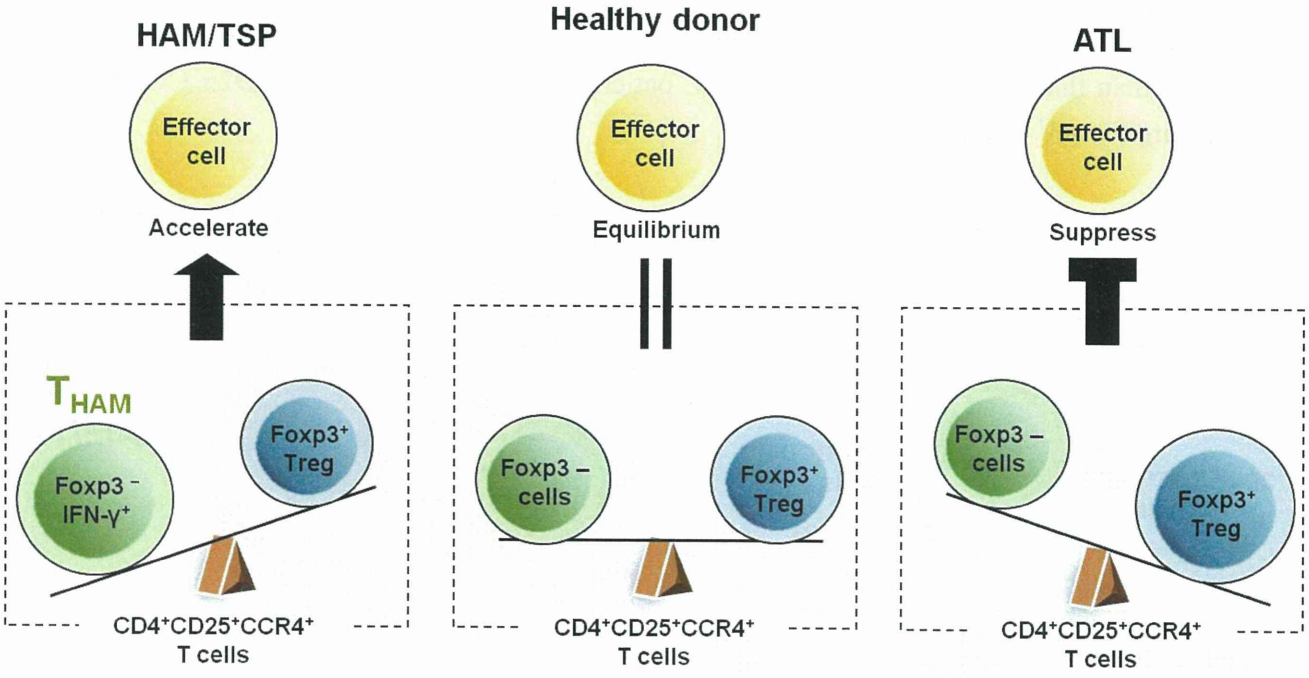


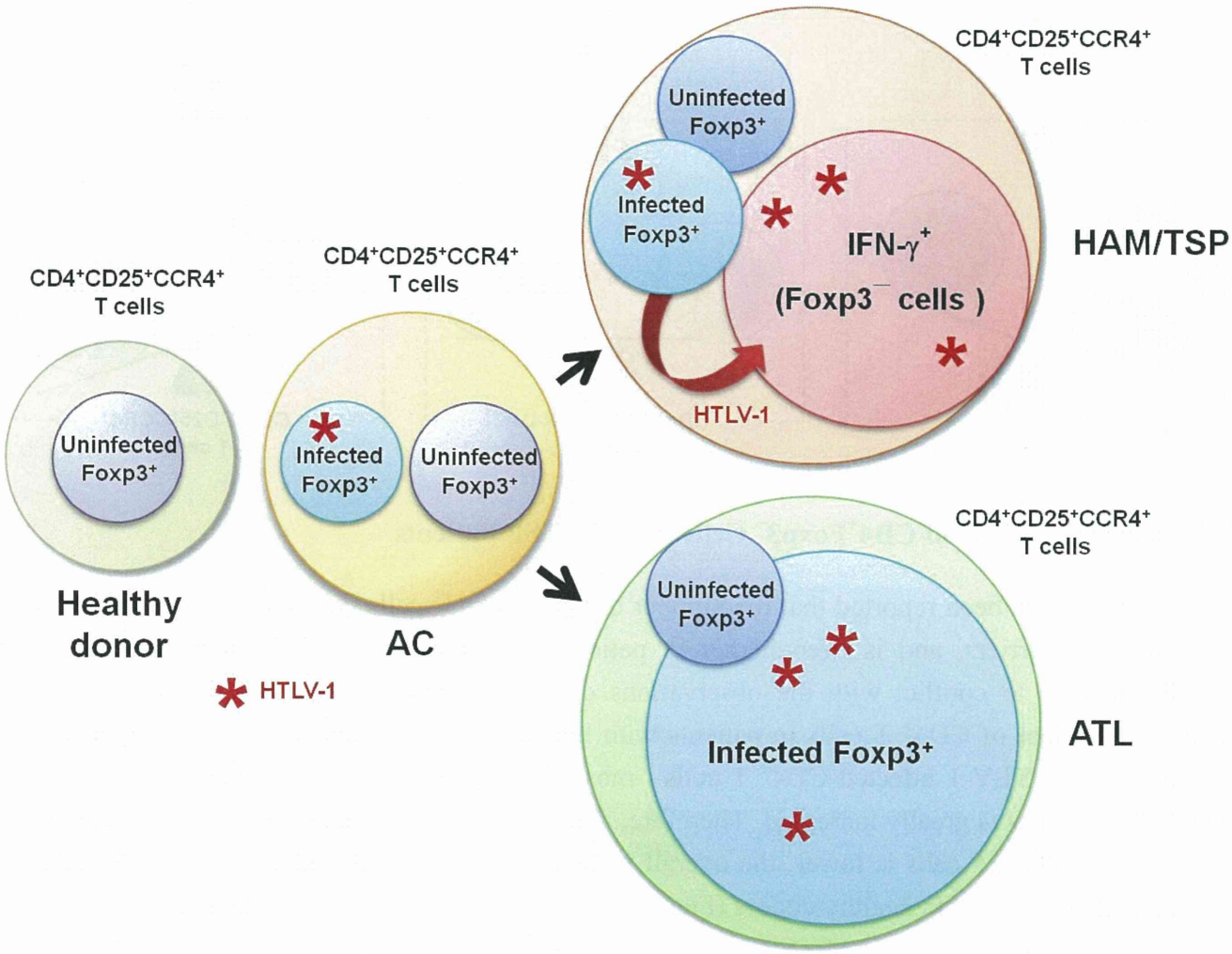
Figure 3. Differential immune responses and T_{HAM}/Treg ratios in $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP and adult T cell leukemia/lymphoma (ATL) patients.



5. Increased Numbers of $CD4^+Foxp3^+$ Cells in HAM/TSP Patients

Recently, it has been reported that the number of $CD4^+Foxp3^+$ cells increases in HTLV-1-infected asymptomatic carriers, and is even higher in patients with HAM/TSP [61]. Although this report initially appears to conflict with the observations described above, it may not. In contrast to the decreased number of $CD4^+$ T cells in patients with human immunodeficiency virus (HIV) infection, the number of HTLV-1 infected $CD4^+$ T cells—most of which are CD4+CD25+CCR4+ T cells—in HAM/TSP patients is greatly increased. Therefore, although the percentage of $Foxp3^+$ cells among the $CD4^+CD25^+CCR4^+$ T cells is lower, the overall number of $CD4^+Foxp3^+$ cells in HAM/TSP patients may be higher than that in healthy donors (Figure 4). Indeed, when we analyzed the number of $Foxp3^+$ cells in healthy donors and HAM/TSP patients, we found it to be nearly equivalent between the two groups or slightly higher in HAM/TSP patients [54]. This difference (from slightly high to higher) would depend on the number of HTLV-1-infected $CD4^+$ T cells in the samples tested. Importantly, Toulza *et al.* demonstrated that the rate of CTL-mediated lysis was negatively correlated with the number of HTLV-1-$Tax^-CD4^+Foxp3^+$ cells, but not with the number of $Tax^+CD4^+Foxp3^+$ cells [61], again suggesting that HTLV-1-infected Treg cells lose their regulatory function, while HTLV-1-uninfected Treg cells contribute substantially to immune control of HTLV-1 infection.

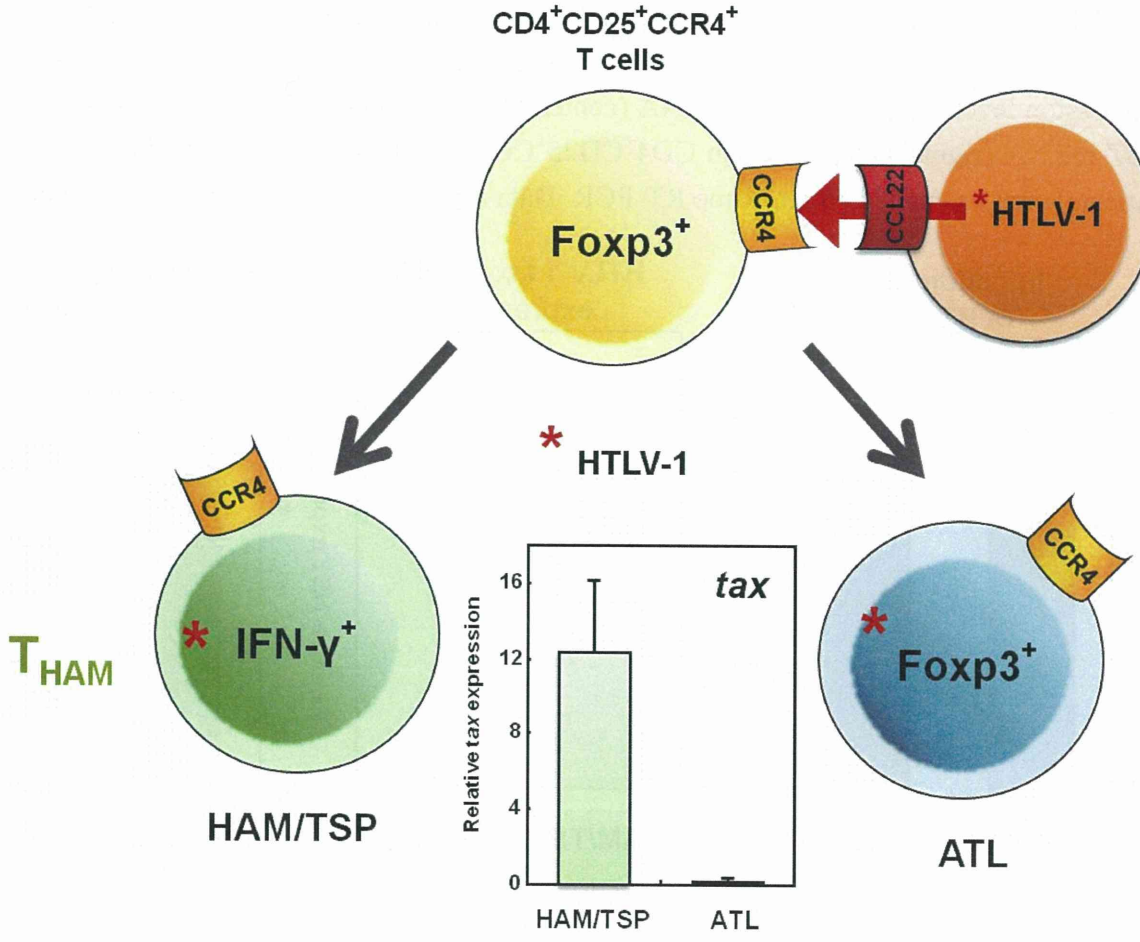
Figure 4. Scheme of proportion of each cellular component in $CD4^+CD25^+CCR4^+$ T cells of healthy donors, asymptomatic carriers (AC), and patients with HAM/TSP or ATL. Although the proportion of $Foxp3^+$ cells among the $CD4^+CD25^+CCR4^+$ T cells is lower in HAM/TSP patients, the overall number of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



6. Does the T_{HAM} Cell Population Include exFoxp3$^+$ Cells?

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

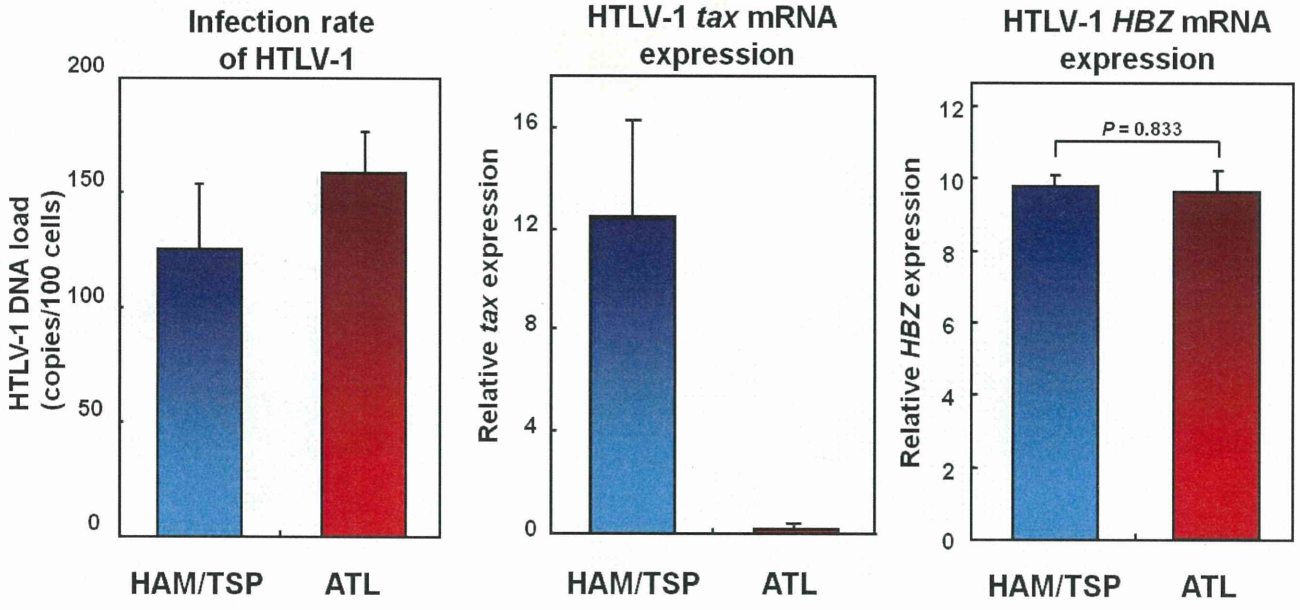
Figure 5. Differential fate of HTLV-1-infected $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP and ATL patients. After HTLV-1 infection, $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP patients, which are primarily Th2 and Treg cells before infection, become IFN-γ^+Foxp3^- T cells (T_{HAM} cells) with high levels of intracellular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



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

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

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



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

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

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

8. Conclusion

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

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labor, and Welfare; the Uehara Memorial Foundation; the Nagao Takeshi Nanbyo Foundation; the Kanagawa Nanbyo Foundation; the Mishima Kaiun Memorial Foundation; the Takeda Science Foundation; the ITSUU Laboratory Research Foundation; the Foundation for Total Health Promotion; and the Sankyo Foundation of Life Science.

References and Notes

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

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

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

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