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Human T-cell Leukemia Virus Type 1 Can Infect a Wide Variety of Cells in Mice

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Analysis of human T-cell leukemia virus type 1 (HTLV-1)-infected cell types and the interplay of these infected cells *in vivo* should provide valuable information to elucidate the pathogenesis of HTLV-1-associated diseases in humans and in animal models. In this study, HTLV-1-infected cell types were identified in HTLV-1-infected C3H/HeJ mice. Pan T, CD_4^+, CD_8^+, granulocyte and pan B cell fractions in the splenocytes of MT-2 cell-inoculated mice were sorted by use of their cell surface high-density expression of CD_{3e}, CD_4, CD_8, Gr-1 and B_{220} antigens, respectively, with a fluorescence-activated cell sorter. The *pX* sequence of HTLV-1 provirus in the lysate of each fraction was amplified by polymerase chain reaction and detected by Southern hybridization. Interestingly, in addition to the CD_4^+ cell fraction, the *pX* sequence was also found in CD_8^+ cell, B cell and granulocyte fractions. The broad cell spectrum of HTLV-1 infection in mice is consistent with the situation in humans. Our findings indicate that HTLV-1 receptor or coreceptor is widely distributed among different cell types in mice.

Key words: HTLV-1 — Cellular tropism — Mouse

It is well known that human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic type C retrovirus and has tropism for T lymphocytes.[1)] HTLV-1 infection is closely associated with adult T-cell leukemia (ATL),[2)] HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and other HTLV-1-associated diseases.[3, 4)] However, the mechanisms of the pathogenesis of HTLV-1-associated diseases are still unclear. In *in vitro* experiments, it was shown that HTLV-1 is able to infect a variety of cell lines, including B cells, endothelial cells, glial cells and fibroblasts.[5–8)] Monkeys, rabbits and rats, besides humans, are susceptible to HTLV-1 infection,[9–11)] but it was reported that the plating efficiency of vesicular stomatitis virus pseudotype bearing HTLV-1 envelope glycoprotein on mouse cells was approximately 1% of that on human cells.[12)] There has been little work on experimental HTLV-1 infection in mouse. Recently we intraperitoneally inoculated MT-2 cells, an HTLV-1-producing human T-cell line, into newborn C3H/HeJ mice. After 15 weeks, the provirus was frequently found in the spleen, lymph nodes and thymus. Integration of the HTLV-1 provirus into the mouse genome was confirmed by determination of the flanking sequence of the HTLV-1 provirus.[13, 14)] The potential of HTLV-1 to infect mice led us to examine the cell types subject to HTLV-1 infection in mice.

We have demonstrated that HTLV-1 can infect pan T cells in HTLV-1-infected mice,[14)] but we did not examine the susceptibility of subpopulations of T cell and non-T cell populations. In this study, we determined the cellular tropism of HTLV-1 in these HTLV-1-infected C3H/HeJ mice.

MATERIALS AND METHODS

Cell lines, animals and antibodies MT-2 cell line was established from normal human cord leukocytes by co-culturing them with leukemic T cells from an ATL patient.[1)] ATL-1K cell line was established from a patient with ATL.[15)] The MT-2 cells and ATL-1K cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The MT-2 cells served as a viral source and ATL-1K cell lysate was used as a standard lysate for determination of the sensitivity of polymerase chain reaction (PCR) in this experiment.

HTLV-1-inoculated mice were prepared by intraperitoneal injection of 2.5×10^6 MT-2 cells into C3H/HeJ mice (Clea, Inc., Tokyo) twice, once within 24 h after birth and 1 week later. Five mice were killed at age 12–16 weeks and 6 mice at age 24–26 weeks. The spleen DNA was analyzed for provirus sequence.

Monoclonal antibodies specific for CD_{3e} (pan T) and B_{220} (pan B) conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE), for CD_4 (CD_4^+) and CD_8 (CD_8^+) conjugated with FITC and PE, were obtained from PHARMINGEN, San Diego, CA, USA. Monoclonal antibody specific for Gr-1 (granulocyte) conjugated with allophycocyanin (APC) was a kind gift from Dr. H. Nakauchi (University of Tsukuba, Tsukuba).

Fluorescence-activated cell sorter (FACS) analysis and cell sorting Spleens were excised from MT-2 cell-inoculated mice. Splenocyte suspensions were prepared by

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pressing the tissue between glass slides followed by filtration through No. 330 nylon mesh. Splenocytes (2.5×10^6) were reacted with monoclonal antibodies (see above) for 30 min at 4°C. The cells were washed three times with staining medium (phosphate-buffered saline with 3% fetal bovine serum and 0.1% sodium azide), then resuspended in the staining medium supplemented with 1 μg/ml propidium iodide at a final concentration of 10^6 cells/ml before sorting for discrimination of dead cells. Antibody-stained cells were analyzed and sorted on a FACS (FACS VANTAGE, Becton Dickinson, San Jose, CA). In order to exclude the remaining MT-2 cells, we set the sorting gate so that the sorted cells contained less than 0.003% MT-2 cells (see "Results").

PCR and subsequent Southern hybridization DNA from a portion of spleen tissue of MT-2 cell-inoculated mice was prepared by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The lysates were prepared from cells sorted by FACS as described above. The lysate of 2.5×10^4 cells or 1 μg of DNA was used for each PCR. The HTLV-1 *pX* sequence was detected by PCR amplification (50 cycles at 95°C for 1 min, 57°C for 1 min and 72°C for 1.5 min) with *taq* polymerase and Southern hybridization, as described previously.[16] The c-*myc* sequence of mouse, which serves as an internal control to assure the quality of DNA, was amplified in the same reaction tube using multiplex PCR.[16] To exclude the possibility of MT-2 cells remaining in the MT-2 cell-inoculated mice, human-specific human endogenous retrovirus-R (HERV-R) sequence[17] in the mouse spleen DNA was examined by PCR amplification (50 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 1 min) with *taq* polymerase and Southern hybridization. The sequences of primers and probes used in the experiments were as follows. The primers for the *pX* region were 5′-CGGATACCCAGTCTACGTGT-3′ (nt 7336–7355) and 5′-GAGCCGATAACGCGTCCATC-3′ (nt 7475–7494), those for mouse c-*myc* were 5′-ACAACGTCTTGGAACGTCAG-3′ (nt 4069–4088) and 5′-GGTTAGGCTTTGAGCATGCA-3′ (nt 4394–4413) and those for HERV-R were 5′-CTGATCAGTCTGACTCACTC-3′ (nt 53–72) and 5′-ATAGTTGTGAGAGCAGAACA-3′ (nt 217–236). The probe for the *pX* region was 5′-CTGTGTACAAGGCGACTGGTG-3′ (nt 7363–7383) and that for the HERV-R region was 5′-CTGCTGGAGCTATGATAACA-3′ (nt 80–99).

RESULTS

Sensitivity of detecting HERV-R and *pX* sequences by PCR and subsequent Southern hybridization MT-2 cells contain 6 copies of HTLV-1 *pX* sequences[18] and 4 copies of HERV-R long terminal repeat (LTR) sequences[17] per cell. When MT-2 cell DNA was serially diluted, the HERV-R LTR sequence was detected in DNA equivalent to 1 MT-2 cell and the *pX* sequence was detected in DNA equivalent to 0.3 MT-2 cell (Fig. 1). ATL-1K cells contain 1 copy of HTLV-1 provirus per cell.[15] Using ATL-1K cell lysate, the *pX* sequence was detected in 1 ATL-1K cell equivalent lysate (data not

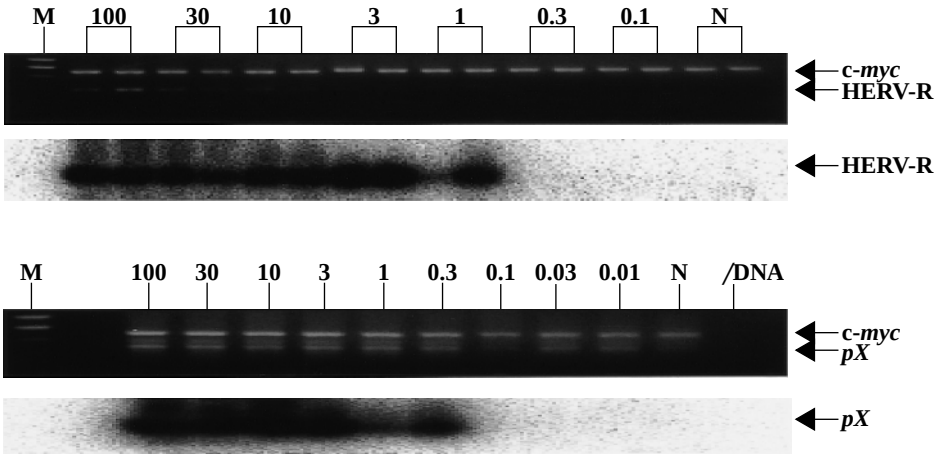

Fig. 1. Sensitivity of detecting HERV-R and *pX* sequences by PCR and Southern hybridization. Using the same lot number of MT-2 cell DNA as the standard DNA, the HERV-R sequence was detected in DNA equivalent to 1 MT-2 cell and the *pX* sequence was detected in DNA equivalent to 0.3 MT-2 cell. The set of ethidium bromide staining patterns and Southern hybridization patterns is presented. Mouse c-*myc* band, which serves as an internal control, was visualized by ethidium bromide staining. The numbers of MT-2 cells are written on the top. M, DNA size marker. N, negative control using normal rat spleen DNA. –DNA, negative control using distilled H_2O.

shown). The present method can detect 1 copy of *pX* and 4 copies of HERV-R LTR sequences per 10^5 cells.

HTLV-1 proviral distribution in splenocytes sorted by FACS Human specific HERV-R LTR sequence was not detected in any of the spleen DNA of the MT-2 cell-inoculated mice examined (Table I, Fig. 2). To identify the cell types harboring HTLV-1 in mouse spleen, the splenocytes of mice 2, 3, 6, 7 and 8 were sorted by FACS into pan T or pan B cells. In a separate set of experiments to exclude the possibility that MT-2 cells remained in the spleen of the MT-2 cell-inoculated mice 9, 10, 11, 12, 13 and 14, the sorting gate of FACS was set to allow less than 0.003% MT-2 cells in the sorted fractions which had been stained with monoclonal antibodies (Fig. 3). Although portions of Gr-1^+ or CD_4^+ cells in mouse splenocytes were lost with this setting, contamination of MT-

Table I. Detection of HTLV-1 *pX* and HERV-R Sequences in MT-2 Cell-inoculated Mice Splenocytes

Mouse No.	DNA of unfractionated splenocytes		Lysate of FACS-sorted splenocytes (*pX*)				
	pX	HERV-R	pan T	pan B	CD_4^+	CD_8^+	granulocytes
2	+	−	+	−	NE	NE	NE
3	−	−	+	−	NE	NE	NE
6	NE	NE	−	−	NE	NE	NE
7	+	−	+	+	NE	NE	NE
8	NE	NE	−	−	NE	NE	NE
9	−	−	NE	NE	+	−	−
10	+	−	NE	NE	+	+	+
11	+	−	NE	NE	+	−	+
12	−	−	NE	NE	+	−	+
13	+	−	+	+	+	+	NE
14	−	−	−	+	−	−	NE
Positive rate	5/9	0/9	4/7	3/7	5/6	2/6	3/4

NE, not examined.

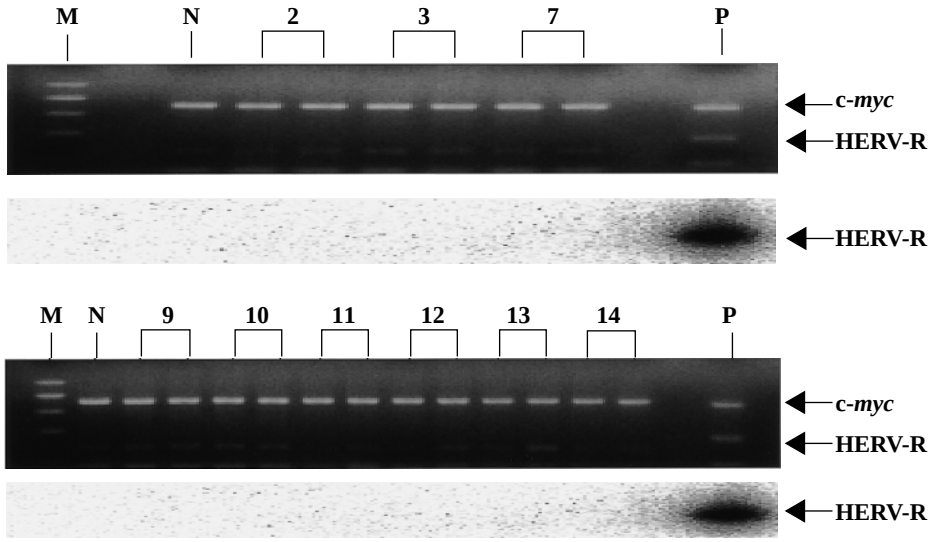

Fig. 2. No HERV-R sequence was detectable in mouse spleen DNA by PCR and Southern hybridization. The set of ethidium bromide staining patterns and Southern hybridization patterns is presented. Mouse c-*myc* band, which serves as an internal control, was seen by ethidium bromide staining. The mouse numbers are written on the top. M, DNA size marker. N, negative control using normal rat spleen DNA. P, positive control using MT-2 cell DNA diluted with normal rat spleen DNA (DNA equivalent to 10 MT-2 cells was contained in the reaction).

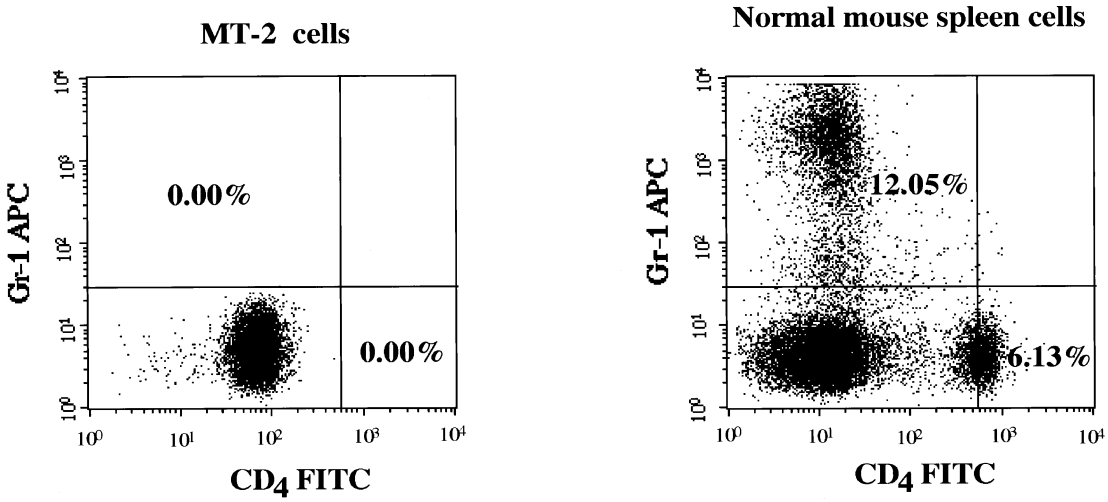

Fig. 3. Two-color flow cytometric analysis of spleen cells of normal C3H/HeJ mice and MT-2 cells. Cells were double-stained with FITC-labeled rat anti-mouse CD_4 monoclonal antibody and APC-labeled rat anti-mouse Gr-1 monoclonal antibody. Vertical and horizontal axes represent immunofluorescence intensity of APC and FITC, respectively. The percentage of Gr-1^+ or CD_4^+ cells is indicated. We analyzed 3×10^4 cells through the strict gate setting, so that MT-2 cell contamination in recovered cells should be excluded.

2 cells in the fractionated cells should be ruled out. The splenocytes of MT-2 cell-inoculated mice were sorted into pan T, pan B, CD_4^+, CD_8^+ and granulocyte cell fractions (Table I). Sorted cells were subjected to PCR and subsequent Southern hybridization to detect the HTLV-1 *pX* sequence in each fraction. The HTLV-1 *pX* sequence was detected not only in CD_4^+ cell fraction, but also in pan T, pan B, CD_8^+ and granulocyte cell fractions (Fig. 4).

DISCUSSION

In the present study, the cellular tropism of HTLV-1 in mice was first identified in HTLV-1-infected mice. HTLV-1 infection of various cell fractions, including pan T, pan B, CD_4^+, CD_8^+ and granulocytes, was shown to occur in MT-2 cell-inoculated C3H/HeJ mice. As granulocytes do not have the ability to divide, they have not been thought to be target cells for HTLV-1 infection. It is possible that HTLV-1-infected myeloid cells differentiated into granulocytes. Human hematopoietic progenitor cells are susceptible to HTLV-1 infection *in vitro*, and HTLV-1 infection could be maintained during differentiation of the HTLV-1-infected hematopoietic progenitor cells into erythroid, myeloid and primitive progenitor colonies.[19] Previous reports had shown that the leukemia cells of one ATL patient expressed myeloid cell phenotypes,[20] and that HTLV-1 could infect human promyelocytic leukemia HL60 cells.[21]

The spleen DNA from mice 2, 3, 7, 9, 10, 11, 12, 13 and 14, that harbored the HTLV-1 *pX* sequence in sorted cell lysates, was checked for the human-specific HERV-R sequence to see whether any MT-2 cells remained in the spleen. However, no HERV-R LTR sequence was detectable in the samples. If MT-2 cells were contained in the unfractionated splenocytes, their content should be less than 1 MT-2 cell in 10^5 splenocytes because the HERV-R LTR sequence was not detected (Table I, Fig. 2). For example, when spleen DNA of mouse 7 was diluted with normal mouse spleen DNA and subjected to triplicate PCR, the HTLV-1 *pX* sequence was positive in 3 out of 3 tubes until 30-fold dilution (data not shown). If the detected *pX* sequence were derived from remaining MT-2 cells, more than 5 MT-2 cells would be present in 10^5 splenocytes (equal to 1 μg of DNA) and the HERV-R LTR sequence would have been detected by our sensitive method. However, the HERV-R LTR sequence was negative in undiluted spleen DNA (Fig. 2). This suggests that the presence of MT-2 cells in the spleen of mouse 7 is unlikely. Moreover the strict parameters of FACS sorting virtually excluded the possibility of residual MT-2 cells, which may result in false-positive signals in mice 9, 10, 11, 12, 13 and 14. For example, 2.1×10^5 and 4.1×10^5 splenocytes were sorted into 2.5×10^4 granulocytes (12.05%) and CD_4^+ cells (6.13%), respectively, and the *pX* sequence was found in these sorted cells of mice 10, 11 and 12 (Table I). Since the HERV-R LTR sequence was not detected in the spleen DNA, which is equivalent to 10^5 cells, the number of MT-2 cells should be less than 2.1 and 4.1 in 2.1×10^5 and 4.1×10^5 splenocytes, respectively. Further, under the strict gate setting of FACS, the probability of MT-2 cell recovery in the sorted granulocytes or CD_4^+ cells is less than 1 cell in 3×10^4 (Fig. 3). The

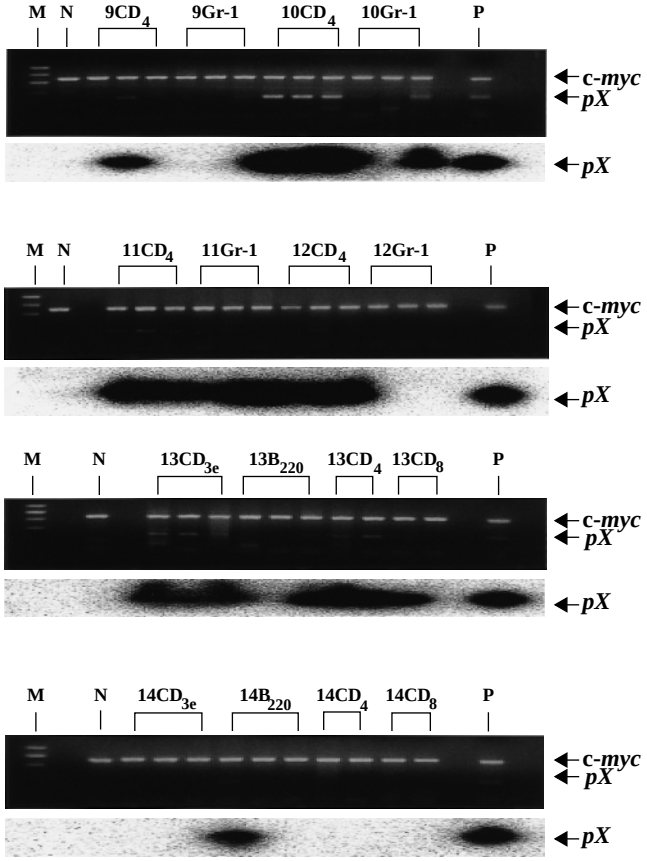

Fig. 4. Detection of the *pX* sequence in FACS-sorted cell lysates by PCR and Southern hybridization. The *pX* sequence was analyzed in each cell type. The set of ethidium bromide staining patterns and Southern hybridization patterns is presented. Mouse c-*myc* band, which serves as an internal control, was visualized by ethidium bromide staining. M, DNA size marker. N, negative control using normal rat peripheral blood mononuclear cell lysate. P, positive control using ATL-1K cell lysate diluted with normal rat peripheral blood mononuclear cell lysate (lysate equivalent to 10 ATL-1K cells was contained in the reaction). The mouse numbers are written on the top. CD_{3e}, B_{220}, CD_4, CD_8 and Gr-1 represent pan T, pan B, CD_4^+, CD_8^+ and granulocyte cell fractions, respectively, after the mouse number.

absence of the HERV-R LTR sequence and the probability of MT-2 cell recovery predict the presence of 0.00007 or 0.00013 MT-2 cell in the PCR reaction tube, which contained 2.5×10^4 sorted granulocytes or CD_4^+ cells. With pan T, pan B and CD_8^+ cells, the sorting gates were set similarly. Thus it is unlikely that MT-2 cells were present in the sorted fractions. The results strongly suggest that HTLV-1 infected various types of mouse cells. This was also supported by our recent finding that HTLV-1 was integrated into the mouse genome.[13, 14] We could not completely exclude the possibility that HTLV-1-infected mouse CD_4^+ cells contaminated other cell fractions. But in mouse 14, the HTLV-1 *pX* sequence was positive only in the B-cell fraction. Thus, the low abundance of HTLV-1-infected mouse CD_4^+ cells argues against the possibility that the HTLV-1-infected mouse CD_4^+ cells contaminated the B-cell fraction in this mouse. However it is desirable to use much more specific methods such as PCR *in situ* hybridization to confirm the above points in future experiments.

mRNA of a *trans*-activator of HTLV-1, Tax, was detected by reverse transcriptase-PCR (RT-PCR) in 0.5 μg of normal mouse RNA containing RNA from 10 to 100 MT-2 cells, as shown by ethidium bromide staining on an agarose gel. But, 0.5 μg of RNA from the spleen of each of MT-2 cell-inoculated mice 2 and 3 did not show the corresponding signal on agarose gel electrophoresis (data not shown). One possibility is that HTLV-1 expression is very low. The low-level expression of HTLV-1 mRNA or its antigen has been reported in HTLV-1 carriers and patients with ATL.[22–24] The mechanism of inhibition of viral expression in HTLV-1 carriers and patients with ATL is not well understood, and the expression of HTLV-1 antigen was reported only when peripheral blood mononuclear cells derived from patients with ATL were cultured *in vitro*.[15, 25] Thus, it might be possible to induce the expression of the viral message under appropriate culture conditions. It is also possible that HTLV-1-infected mouse cells lack the cellular factors required for HTLV-1 expression. When a similar retrovirus, HIV-1, was used to infect human CD4/CCR5 transgenic mice, *tat*/*rev* RNA could not be detected by RT-PCR from spleen or lymphnode RNA.[26] When HIV-1 was used to infect rodent cells, viral expression could be observed only when human chromosome 12 was introduced into the cells.[27] Recently, overexpression of human cyclin T, which is located in human chromosome 12, was reported to rescue the activity of a *trans*-activator of HIV, Tat, in nonpermissive rodent cells.[28] Further study is necessary to clarify the reasons for the low-level expression of Tax RNA in HTLV-1-infected mice.

HTLV-1 infection is associated not only with ATL,[2] but also with various other diseases such as HAM/TSP,[3] HTLV-1 uveitis,[29] infective dermatitis,[30] arthropathy[31] and myositis.[32] The latent period from HTLV-1 infection to the manifestation of HTLV-1-associated diseases is 40 years or more. A statistical model analysis indicated that age-dependent accumulation of 5 supposedly independent leukemogenic events within HTLV-1-infected T cells might be involved in the occurrence of ATL.[33] However, the mechanism of pathogenesis by HTLV-1 is poorly understood. Although CD_4^+ cells are the major target cells of HTLV-1 infection in humans,[34] HTLV-1 is successfully transmitted to various cell lines other than CD_4^+ cells *in vitro*.[5–8] Our *in vivo* results suggest that there is similar

tropism of HTLV-1 in mice to that in humans. It was reported that HTLV-1 provirus could be detected in CD_4^+, CD_8^+, monocytes and B cells in patients with ATL and HAM/TSP and HTLV-1-infected healthy carriers.[34] It is worth noting that those reported human cases are in the relatively late stage of HTLV-1 infection and no data are available on human carriers in the early stage of infection. In the present study with HTLV-1-infected mice, we found that various cell types are infected with HTLV-1 in the early stage of infection. In order to elucidate the mechanism of pathogenesis of HTLV-1-associated diseases, it is important to identify the various infected cell types and the interplay of these infected cell fractions present from the early stage of infection.

In conclusion, our results suggest that HTLV-1 has tropism for various cell types in mice, which is consistent with the case in humans, and the receptor or coreceptor is distributed in a variety of cell types. The knowledge of which cell types harbor the virus in infected mice is important for understanding the natural history of HTLV-1 infection, which is associated with various diseases in humans.

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