Reduction of human T-cell leukemia virus type-1 infection in mice lacking nuclear factor-κB-inducing kinase

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(Received August 29, 2007/Revised December 21, 2007/Accepted January 3, 2008/Online publication February 29, 2008)

Human T-cell lymphotropic virus type 1 (HTLV-1) causes adult T-cell leukemia and inflammatory disorders. Aberrant activation of nudear factor-κB (NF-κB) has been linked to HTLV-1 pathogenesis and to various kinds of cancers, including adult T-cell leukemia. NF-κB-inducing kinase (NIK) is critical for non-canonical activation of NF-κB and for the development of lymphoid organs. HTLV-1 activates NF-κB by the non-canonical pathway, but examination of the role of NIK in proliferation of HTLV-1-infected cells *in vivo* has been hindered by lack of a suitable animal model. Alymphoplasia (*aly/aly*) mice bear a mutation of NIK, resulting in defects in the development of lymphoid organs and severe deficiencies in both humoral and cell-mediated immunity. In the present study we therefore used a mouse model of HTLV-1 infection with *aly/aly* mice. The number of HTLV-1-infected cells in the reservoir organs in *aly/aly* mice was significantly smaller than in the control group 1 month after infection. In addition, *aly/aly* mice did not maintain provirus for 1 year and antibodies against HTLV-1 were undetectable. These results demonstrate that the absence of functional NIK impairs primary HTLV-1 proliferation and abolishes the maintenance of provirus. Interestingly, clonal proliferation of HTLV-1-infected mouse cells was not detected in *aly/aly* mice, which is consistent with the lack of HTLV-1 persistence. These observations imply that the clonal proliferation of HTLV-1-infected cells in secondary lymphoid organs might be important for HTLV-1 persistence. (*Cancer Sci* 2008; 99: 872–878)

Human T-cell lymphotropic virus type 1 infection causes various diseases, including ATL, HAM/TSP, uveitis, and arthropathy.[1] Other diseases reported to be associated with HTLV-1 infection include hepatitis C virus-associated hepatocellular carcinoma,[2] gynecologic malignancies,[3] polymyositis,[4] chronic respiratory diseases,[5] and dermatitis.[6] These observations indicate that HTLV-1 infection places individuals at high risk of developing certain malignancies and inflammatory diseases.

Adult T-cell leukemia occurs in 2–6% of people infected with HTLV-1 after a long latency of 20–50 years. It has been suggested that approximately five independent leukemogenic events, including immortalization of T lymphocytes, must occur prior to the development of ATL.[7] These events are not well understood; however, NF-κB is considered critical for transformation and survival of HTLV-1-infected cells as well as for other transformed cells.[8,9] In fact, both lymphocytes isolated from ATL patients and HTLV-1-transformed T-cell lines show constitutive activation of NF-κB.[8–10] In addition, NF-κB inhibitors have been shown to induce apoptosis of ATL cells and HTLV-1-transformed cells,[11] and to prevent growth of HTLV-1-transformed T cells transplanted into NOD/SCID/γc^{null} and SCID mice.[12,13]

Nuclear factor-κB complexes can be activated by two distinct signaling pathways: the canonical (classical) pathway and the non-canonical (alternative) pathway.[9,14] NIK phosphorylates IκB kinase α, resulting in the processing of the *nf-κB* gene product p100 to generate the transcriptional regulator p52, a pivotal component of the non-canonical NF-κB pathway. The non-canonical pathway is stimulated by CD40 ligand, lymphotoxin-β, and B-cell-activating factor.[8,9,14] Alymphoplasia (*aly*/*aly*) mice are a natural strain identified originally in a colony of C57BL/6 J mice and bear a dominant-negative NIK mutation.[15,16] These mutant mice lack lymph nodes and Peyer's patches, and the structures of their spleen and thymus are abnormal.[15,17] Although *aly*/*aly* mice have a normal number of erythrocytes, leukocytes, and lymphoid cells harboring typical surface markers, they display diverse immune defects, including reduction in certain humoral and cell-mediated immunity leading to delayed or ablated elimination of vaccinia virus and lymphocytic choriomeningitis virus.[15,17–19] These observations indicate that *aly*/*aly* mice offer a unique possibility to assess the role of NIK in viral infection.

Not all individuals exposed to HTLV-1 develop persistent infection. Previous reports indicate that approximately 60% of individuals who had received infected blood or blood components, and 15–30% of breast-fed children born to infected mothers, developed persistent HTLV-1 infection.[20,21] Once HTLV-1 infection is established, the proviral load is maintained for a long period in asymptomatic carriers.[22,23] Importantly, a high proviral load in asymptomatic carriers is associated with the onset of ATL,[24] and the progression of motor disability in HAM/TSP.[25] Thus, a detailed understanding of the early events involved in the proliferation of HTLV-1-infected cells in primary infection and the maintenance of proviral load in the asymptomatic carrier stage is essential for the development of effective strategies to decrease the number of patients with HTLV-1-associated diseases.

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Abbreviations: ATL, adult T-cell leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAM/TSP, human T-cell leukemia virus type 1-associated myelopathy/tropical spastic paraparesis; HTLV-1, human T-cell leukemia virus type 1; LMPCR, linker-mediated polymerase chain reaction; LTR, long terminal repeat; NF-κB, nuclear factor-κB; NIK, nuclear factor-κB-inducing kinase; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; RT, reverse transcription; SCID mice, severe combined immunodeficient mice.

doi: 10.1111/j.1349-7006.2008.00766.x

Understanding of the mechanism of HTLV-1 proliferation in asymptomatic carriers has been hindered by a lack of human specimens. Therefore, a variety of animal models of HTLV-1 infection, including rabbits, monkeys, and rats, have been developed to understand viral and host factors controlling viral proliferation.[26] However, more information regarding genetics and genetics-related factors are available for mice. Thus, we established a mouse model for natural HTLV-1 infection using various syngeneic mouse strains. Some of these mouse strains have shown proliferation of naturally infected cells,[27–29] and some have not, suggesting that these mice could be used to investigate the role of genetic backgrounds on proliferation of HTLV-1-infected cells in the carrier stage. This mouse model is different from the other mouse models that have only allowed examination of proliferation after transplantation into SCID mice of human cells already transformed by HTLV-1.[30,31] In the present study, we used the *aly*/*aly* mouse model to examine the role of NIK on primary and persistent infection of HTLV-1.

Materials and Methods

Cells and animals. The HTLV-1-producing human T-cell line MT-2, which was established by coculture with umbilical cord leukocytes from a normal male infant and human leukemic T cells,[32] was cultured in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Female BALB/*c* mice were obtained from Charles River (Tokyo, Japan), and female *aly*/*aly* and C57BL/6 J mice were purchased from Clea Japan (Tokyo, Japan). The mice were inoculated intraperitoneally with 10^7 MT-2 cells at 4 weeks of age, and were sacrificed using ethyl ether anesthesia at 12 h, 2 days, 1 week, 1 month, or at 1 or 2 years after inoculation. All experiments were conducted in line with the Regulations on Animal Experiments of the University of Tsukuba and approved by the Animal Experiment Committee, University of Tsukuba.

DNA extraction. PBMC were prepared from peripheral blood according to the standard Ficoll-Hypaque method (Lymphosepal II; Immuno-Biological Laboratories, Gunma, Japan). PBMC and pieces of mouse organs were suspended in 0.5 mL of the digestion buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid, 0.3% sodium dodecyl sulfate, and 0.05 mg/mL proteinase K at 50°C for 6 h. Total genomic DNA was prepared from the lysate by DNase-free RNase A digestion, phenol extraction, and ethanol precipitation.

Detection of inoculated MT-2 cells. We previously cloned and sequenced a cellular DNA fragment flanking the 3′ LTR of the HTLV-1 provirus in MT-2 cells using LMPCR.[29] To detect the presence of MT-2 cells in mice after inoculation, we utilized a set of oligonucleotide primers designed to amplify the 3′ LTR of HTLV-1 and the human flanking sequence of MT-2 cells via PCR. The PCR conditions were as described previously.[29] Genomic DNA samples (500 ng) isolated from mouse organs and HTLV-1-producing cell lines were subjected to PCR followed by agarose gel electrophoresis. The mouse c-*myc* sequence was amplified as an internal control.[29]

Quantification of proviral load. The PCR conditions for quantification of HTLV-1 proviral load were as described previously.[29] Briefly, the number of *tax* (viral gene) and mouse c-*myc* molecules (cellular gene control) were quantified using real-time PCR, and the HTLV-1 proviral load per 10^5 mouse cells was calculated as follows:

$$(\text{Number of } tax \text{ molecules/number of mouse c-}myc \text{ molecules}/2) \times 10^5.$$

Proviral load was defined as zero when there was no amplification of the *tax* product after 50 cycles of PCR under conditions where mouse c-*myc* was amplified correctly.

Antibody detection. Antibodies against HTLV-1 proteins in mouse plasma were assayed using a particle agglutination kit (Serodia HTLV-1; Fujirebio, Tokyo, Japan) according to the manufacturer's instructions.

Identification and sequence analysis of mouse cell clones containing HTLV-1 provirus. To identify the cellular sequences flanking the provirus in HTLV-1-infected mouse cell clones, triplicate LMPCR analyses were carried out.[33] Total DNA (5 μg) was digested with 15 units *Nla*III (New England Biolabs, Ipswich, MA, USA) for 3 h at 37°C. DNA was extracted with a phenol–chloroform mixture and precipitated with ethanol. Digested DNA (1 μg) was ligated with 100 pmol Bio-1 primer (5′-TCATGATCAATGGGACGATCACATG) with 2.8 Weiss units of T4 DNA ligase (Takara Biochemical, Tokyo, Japan) for 4 h at 15°C. Ligated DNA was amplified in 50 μL of the solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 20 pmol Bio-2 (5′-CTGTTCTGCGCCGTTACAGATCGA), 200 μM of each deoxynucleoside triphosphate, and 1.25 units of *Taq* Gold polymerase (Perkin Elmer, Waltham, MA, USA). A thermal cycling reaction was carried out with Robocycler (Stratagen, La Jolla, CA, USA) at 95°C for 12 min and 99 cycles at 95°C for 30 s, at 61°C for 30 s, and at 72°C for 30 s, followed by a final elongation step for 7 min at 72°C. This linear PCR mixture (0.5 μL) was added to a 25-μL solution containing 20 pmol Bio-3 (5′-CCTTTCATTCACGACTGACTGCCG), bio-4 (5′-TCATGATCAATGGGACGATCA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, and 0.25 unit *Taq* polymerase (Wako Chemicals, Osaka, Japan). Thermal cycling was carried out at 95°C for 5 min and 50 cycles at 95°C for 30 s, 61°C for 30 s, and 72°C for 1 min, and was followed by a final elongation step for 7 min at 72°C.

The final LMPCR products (10 μL) were subjected to 2% agarose gel electrophoresis. DNA fragments were transferred under alkaline conditions onto a nylon membrane, and hybridized with a digoxigenin-labeled 3′ LTR-specific bio-5 probe (5′-TGGCTCGGAGCCAGCGACAGCCCAT). The filter was then washed three times for 15 min at 63°C with a mixture of 30 mM NaCl, 3 mM sodium citrate, and 0.1% sodium dodecylsulfate. The bio-5 probe was detected with antidigoxigenin antibody conjugated with alkaline phosphatase in 1 mL of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 280 μM nitroblue tetrazolium chloride, and 400 μM 5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI, US, USA).

DNA fragments containing the 3′ LTR of the HTLV-1 provirus and its flanking sequence were then subcloned into the pCR-TOPO vector (Invitrogen, San Diego, CA, USA). Screening of clones was carried out by hybridization with the digoxigenin-labeled bio-5 probe as described above. Each LTR-containing fragment was then sequenced to determine the nucleotide sequence flanking the 3′ LTR of the HTLV-1 provirus by autosequencer ABI377 (Perkin Elmer).

Confirmation of cell clones harboring HTLV-1 provirus. The cellular mouse sequences flanking the 3′ LTR were compared with the GenBank database, and their chromosomal locations were determined by BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Mouse cell clones harboring HTLV-1 provirus were confirmed by PCR using a forward primer complementary to the 3′ LTR of HTLV-1 (bio-2 primer) and reverse primers deduced from the host flanking sequence (Table 4). PCR conditions were the same as for the provirus detection except for the annealing temperatures, which varied for each primer set.

Detection of HTLV-1 expression ex vivo. Mouse lymphocytes isolated from the spleen and the mesenteric lymph nodes were cultured at 1×10^6 cells/well for 24 h in 200 μL RPMI medium supplemented with 10% fetal bovine serum, 5 μg/mL anti-CD3 antibody (145-2C11; Pharmingen, Franklin Lakes, NJ, USA), 5 μg/mL anti-CD28 antibody (37.51; Pharmingen), and 10 U/mL

recombinant interleukin-2 (eBioscience, San Diego, CA, US). Total RNA was extracted from the cultivated cells with TRIzol reagent (Invitrogen), and 1 μg was used for RT-PCR analysis. PCR was carried out for 50 cycles using RPX3 (5′-ATCCCGT GGAGACTCCTCAA) and RPX4 (5′-AACACGTAGACTGGG TATCC) primers located at the *tax* region. The PCR products were detected with the digoxigenin-labeled probe RPXPR1 (5′-AACACCATGGCCCACTTCCC). Amplification of a cDNA fragment from the *GAPDH* gene was carried out as an internal control.

Statistical analysis. Welch's *t*-test was used to detect any difference between the mean scores in two groups, based on the equality test of two variances.

Results

Secondary lymphoid organs are the principal reservoirs of HTLV-1-infected mouse cells. Although HTLV-1-infected cells are distributed mainly in the blood or secondary lymphoid organs in rats, rabbits, and squirrel monkeys,[34–37] the major reservoir organs in HTLV-1-infected mice with different genetic backgrounds are unknown. Thus, we first examined whether different strains of syngeneic mice show the same organ distribution of HTLV-1-infected cells. For infecting BALB/*c* and C57BL/6 J mice we inoculated MT-2 cells and quantified the proviral load at 1 month after infection (Table 1). Provirus was detected in all organs, but the spleen and the mesenteric lymph nodes had higher proviral loads than other organs. Next we isolated mouse splenocytes from HTLV-1-infected BALB/*c* mice 1 month after infection. The 10^7 splenocytes isolated from the infected mice were inoculated intraperitoneally into uninfected BALB/*c* mice. At 1 month after inoculation, the BALB/*c* mice inoculated with the infected splenocytes showed a similar pattern of proviral distribution and a similar amount of provirus as the host mice inoculated with human MT-2 cells (Table 2). These results suggest that the secondary lymphoid organs are the major reservoir organs for HTLV-1 provirus in mice and that the organ distribution of HTLV-1-infected cells is similar among mouse strains.

To analyze the proliferation of HTLV-1-infected cells in animal models, it is important to distinguish the inoculated HTLV-1-producing cells from newly infected host cells. We previously cloned a human cellular DNA sequence flanking the 3′ LTR of HTLV-1 from MT-2 cells and established a sensitive PCR system to detect these sequences.[29] Using this PCR-based system, a single molecule of human-specific MT-2 sequence can be detected in sample DNA. The MT-2 sequence was detected in DNA samples isolated from secondary lymphoid organs of BALB/*c* mice at 12 h and 2 days after infection (data not shown). However, amplification of the human MT-2 sequence was not detected in any of the mouse organs at 1 month after infection (data not shown), indicating that the inoculated human MT-2 cells were rejected within 1 month and that the presence of provirus in the inoculated mice represents HTLV-1-infected mouse cells.

***aly/aly* mice have reduced primary HTLV-1 proliferation and failed to maintain proviral load.** To clarify whether mice with non-functional NIK display altered proliferation of HTLV-1-infected cells, we used quantitative PCR to examine the proviral load in *aly*/*aly* mice 1 month after MT-2 inoculation (Table 1). Similar to BALB/*c* and C57BL/6 J mice, the major reservoir organ of HTLV-1-infected cells in *aly*/*aly* mice was the spleen, but proviral load in the spleen was significantly lower than that in C57BL/6 J mice ($P < 0.005$; Welch's *t*-test). PCR to detect the MT-2 sequence confirmed that the disappearance of human MT-2 cells in all *aly*/*aly* mice examined occurred within 1 month of inoculation. We next analyzed proviral load in the mice at 1 year after infection (Table 3). Provirus in BALB/*c* and C57BL/6 J mice was distributed mainly in the mesenteric lymph nodes and in PBMC. In addition, proviral load in these mice at 1 year after infection tended to be higher than that seen at 1 month after infection. In contrast, by 1 year, *aly*/*aly* mice had failed to maintain proviral load. These data suggest that in *aly*/*aly* mice NIK deficiency impairs both proliferation of HTLV-1-infected cells in primary infection and the maintenance of HTLV-1 proviral load.

***aly/aly* mice have impaired production of anti-HTLV-1 antibody.** The aggregate of diverse immune defects, such as impaired activation of T and B lymphocytes, combined with the absence of secondary lymphoid organs in *aly*/*aly* mice leads to very weak or inactive immune responses to viral infection.[18,19] To confirm the impairment of immune responses in *aly*/*aly* mice, we measured the levels of antibodies against HTLV-1 in mice at 1 month after infection. As expected, BALB/*c* and C57BL/6 J mice had antibodies against HTLV-1 (682.7 ± 264.4, $n = 6$ and 384.0 ± 140.2, $n = 6$), but the antibodies were not detected in *aly*/*aly* mice (<16, $n = 5$). These results are consistent with the previous reports in which *aly*/*aly* mice produced a minimal

Table 1. Organ distribution of proviral load‡ in human T-cell leukemia virus type 1-1-infected mice at 1 month after infection

Organ	BALB/c†	C57BL/6J†	*aly/aly*†
Peripheral blood mononuclear cells	20.4 ± 8.9	58.3 ± 23.1	0
Spleen	56.1 ± 29.0	115.5 ± 44.2	10.3 ± 2.8
Mesenteric lymph node	38.0 ± 16.1	110.8 ± 31.1	NA
Thymus	2.7 ± 2.9	7.0 ± 3.0	0
Liver	7.0 ± 6.7	27.9 ± 15.4	0
Kidney	7.8 ± 4.9	10.4 ± 7.5	0

†Mouse strain.
‡Values represent the mean ± SD. Proviral load was calculated as described in the Materials and Methods section and represents copy number per 10^5 cells.
NA, not applicable because of structural defects in *aly/aly* mice.

Table 2. Organ distribution of proviral load‡ in mice inoculated with human T-cell leukemia virus type 1-infected mouse splenocytes

Organs	BA51†	BA20	BA50	BA60†	BA61	BA62	BA63
Peripheral blood mononuclear cells	24.4	22.4	15.6	15.4	13.2	26.6	9.3
Spleen	68.3	66.8	68.0	52.0	38.5	50.2	26.9
Mesenteric lymph nodes	38.9	35.5	43.3	57.7	56.3	61.1	41.3
Thymus	2.4	0	0	0	0	0	0
Liver	6.8	2.7	5.8	2.4	7.4	4.8	0.4
Kidney	11.8	4.1	9.6	4.8	9.9	4.1	2.4

†Two BALB/*c* mice, BA51 and BA60, were inoculated with 10^7 MT-2 cells intraperitoneally. At 1 month after inoculation, 10^7 splenocytes were isolated from BA51 and BA60 and these cells were introduced into the uninfected mice BA20, BA50, and BA61-63 via intraperitoneal injection. Proviral load of the mice inoculated with the infected mouse splenocytes was quantified at 1 month after infection.
‡Proviral load was calculated as described in the Materials and Methods section and represents copy number per 10^5 cells.

doi: 10.1111/j.1349-7006.2008.00766.x

Table 3. Organ distribution of proviral load[§] in human T-cell leukemia virus type 1-infected mice at 1 year after infection

Organs	BALB/*c*[†]		C57BL/6 J[†]		*aly/aly*[†]		
	BA216[‡]	BA217[‡]	CB40[‡]	CB41[‡]	A50[‡]	A51[‡]	A52[‡]
Peripheral blood mononuclear cells	1404	291	353	1440	0	0	0
Spleen	428	271	263	808	0	0	0
Mesenteric lymph nodes	4978	958	585	2886	NA	NA	NA
Thymus	0	0	26	116	0	0	0
Liver	322	54	62	701	0	0	0
Kidney	35	62	126	459	0	0	0

[†]Mouse strain.
[‡]Mouse identification number.
[§]Proviral load was calculated as described in the Materials and Methods section and represents copy number per 10^5 cells.
NA, not applicable because of structural defects in *aly/aly* mice.

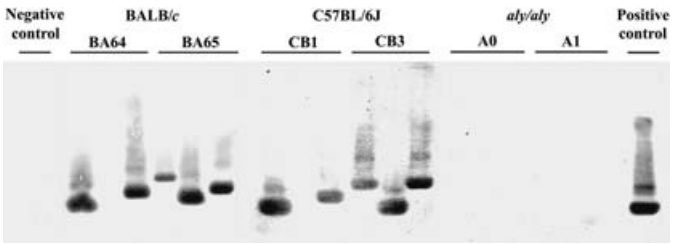

Fig. 1. Triplicate analyses of linker-mediated polymerase chain reaction (LMPCR) using splenic DNA from human T-cell leukemia virus type 1 (HTLV-1)-infected mice. DNA fragments comprising a portion of the 3′ long terminal repeat (LTR) of HTLV-1 and the host flanking sequence were amplified in triplicate by LMPCR using genomic DNA extracted from the spleen of BALB/*c*, C57BL/6 J, and *aly/aly* mice at 1 month after infection. Amplified polymerase chain reaction products were subjected to agarose gel electrophoresis, transferred to a membrane, and then detected by hybridization with a probe specific for digoxigenin-labeled HTLV-1 3′ LTR. Representative data for each mouse strain is shown (mouse BA64 and BA65, BALB/*c*; mouse CB1 and CB3, C57BL/6 J; mouse A0 and A1, *aly/aly*). Splenic DNA in an uninfected mouse was used as a negative control, and MT-2 DNA was used as a positive control.

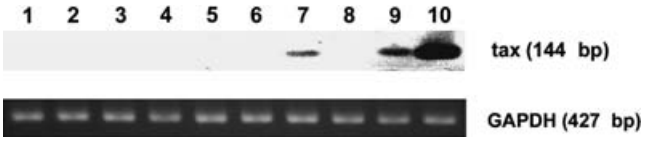

Fig. 2. Detection of human T-cell leukemia virus type 1 (HTLV-1) tax mRNA in infected mouse lymphocytes by reverse transcription–polymerase chain reaction (RT-PCR). HTLV-1 expression was surveyed in the BALB/*c* mice at 1 month (lanes 2–5) or 2 years (lanes 6–9) after infection. Lymphocytes isolated from the spleen or the mesenteric lymph nodes were cultivated for 24 h *ex vivo*, and total RNA was extracted for RT-PCR. Amplified RT-PCR products were subjected to agarose gel electrophoresis, transferred to a membrane, and then detected by hybridization with a digoxigenin-labeled probe that anneals within the *tax* region. Amplification of a cDNA fragment of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was carried out as an internal control. GAPDH mRNA was detected by ethidium bromide staining after agarose gel electrophoresis. The mouse identification number (ID) and the organs used for RT-PCR assay were as follows: 1, spleen of non-infected mouse (control); 2, ID BA43, spleen; 3, ID BA43, mesenteric lymph nodes; 4, ID BA44, spleen; 5, ID BA44, mesenteric lymph nodes; 6; ID BA214, spleen, 7; ID BA214, mesenteric lymph nodes; 8, ID BA215, spleen; 9, ID BA215, mesenteric lymph nodes; 10, MT-2 cells (positive control).

immune response to lymphocytic choriomeningitis virus or vaccinia virus.[18,19]

No cell clone with integrated HTLV-1 provirus was detected in *aly/aly* mice at 1 month after infection. One of the hallmarks of HTLV-1 infection in humans is the presence of cell clones with integrated provirus, which indicates that HTLV-1-infected cells proliferate clonally in asymptomatic carrier stages.[38] We therefore carried out LMPCR using DNA isolated from the spleen of BALB/c, C57BL/6 J, and aly/aly mice at 1 month after infection. This LMPCR method can detect clonal proliferation of HTLV-1-integrated cells if samples have 10 or more provirus molecules that are located at the same integration site.[28] Representative data of triplicate LMPCR analyses are shown in Figure 1. Cell clones with integrated HTLV-1 provirus were detected in all six BALB/c and all five C57BL/6 J mice but not in any of the five aly/aly mice analyzed (Fig. 1).

Clonal proliferation of HTLV-1-infected cells occurs in secondary lymphoid organs within 1 week of inoculation. To determine the period required for HTLV-1-infected cell clones to proliferate in secondary lymphoid organs, we used LMPCR with DNA isolated from the spleen, the mesenteric lymph nodes, and the Peyer's patches of BALB/*c* mice at 1 week after infection ($n = 2$). One mouse (ID BA206) harbored cell clones with integrated provirus in the Peyer's patches, and another mouse (ID BA207) harbored a HTLV-1 cell clone with integrated provirus in the mesenteric lymph nodes (Table 4). We obtained direct evidence of the integration of HTLV-1 in the mouse genome by cloning the DNA fragments amplified by LMPCR and subsequently sequencing the DNA flanking the 3′ LTR of HTLV-1 (Table 4). Comparison of the flanking sequence of each clone with the GenBank database indicated that each had 100% identity to mouse genomic DNA. To further confirm that the HTLV-1-infected cells proliferating clonally were derived from mouse cells and not from human MT-2 cells, we carried out PCR using reverse primers specific for the flanking sequence of each cell clone (Table 4) and a forward primer specific for a sequence within the 3′ LTR (bio-2 primer).[33] We observed PCR products of the expected size using DNA from the organs of infected mice but not from the control human MT-2 cells (data not shown).

Viral RNA is expressed in the mesenteric lymph nodes cultured *ex vivo*. In HTLV-1 carriers, HTLV-1 provirus in PBMC is transcriptionally silent, but a portion of these cells is capable of expressing viral genes after short-term cultivation *ex vivo*.[39] We therefore surveyed the expression of viral tax mRNA in secondary lymphoid organs by RT-PCR. We used RNA extracted from the splenocytes and the cells isolated from the mesenteric lymph nodes that had been cultivated for 24 h. GAPDH mRNA was also amplified via RT-PCR as a control for RNA integrity. Although we did not find tax mRNA in the splenocytes, we did find tax mRNA in cells of the mesenteric lymph nodes of mice at 2 years after infection (Fig. 2). But tax

Table 4. Human T-cell leukemia virus type 1 (HTLV-1) integration sites and primers used to detect cell clones harboring HTLV-1 provirus

Mouse ID	Clone ID	Segment of host sequence immediately flanking the 3′ LTR of HTLV-1 provirus	Position‡	Candidate genes with integration or nearest genes	Reverse primer sequence and product size (bp)§
BA206	206C1	5′-AACCAAAGGCTTAACATTTAACGTCTTAAG	NT_078297.4 (11853191–11853323)	2310035C23Rik (hypothetical gene) intron	5′-CATGCAATAAAAAATGGTTTGGTA (290 bp)
	206C2	5′-GACATCTCTTCAGCCCAGCAGCTCCTGTCCC	NT_039305.5 (861623–861694)	Serine/threonine kinase 32B intron	5′-CATGGTTAAGAAGCCCCACACCCA (229 bp)
BA207	207C1	5′-GATGCCCACTTCCTCCTCTATTCGGTTAACG	NT_163365.1 (5391343–5391501)	8 kb downstream of similar to glyceraldehyde-3-phosphate dehydrogenase	5′-CATGGTGAAGACAACTTCCTTTTC (316 bp)

Each host sequence flanking the 3′ LTR of HTLV-1 provirus was identified using linker-mediated polymerase chain reaction. Standard polymerase chain reaction was then carried out using a 3′ LTR-specific forward primer and the site-specific reverse primer (above) to confirm that each cell clone harboring provirus was of mouse origin.
‡The RefSeq number is indicated, and the position corresponding to the sequence flanking the 3′ LTR of the HTLV-1 provirus is shown in parentheses.
§Each reverse primer was deduced from comparison of sequences flanking the 3′ LTR with GenBank mouse genomic DNA. (The expected size of each polymerase chain reaction product from amplification using the bio-2 primer, a primer complementary to the 3′ LTR of HTLV-1, 5′-CTGTTCTGCGCCGTTACAGATCGA-3′, and each reverse primer is shown in parentheses.)

mRNA was not detected without cultivation (data not shown). These data demonstrate that HTLV-1-infected mouse cells in secondary lymphoid organs have the potential to produce viral components, as PBMC isolated from human carriers.

Discussion

The NIK mutation in *aly*/*aly* mice disrupts NF-κB activation as well as the development of lymphoid organs. HTLV-1 is a T-lymphotropic retrovirus, and clonal proliferation of infected cells in asymptomatic carriers has been observed.[38] Thus, *aly*/*aly* mice provide a unique model for assessing the effects of defective NIK on HTLV-1 proliferation in the asymptomatic carrier stage. In the present study, we found that the proviral load in the spleen of *aly*/*aly* mutant mice at 1 month after infection was significantly lower than that in C57BL/6 J mice with wild-type NIK. In addition, in *aly*/*aly* mice, provirus was no longer detectable at 1 year after infection, which is consistent with the fact that clonal proliferation of HTLV-1-infected cells was undetectable in these mice at 1 month after infection. These results demonstrate that NIK signaling is critical for the proliferation of HTLV-1-infected cells in primary infection and for the establishment of persistent infection of HTLV-1 in mice.

Here, we found that infected cells were primarily distributed in the PBMC and secondary lymphoid organs irrespective of the mouse strain (Tables 1,3), which is consistent with previous reports examining HTLV-1-infected rats and rabbits.[34–36] Interestingly, we also showed that provirus was concentrated in the spleen of *aly*/*aly* mice at 1 month after infection (Table 1), even though this mouse strain has structural alterations in the splenic white pulp, the inner region of which is compartmentalized into a periarteriolar lymphoid sheath containing T cells and a flanking B-cell corona.[17] This result indicates that clustered immune structures, such as the germinal centers in lymphoid organs, are not required for the localization of HTLV-1-infected donor cells and transmission of HTLV-1.

One of the most important characteristics of *aly*/*aly* mice is the dysfunctional secondary lymphoid organs induced by impaired NIK signaling.[15,16] Consistent with this, we found that *aly*/*aly* mice did not produce antibodies against HTLV-1 and had lower proviral load in the spleen compared to C57BL/6 J control mice (Table 1). Considering the previous reports indicating that *aly*/*aly* mice have an impaired cell-mediated immune response to lymphocytic choriomeningitis virus and vaccinia virus leading to unsuccessful or delayed elimination of these viruses,[18,19] and reduced cytokine production in response to *Listeria monocytogenes* infection,[40] it is interesting that our data suggest that the initial immune activation of T and B cells in secondary lymphoid organs may be important for the proliferation of HTLV-1-infected cells in the primary infection. Consistent with this concept, we previously found that the proviral load in mice infected with HTLV-1 at 4 weeks of age tended to be higher than in mice infected within 24 h of birth.[29] In addition, certain retroviruses, such as mouse mammary tumor virus and human immunodeficiency virus type 1, activate host immune responses in the early phase of infection to enhance viral proliferation.[41,42] Thus, in *aly*/*aly* mice, inefficient primary activation of T and B cells might result in failed proliferation of virally infected cells. However, there is another possibility that abnormal signal transduction by mutated NIK in *aly*/*aly* mice would disturb the generation of primary target cells for HTLV-1 or the entry steps of infection, which should be clarified in future work.

Although some animal models demonstrated that secondary lymphoid organs are major reservoirs of HTLV-1-infected cells, the characteristics of the infected cells in secondary lymphoid organs in the asymptomatic carrier stage are still obscure.[34–37] In the mice 2 years after infection, here we found tax mRNA expression in the mesenteric lymph nodes but not in the spleen

doi: 10.1111/j.1349-7006.2008.00766.x

by RT-PCR after short-term cultivation. Recently, we also isolated lymphocytes from the mesenteric lymph nodes and the spleen of two C3H/HeN mice 5 months after infection, and checked Tax expression by immunocytochemistry after short-term cultivation. Weak expression of the Tax protein was found in the cells isolated from the mesenteric lymph nodes but rarely in the splenocytes (our unpublished data). These results might reflect the differences of the proviral load or the microenvironment in each reservoir organ.[43]

In human carriers and experimentally infected animals, cell clones with integrated HTLV-1 provirus persist for a long period and are believed to maintain proviral load.[22,44,45] Using LMPCR, we demonstrated that BALB/*c* and C57BL/6 J mice, but not *aly*/*aly* mice, harbored cell clones with integrated provirus at 1 month after infection and that clonal proliferation was initiated as early as 1 week after viral exposure (Fig. 1; Table 4). These results imply that NIK may also be involved in clonal proliferation of cells containing integrated provirus in primary infection.

In persistently infected individuals, HTLV-1 proviral load is considered to be maintained by the equilibrium between proliferation and eradication of the virus or virus-infected cells by host immune responses against HTLV-1.[46] It was previously shown that the proviral load in carriers correlates with a certain haplotype of human leukocyte antigen that recognizes Tax.[46] In the present study, we found that the *aly*/*aly* mice did not maintain proviral load over time in contrast to BALB/*c* and C57BL/6 J mice (Table 3), even though the latter two strains show vigorous humoral and cell-mediated immune responses against HTLV-1.[29,47] These results have raised an interesting possibility that HTLV-1 persistence might be supported in part by continuous immune activation. HTLV-1 can infect regulatory $CD4^+$ $CD25^+$ T cells, memory $CD4^+$ $CD45RO^+$ T cells, and $CD8^+$ $CD25^+$ T cells, and it has been hypothesized that these T cells may be the HTLV-1 reservoir *in vivo*.[48–50] We therefore suggest that persistent activation and expansion of certain kinds of T cells might be important for the maintenance of proviral load in the asymptomatic carrier stage.

Although many studies have used immune-competent animals to study the proliferation of HTLV-1-infected cells, few studies have analyzed the transmission of HTLV-1 to host cells. To exclude the possibility that inoculated cells may remain in the animals over time, we demonstrated that human MT-2 cells disappear by 1 month after infection. Furthermore, we confirmed that the host sequence at the 3′ end of the provirus was of mouse origin and not of human origin. We also used site-specific PCR primers targeted to the 3′ LTR and the flanking cellular sequences and successfully amplified these sequences from the DNA of various HTLV-1-infected mouse organs but not from DNA isolated from MT-2 cells. Furthermore, our data corroborate previous reports showing that MT-2 cells cannot proliferate in various mouse strains having natural killer activity, including SCID mice.[30,31]

In summary, our results provide evidence that functional NIK is required for the initial proliferation and maintenance of HTLV-1-infected cells in mice. In addition to NIK, identification and analysis of other host genes that control the proliferation of HTLV-1-infected cells may be crucial for the development of effective preventive therapies for HTLV-1-associated diseases.

Acknowledgments

We thank Drs T. Ishii, A. Shibuya, K. Takano, and K. Yagami for kind suggestions. We appreciate Drs H. Sakai and S. Jahid for critical reading of the manuscript. We also thank A. Kabayama for technical support. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, and Science (14570260), Japan. T. N. was supported in part by individual awards from the Ishidu Shun Memorial Scholarship and the YASUDA Medical Foundation. M. T. and E. S. were supported in part by the JSPS fellowship program.

References

1 Uchiyama T. Human T cell leukemia virus type I (HTLV-I) and human diseases. *Annu Rev Immunol* 1997; **15**: 15–37.
2 Boschi-Pinto C, Stuver S, Okayama A *et al*. A follow-up study of morbidity and mortality associated with hepatitis C virus infection and its interaction with human T lymphotropic virus type I in Miyazaki, Japan. *J Infect Dis* 2000; **181**: 35–41.
3 Miyazaki K, Yamaguchi K, Tohya T, Ohba T, Takatsuki K, Okamura H. Human T-cell leukemia virus type I infection as an oncogenic and prognostic risk factor in cervical and vaginal carcinoma. *Obstet Gynecol* 1991; **77**: 107–10.
4 Morgan OS, Rodgers-Johnson P, Mora C, Char G. HTLV-1 and polymyositis in Jamaica. *Lancet* 1989; **2**: 1184–7.
5 Kimura I, Tsubota T, Tada S, Sogawa J. Presence of antibodies against adult T cell leukemia antigen in the patients with chronic respiratory diseases. *Acta Med Okayama* 1986; **40**: 281–4.
6 LaGrenade L, Hanchard B, Fletcher V, Cranston B, Blattner W. Infective dermatitis of Jamaican children: a marker for HTLV-I infection. *Lancet* 1990; **336**: 1345–7.
7 Tajima K. The 4th nation-wide study of adult T-cell leukemia/lymphoma (ATL) in Japan: estimates of risk of ATL and its geographical and clinical features. The T- and B-Cell Malignancy Study Group. *Int J Cancer* 1990; **45**: 237–43.
8 Sun SC, Yamaoka S. Activation of NF-κB by HTLV-I and implications for cell transformation. *Oncogene* 2005; **24**: 5952–64.
9 Inoue J, Gohda J, Akiyama T, Semba K. NF-κB activation in development and progression of cancer. *Cancer Sci* 2007; **98**: 268–74.
10 Mori N, Fujii M, Ikeda S *et al*. Constitutive activation of NF-κB in primary adult T-cell leukemia cells. *Blood* 1999; **93**: 2360–8.
11 Mori N, Yamada Y, Ikeda S *et al*. Bay 11-7082 inhibits transcription factor NF-κB and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells. *Blood* 2002; **100**: 1828–34.
12 Dewan MZ, Terashima K, Taruishi M *et al*. Rapid tumor formation of human T-cell leukemia virus type 1-infected cell lines in novel NOD-SCID/γc (null) mice: suppression by an inhibitor against NF-κB. *J Virol* 2003; **77**: 5286–94.
13 Ohsugi T, Horie R, Kumasaka T *et al*. *In vivo* antitumor activity of the NF-κB inhibitor dehydroxymethylepoxyquinomicin in a mouse model of adult T-cell leukemia. *Carcinogenesis* 2005; **26**: 1382–8.
14 Pomerantz JL, Baltimore D. Two pathways to NF-κB. *Mol Cell* 2002; **10**: 693–5.
15 Miyawaki S, Nakamura Y, Suzuka H *et al*. A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur J Immunol* 1994; **24**: 429–34.
16 Shinkura R, Kitada K, Matsuda F *et al*. Alymphoplasia is caused by a point mutation in the mouse gene encoding NF-κB-inducing kinase. *Nat Genet* 1999; **22**: 74–7.
17 Koike R, Nishimura T, Yasumizu R *et al*. The splenic marginal zone is absent in alymphoplastic *aly* mutant mice. *Eur J Immunol* 1996; **26**: 669–75.
18 Karrer U, Althage A, Odermatt B *et al*. On the key role of secondary lymphoid organs in antiviral immune responses studied in alymphoplastic (*aly*/*aly*) and spleenless ($Hox11^{-/-}$) mutant mice. *J Exp Med* 1997; **185**: 2157–70.
19 Karrer U, Althage A, Odermatt B, Hengartner H, Zinkernagel RM. Immunodeficiency of alymphoplasia mice (*aly*/*aly*) *in vivo*: structural defect of secondary lymphoid organs and functional B-cell defect. *Eur J Immunol* 2000; **30**: 2799–807.
20 Hino S. Maternal transmission: diagnosis and prevention of the infected. In: Takatsuki K, Hinuma Y, Yoshida M, eds. *Advances in Adult T-Cell Leukemia and HTLV-1 Research Japan*. Tokyo: Scientific Societies Press, 1992; 163–73.
21 Okochi K, Sato H, Hinuma Y. A retrospective study on transmission of adult T cell leukemia virus by blood transfusion: seroconversion in recipients. *Vox Sang* 1984; **46**: 245–53.
22 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**: 4862–7.
23 Manns A, Miley WJ, Wilks RJ *et al*. Quantitative proviral DNA and antibody levels in the natural history of HTLV-I infection. *J Infect Dis* 1999; **180**: 1487–93.
24 Okayama A, Stuver S, Matsuoka M *et al*. Role of HTLV-1 proviral DNA load and clonality in the development of adult T-cell leukemia/lymphoma in asymptomatic carriers. *Int J Cancer* 2004; **110**: 621–5.

25 Matsuzaki T, Nakagawa M, Nagai M *et al*. HTLV-I proviral load correlates with progression of motor disability in HAM/TSP: analysis of 239 HAM/TSP patients including 64 patients followed up for 10 years. *J Neurovirol* 2001; **7**: 228–34.
26 Lairmore MD, Silverman L, Ratner L. Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation. *Oncogene* 2005; **24**: 6005–15.
27 Fang J, Kushida S, Feng R *et al*. Transmission of human T-cell leukemia virus type 1 to mice. *J Virol* 1998; **72**: 3952–7.
28 Tanaka M, Sun B, Fang J *et al*. Human T-cell leukemia virus type 1 (HTLV-1) infection of mice: proliferation of cell clones with integrated HTLV-1 provirus in lymphoid organs. *J Virol* 2001; **75**: 4420–3.
29 Nitta T, Tanaka M, Sun B, Hanai S, Miwa M. The genetic background as a determinant of human T-cell leukemia virus type 1 proviral load. *Biochem Biophys Res Commun* 2003; **309**: 161–5.
30 Imada K, Takaori-Kondo A, Akagi T *et al*. Tumorigenicity of human T-cell leukemia virus type I-infected cell lines in severe combined immunodeficient mice and characterization of the cells proliferating *in vivo*. *Blood* 1995; **86**: 2350–7.
31 Ishihara S, Tachibana N, Okayama A, Murai K, Tsuda K, Mueller N. Successful graft of HTLV-I-transformed human T-cells (MT-2) in severe combined immunodeficiency mice treated with anti-asialo GM-1 antibody. *Jpn J Cancer Res* 1992; **83**: 320–3.
32 Miyoshi I, Kubonishi I, Yoshimoto S *et al*. Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature* 1981; **294**: 770–1.
33 Fang J, Kushida S, Feng R *et al*. Integration of HTLV-1 provirus into mouse transforming growth factor-α gene. *Biochem Biophys Res Commun* 1997; **233**: 792–5.
34 Ibrahim F, Fiette L, Gessain A, Buisson N, de-The G, Bomford R. Infection of rats with human T-cell leukemia virus type-I: susceptibility of inbred strains, antibody response and provirus location. *Int J Cancer* 1994; **58**: 446–51.
35 Zhao TM, Hague B, Caudell DL, Simpson RM, Kindt TJ. Quantification of HTLV-I proviral load in experimentally infected rabbits. *Retrovirology* 2005; **2**: 34.
36 Cockerell GL, De Lairmore MB, Rovnak J, Hartley TM, Miyoshi I. Persistent infection of rabbits with HTLV-I: patterns of anti-viral antibody reactivity and detection of virus by gene amplification. *Int J Cancer* 1990; **45**: 127–30.
37 Kazanji M, Ureta-Vidal A, Ozden S *et al*. Lymphoid organs as a major reservoir for human T-cell leukemia virus type 1 in experimentally infected squirrel monkeys (*Saimiri sciureus*): provirus expression, persistence, and humoral and cellular immune responses. *J Virol* 2000; **74**: 4860–7.
38 Wattel E, Vartanian JP, Pannetier C, Wain-Hobson S. Clonal expansion of human T-cell leukemia virus type I-infected cells in asymptomatic and symptomatic carriers without malignancy. *J Virol* 1995; **69**: 2863–8.
39 Hanon E, Hall S, Taylor GP *et al*. Abundant tax protein expression in $CD4^+$ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes. *Blood* 2000; **95**: 1386–92.
40 Nishikawa S, Nakane A. Host resistance against *Listeria monocytogenes* is reciprocal during the course of infection in alymphoplastic *aly* mutant mice. *Cell Immunol* 1998; **187**: 88–94.
41 Czarneski J, Rassa JC, Ross SR. Mouse mammary tumor virus and the immune system. *Immunol Res* 2003; **27**: 469–80.
42 Lawn SD, Butera ST, Folks TM. Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. *Clin Microbiol Rev* 2001; **14**: 753–77.
43 Gonzalez-Dunia D, Grimber G, Briand P, Brahic M, Ozden S. Tissue expression pattern directed in transgenic mice by the LTR of an HTLV-I provirus isolated from a case of tropical spastic paraparesis. *Virology* 1992; **187**: 705–10.
44 Mortreux F, Kazanji M, Gabet AS, de Thoisy B, Wattel E. Two-step nature of human T-cell leukemia virus type 1 replication in experimentally infected squirrel monkeys (*Saimiri sciureus*). *J Virol* 2001; **75**: 1083–9.
45 Tanaka G, Okayama A, Watanabe T *et al*. The clonal expansion of human T lymphotropic virus type 1-infected T cells: a comparison between seroconverters and long-term carriers. *J Infect Dis* 2005; **191**: 1140–7.
46 Bangham CR. The immune control and cell-to-cell spread of human T-lymphotropic virus type 1. *J Gen Virol* 2003; **84**: 3177–89.
47 Furuta RA, Sugiura K, Kawakita S *et al*. Mouse model for the equilibration interaction between the host immune system and human T-cell leukemia virus type 1 gene expression. *J Virol* 2002; **76**: 2703–13.
48 Richardson JH, Edwards AJ, Cruickshank JK, Rudge P, Dalgleish AG. *In vivo* cellular tropism of human T-cell leukemia virus type 1. *J Virol* 1990; **64**: 5682–7.
49 Satoh M, Toma H, Sugahara K *et al*. Involvement of IL-2/IL-2R system activation by parasite antigen in polyclonal expansion of $CD4^+25^+$ HTLV-1-infected T-cells in human carriers of both HTLV-1 and *S. stercoralis*. *Oncogene* 2002; **21**: 2466–75.
50 Yamano Y, Cohen CJ, Takenouchi N *et al*. 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**: 1367–77.

doi: 10.1111/j.1349-7006.2008.00766.x