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Induction of APOBEC3B cytidine deaminase in HTLV-1-infected humanized mice

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Abstract. Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL). Following viral infection with HTLV-1, certain infected cells exhibit clonal proliferation. Additional genetic and epigenetic changes in these clonally proliferating cells provide them with the selective advantage of growth, which eventually results in ATL. The precise mechanism, however, has yet to be completely elucidated. It has previously been established that APOBEC3 enzymes are potent host-antiviral restriction factors. Conversely, previous studies have reported that the A3B level is increased in tumor virus infections, such as those caused by HBV and HPV, suggesting that A3B exerts a function as a mutagen. Therefore, the present study analyzed the expression of APOBEC3 family members in various HTLV-1 infection states. No significant differences were observed in the expression between healthy donors and patients with HTLV-1-associated myelopathy. Although no significant changes in the expressions of A3C, A3D, A3F and A3G between uninfected and HTLV-1-infected mice were observed, an increased A3B expression was observed in a short-term humanized mouse model following HTLV-1 infection. In a long-term humanized mouse model following HTLV-1 infection, the gene expression array data exhibited an apparent increase in A3B and CADM1, which are indicators of ATL. Collectively, the results of the present study suggest that A3B is likely involved in the development of ATL in HTLV-1-infected humanized mice.

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL) (1), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (2), and HTLV-1-associated uveitis (3). Moreover, HTLV-1 virions are thought to be very poorly infectious (4,5). It is still unclear whether the virions are unusually unstable, are poorly released from the infected cell, or inefficiently bind and penetrate into the host cell. After HTLV-1 infection, some of the infected cells exhibit clonal proliferation, albeit slowly, to achieve carrier conditions. Additional genetic and epigenetic changes in these clonally proliferating cells provide them with the selective advantage of growth, which eventually leads to leukemia/lymphoma, including ATL (6).

APOBEC3 (A3) enzymes have been reported to be potent host-antiviral restriction factors that encode DNA-editing deaminase enzymes. A3F and A3G are the most studied of the seven A3 genes (A3A, A3B, A3C, A3D/E, A3F, A3G, and A3H) encoded in the human genome (7). The APOBEC3B mutation signature is remarkably enriched in six types of cancers, including those of the cervix, bladder, lung (adeno and squamous cell), head and neck, and breast (8,9). There are several reports that A3B level is increased in tumor virus infections, such as those caused by HBV and HPV (10,11). On the other hand, in HIV infection, whose causative agent is a retrovirus, the levels of A3C, A3F, and A3G are increased, but that of A3B is not (12). However, APOBEC3 superfamily has been found to exert varying levels of activity under different experimental conditions. We analyzed APOBEC3 superfamily expression in HTLV-1 infection because HTLV-1 is a retrovirus and causative agent for malignancy.

Recently, we established a humanized mouse model of HTLV-1 infection in the presence of specific adaptive immune responses. These HTLV-1-infected humanized mice showed distinct ATL-like features, including hepatosplenomegaly and the appearance of flower cells (13). In this report, we provide evidence of the pathogenesis of different expressions of APOBEC superfamily in patients with HAM, who were non-ATL patients, and HTLV-1-infected humanized mice. Moreover, we discuss the role of APOBEC superfamily in the antiviral resistance realized by the innate immunity of HTLV-1-infected humanized mice.

Materials and methods

Patients. A total of 5 patients with HAM and 5 healthy donors were included in the present study between April 2013 and December 2016 at Kansai Medical University Hospital (Hirakata, Japan). All patients with HAM were of Japan. Another 5 healthy subjects were included in the normal control group. The age and sex composition were not significantly different (P>0.05; Table I). Human leukocytes (derived from patients with HAM and normal donors) were separated from the peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using Ficoll Histopaque. The cells were maintained in a RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. All procedures were approved by the Ethics Committee of Kansai Medical University. Written informed consent was obtained from all patients or their families.

HTLV-1-infected humanized mice. Female 4-week-old NOD/Shi-scid/IL-2Rγc null (NOG) mice were purchased from the Central Institute of Experimental Animals (Kawasaki, Japan). Mice were handled under sterile conditions and maintained in germ-free isolators. The Animal Care Committees of Kansai Medical University approved all animal experiments. Five-week-old NOG mice were sublethally irradiated with 250 cGy from a 137Cs source (Gammacell 40 exactor; Nordion International). Within 24 h of irradiation, each mouse was injected with $5x10^4$ human $CD133^+$ cells by intrabone marrow injection. After 5-6 months, the HTLV-1-infected T-cell line JEX28, HTLV-1 molecular clone pX1 MT-M was prepared by electroporation into jurkat cells, was irradiated with 10 Gy from a 137Cs source irradiator. Then, the irradiated JEX28 cells ($2.5x10^6$) or phosphate-buffered saline were intraperitoneally inoculated (Table II). All infections were performed in a bio-safety P2A Laboratory level in accordance with the guidelines of Kansai Medical University.

Preparation of cDNA samples. The RNeasy kit (Qiagen, Inc., Valencia, CA, USA) was employed for the isolation of human total RNA. A concentration of 0.5 μg of total RNA was used for the synthesis of complementary DNA (cDNA) samples by the reverse transcriptase ReverTra Ace (MMLV Reverse Transcriptase RNase H-; Toyobo Co., Ltd., Osaka, Japan). The RNA concentration was determined by using an IMPLEN NanoPhotometer. Further, humanized mouse RNA was isolated by using the ZR-Duet™ DNA/RNA MiniPrep kit, and humanized mouse cDNA samples were synthesized using the same method as in human cDNA synthesis.

RT-qPCR. The primers used for RT-PCR are listed in Table III. cDNA levels were quantified by using the RT-PCR method (MyiQ Single color real-time PCR detection system; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Reactions were performed in 96-well plates each of which containing 10 μl of SYBR premix Ex Taq GC (Takara Bio, Inc., Otsu, Japan). The cycling conditions used were as follows: initial denaturation at 95˚C for 10 min, followed by 15 cycles at 95˚C for 10 sec and 60˚C for 30 sec, and 35 cycles at 95˚C for 10 sec and 59˚C for 30 sec. The relative RNA expression levels were calculated by the MyiQ system (Bio-Rad Laboratories, Inc.).

DNA isolation and HTLV-1 proviral load measurement. Genomic DNA was extracted from splenocyte or peripheral blood by using ZR-Duet™ DNA/RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA). Proviral loads (PVLs) were measured by real-time PCR using the previously described protocol with some modifications (TaqMan Fast Advanced Master Mix; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (MyiQ or CFX96 real-time PCR system; Bio-Rad Laboratories, Inc.) (13). The primers and probes targeting HTLV-1 pX and human β-globin (HBB; as an internal control) are listed in Table III. PVLs were calculated by the following formula: [(copy number of pX)/(copy number of HBB/2)] x 100.

Microarray hybridization and data analyses. RNA was extracted from each sample and purified by using an RNeasy mini kit (Qiagen, Inc.) following the manufacturer's instructions. RNA labeling and hybridization were performed using a commercial kit from Hokkaido System Science Co., Ltd., Sapporo, Japan. A 200-ng aliquot of total RNA was converted to double-stranded cDNA, and Cyanie3-labeled cRNA was produced using the Low Input Quick Amp Labeling kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The resulting labeled cRNA was purified by using the RNeasy mini kit (Qiagen, Inc.) and fragmented into strands in length in accordance with the protocols of the Agilent Technologies, Inc. These strands were then hybridized to GeneChip Array, and the hybridization data were analyzed using SurePrint G3 Human GE 8x60k 1color (Agilent Technologies, Inc.).

Flow cytometric analysis and cell sorting. Mice with a body weight of below 17 g were euthanized. Single-cell spleen suspensions were prepared as described previously (14). To stain surface markers, anti-human CD45-PerCP or APC-Cy7, CD3-fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-Cy7, CD4-PE, CD25-FITC antibodies were used, along with mouse immunoglobulin G1 and FITC as isotype controls (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometric analysis was performed on a BD FACSCan for 3-color staining and a BD FACSCant II (BD Biosciences) for 7-color staining. The CellQuest and Diva software programs were used for data acquisition (BD Biosciences), and the collected data were analyzed by using FACS express 3 (De Novo Software, Piscataway, NJ, USA). T cells expressing human $CD4^+$, $CD8^-$, and CD25 were sorted from splenic mononuclear cells by using FACSAria or FACSAria III (BD Biosciences).

Statistical analysis. Mann-Whitney U-test and Wilcoxon's signed-rank test were used to determine the significance of differences and data comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

Differences in APOBEC3 superfamily expression. The APOBEC superfamily proteins has several and important functions in human health and disease (15). We first analyzed the mRNA expression of the APOBEC3 superfamily of *in vivo* samples from healthy donors and patients with HAM. However, we found no significant differences between these

Table I. Characteristics of the study sample from human.

Patient	Status	Sex	Age (year)	Proviral DNA loads per 100 PBMCs
Case 1	Healthy donor	M	47	N.D.
Case 2	Healthy donor	F	63	N.D.
Case 3	Healthy donor	F	67	N.D.
Case 4	Healthy donor	M	70	N.D.
Case 5	Healthy donor	M	54	N.D.
Average/SD			60.2±7.8	N.D.
Case 6	HAM patient	F	69	11.88
Case 7	HAM patient	M	63	7.89
Case 8	HAM patient	F	44	8.4
Case 9	HAM patient	M	66	13.8
Case 10	HAM patient	M	62	9.79
Average/SD			60.8±8.0	10.4±2.5

HAM, HTLV-1-associated myelopathy; PBMC, peripheral blood mononuclear cell; SD, standard deviation; N.D. not detected.

Table II. Characteristics of the study sample from mouse.

Patient	Status	Week after infection	Cell type	Proviral DNA loads per 100 PBMCs
Case 11	Uninfected	-	PBMC	-
Case 12	Uninfected	-	PBMC	-
Case 13	Uninfected	-	PBMC	-
Case 14	Uninfected	-	PBMC	-
Case 15	Uninfected	-	PBMC	-
Case 16	HTLV-1 infected	7	PBMC, splenocyte	97.7
Case 17	HTLV-1 infected	5	PBMC, splenocyte	99.4
Case 18	HTLV-1 infected	9	PBMC, splenocyte	75.3
Case 19	HTLV-1 infected	9	PBMC, splenocyte	76.8
Case 20	HTLV-1 infected	5	PBMC, splenocyte	96.7

HTLV-1, human T-cell leukemia virus type 1; PBMC, peripheral blood mononuclear cell.

groups (Fig. 1A). HTLV-1 tax is known as a transactivator that can activate many genes (16) and its expression is suppressed *in vivo* (17). Therefore, we hypothesized that the tax might influence the expression of the APOBEC3 superfamily. As the HTLV-1 tax expression increased after 24 h of *ex vivo* culture, we compared the mRNA expression of the APOBEC3 superfamily in the samples of patients with HAM before and after the *ex vivo* culture. After culture, tax expression was apparently increased as well as that of A3C, A3D, A3F, and A3G. On the other hand, there was a tendency of decreasing A3B expression (Fig. 1B). As a negative control, we conducted the same experiment by using samples from healthy donors. We found that after 24 h of *ex vivo* culture, A3A expression was decreased; a tendency of decrease was also exhibited by the level of A3B expression. However, no changes were observed in the expression of A3C, A3D, A3F, A3G, and A3H (Fig. 1C). There are reports that A3B is a mutagen (8,9), whereas ATL is known as a malignant disease that is caused by HTLV-1 infection. Therefore, A3B expression might be increased in HTLV-1-infected individuals. However, these results did not show the tendency. HTLV-1 infection might not influence A3B expression because we used samples from the patients with HAM in the first experiment, which is an inflammatory disease not a malignant disease. Recently, we developed a humanized mouse model similar to ATL (13). For further research, we used PBMCs that were obtained from uninfected humanized mouse and 4-6 weeks postinfected humanized mouse and analyzed the mRNA expression of the APOBEC3 superfamily in these

Table III. The primer sets for this study.

Gene	Forward primer	Reverse primer	Probes
HTLV-1 pX	5'-acaaagttaaccatgcttattatcagc-3'	5'-tctccaaacacgtagactgggt-3'	5'FAM-acaaagttaaccatgcttattatcagc-BHQ3'
Human β-globin	5'-tgaggagaagtctgccgttac-3'	5'-tggtctccttaaacctgtcttg-3'	5'FAM-tgaggagaagtctgccgttac-BHQ3'
Gene	Forward primer	Reverse primer	
APOBEC3A	5'-gagaagggacaagcacatgg-3'	5'-tggatccatcaagtgtctgg-3'	
APOBEC3B	5'-gacccttggtccttcgac-3'	5'-gcacagccccaggagaag-3'	
APOBEC3C	5'-agcgcttcagaaaagagtgg-3'	5'-aagtttcgttccgatcgttg-3'	
APOBEC3D	5'-acccaaacgtcagtcgaatc-3'	5'-cacatttctgcgtggttctc-3'	
APOBEC3F	5'-ccgtttggacgcaaagat-3'	5'-ccaggtgatctggaaacactt-3'	
APOBEC3G	5'-ccgaggacccgaaggttac-3'	5'-tccaacagtgctgaaattcg-3'	
APOBEC3H	5'-agctgtggccagaagcac-3'	5'-cggaatgtttcggctgtt-3'	
HTLV-1 tax	5'-atcccgtggagactcctcaa-3'	5'-aacacgtagactgggtatcc-3'	
HPRT	5'-ccttggtcaggcagtataatcca-3'	5'-ccaacaaagtctggcttatatccaa-3'	

HTLV-1, Human T-cell leukemia virus type 1.

humanized mouse models. Although no significant changes in the expression of A3C, A3D, A3F, and A3G were observed, an increase in the level of expression of A3B and a decrease in the level of expression of A3A was noted. In contrast, the level of expression of A3H was significantly increased (Fig. 2A). As it is difficult to extract sufficient qualities of PBMCs from the animals in humanized mouse model, we used splenocytes instead of PBMCs for *ex vivo* culture experiments. After 24-h *ex vivo* culture, the levels of the expression of tax and A3A apparently increased. There was a tendency of decreasing A3B expression and increasing A3C, A3D, A3F, A3G, and A3H expression (Fig. 2B). Almost the data, except A3A, showed the same tendency with the samples from the patients with HAM.

A3B expression increased in 18 weeks post infection humanized mouse model. The levels of the expression of A3B did not change in patients with HAM (Fig. 1A). In contrast, it decreased after *ex vivo* culture in both the samples from patients with HAM and infected humanized mouse model (Figs. 1B and 2B). On the other hand, there was a tendency of increasing A3B expression in the *in vivo* humanized mouse samples (Fig. 2A). It was too short to investigate the change in A3B expression, because we used the ATL-like humanized mouse model samples with 4-6 weeks post infection. Therefore, we analyzed the long-term humanized mouse model 18 weeks after HTLV-1 infection. Initially, we checked the phenotype of the humanized mouse splenocytes by using FACS. In the infected mice, the expression of $CD4^+$ $CD25^+$ cells was higher compared with that in the uninfected (Fig. 3). When the ATL-like humanized mouse model was developed, $CD4^+$ $CD25^+$ cells was quite increased (13). It is hard to collect sufficient quantities of $CD4^+$ $CD25^+$ cells from uninfected control mice because this population is exceedingly small. There is a report that $CD4^+$ $CD25^-$ cells are also infected with HTLV-1 in HTLV-1-infected individual (18). Therefore, instead of $CD4^+$ $CD25^+$ cells, we analyzed and compared $CD4^+$ $CD25^-$ cells. Gene expression array of $CD4^+$ $CD25^-$ splenocytes showed a 16.3-fold increase of CADM1 and a 12.6-fold increase of APOBEC3B levels in the long-term humanized mouse model after HTLV-1 infection compared with the uninfected one (Table IV). In the humanized mouse model, both CADM1 and APOBEC3B were increased in $CD4^+$ $CD25^+$ cells compared with those in $CD4^+$ $CD25^-$ cells. On the other hand, APOBEC3 superfamily expressions except APOBEC3B were not so increased in this humanized mouse model (Table IV). These data showed that APOBEC3B was increased in the ATL-like humanized mouse model as well as such as CADM1, which are ATL indicators (19,20).

Table IV. Gene expression array of $CD4^+$ $CD25^-$/$CD4^+$ $CD25^+$ splenocytes.

Gene	$CD4^+$ $CD25^-$	$CD4^+$ $CD25^+$
CADM1	16.3	42.3
APOBEC3A	0.9	1.0
APOBEC3B	12.6	34.2
APOBEC3C	2.2	1.8
APOBEC3D	1.0	1.2
APOBEC3F	2.0	2.0
APOBEC3G	2.4	1.7
APOBEC3H	3.2	3.0

Fold change in infected two mice splenocytes compared to uninfected two mice $CD4^+$ $CD25^-$ splenocytes.

Discussion

The host factor taking the role that is important to the virus replication is targets to the development of a new virus therapeutic drug (21). Since it has been reported that APOBEC3G combines with HIV-1 vif and influences infectivity of a virus

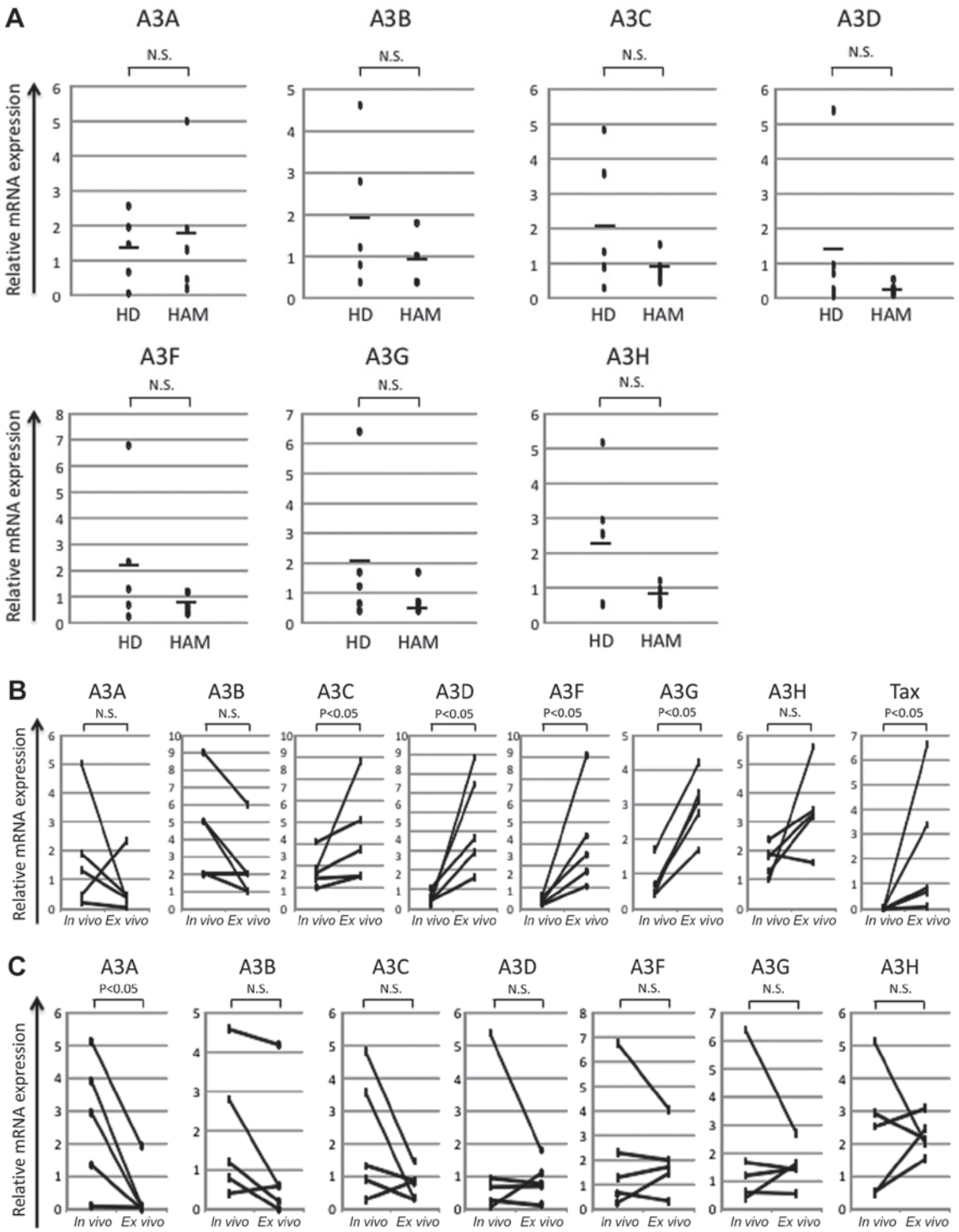

Figure 1. Analysis of mRNA expression in human samples. (A) mRNA expression of *in vivo* samples of healthy donors and HAM patients. There was no significant difference in APOBEC family expression; (B) mRNA expression of *in vivo* and *ex vivo* samples of HAM patients. After 24 h of *ex vivo* culture, tax expression was apparently increased (P<0.05); the expression levels of A3C, A3D, A3F, and A3G were also increased (P<0.05); on the other hand, there was a tendency of decreasing A3B expression (0.05<P<0.1); (C) mRNA expression of *in vivo* and *ex vivo* samples of healthy donor. After 24 h of *ex vivo* culture, A3A was decreased (P<0.05), A3B showed the tendency of decreasing (0.05<P<0.1); however, no changes were observed in A3C, A3D, A3F, A3G, and A3H expression.

in the past, new host-virus relations came to be understood by through these molecules (22,23). Previously it has been reported that multiple APOBEC3 enzymes may limit the infectivity of HTLV-1 (24,25). APOBEC3 genes are interferon-stimulated genes whose expression is increased in response to various stimuli and exposure, including the ligands of toll-like receptors TLR3, TLR4 and TLR7 (26,27). Thus, the activation of innate immune responses that occurred after infection with many different viruses could lead to increased APOBEC3 activity. Therefore, APOBEC3 enzymes are involved in the intrinsic/innate response to and the subsequent control of early virus infection. Furthermore, there is increasing evidence

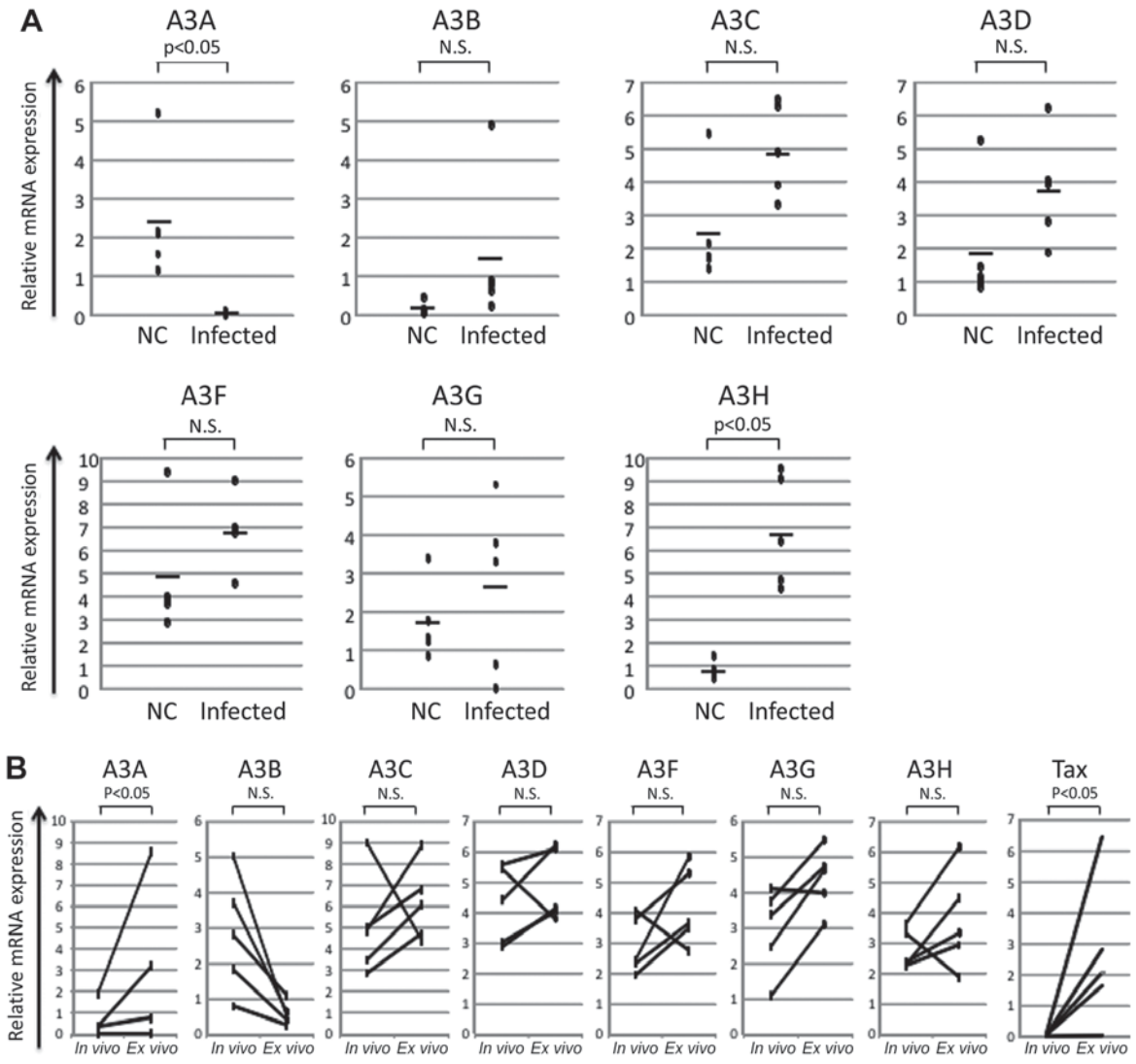

Figure 2. Analysis of mRNA expression in the humanized mice samples. (A) mRNA expression of *in vivo* samples in both uninfected humanized mice and a 4-6-week post-infection humanized mouse model. Although there were no significant changes in A3C A3D A3F and A3G expression, there was a tendency of increasing A3B expression (0.05<P<0.1). A3A was significantly decreased (P<0.05). A3H expression was significantly increased (P<0.05); (B) mRNA expression of *in vivo* and *ex vivo* samples of 4-6-week post-infection. After 24 h of *ex vivo* culture, tax and A3A expression levels were apparently increased (P<0.05), but there was a tendency of decreasing A3B expression (0.05<P<0.1) and increasing A3C, A3D, A3F, A3G, and A3H expression levels (0.05<P<0.1).

that APOBEC3 enzymes contribute to the adaptive immune response, more specifically by affecting the generation of cytotoxic T lymphocytes that recognize viral peptides and B-cell production of antiviral antibodies. We expected that APOBEC superfamily expression was increased in patients with HAM due to persistent viral infection. Surprisingly, our results indicated that no significant differences were observed in the APOBEC superfamily expression between the PBMCs of healthy donors and patients with HAM (Fig. 1A). Similarly, Kataoka *et al* reported that A3B was increased in both ATL patients and asymptomatic HTLV-1 carriers (28). On the other hand, Fan *et al* reported that A3G levels were increased in patients with ATL (24). The results were contradictory because we used samples from the patients with HAM.

A previous study has demonstrated that APOBEC3B is enriched in the genomics of many human cancers. APOBEC enzymes normally function in innate immune responses in according to viral infection in the case of cancer developments (29). In ATL, it was reported that APOBEC3B expression increases in comparison with healthy donor (28). In contrast, the expression of APOBEC3B did not change significantly in HAM without tumor characteristics (Fig. 1A). These results suggest that A3B rises by malignant transformation not infection itself. To solve this question, we should analyze the patient sample in the process when ATL develops. It is, however, quite difficult, because the onset rate of ATL is low (approximately 5%), and extremely long periods of time are necessary until the disease develops (30). The experimental system by an animal model is needed to analyze a cause of the diseases of a virus. The experimental limit is considered from individual's number and cost side by an experimental animal by an ape. Thus, when it was seen historically, a trial by the HTLV-1-infected animal model for which mice, rats, rabbits were used had been performed, but there was process, which didn't even come to clinical condition progress to ATL (31). Recently, we developed a humanized mouse model that showed ATL-like symptoms (13). Therefore, we analyzed changes of APOBEC superfamily expression with HTLV-1

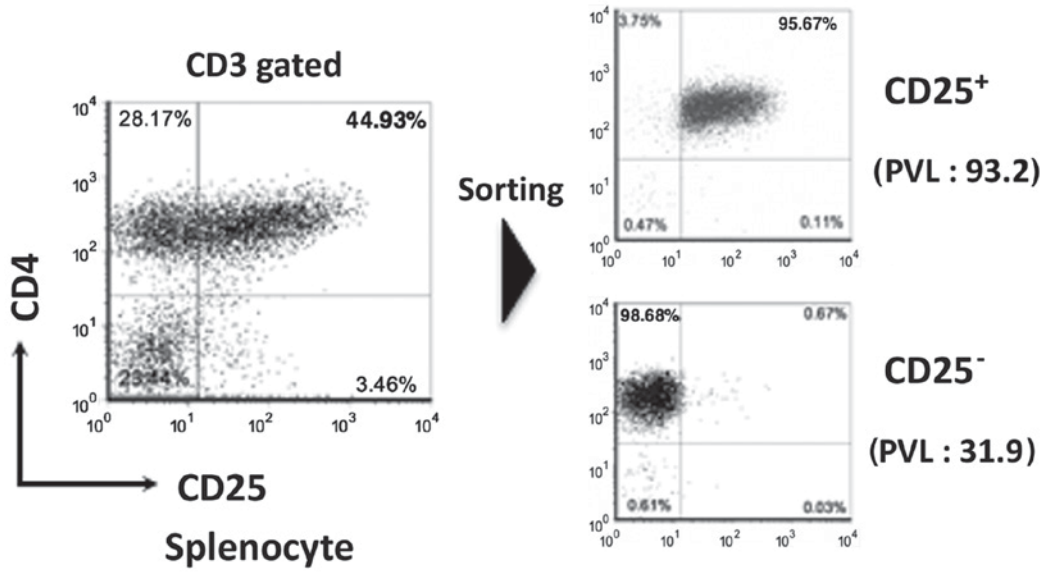

Figure 3. Analysis of long-term humanized mouse model after HTLV-1 infection. Splenocytes from HTLV-1-infected humanized mouse were sorted into $CD4^+$ $CD25^+$ cells and $CD4^+$ $CD25^-$ cells by FACS. Genomic DNA isolated from each T-cell population was analyzed for PVL by real-time PCR. PVL is expressed as number of pX copies per 100 cells.

infected humanized mouse. As expected, in the long-term humanized mouse model, A3B was apparently increased after HTLV-1 infection (Table IV). In contrast, A3B increased only slightly in the short-term model (Fig. 2A). We speculate that these different results might be caused by the different lengths of the infection periods. Based on the above consideration, we hypothesized that our animal model is ATL pre-oncogenic, based on APOBEC3B, which induces mutations in various human cancers (32) and that this may lead to genomic instability and ultimately to tumorigenesis. Further research is necessary in the future. In fact, CADM1, known as an indicator of ATL, was apparently increased in the long-term humanized mouse model after HTLV-1 infection (Table IV). HTLV-1 infectivity to the animal besides humans was very low and animal immunity systems were so different from humans. The treatment development, which also vaccinates with first as preclinical study is performed even and it seems different to adaptation to humans. We tried using HTLV-1-infected humanized mouse system. This study, which argued to follow up a change with the passage of time using HTLV-1 infection in humanized mouse models *in vivo*, was for the first time.

To gain insights into the mechanisms underlying the long latency and onset of HTLV-1-related diseases, we investigated the interaction between Tax and APOBECs expression. We have already reported when the cells were transferred to *in vitro* conditions, tax expression *in vivo* resumed. Therefore, by the change induced in Tax expression, we confirmed whether APOBECs expression changed or not. In humanized mouse spleen, after 24 h of *ex vivo* culture, tax expression apparently increased. The levels of A3C, A3D, A3F, A3G, and A3H expression were also increased. On the other hand, the expression of A3B was decreased (Fig. 2B). Almost all the data except those concerning A3A showed the same tendency as that in HAM patient samples. These results suggest that tax expression does not influence A3B expression directly. Therefore, other viral proteins, such as HBZ, might likely be involved in APOBEC protein expression, a subject we will push investigate further in the future.

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Availability of data and materials

The analyses data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JY was the guarantor for integrity of the study and contributed to study concepts, experimental studies, data analysis and manuscript preparation. MT contributed to study design and prepared and revised the manuscript. NT contributed to manuscript review, statistical analysis and clinical studies. YR and SL contributed to established and maintained humanized mice. JIF contributed to study design and

provided intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Kansai Medical University (Hirakata, Japan). All patients provided informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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