a clone[29]; the HTLV-1 genomic integration site may contribute a similar advantage.[26]

A further unexpected observation was the preferential survival in vivo of the HTLV-1 provirus in the acrocentric chromosomes 13, 14, 15, 21, and (although not reaching formal significance) 22. Throughout most of the cell cycle, these chromosomes are physically associated with the nucleolus, and they encode the machinery of the ribosome on the short (p) arm. Because the HTLV-1 proviral integration sites are found only in the long (q) arm of these chromosomes, we postulate that the selective advantage enjoyed by these clones derives not from the proviral integration near the ribosome-coding genes but rather from the physical location of the provirus-containing chromatin in the nucleus, perhaps by coupling proviral transcription to transcription of the acrocentric chromosomes. Experiments are underway to test this hypothesis.

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Authorship

Contribution: L.B.C., G.P.T., M.M., and C.R.M.B. conceived and designed the experiments; M.M. performed the clinical diagnosis; L.B.C. performed the experiments; M.V. and L.F. performed and interpreted TCR studies; L.B.C. analyzed the data; A.M., H.N., and D.J.L. contributed to the bioinformatic and statistical analysis, tools, and data sets; and L.B.C. and C.R.M.B. wrote the paper.

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The role of HTLV-1 clonality, proviral structure, and genomic integration site in adult T-cell leukemia/lymphoma

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Reevaluation of confirmatory tests for human T-cell leukemia virus Type 1 using a luciferase immunoprecipitation system in blood donors

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BACKGROUND: Recently, Japanese Red Cross blood centers have changed the confirmatory test method from an indirect immunofluorescence (IF) technique to Western blotting (WB) for antibodies against human T-cell leukemia virus Type 1 (HTLV-1). In this study, these HTLV-1 tests were assessed using another sensitive method, that is, a luciferase immunoprecipitation system (LIPS), to identify a better confirmatory test for HTLV-1 infection.

STUDY DESIGN AND METHODS: Plasma samples from 54 qualified donors and 114 HTLV-1 screening–positive donors were tested by LIPS for antibodies against HTLV-1 Gag, Tax, Env, and HBZ recombinant proteins. The donors were categorized into six groups, namely, (Group I) qualified donors, screening positive; (Group II) IF positive; (Group III) IF negative; (Group IV) WB positive; (Group V) WB negative; and (Group VI) screening positive in the previous blood donation, but WB-indeterminate during this study period.

RESULTS: In Groups II and IV, all plasma samples tested positive by LIPS for antibodies against Gag and Env proteins. In Group V, all samples tested negative by LIPS, whereas some Group III samples reacted with single or double antigens in LIPS. In Group VI, the LIPS test identified a donor with suspected HTLV-1 infection. The first case of a blood donor with plasma that reacted with HBZ was identified by LIPS.

CONCLUSION: Reevaluation of the current HTLV-1 screening method using the LIPS test showed that both confirmatory tests had similar sensitivity and specificity only when WB indeterminate results were eliminated. LIPS is a promising method for detecting and characterizing HTLV-1 antibodies.

On September 19, 2012, Japanese Red Cross (JRC) blood centers changed the confirmatory test method for blood donors testing positive for human T-cell leukemia virus Type 1 (HTLV-1) antibodies during screening using an automated chemiluminescence enzyme-linked immunoassay (CLEIA) test (CL4800 testing system, Fujirebio, Shinjuku Ward, Tokyo, Japan) from an in-house cell-based indirect immunofluorescence technique (IF) test[1] to a commercial Western blotting (WB) test (ProBlot HTLV-1, Fujirebio) to reduce the number of false-positive results. In our donor screening method, all blood samples were disqualified if they were collected from donors who tested positive in CLEIA screening tests for transfusion-transmitted infections, including hepatitis B and C viruses, human immunodeficiency virus Types 1 (HIV-1) and 2 (HIV-2), HTLV-1, *Treponema pallidum*, and parvovirus B19. These screening tests were established with high sensitivities while

ABBREVIATIONS: ATL = adult T-cell leukemia; CLEIA = chemiluminescence enzyme-linked immunoassay; HAM/TSP = HTLV-1–associated myelopathy/tropical spastic paraparesis; IF = indirect immunofluorescence; JRC = Japanese Red Cross; LIPS = luciferase immunoprecipitation system; RLU(s) = relative luciferase unit(s); WB = Western blotting.

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sacrificing their specificities to ensure the safety of transfusion medicine, yielding a substantial amount of false-positive results. A confirmatory test for HTLV-1 was implemented to identify infected donors, who were notified about their test result if they wished. However, the WB test for HTLV-1 still occasionally produced unclear results, represented as WB indeterminate.[2]

The luciferase immunoprecipitation system (LIPS) is an antibody detection method with high sensitivity, which uses recombinant antigens fused with the luciferase protein. The amount of luciferase-fused antigen captured by antibodies in a test sample is measured as the luciferase activity without using secondary antibodies for detection. LIPS was originally developed to analyze whole proteome antibody response profiles,[3] but it was subsequently applied to the detection of anti-HTLV in asymptomatic carriers and patients with adult T-cell leukemia (ATL) or HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP).[4] In the latter study, all HTLV-1–infected subjects, including 15 asymptomatic carriers, tested positive for antibodies against the Gag protein by LIPS, while 62 of 73 and 71 of 73 tested positive for antibodies against Env or Tax proteins, respectively. These results suggested that the LIPS test for Gag antibodies would exclude donors with false-positive or WB-indeterminate results in our HTLV-1 confirmatory tests. Therefore, we constructed a series of plasmids that encoded the genes for HTLV-1 Gag, Tax, Env, or HBZ fused with *Renilla* luciferase (Promega, Madison, WI) and reevaluated the positive plasma samples collected during the screening period immediately before and after changing the confirmatory test method. We analyzed 114 HTLV-1 screening–positive plasma samples and 54 screening-negative plasma samples by LIPS for antibodies against four viral antigens to determine the quality of our confirmatory test for HTLV-1 and explore the potential utility of HTLV-1 LIPS tests for blood donors.

MATERIALS AND METHODS

Test subjects

In JRC blood centers, the routine tests applied to detect transfusion-transmitted infections are performed using donor sera. This study used plasma that was stored at –40°C in all the LIPS tests. Plasma samples that tested positive for HTLV-1 antibodies in the screening tests were collected between August 1 and October 23, 2012. In addition, 54 plasma samples were collected from qualified donors as negative controls (categorized as Group I). Among the screening-positive plasma samples, 17 tested positive by IF (Group II), 36 tested negative by IF (Group III), 21 tested positive by WB (Group IV), and 14 tested negative by WB (Group V). Group VI comprised 26 plasma samples collected from donors who tested positive by CLEIA during the previous blood donation and showed indeterminate test results by WB during the study period. Thirteen of the 26 plasma samples in Group VI tested positive and the remaining were negative by CLEIA at the time of donation during the study period. The identifiers used for the positive plasma samples in Group VI were VI-1, -3, -5, -6, -9, -10, -12, -15, -18, -22, -24, -25, and -26. In addition, plasma samples from Groups II and III were examined by WB and those from Groups IV to VI were examined by IF. All the plasma samples were analyzed after receiving informed consent from the corresponding donors during blood donation.

Expression vectors for HTLV-1 LIPS antigens

To express HTLV-1 antigens fused with *Renilla* luciferase, we eliminated the codon of the first methionine in *Renilla* luciferase in the pGL4.75 plasmid (Promega) via site-directed mutagenesis. A synthetic cDNA fragment of the HTLV-1 Gag precursor, Env precursor, Tax, or HBZ, from which the stop codons were eliminated, was then inserted at the N-terminus of the mutated *Renilla* luciferase plasmid (pGL4.75ΔMet) with the spacer amino acids "Gly-Gly-Arg-Gly," thereby generating the plasmids designated as pGagRLuc, pTaxRLuc, pEnvRLuc, and pHBZRLuc, respectively. To produce an additional HBZ protein, which was fused with *Renilla* luciferase in the opposite order to that of pHBZRLuc, we also mutagenized the pGL4.75 plasmid to eliminate a stop codon in *Renilla* luciferase cDNA and replaced it with a new Asp718 enzyme recognition sequence, thereby generating the pGL4.75Asp718 plasmid. The HBZ cDNA was amplified by PCR using a forward primer containing the Asp718 recognition sequences and a reverse primer containing the *Xba*I recognition sequence, which was originally located at the end of the *Renilla* luciferase cDNA. The resultant plasmid, pRLucHBZ, expressed *Renilla* luciferase fused with the HBZ protein at the C-terminus with Gly-Gly-Thr spacer amino acids. The GenBank accession numbers of the HTLV-1 sequences used in this study are NC_001436 for Gag, Env, and Tax and DQ273132 for HBZ. The oligonucleotide sequences used for site-directed mutagenesis were as follows: 5′-gaattcgcactcgagtggcttccaaggtgtacg-3′ and 5′-gagctcacgcttaagaccaccgaaatggttgtc-3′ for pGL4.75ΔMet and 5′-cagggaggtaccttctagagtcggggcggc-3′ and 5′-gaaggtacctccctgctcgttcttcagcac-3′ for pGL4.75Asp718. The sequences of the PCR primers used to amplify HBZ cDNA with an Asp718 enzyme recognition sequence were as follows: forward primer, 5′-ggtaccatggctgcaagcggactg-3′ and reverse primer, 5′-tctagattactgcagccacatagcctcca-3′.

Preparation of antigens

Human kidney 293T cells were obtained from ATCC (ATCC CRL-3216) and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA), which

was supplemented with 10% fetal bovine serum and antibiotics. To transfect semiconfluent human 293T cells in a 10-cm culture dish, 12.5 μg of the *Renilla* luciferase–fused HTLV-1 antigen expression plasmid was introduced using Lipofectamine LTX (Life Technologies), according to the manufacturer's instruction. Two days after transfection, the cells were lysed with 1 mL of *Renilla* luciferase assay lysis buffer (Promega). After two cycles of freezing at –80°C and thawing at room temperature, the lysate was clarified by centrifugation at 13,000 × *g* for 5 minutes at 4°C. Aliquots of the lysate were stored at –80°C until use.

LIPS test

The LIPS tests were performed as described previously,[4] with slight modifications. In brief, 10 μL of plasma diluted 1:10 with assay Buffer A (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1% Triton X-100) was used in 100 μL of a LIPS test mixture in which 50 μL of the equivalent of 1.5×10^7 relative luciferase units (RLUs) of *Renilla* luciferase–fused antigen (293T cell lysate) and 40 μL of Buffer A were added. After being incubated for 30 minutes at room temperature, the antigen–antibody complex was captured using 7 μL of 30% protein A/G resin (Pierce, Rockford, IL) for 30 minutes at room temperature in a 96-well filter plate (MultiScreen HTS, Merck Millipore, Darmstadt, Germany). After being washed six times with Buffer A using a vacuum manifold (MultiScreen HTS, Merck Millipore), the luciferase activities were measured with a detection instrument (Glomax Multi, Promega) using 100 μL of *Renilla* luciferase assay substrate (Promega).

Immunoblotting

To determine the LIPS antigen expression levels, 1×10^6 of 293T cells were seeded 1 day before transfection and then transfected with 2.5 μg of empty pcDNA3.1(–)/myc-His (Life Technologies), or the antigen-expressing vector mentioned. Two days after transfection, the cells were lysed with 200 μL of lysis buffer (50 mmol/L Tris-Cl, pH 7.6, 150 mmol/L NaCl, 0.5% NP-40, 0.1% Na-deoxycholate, 0.1% Triton X-100). The proteins were analyzed by 5% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Wako, Osaka, Japan). The recombinant LIPS antigens were detected using an anti-*Renilla* luciferase rabbit polyclonal antibody (PM047; MBL, Nagoya, Japan). To confirm the anti-HBZ in the donor plasma samples, 293T cells were transfected with 2.5 μg of pcDNA3.1(–)/myc-His, MycHis-HBZ,[5] pME-HBZ,[5] MycHis-HBZ,[5] pHBZRLuc, or pRLucHBZ using Lipofectamine LTX, according to the manufacturer's instructions. MycHis-HBZ and pME-HBZ are expression vectors for myc-6× His-tagged and wild-type HBZ proteins, respectively. SDS-PAGE and membrane transfer were performed as described above and probed with 1:500-diluted anti-HBZ rabbit polyclonal antisera[6] or 1:100-diluted donor plasma.

Statistical analysis

Computer software (IBM SPSS Statistics, Version 21.0, IBM Corp., Armonk, NY) was used for the statistical calculations. The cutoff limit for each antigen was derived from the mean value of 54 control samples plus five standard deviations (SDs), as reported previously.[4]

RESULTS

Expression of LIPS antigens

We constructed a series of plasmid vectors to express recombinant HTLV-1 antigens, which were fused with *Renilla* luciferase to detect anti-HTLV-1 by measuring the luciferase enzyme activity levels. In previous studies that used the LIPS method to detect HTLV-1 antibodies, the viral antigens were prepared as fusion proteins, where the *Renilla* luciferase protein was located at the N-terminus of each HTLV-1 protein.[4,7] We expected that the tertiary structure of the viral protein, particularly that of the envelope glycoprotein, would be less affected by the fused luciferase protein when it was placed in the opposite order because a signal sequence that mediates targeting and translocation to endoplasmic reticulum[8-10] is located at the N-terminus of the envelope glycoprotein precursor gp62. Therefore, we placed the *Renilla* luciferase protein at the C-terminus of each viral protein. Figure 1A shows the expression levels of recombinant HTLV-1 proteins fused with *Renilla* luciferase in 293T cells. First, we eliminated the codon for the first methionine of *Renilla* luciferase in the original vector (Fig. 1A, Lane 1, pGL4.75) using site-directed mutagenesis to minimize the background luciferase signals produced by intrinsic luciferase proteins in the fusion protein constructs. A small amount of the luciferase protein was detected in the mutated *Renilla* luciferase expression vector (Fig. 1A, Lane 3, pGL4.75ΔMet), which migrated slightly faster, thereby suggesting that a small amount of the luciferase protein was translated from the second (+39 bp) or third (+78 bp) codons of methionine in cDNA of *Renilla* luciferase with low efficiency. The Gag–*Renilla* luciferase and Env–*Renilla* luciferase fusion proteins were detected at the expected sizes of their precursors (Fig. 1A, Lanes 4 and 5). In our plasmids, Gag and Env proteins were expressed as the precursor Pr53 and gp62, respectively, and only small fractions of these proteins were processed in the plasmid-transfected cells. The Tax–*Renilla* luciferase and HBZ–*Renilla* luciferase fusion proteins were expressed in an efficient manner (Fig. 1A, Lanes 6 and 7). Figure 1B shows the luciferase enzyme activities (indicated as RLUs) of the

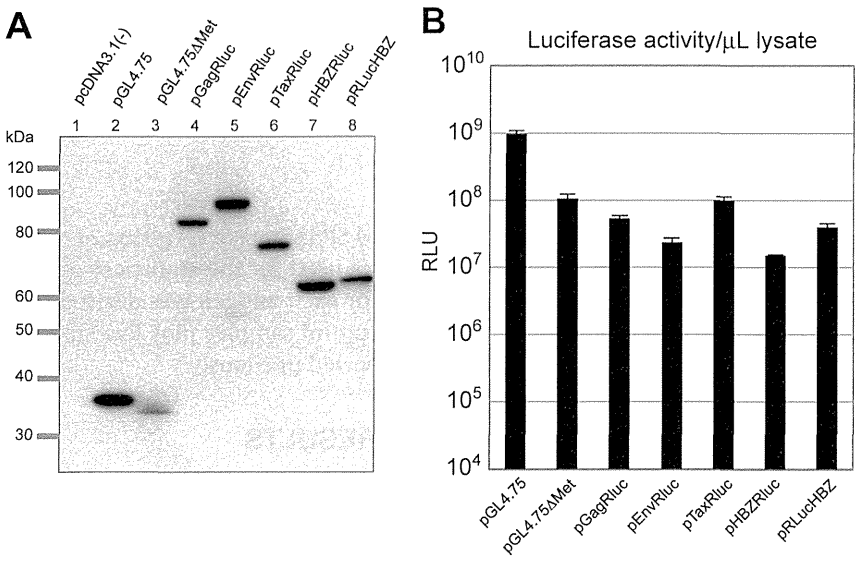

Fig. 1. Expression antigens used in HTLV-1 LIPS. Human 293T cells were transfected with each indicated plasmid, which encoded LIPS antigens, and we analyzed the protein expression levels in the cell lysates by immunoblotting with anti-*Renilla* luciferase (A) and by measuring luciferase activities (B). (A) Lanes 1-3 = pcDNA3.1(–)/myc-His, empty vector; pGL4.75, *Renilla* luciferase expression vector; pGL4.74ΔMet, pGL4.75 with a mutation in the first methionine codon. Lanes 4-8 = antigen–*Renilla* luciferase fusion protein expression vectors: Gag (Lane 4), Env (Lane 5), Tax (Lane 6), and HBZ (Lane 7). pRLucHBZ in Lane 8 = an expression vector for the *Renilla* luciferase–HBZ fusion protein. RLU, measured for 1 second.

lysates of cells transfected with each of the vectors. We also constructed a plasmid that encoded the HBZ protein fused with *Renilla* luciferase at the N-terminus of HBZ (RLucHBZ), as reported previously,[11] to examine whether the antibody responses were affected by the order of the HBZ and *Renilla* luciferase proteins. The expression level of RLucHBZ was slightly less than that of HBZRLuc (Fig. 1B, Lanes 7 and 8); however, the luciferase activity of RLucHBZ was higher than that of its counterpart (Fig. 1B).

HTLV-1 LIPS tests of blood donors

To evaluate the two types of confirmatory tests for HTLV-1, we performed the HTLV-1 LIPS test using donor plasma that had been screened by CLEIA and confirmed by IF or WB. First, we examined the reproducibility of our LIPS tests. Using one control plasma sample that tested negative for HTLV-1 by CLEIA screening and two plasma samples that tested positive by both IF and WB, we performed the LIPS test four times independently against the Gag, Env, Tax, and HBZ antigens. As shown in Fig. 2A, each LIPS result obtained from the same test subject exhibited good reproducibility in multiple tests against all four antigens, whereas the antibody responses of the same test subject varied widely between antigens. The mean of the SD was 4.9% (0.04% to 11.2% at minimum and maximum, respectively) of the mean of four test results against the same antigen. Based on these results, we performed the LIPS test once on one plasma sample in the subsequent analysis.

The luciferase activities of the individual plasma samples according to LIPS tests are shown in Fig. 2B, and the numbers of positive test results are summarized in Table 1. We categorized the donors into six groups. Group I comprised screening test–negative donor samples (negative controls). Groups II to V comprised donors who tested positive by CLEIA screening and who tested positive by IF, negative by IF, positive by WB, and negative by WB, respectively. In Group VI, the plasma collected from donors tested positive by CLEIA at the previous donation. However, indeterminate results were obtained in the WB confirmatory test during the study period; that is, the CLEIA screening results of the donors in Group VI were positive or negative during the study period. Moreover, we reexamined the plasma samples from Groups II and III by WB and those from Groups IV to VI by IF (Table 1, right-end column). As shown in Table 1, the plasma samples that tested positive in both the antibody tests, that is, screening and confirmatory tests (Groups II and IV), exhibited 100% positivity for antibodies against both Gag and Env antigens. The donors in these categories tested positive for antibodies in all three different tests, that is, CLEIA, IF or WB, and LIPS, which strongly suggests that these donors were asymptomatic carriers of HTLV-1. One plasma sample from Group II showed a weak binding to the gp46 Env antigen, as assessed in the additional WB, resulting in an indeterminate test result (Fig. 2B, asterisk). In JRC blood centers, the positive diagnosis of the HTLV-1 WB test is determined as follows: antibody responses against multiple antigens including Env gp46 and at least one antigen from Gag p19, p24, or GagPr53. Otherwise, the test results are categorized as indeterminate. In Groups II and IV, the LIPS results for the antibodies against the Gag and Env antigens indicated that both the confirmatory antibody tests had equivalent sensitivity when we excluded the results for Group VI. In Groups II and IV, we found that all the antibody responses against Env in the LIPS test were strong, whereas some plasma samples had much weaker responses against Gag. The detection results for Tax antibodies showed that more than half of the plasma samples in Groups II and IV tested positive with various intensities, as reported previously.[4,7] Among the donors who tested

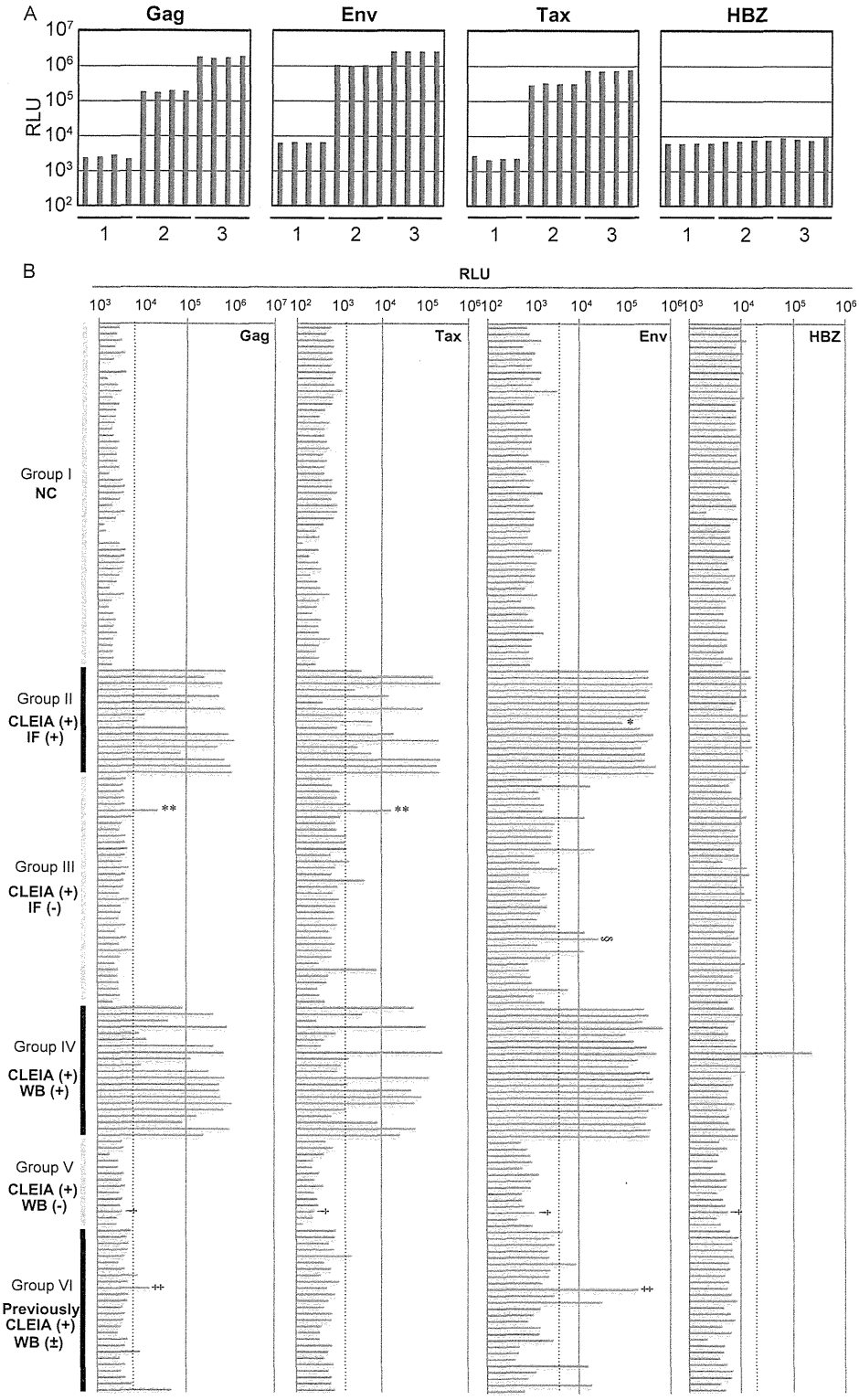

Fig. 2. Antibody responses against four viral antigens, that is, Gag, Tax, Env, and HBZ measured by LIPS. (A) HTLV-1 LIPS of representative samples. One negative control (1) and two positive control (2 and 3) plasma samples were examined by LIPS against the Gag, Env, Tax, and HBZ antigens in four independent experiments. (B) HTLV-1 LIPS of all samples. Data in the same vertical position indicate results from the same test plasma sample. Dotted lines = cutoff values (6938, 15620, 2379, and 30119 for Gag, Tax, Env, and HBZ, respectively); *plasma samples that showed a WB-indeterminate result; **plasma sample that tested positive for antibodies against Gag and Tax by LIPS; §plasma sample that tested positive by WB; †plasma sample that tested positive by IF; ‡plasma

TABLE 1. Positive results using the HTLV-1 LIPS tests

Group	Screening test (CLEIA)	Confirmatory Test 1*	Positive number/test number by LIPS (%)				Positive number/test number by Confirmatory Test 2†
			Gag	Tax	Env	HBZ	
I	Negative	ND	0/54 (0%)	0/54 (0%)	0/54 (0%)	0/54 (0%)	ND
II	Positive	Positive by IF	17/17 (100%)	14/17 (82.4%)	17/17 (100%)	0/17 (0%)	16/17 by WB (94.1%)
III	Positive	Negative by IF	1/36 (2.8%)	7/36 (19.4%)	8/36 (22.2%)	0/36 (0%)	1/36 by WB (2.8%)
IV	Positive	Positive by WB	21/21 (100%)	13/21 (61.9%)	21/21 (100%)	1/21 (4.8%)‡	21/21 by IF (100%)
V	Positive	Negative by WB	0/14 (0%)	0/14 (0%)	0/14 (0%)	0/14 (0%)	1/14 by IF (7.1%)
VI	Positive at the previous donation	WB indeterminate	4/26 (15.4%)	1/26 (3.8%)	5/26 (19.2%)	0/26 (0%)	4/26 by IF (15.4%)

* Examined through blood screening.
† Examined after the blood screening performed in this study.
‡ Confirmed using an in-house WB test.
ND = not determined.

positive only by CLEIA, one, seven, and eight plasma samples were positive by LIPS for antibodies against Gag, Tax, and Env, respectively, for 36 plasma samples in Group III; however, there were no positive results for the 14 plasma samples in Group V (Table 1). By LIPS in Group III, one plasma sample (Fig. 2B, double asterisks) exhibited positive responses against Gag and Tax. Other positive plasma samples in Group III exhibited responses against only a single antigen, either Tax or Env. The antibody responses in Group III did not reach the maximum level of those observed in Groups II and IV. According to the criteria of WB indeterminate, the weak responses detected in Group III by LIPS would be considered "indeterminate." Among them, one plasma sample (Fig. 2B, section symbol) tested positive in the additional WB. In Group V, one plasma sample tested positive in the additional IF test, although no antigen reacted with this plasma sample in LIPS (Fig. 2B, dagger). Among the 26 plasma samples in Group VI, one exhibited antibody responses against both Gag and Env (Fig. 2B, double dagger; and Sample Identifier VI-10). The intensity of the luciferase signal for this plasma sample was almost equivalent to that of the plasma samples in Groups II and IV by LIPS for Env, whereas the antibody response against Gag was relatively low. Previously and in this study period, donor VI-10 tested positive for HTLV-1 by CLEIA screening (see Materials and Methods), and this donor tested positive by LIPS for antibodies against both Gag and Env and tested positive in the additional IF test; thus, this donor was probably infected with HTLV-1, although the donor was indeterminate according to the WB test.

Detection of an anti-HBZ response

Recently, HBZ antibody responses were reported for the first time in both asymptomatic HTLV-1 carriers and patients with ATL or HAM/TSP in two cohorts that comprised multiple ethnicities other than Japanese.[11] We also examined the test plasma samples collected in Japan by LIPS for antibodies against HBZ. Among 114 HTLV-1 screening–positive plasma samples, we detected a single case of HBZ positivity in Group IV (Fig. 2B and Sample Identifier IV-8 in Fig. 3A). We confirmed the presence of anti-HBZ in this plasma sample using an in-house WB test with a human 293T cell lysate that transiently expressed HBZ proteins because no commercial tests are available for detecting HBZ antibodies. As shown in Fig. 3A, Plasma Sample IV-8, but not plasma samples from Group I (I-24 and I-25), exhibited a specific band against wild-type and myc-His–tagged HBZ proteins, HBZRLuc, and RLucHBZ with the appropriate estimated molecular weight (Fig. 3A, Lanes 2-5). This is the first identification of an anti-HBZ–positive blood donor in Japan. Plasma Sample IV-8 exhibited consistently high luciferase activities for the antibody responses against all three other antigens in our LIPS test

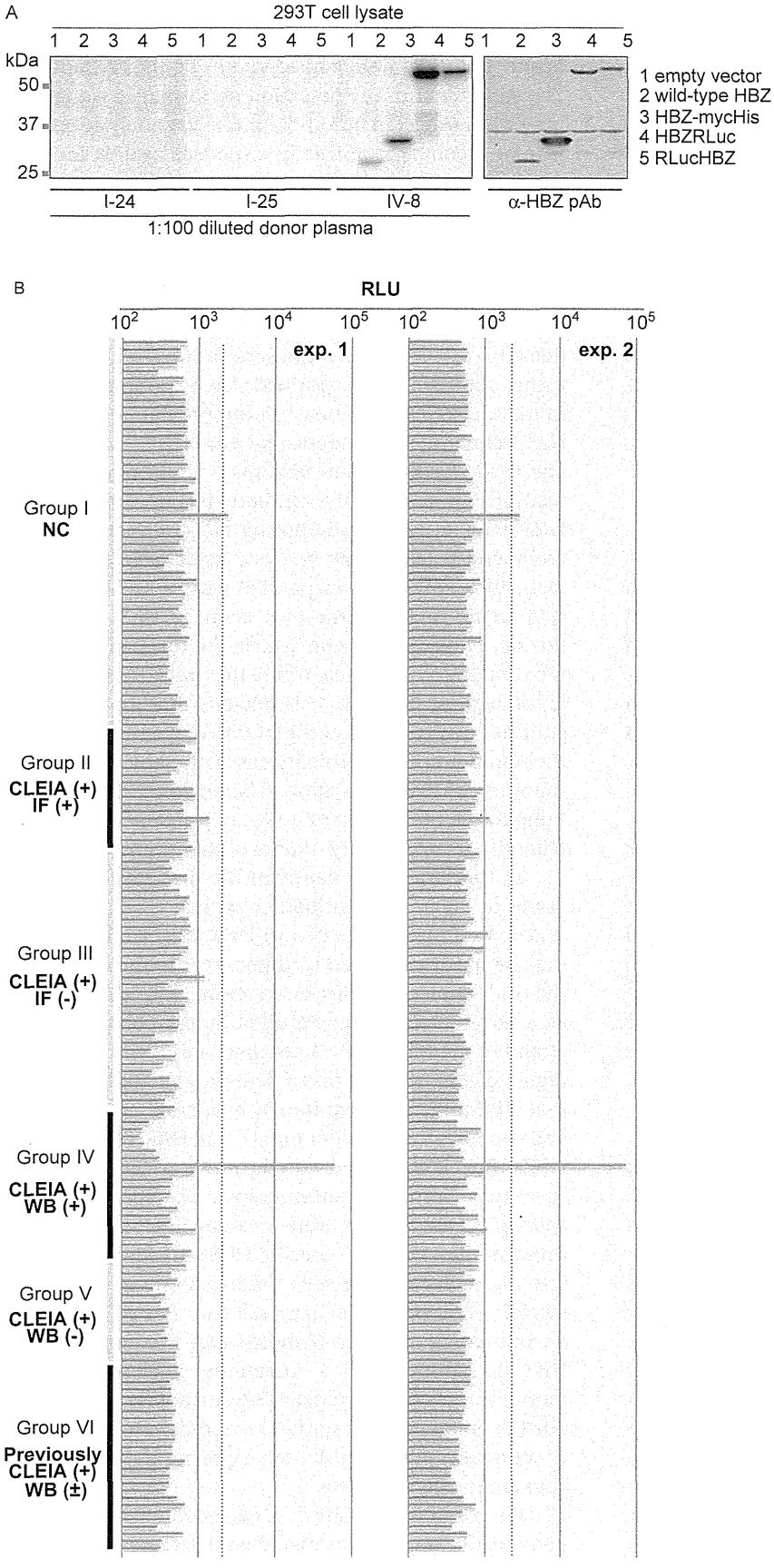

Fig. 3. Characterization of antibody responses against HBZ. (A) Immunoblot analysis against HBZ proteins identified in plasma samples by LIPS. Human 293T cell lysates transfected with an empty vector (Lane 1), wild-type HBZ expression vector (Lane 2), Myc-His-tagged HBZ expression vector (Lane 3), HBZRLuc expression vector (Lane 4), or RLucHBZ expression vector (Lane 5) were analyzed by SDS-PAGE and probed with 1:100-diluted plasma from donors, as indicated by their identifier numbers (I-24, I-25, and IV-8) or with an anti-HBZ rabbit polyclonal antibody (right). (B) Antibody responses against RLucHBZ protein were measured on a separate day by LIPS (Experiments 1 and 2). RLU, measured for 1 second; dotted lines = cutoff values (2071 in Experiment 1 and 2308 in Experiment 2).

(Fig. 2B). We observed that several other plasma samples had relatively strong responses by LIPS for three antigens, that is, Gag, Tax, and Env, in Groups II and IV, whereas they did not bind to the HBZ antigen, except for Plasma Sample IV-8. The frequency of the HBZ antibody was 0.88% (1/114) among the screening-positive donors and 2.63% (1/38) among the confirmatory test–positive donors. Enose-Akahata and coworkers[11] reported that 10.34% (15 in 145) of asymptomatic HTLV-1 carriers exhibited antibody responses against the HBZ protein. To examine whether the low frequency of antibody responses against the HBZ protein in our study was attributable to the C-terminus *Renilla* luciferase fusion structure of our HBZ antigen, that is, HBZRLuc, we analyzed the same test plasma samples using RLucHBZ, where the order of *Renilla* luciferase and HBZ was the same as that reported by Enose-Akahata and coworkers.[7] As shown in Fig. 3B we found that Plasma Sample IV-8 tested positive and exhibited the same high luciferase activity as that detected in tests using HBZRluc. In addition, another positive plasma sample in Group I, that is, I-25, tested negative by CLEIA screening, although the RLU value was much lower than that

of IV-8. To exclude the possibility that the antibody response of I-25 against RLucHBZ was caused by contamination during handling, we repeated the same LIPS test on another day and obtained the same results (Experiment 2 in Fig. 3B). There was no specific signal for the I-25 plasma sample in the immunoblot analysis against the four HBZ proteins (Fig. 3A). Therefore, we consider that the screening-negative plasma sample, I-25, contained antibodies that could weakly bind to a nonlinear epitope in the RLucHBZ antigen but not in the HBZRLuc antigen. These results indicate that the HBZRLuc antigen did not have an impaired capacity for binding HTLV-1–induced antibodies but the structure of HBZRLuc led to a greater specificity. We conclude that the frequency of asymptomatic HTLV-1 carriers who retain anti-HBZ responses in Japan is lower than that in other endemic areas.

DISCUSSION

To explore more reliable tests to identify asymptomatic HTLV-1 carriers among blood donors, we reevaluated two confirmatory tests for HTLV-1 antibodies at our blood center. The LIPS test results for antibodies against the Gag and Env proteins in Groups II and IV were perfectly consistent with those of our previous (IF in Group II) and present (WB in Group IV) HTLV-1 confirmatory test results. These results indicate that the validity of the positive results obtained with both the confirmatory tests used at JRC was further assured using an independent antibody test, that is, LIPS tests. In confirmatory test–negative samples from Groups III and V, LIPS did not identify any plasma samples that were positive for both the Env and the Gag antibodies, indicating that the specificity of both confirmatory tests is equivalent. However, we observed many other types of positive responses via LIPS in Group III, which suggests a particular property of IF in antibody detection. It is likely that some of these antibody responses would be categorized as "indeterminate" in WB, which remains to be fully characterized to assess HTLV-1 confirmatory tests.

Interestingly, our results slightly disagreed with those reported previously, where only LIPS for antibodies against Gag but not Env exhibited 100% positivity in asymptomatic HTLV-1 carriers.[4,7] In our study, all the plasma samples that tested positive in confirmatory tests exhibited unambiguously strong antibody responses against Env (Fig. 2). We assume that the tertiary structure of Env antigens in the Env–*Renilla* luciferase fusion protein in our LIPS was the main contributor to this improvement. This discrepancy may be explained by the different ethnicities of the participants and different distribution of human leukocyte antigen types. The ethnicities of the archived samples and cohorts used in previous studies were mainly Caucasian, African-descent, and Hispanic (Jamaican).[4,7] Our test samples were collected from donors who donated their blood in the Kinki area, which is in the middle part of the Japanese Main Island, and they were considered to be mostly Japanese. The superior anti-Env detection performance is the most important aspect of our LIPS for HTLV-1 analysis because zero or incomplete antibody responses against Env proteins frequently lead to WB-indeterminate results in confirmatory tests.[12-14]

During the screening of donated blood, the most severe issue when determining the HTLV-1 infection status is the lack of gold standard tests, such as the highly sensitive nucleic acid amplification test (NAT) used for detecting HIV-1 and HIV-2 in serum. However, even when using genomic DNA purified from peripheral blood mononuclear cells to measure the proviral load, HTLV-1 NAT frequently fails to detect proviral DNA in asymptomatic HTLV-1 carriers and patients.[15-18] To understand the relationship between the antibody response profiles and viral loads of HTLV-1–harboring blood donors, we have commenced large-scale analysis using several antibody tests, including LIPS, in parallel with a highly sensitive NAT using archived samples from HTLV-1 screening–positive blood donors who lived in the Tokyo metropolitan area and the Kyusyu area, where the prevalence of HTLV-1 is the highest in Japan. It is obvious that more accurate confirmatory tests or a combination of tests would reduce the number of indeterminate cases, thereby reducing the unnecessary disqualification of blood donors and contributing to the prevention of unaware transmission by notifying the corresponding donors of their infection status.

In this study, we identified the first case of a blood donor in Japan with antibodies against HBZ (Figs. 2 and 3). The lower frequency of anti-HBZ detected by our LIPS may be partly explained by differences in the ethnicity of the study subjects, as discussed above. HBZ was identified as a novel viral protein encoded by the complementary strand of the HTLV-1 RNA genome, and it was thought to regulate viral transcription.[19] Subsequent studies showed that HBZ mRNA is ubiquitously expressed in all ATL cells and supports their proliferation.[20,21] In HAM/TSP patients, HBZ mRNA is correlated with the proviral load and disease severity.[22] From an immunologic perspective, HBZ-specific CD8+ T cells were recently identified.[21,23,24] In these studies, the HBZ-specific CD8+ T cells were able to lyse naturally infected cells isolated from asymptomatic carriers and HAM/TSP patients but not ATL patients. Accumulating evidence demonstrates the importance of HBZ in natural HTLV-1 infections; however, humoral immune responses against HBZ are poorly understood. Further epidemiologic studies are required to confirm our observations of HBZ antibody responses in blood donors who are mostly Japanese.

We consider that LIPS has two advantages as an antibody test in transfusion medicine. First, we were able to detect structure-specific antibodies by LIPS without cell

fixation. This feature is particularly useful in confirmatory tests because it complements WB, which only detects the linear epitopes of an antigen. Second, we were able to establish a new test or modify the antigens very easily when we performed the HBZ tests because LIPS does not require the purification of antigens or any secondary antibodies. Only amino acid sequences of the antigens involved are required to implement a new test. This advantage is particularly useful when detecting antibodies against newly emerging infectious diseases, as performed recently in animals[25] and human populations.[26] It would also allow us to expand the existing tests easily by adding new antigens, such as those of HTLV-2. To evaluate the capability of LIPS for detecting antibodies that are critical for blood screening, we must further examine whether LIPS tests can be easily automatized, in addition to testing their sensitivity, specificity, and cost.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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BRIEF REPORT

Development of T cell lymphoma in HTLV-1 bZIP factor and Tax double transgenic mice

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Abstract Adult T-cell leukemia (ATL) is an aggressive T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1). ATL cells possess a CD4+ CD25+ phenotype, similar to that of regulatory T cells (Tregs). Tax has been reported to play a crucial role in the leukemogenesis of HTLV-1. The HTLV-1 bZIP factor (HBZ), which is encoded by the minus strand of the viral genomic RNA, is expressed in all ATL cases and induces neoplastic and inflammatory disease *in vivo*. To test whether HBZ and Tax are both required for T cell malignancy, we generated HBZ/Tax double transgenic mice in which HBZ and Tax are expressed exclusively in CD4+ T cells. Survival was much reduced in HBZ/Tax double-transgenic mice compared with wild type littermates. Transgenic expression of HBZ and Tax induced skin lesions and T-cell lymphoma in mice, resembling diseases observed in HTLV-1 infected individuals. However, Tax single transgenic mice did not develop major health problems. In addition, memory CD4+ T cells and Foxp3+ Treg cells counts were increased in HBZ/Tax double transgenic mice, and their proliferation was enhanced. There was very little difference between HBZ single and HBZ/Tax double transgenic mice. Taken together, these results show that HBZ, in addition to Tax, plays a critical role in T-cell lymphoma arising from HTLV-1 infection.

Keywords HTLV-1 · HBZ · Tax · Transgenic mice · Lymphoma

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Introduction

Human T-cell leukemia virus type1 (HTLV-1) was the first retrovirus proven to be associated with human disease. Infection with HTLV-1 causes adult T-cell leukemia (ATL) [20, 24]. ATL cells possess a CD4+ CD25+ phenotype, similar to that of regulatory T cells (Tregs). Previous report showed that HTLV-1 provirus is detected mainly in CD4+ memory T cells and Treg cells, suggesting that HTLV-1 favors Treg cells and memory T cells *in vivo* [10, 23, 26].

HTLV-1 encodes several regulatory (*tax* and *rex*) and accessory (*p12*, *p13*, and *p30*) genes in the pX region between the *env* gene and the 3′ Long terminal repeat (LTR) [19]. Another gene, the *HTLV-1 bZIP factor* (*HBZ*), is encoded by the minus strand of the HTLV-1 genome [4]. Among the proteins encoded by these genes, Tax and HBZ play critical roles in ATL [5, 16]. Accumulating evidence shows that Tax can immortalize human primary T cells, enhance viral replication and support cellular proliferation [5]. However, the expression of Tax cannot be detected in approximately 60 % of fresh ATL cells because of genetic and epigenetic changes in the HTLV-1 provirus, which indicated that Tax may not be essential for the development of ATL [15]. We reported previously that HBZ is

consistently expressed in all ATL cells and promotes proliferation of ATL cells [21]. Non-sense mutations of all HTLV-1 genes except HBZ were generated by APOBEC3G (A3G), suggesting that HBZ is indispensable for the growth and survival of HTLV-1 infected cells [3].

It is noteworthy that Tax and HBZ synergistically regulated the viral transcription and cellular signaling pathways in ATL [29]. HBZ suppressed Tax-mediated HTLV-1 viral transcription through interaction with cAMP response element-binding protein (CREB) [12]. Additionally, HBZ selectively inhibited the classical nuclear factor-κB (NF-κB) pathway which was activated by Tax [27]. We reported that HBZ induced the differentiation of Treg cells by activating the transforming growth factor-β (TGF-β) pathway [28]. Contrariwise, three distinct mechanisms by which Tax suppressed TGF-β–mediated signaling were reported [1, 11, 17]. Taken together, we speculated that the complementary effect of Tax and HBZ on regulating signaling pathways may facilitate better survival of HTLV-1 infected cells and help the cancer cells escape immune attack.

To test the effect of synchronous expression of HBZ and Tax on T cell malignancy in vivo, we generated double transgenic mice expressing HBZ and Tax under the control of the CD4 promoter. In the present study, we found that HBZ/Tax mice have increased memory CD4+ T cells and Foxp3+ Treg cells, resulting in the development of skin lesions and T-cell lymphoma. Both the skin lesions and the lymphoma resemble diseases observed in HTLV-1 infected individuals.

Material and methods

Mice and cell cultures

C57BL/6 J mice were purchased from CLEA Japan. Transgenic HBZ mice expressing HBZ specifically in CD4+ cells have been described elsewhere [22, 25]. Tax single transgenic mice were generated as previously reported [22]. Male HBZ transgenic mice were mated with female Tax transgenic mice, and offspring were typed for the presence of each transgene. Wild-type, HBZ, and Tax single transgenic mice were maintained as controls along with experimental HBZ/Tax transgenic offspring.

All animal experimentation was performed in strict accordance with the Japanese animal welfare bodies, and the Regulation on Animal Experimentation at Kyoto University. The protocol was approved by the Institutional Animal Research Committees of Kyoto University and Zhejiang Normal University. All efforts were made to minimize suffering.

ATL cell lines, ATL-43T and MT-1, were cultured in RPMI-1640 containing 10 % FBS and antibiotics. 293FT cells were maintained as described previously [27].

Semiquantitative RT-PCR and real-time PCR

Total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. We reverse-transcribed total RNA into single-stranded cDNA with SuperScript III reverse transcriptase (Invitrogen). For semiquantitative PCR, cDNA was amplified by increasing PCR cycles using forward (F) and reverse (R) primers specific to the target genes. The expression of transgenic genes was quantified by real-time PCR using the Taqman Universal PCR Master Mix (PE Applied Biosystems) according to the manufacturer's instructions.

Lentiviral vector construction and transfection of recombinant lentivirus

We cloned Tax cDNA into a lentiviral vector, pCSII-EF-MCS. Recombinant lentivirus was produced as described. ATL-43T cells were incubated with concentrated vector stocks in the presence of 4 μg/mL polybrene.

Cell isolation and flow cytometric analysis

Murine spleen was carefully crushed to release the lymphocytes. Splenic erythrocytes were eliminated with NH_4Cl. Cells were washed with PBS containing 1 % FBS. After centrifugation, cells were incubated with antibodies for 30 min at 4 °C, and then analyzed with a flow cytometer (BD FACSCanto II, BD Biosciences). For intracellular staining, we used a mouse Foxp3 staining kit according to its protocol (eBioscience).

BrdU staining

In vivo proliferation was measured by BrdU incorporation. BrdU (Nacalai Tesque) was dissolved in PBS (3 μg/ml), and then 200 μl was injected intraperitoneally into transgenic and non-transgenic mice twice a day for three days. BrdU incorporation in CD4+ splenocytes was detected using FITC BrdU Flow Kits (BD Pharmingen) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the unpaired student *t* test.

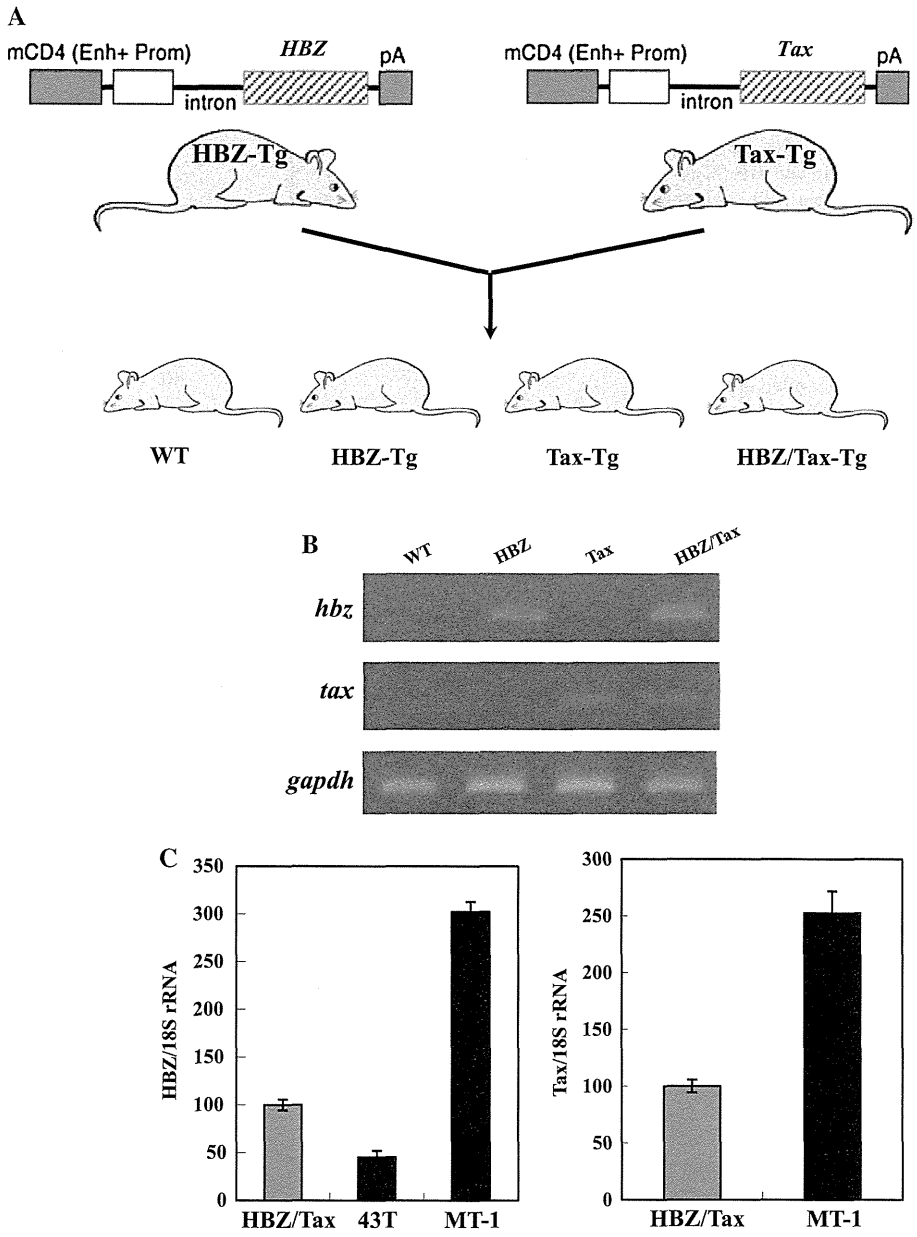

Fig. 1 Generation of HBZ/Tax transgenic (Tg) mice. (A) Schematic representation of the HBZ and Tax transgene. The promoter (Prom) and enhancer (Enh) of the mouse CD4 (mCD4) gene were ligated to HBZ and Tax cDNA plus the polyadenylation signal sequence of SV40. (B) Expression of HBZ and Tax transcripts was detected by RT-PCR in purified CD4+ splenocytes from transgenic mice. (C) Transcripts of the HBZ and Tax genes in CD4+ splenocyte from HBZ/Tax-transgenic mice or ATL cell lines were quantified by real time PCR. ATL-43T and MT-1 are derived from ATL cells

Results

In the development of ATL, Tax is thought to play critical role in leukemogenesis because of its pleiotropic functions [5]. HBZ was constitutively expressed in all ATL cases, and involved in cell proliferation [21]. Additionally, HBZ modulated Tax-mediated viral gene transcription and cellular signaling, suggesting that HBZ cooperates closely with Tax in ATL [2, 7, 12–14, 27, 28]. However, the relevance to T cell malignancy of combined up-regulation of Tax and HBZ has not been established. Since HTLV-1 mainly infects CD4+ T cells, we generated transgenic mice expressing HBZ and Tax under the control of the murine CD4-specific promoter/enhancer/silencer. To test whether HBZ and Tax could cooperate in the development of T cell malignancies, we took advantage of the availability of HBZ and Tax transgenic mice and produced double-transgenic mice expressing both HBZ and Tax in T lymphocytes (Fig. 1A). As shown in Fig. 1B, HBZ and Tax transgene expression was detected by RT-PCR by using CD4+ splenocytes from age-matched mice of the different genotypes. Moreover, HBZ did not interfere with the expression of Tax in double transgenic mice. As shown in Figure 1C, the expression level of HBZ and Tax in transgenic mice was similar to that of ATL cell lines.

Tax single transgenic mice did not develop major health problems, and had a normal life span, as shown in Fig. 2A. In contrast, survival of HBZ and HBZ/Tax double

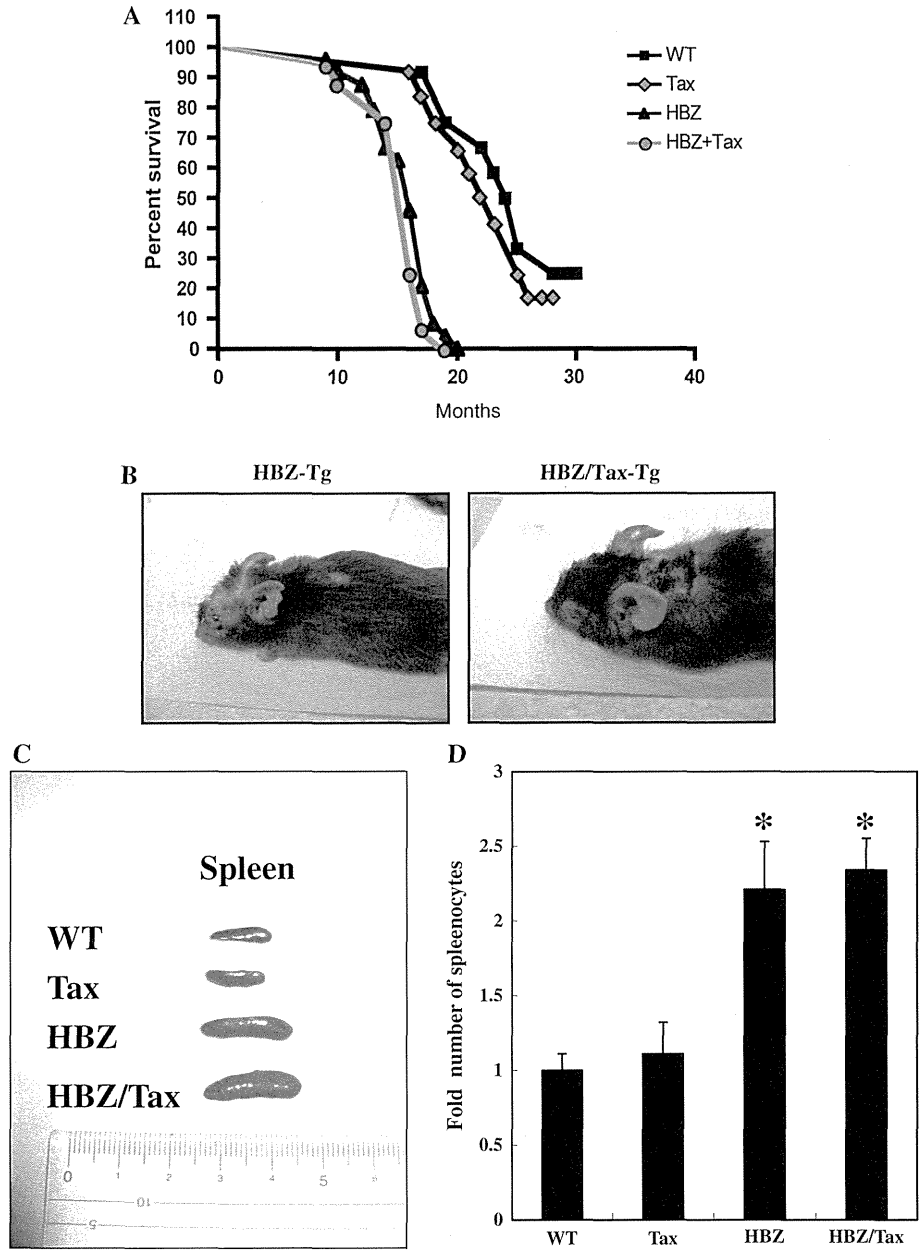

Fig. 2 Characterization of transgenic mice. (A) Kaplan–Meier analysis of survival of wild-type, HBZ single-, Tax single-, and HBZ/Tax double transgenic mice. (B) An HBZ-transgenic and HBZ/Tax mouse with typical skin symptom. (C) Representative examples of spleens from age-matched transgenic mice. (D) Total number of splenocytes of transgenic mice are compared.*, $P < 0.05$ when transgenic mice are compared with wild type littermates by unpaired t test

transgenic mice was much reduced. Some of these mice died as early as ten months after birth. By 20 months, <5% of the HBZ and HBZ/Tax mice remained alive. Interestingly, there is not much difference in survival time between HBZ single transgenic mice and HBZ/Tax double transgenic mice.

Consistent with previous reports, HBZ transgenic mice developed skin lesions by 4 months of age, non-transgenic littermates developed no disease. As shown in Fig. 2B, HBZ/Tax double transgenic mice had similar skin symptoms as HBZ transgenic mice. In Tax transgenic mice, we did not observe inflammatory lesions in the skin (data not shown). Analysis of spleen size in transgenic mice expressing HBZ or Tax alone or in combination revealed splenomegaly in HBZ and HBZ/Tax lines, while tax transgenic mice present spleens of normal size. Moreover, age-matched HBZ/Tax double-transgenic mice had spleens similar in size and weight to HBZ single transgenic mice. Representative examples of the spleens from the various transgenic mice are shown in Fig. 2C. Next, we analyzed the total number of splenic CD4+ T cells in each transgenic line. As shown in Fig. 2D, HBZ and HBZ/Tax mice had, on average, twice the CD4+ splenocytes of wild-type littermates, whereas Tax mice had equal numbers of splenocytes as non-transgenic mice.

Table 1 The incidence of lymphoma in transgenic mice

	WT	Tax-Tg	HBZ-Tg	HBZ/Tax-Tg
Total	52	42	43	32
Lymphoma	2.7 %	2.2 %	32.2 %	34.7 %

The total number of mice and percentage of transgenic mice with lymphoma are listed

Analogous to the incidence of ATL in humans, 32.2 % of HBZ transgenic mice developed T-cell lymphomas after 14 months, in contrast with 2.7 % of non-transgenic mice. As shown in Table 1, the incidence of T-cell lymphomas in HBZ/Tax double transgenic mice is similar to that in HBZ transgenic mice. On the other hand, excessive Tax gene expression did not induce T-cell lymphoma in Tax transgenic mice.

To study the cellular basis of lymphomagenesis in HBZ/Tax double transgenic mice, we next analyzed the phenotype and FoxP3 expression in three month old mice before they developed pathological manifestations. A previous report showed that effector/memory and CD4+ FoxP3+ regulatory T cells were increased in the HBZ-transgenic mice [22]. We found in this study that the percentage of CD4 single positive T cells increased in the HBZ/Tax transgenic mice (Fig. 3). Moreover, transgenic expression of Tax and HBZ induced Foxp3 expression. We also observed an increased population of effector/memory T cells in the HBZ/Tax transgenic mice, yet the percentage of effector/memory T cells or Treg cells in the Tax-transgenic mice did not change significantly. In addition, there is not much difference in the enhancement of memory and FoxP3+ CD4+ regulatory T-cell populations between HBZ and HBZ/Tax transgenic mice. Taken together, these observations demonstrate that HBZ increased memory T cells and Foxp3 induction in CD4+ T cells regardless of Tax.

We further studied whether Tax has any influence on the generation of Foxp3+ T cells *in vivo*. As shown in Supplemental Fig. 1, retrovirally expressed Tax protein could not increase the level of Foxp3 in ATL-43T, an HTLV-1-associated cell line, which does not express Foxp3.

To study the growth-promoting activity of the HBZ and Tax genes, we assessed the proliferation of CD4+ T cells in transgenic mice by incorporation of BrdU. We found that, in HBZ and HBZ/Tax transgenic mice, the proliferation of CD4+ T cells was three fold-higher than in non-transgenic mice, whereas the proliferation of CD4+ T cells of Tax transgenic mice was similar to that in non-transgenic mice (Fig. 4).

All of these results suggest an important role for HBZ, in addition to Tax, in the oncogenic activity of HTLV-1.

Discussion

Over the past 35 years, substantial effort has been made toward investigating the HTLV-1 associated viral proteins and their regulatory functions [15]. Tax has been shown to be a viral oncogene, since it transforms and immortalizes rodent cells and human T-lymphocytes [15]. HBZ is the only viral gene which is conserved and expressed in all ATL cases, indicating that HBZ is indispensable for HTLV-1 infection and development of ATL [16]. Accumulating evidences shows that HBZ and Tax synergistically dysregulate cell signaling pathways in ATL and determine the cell fate, despite the fact that HBZ and Tax have opposite effects on regulation of cellular activity [2, 13, 14, 27, 28]. For example, HBZ was found to inhibit the Tax-mediated transactivation of viral transcription from the 5′LTR by interacting with JUN and CREB. HBZ overcame the suppression function of Tax on the TGF-β pathway, leading to the activation of TGF-β signaling and differentiation of Foxp3+ CD4+ regulatory T cells. Thus, we proposed that HTLV-1 may take advantage of the complementary functions of HBZ and Tax to facilitate the onset of ATL. So far, there has been no direct evidence for HBZ and Tax together inducing T cell malignancy in vivo. The present study demonstrated that HBZ/Tax double transgenic mice indeed developed T cell lymphoma and inflammation via increased memory T cells and Treg cells.

Since Tax is the major target of cytotoxic T-lymphocytes (CTLs), host cells have developed several mechanisms to silence the expression of Tax [9]. Tax transcripts are detected in only ~40 % of ATL patients. Therefore, HBZ maintains cell proliferation in the late stage of ATL when Tax expression is lost. In this study, we demonstrated that HBZ/Tax double and HBZ single transgenic mice developed neoplastic and inflammatory diseases, while mice expressing only Tax did not. Thus, we suggest that constitutively expressed HBZ is the predominant driver of leukemogenesis of ATL.

It has been reported that Tax transgenic mice develop tumors [6, 8, 18]. In those reports, the type of tumor induced by Tax depended on the promoter used. In this study, we generated Tax transgenic mice using the CD4-specific promoter/enhancer/silencer which has previously been used in generating HBZ-transgenic mice. The Tax transgenic mice thus generated did not show any change in the number of Foxp3+ Treg cells or memory T cells, in contrast to mice expressing HBZ, who developed T cell lymphomas. Moreover, since there was no difference in disease between HBZ single and HBZ/Tax double transgenic mice, expression of Tax did not synergistically enhance the lymphomagenesis by HBZ. These data

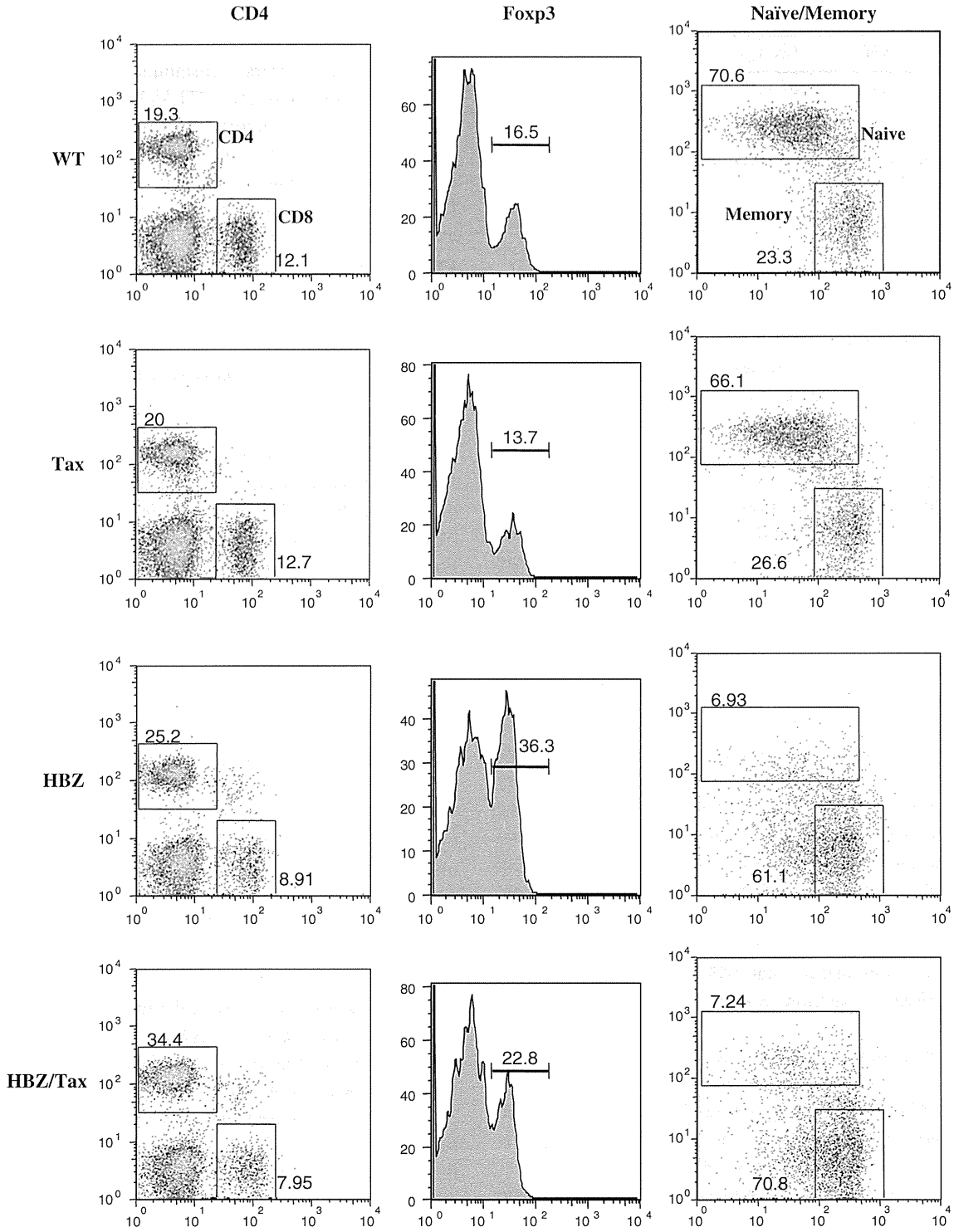

Fig. 3 Transgenic expression of HBZ/Tax in CD4+ T cells increases Foxp3+ Treg and memory T cells. Mouse splenocytes were stained with the indicated antibodies, and analyzed by flow cytometry. Representative dot plots gated on the CD4+ population are shown. For these experiments, HBZ and HBZ/Tax transgenic mice without any symptoms were used

suggest that HBZ, rather than Tax, is responsible for conferring the specific phenotype of HTLV-1 infected cells, and for triggering the development of ATL.

In conclusion, we showed that HBZ single and HBZ/Tax double transgenic mice spontaneously develop T cell lymphomas and inflammatory diseases similar to those in

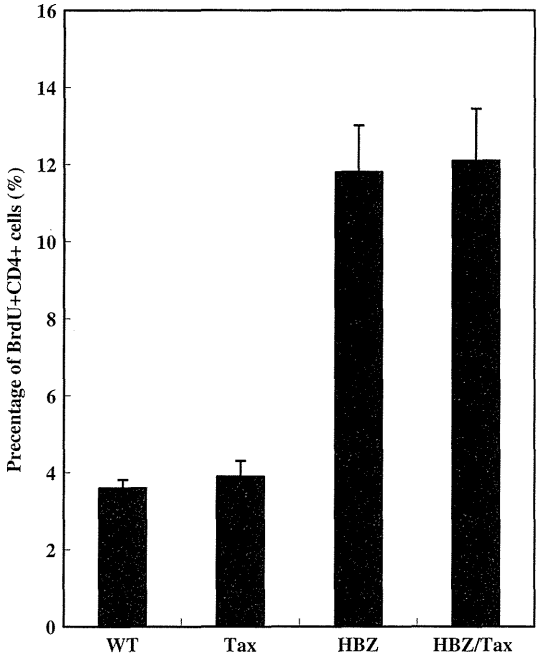

Fig. 4 CD4+ T cells proliferated in HBZ/Tax transgenic mice. BrdU was injected into mice twice a day for three days, and splenocytes were stained with antibodies to BrdU and CD4

HTLV-1 infected individuals. However, Tax single transgenic mice did not develop major health problems. This study highlights the importance of HBZ in HTLV-1-associated disease.

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Conflict of interest The authors declare that they have no competing interests.

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