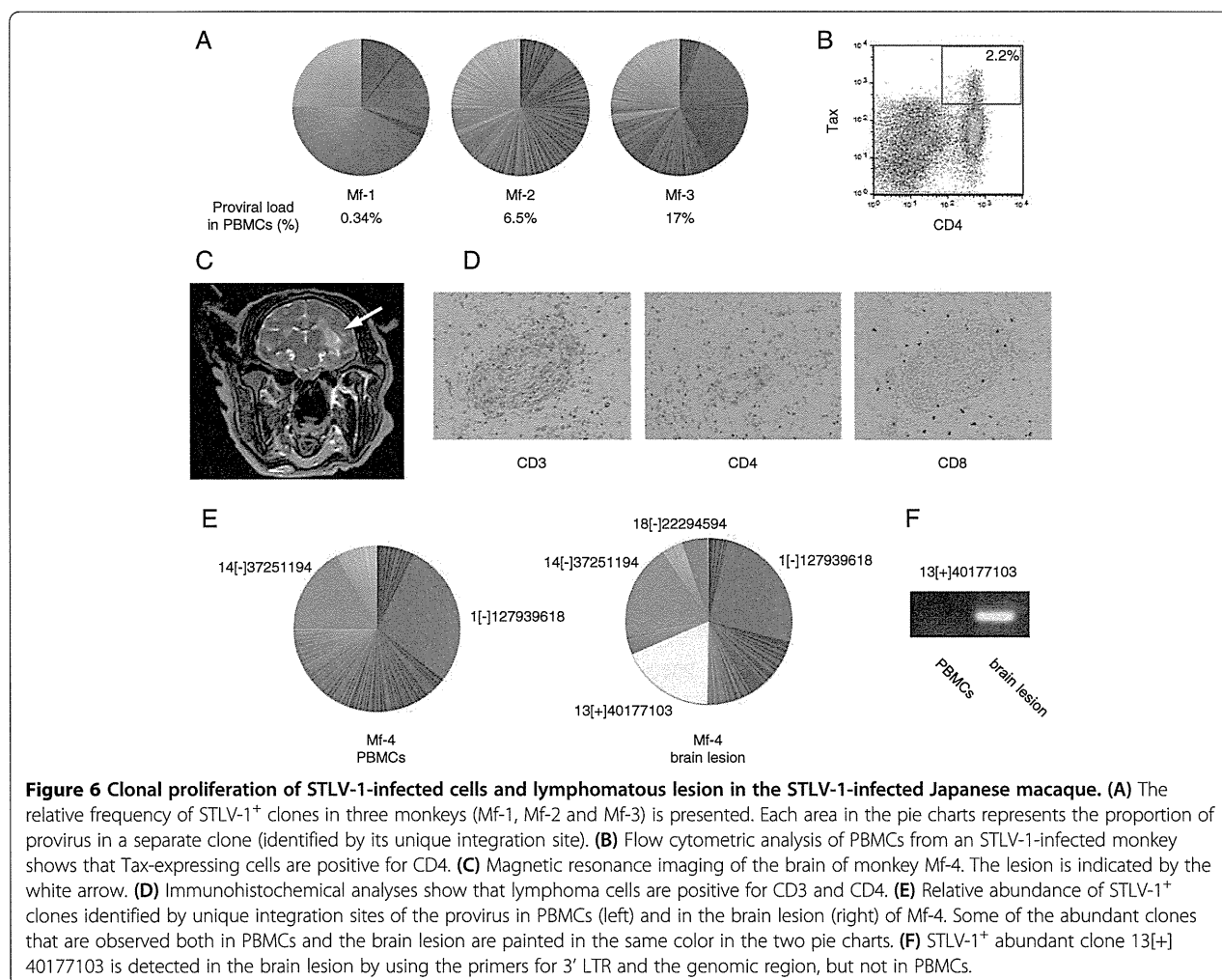


and middle). On the other hand, another monkey, Mf-3, which had higher proviral load (17%), possessed two major STLV-1-infected clones (Figure 6A, right). To study which cell types are infected by STLV-1, Tax expression in PBMCs obtained from one monkey (Mf-4) was analyzed by flow cytometry. The Tax-expressing cells were largely found to be CD4<sup>+</sup> T cells, as is the case with HTLV-1 infection in humans (Figure 6B).

### STLV-1-associated T-cell lymphoma in a Japanese macaque

A monkey (Mf-4) developed anorexia and had paralysis of the lower limbs. This monkey had high proviral load (53%) in PBMCs. We suspected that this monkey has developed a disease similar to HAM/TSP because paralysis of the lower limbs is one of the major symptoms of HAM/TSP patients. Magnetic resonance imaging (MRI) revealed a high intensity lesion in the brain on a T2-weighted image (Figure 6C). Pathological analysis showed

that this tumor was a lymphoma with atypical morphology, and by immunohistochemical methods, it was found that these cells were CD3<sup>+</sup> CD4<sup>+</sup> (Figure 6D). In contrast, no obvious demyelination was observed in the spinal cord. Thus, this monkey was diagnosed with T-cell lymphoma in the brain rather than the disease like HAM/TSP. In this monkey, some major clones had proliferated in peripheral blood (Figure 6E, left). We found that the major clones in peripheral blood were also detected in the brain lesion (Figure 6E, right). These observations demonstrate that STLV-1 causes lymphoma in Japanese macaques. Notably, one of the major clones in the brain, which had its provirus integration site in chromosome 13, was not detected in PBMCs. This was confirmed by conventional PCR using the primers for the 3'LTR and the host genome proximal to the integration site (Figure 6F). Moreover, a clone with the integration site in chromosome 18 was also detected only in the brain lesion. These tumor-specific

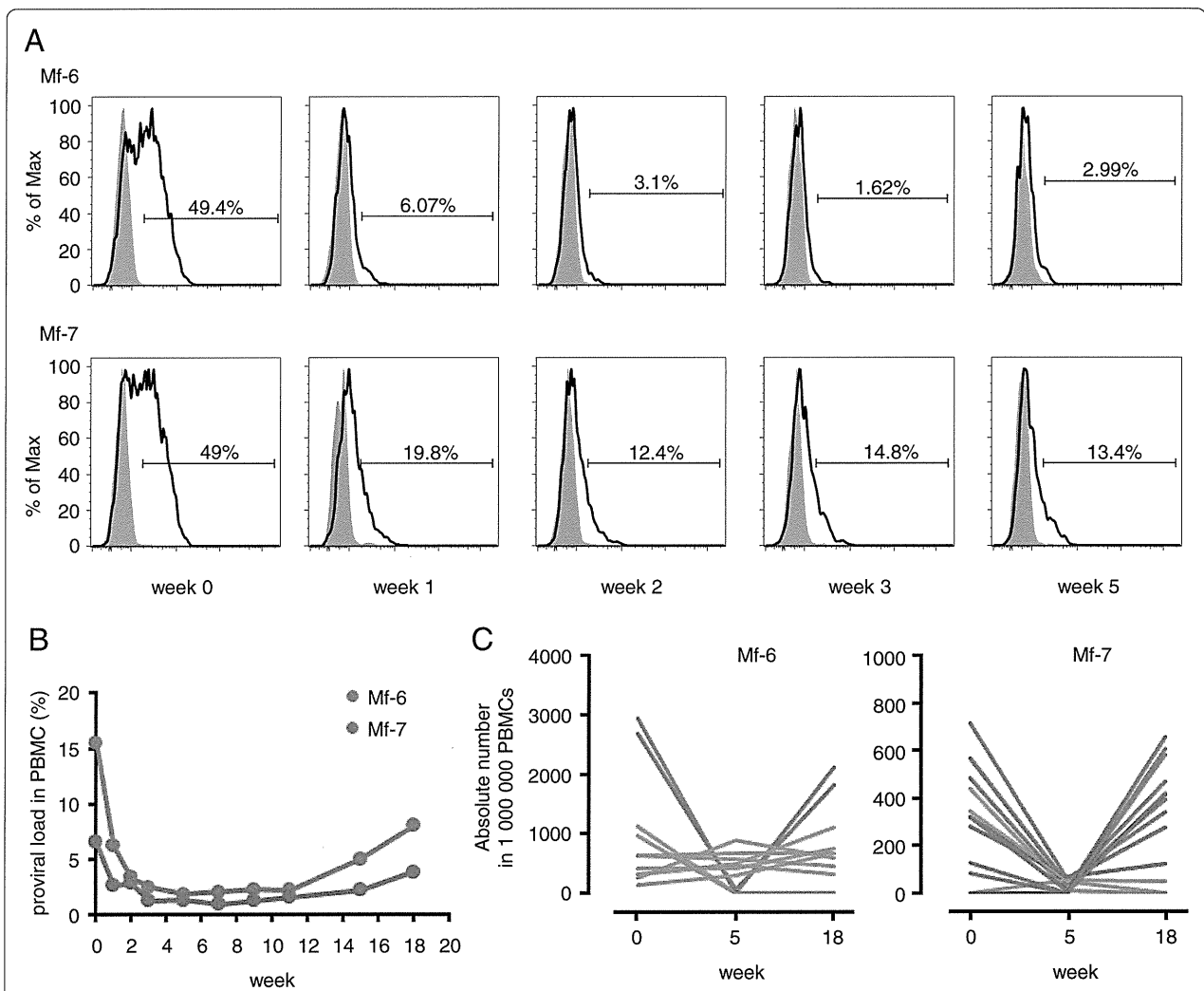


STLV-1-infected clones are thought to contribute to the formation of the tumor.

**Treatment with anti-CCR4 antibody decreased proviral load in STLV-1-infected Japanese macaques**

ATL cells express high levels of CC chemokine receptor 4 (CCR4) [28]. Recently, mogamulizumab, a humanized IgG1 monoclonal antibody against CCR4 [29], was approved in Japan for the treatment of relapsed ATL patients. HTLV-1-infected cells of healthy carriers also express CCR4, which indicates that mogamulizumab likely reduces the proviral load in HTLV-1-infected asymptomatic individuals [30]. High proviral load has been reported to be associated with HAM/TSP, HTLV-1 uveitis, and risk of ATL, indicating that mogamulizumab

may potentially be used for the treatment of HTLV-1-associated diseases and the prevention of ATL. However, it is not clear whether mogamulizumab can reduce the proviral load in HTLV-1-infected individuals. We confirmed that mogamulizumab also recognizes macaque CCR4 by staining Japanese macaque PBMCs *in vitro* with the fluorescently labeled antibody (see Additional file 3). Then, we tested the efficacy of mogamulizumab to reduce the proviral load in STLV-1-infected Japanese macaques. Mogamulizumab was administered to two monkeys with high proviral load (Mf-6 and Mf-7), once a week for 4 weeks. As shown in Figure 7A, nearly half of the CD4<sup>+</sup> T cells expressed CCR4 before the treatment (week 0). After the treatment, the CCR4 positivity decreased to 1.62% and 12.4% respectively. We also



**Figure 7** Effect of anti-CCR4 antibody on STLV-1 dynamics *in vivo*. **(A)** CD3<sup>+</sup>CD4<sup>+</sup> T cells were gated and the expression of CCR4 was analyzed by flow cytometry. **(B)** Changes in STLV-1 proviral load in two monkeys treated with anti-CCR4 antibody until week 3. **(C)** Absolute cell numbers of the five most abundant clones in 1,000,000 PBMCs at weeks 0, 5 and 18 are shown.

measured proviral load over the course of the treatment and found that it decreased dramatically within 2 weeks (Figure 7B). Thus, this demonstrates that mogamulizumab can indeed reduce the number of STLV-1-infected cells *in vivo*.

Eight weeks after the final administration of mogamulizumab, the proviral load started to recover (Figure 7B). To investigate whether mogamulizumab influences the clonality of STLV-1-infected cells, we evaluated the absolute number of each clone by high-throughput sequencing of provirus integration sites. Figure 7C shows changes of the five most abundant clones at weeks 0, 5 and 18. The major clones before the treatment (week 0) recovered at week 18 (red lines in Figure 7C), while some clones were present constantly during the treatment (grey lines) or diminished after the treatment (blue lines). Interestingly, some clones (green lines) that emerged in a monkey after treatment were rare or even not detected before treatment (Figure 7C).

## Discussion

HTLV-1 is thought to originate from STLV-1. In STLV-1-infected monkeys, investigators found clonal proliferation of STLV-1-infected cells and the preferential infection of CD4<sup>+</sup> T cells by the virus [15,31]. Moreover, several groups reported the development of lymphomas in STLV-1-infected monkeys [16,17,32-35]. Monoclonal integration of STLV-1 in the lymphoproliferative disease of African green monkeys was detected by Southern blot [16,33], demonstrating the direct causative role of STLV-1. Thus STLV-1-infected non-human primates have been thought to be a useful animal model for HTLV-1 research. The dynamics of infected cells after treatment with histone deacetylase inhibitors and reverse transcriptase inhibitors has been analyzed in STLV-1-infected baboons, and it was found that this combination significantly decreased proviral load in treated animals [36]. However, there have been no detailed studies on functions of STLV-1-encoded genes. Analyses of the functions of its accessory and regulatory proteins are necessary if we are to use STLV-1-infected monkeys as a model of HTLV-1 infection. In the present study, we focused on Japanese macaques naturally infected with STLV-1.

The amino acid sequence of STLV-1 Tax is closely homologous to that of HTLV-1 Tax, and this study demonstrated that their functions on various transcriptional pathways are similar as well. This study was the first to identify SBZ as an antisense transcript of STLV-1 and a homolog of HBZ. SBZ and HBZ share only approximately 73% identity at the amino acid level. Nevertheless, for all the functions we examined, SBZ behaves similarly to HBZ. In particular, SBZ expression could induce Foxp3 expression like HBZ expression does. This might be attributed to the following reasons. First, the N-terminal region, as well

as the heptad repeats of hydrophobic amino acids in the basic leucine zipper domain, are conserved between HBZ and SBZ. This may allow SBZ to interact with and suppress NF- $\kappa$ B, AP-1 and other transcription factors with basic leucine zipper motifs [37,38]. Second, the LXXLL-like region (Leu27, Leu28, Leu48 and Leu49), which is critical for the interaction with p300 and Smad3 protein, is also conserved between HBZ and SBZ [22,39]. Some lysine residues present in HBZ are substituted with different amino acids in Japanese macaque SBZ. This study showed that SBZ has similar functions compared with HBZ, suggesting that these lysine residues are not critical for their functions. However, further studies are necessary for deep understanding of implication of these amino acid sequences.

HTLV-1 increases the number of infected cells by clonal proliferation of infected cells, which likely facilitates cell-to-cell transmission of this virus. Clonal proliferation of STLV-1-infected cells in Celebes macaques was demonstrated by the conventional inverse PCR method [15]. However, this technique could detect only a limited population of the clones because of its limited sensitivity or the stochastic amplification of the integration sites. In the present study, we investigated more comprehensively the clonal proliferation of infected cells in Japanese macaques naturally infected with STLV-1 by massively sequencing the unique integration sites of the provirus. The finding that STLV-1-infected cells proliferated clonally in the monkeys with higher proviral loads resembles the finding for HTLV-1. Furthermore, one monkey had lymphoma in the brain, showing that STLV-1 induces lymphoma in Japanese macaques. Analyses of STLV-1 integration sites in this T-cell lymphoma showed that one of the major clones in the brain was unique to this tumor, suggesting that this clone played an important role in the lymphomagenesis of this tumor.

This study also revealed a remarkable difference in STLV-1 seroprevalence between Japanese macaques (320/533: 60%) and rhesus macaques (1/163: 0.6%). Previous studies showed that the seroprevalence in rhesus macaques was 25%, and that in Japanese macaques was quite high [40-42]. Similarly, high seroprevalence was reported in baboons [43]. Furthermore, many studies reported the development of lymphoma in baboons [17,44,45]. The high seroprevalence and the development of lymphomas in Japanese macaques and baboons may suggest a higher susceptibility of these species to STLV-1 infection. Japanese macaques and baboons infected with STLV-1 may be suitable models for HTLV-1 research.

In this study, we also demonstrated that mogamulizumab strongly suppressed proviral load in STLV-1-infected Japanese macaques. Proviral load was suppressed for 4 weeks after the final administration of mogamulizumab, which seems reasonable when considering that the half-life of the

antibody administered at 1.0 mg/kg is approximately 18 days as measured in a clinical trial [46]. Some STLV-1-infected major clones recovered after the treatment, while other clones were still suppressed or even not detected. In HTLV-1-infected individuals, HTLV-1 proviral load is relatively constant in the chronic phase, although some minor clones fluctuate [25]. This study is the first to report that most of the major clones recover after the withdrawal of mogamulizumab. This observation suggests that the major clones may have some growth advantages that allow them to proliferate robustly *in vivo*. These growth advantages may be due to the integration site of the provirus, accumulation of genetic mutations, or epigenetic changes. The population of some clones remained constant over the course of the treatment. We speculate that these clones are negative for CCR4 expression. High proviral load is associated with risk of ATL and inflammatory diseases. Therefore, suppression of proviral load by mogamulizumab is a possible treatment for HTLV-1-associated inflammatory diseases such as HAM/TSP.

## Conclusions

In summary, this study is the first to show that STLV-1 Tax and SBZ have activities similar to those of Tax and HBZ, activities which likely induce clonal proliferation and T-cell lymphoma in infected monkeys. STLV-1-infected Japanese macaques appear to be a good model for studying the effects of anti-viral drugs and the immunological aspects of HTLV-1 infection.

## Methods

### Biological samples of macaques

Japanese macaques (*Macaca fuscata*) and rhesus macaques (*Macaca mulatta*) used in this study were reared in the Primate Research Institute, Kyoto University. Blood samples were obtained from the macaques (for routine veterinary and microbiological examination) under ketamine anesthesia. All animal studies were conducted in accordance with the protocols of experimental procedures that were approved (2011–095) by the Animal Welfare and Animal Care Committee of the Primate Research Institute of Kyoto University, Inuyama, Japan.

### Antibody screening and measurement of proviral load

Plasma samples were screened for the presence of antibodies against HTLV-1 by particle-agglutination test using SERODIA-HTLV-1 (Fujirebio). Proviral load was measured by real-time PCR quantifying the copy number of *tax* and *RAG1* as previously described [47]. Primers and probes are available in Additional file 4.

### Detection of STLV-1 transcripts

Total RNA was extracted from STLV-1-infected Japanese macaque cell line Si-2 [48] with Trizol (Invitrogen), then

cDNA was synthesized with SuperScript III (Invitrogen) using oligo dT primer. STLV-1 *tax* and SBZ was detected by PCR using primers (see Additional file 4) from the synthesized Si-2 cDNA: for STLV-1 *tax*, 2 min at 95°C, followed by 35 cycles of 20 seconds at 95°C, 10 seconds at 61°C, and 30 seconds at 72°C, and additional 5 min at 72°C; for SBZ, 2 min at 95°C, followed by 35 cycles of 20 seconds at 95°C, 10 seconds at 58°C, and 30 seconds at 72°C, and additional 5 min at 72°C. For comparison, HTLV-1 *tax* and *HBZ* were also amplified by PCR using cDNA of HTLV-1-infected cell lines (MT-1 or MT-2) with the same conditions. The primers used are shown in Additional file 4.

### Plasmids

The PathDetect pNFκB-Luc, pAP-1-Luc and pNFAT-Luc plasmids were purchased from Stratagene. The 3TP-Lux, TopFlash reporter plasmids and WT-Luc were described previously [22,49]. The coding sequences of STLV-1 Tax and SBZ were amplified from STLV-1 provirus using oligos (see Additional file 4) and cloned into pME18Sneo to generate expression plasmids of STLV-1 Tax and SBZ. HTLV-1 *tax* was amplified using flanking primers (see Additional file 4) from pCGTax [50] and subcloned into pME18Sneo. The expression vector of HBZ cloned into pME18Sneo was described previously [11]. For the reporter assay, Jurkat cells or HepG2 cells were co-transfected with the reporter plasmid and the viral protein expression plasmids specified in each experiment, as previously described [22,24,51]. The activity of firefly luciferase was represented by normalizing to that of Renilla luciferase.

### Retroviral vectors

The SBZ coding fragment was inserted into pGCDNSamI/N utilizing the NotI and SalI sites and SBZ-expressing retroviral vector was prepared as described previously [22].

### Transduction of primary T-cells with retroviral vectors

CD4<sup>+</sup>CD25<sup>-</sup> mouse T lymphocytes were stimulated and transduced with SBZ-expressing retroviral vector as previously described [22]. Forty-eight hours after the transduction, cells were harvested and analyzed by flow cytometry.

### Flow cytometry

Antibodies used in this study were as follows: anti-human CD4 (OKT4), anti-Tax MI-73 [52], anti-mouse CD4 (RM4-5), anti-human CD271 (NGFR) (C40-1457), anti-mouse Foxp3 (FJK-16s), anti-human CD3 (SP34-2) and anti-human CCR4 (1G1, which recognizes a different epitope from that recognized by mogamulizumab). Intracellular staining was performed as previously described for Tax [52] and Foxp3 [22]. Cells were analyzed

by BD FACSCanto II with FACS Diva Software (BD Biosciences) or BD FACSVerse with FACSuite software (BD Biosciences).

#### Deep sequencing of provirus integration sites

The provirus integration sites in the Japanese macaque genome were amplified by linker-mediated PCR as previously described [27], with some modifications. Japanese macaque PBMC genomic DNA (3 µg) was sheared by sonication with a Bioruptor UCD-200 TM to obtain DNA fragments of approximately 200–500 bp. The ends of the DNA fragments were repaired to generate blunt ends using 18 units of T4 DNA polymerase, 5.3 units of DNA Klenow Polymerase I and 18 units of T4 polynucleotide kinase (TOYOBO) in T4 DNA ligase buffer (NEB) supplemented with 300 µM each of dNTP (TAKARA Bio). Adenine nucleotides were added to the blunt ends, and then linkers were ligated using 24 units of T4 DNA ligase (TOYOBO) in T4 DNA ligase buffer (NEB) utilizing the overhang of one thymidine nucleotide at the 3' end of the linker. The linker was generated by annealing two oligonucleotides (see Additional file 4). The first round of PCR was performed with the primers, STLV-1 Bio5 and Bio4. STLV-1 Bio5 anneals to the sequence within LTR of the STLV-1 provirus and Bio4 is the sequence present in the linker (see Additional file 4). Then, nested PCR was performed with the primers, Ion A-Bio7 and P1. In Ion A-Bio7, uppercase letters denote the sequence that anneals to the viral LTR downstream of STLV-1 Bio5, whereas the sequence in lowercase letters represents a tag specific for the Ion Torrent Personal Genome Machine (Ion PGM). P1 is also a tag specific for Ion PGM, which appears in the linker sequence (see Additional file 4). The amplification conditions of both the first and second PCR were 96°C for 30 sec, 7 cycles of 94°C for 5 sec and 72°C for 1 min, 23 cycles of 94°C for 5 sec and 68°C for 1 min, followed by additional 68°C for 9 min. Amplified fragments of approximately 150–300 bp were size-selected with E-Gel SizeSelect Agarose Gel (Life Technologies) and used as a DNA library in subsequent deep sequencing. Template beads to be sequenced with Ion Torrent Personal Genome Machine (Ion PGM) were prepared with the DNA library using the Ion PGM 200 Xpress Template Kit (Applied Biosystems) and subjected to sequencing on Ion Torrent 314 or 316 semiconductor chip using Ion PGM 200 Sequencing Kit (Applied Biosystems).

#### Deep sequencing data analysis

The host genomic sequences, located between the region immediately adjacent to the viral 3' LTR (ACACA) and the linker sequence (AGATCG), were extracted from the reads. Reads that started with GTTGGG (viral 5' LTR) were removed. Remaining reads were mapped to the reference genome of *Macaca mulatta* (MMUL 1.0) using the Burrows-Wheeler Aligner (BWA) [53]. Reads that

were mapped only to single sites were analyzed. In order to obtain the absolute frequency of each provirus clone (the number of sister cells of the clone), the end position of each mapped read was obtained from the start position and cigar code in the SAM file generated by BWA. The reads with an identical start position and end position (integration site and shear site) were judged to derive from a single DNA fragment amplified by PCR, while reads with identical integration sites but distinct shear sites were judged to derive from different cells in a clone. In other words, the number of reads in the second category reflects the absolute frequency of each clone. Relative frequency represents the proportion of the absolute frequency of a clone to the number of all the sister cells observed. In order to minimize the distortion of relative frequencies of major clones, 6,000 reads that were mapped only to single sites were randomly selected for each specimen and analyzed (see Additional file 2).

#### Treatment of STLV-1<sup>+</sup> Japanese macaques with humanized anti-CCR4 antibody

Two Japanese macaques infected with STLV-1 were treated with mogamulizumab, which is an antibody against CCR4 and is approved in Japan as a drug to treat relapsed ATL. Mogamulizumab was provided by Kyowa Hakko Kirin Co Ltd. One mg/kg mogamulizumab was diluted in 40 ml saline and infused into each monkey intravenously for 20 min. Administration was performed once a week for 4 times. Before each administration, a 10 ml of blood sample was obtained. After the fourth administration, blood samples were collected every 2 weeks until week 11. Extra samples were collected on week 15 and week 18. The two monkeys were observed for any adverse effects during the experiment.

#### Additional files

**Additional file 1:** Phylogenetic analyses of HTLV-1 subtypes and Japanese macaque STLV-1.

**Additional file 2:** Deep sequencing data analysis.

**Additional file 3:** In vitro staining of Japanese macaque PBMCs with mogamulizumab.

**Additional file 4:** Primers and oligonucleotides.

#### Competing interests

Kyowa Hakko Kirin provided us the monoclonal antibody (mogamulizumab) that was used in this study.

#### Authors' contributions

JY and M. Matsuoka conceived of this study. JT carried out antibody screening and proviral load measurement. M. Miura, KS, GM and TZ carried out the molecular experiments and the reporter assays. AK, AW, AS and HA coordinated the macaque experiments and collected the macaque specimens. PM analyzed viral protein and surface marker expression. KO carried out immunohistochemistry and pathological analyses. M. Miura carried out massive sequencing and its data analysis. M. Miura, JY and M.

Matsuoka prepared the manuscript. All the authors approved the final manuscript.

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# HTLV-1 bZIP factor supports proliferation of adult T cell leukemia cells through suppression of C/EBP $\alpha$ signaling

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## Abstract

**Background:** Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus etiologically associated with adult T-cell leukemia (ATL). The HTLV-1 bZIP factor (HBZ), which is encoded by minus strand of provirus, is expressed in all ATL cases and supports the proliferation of ATL cells. However, the precise mechanism of growth promoting activity of HBZ is poorly understood.

**Results:** In this study, we showed that HBZ suppressed C/EBP $\alpha$  signaling activation induced by either Tax or C/EBP $\alpha$ . As mechanisms of HBZ-mediated C/EBP $\alpha$  inhibition, we found that HBZ physically interacted with C/EBP $\alpha$  and diminished its DNA binding capacity. Luciferase and immunoprecipitation assays revealed that HBZ repressed C/EBP $\alpha$  activation in a Smad3-dependent manner. In addition, C/EBP $\alpha$  was overexpressed in HTLV-1 infected cell lines and fresh ATL cases. HBZ was able to induce C/EBP $\alpha$  transcription by enhancing its promoter activity. Finally, HBZ selectively modulated the expression of C/EBP $\alpha$  target genes, leading to the impairment of C/EBP $\alpha$ -mediated cell growth suppression.

**Conclusion:** HBZ, by suppressing C/EBP $\alpha$  signaling, supports the proliferation of HTLV-1 infected cells, which is thought to be critical for oncogenesis.

**Keywords:** HTLV-1, HBZ, C/EBP $\alpha$

## Background

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL) [1,2]. HTLV-1 encodes several regulatory (*tax* and *rex*) and accessory (*p12*, *p13* and *p30*) genes in the pX region located between the *env* and 3' long terminal repeat (LTR) [3]. Among the viral genes, Tax is thought to play a central role in the pathogenesis of HTLV-1 [4]. Yet the expression of Tax cannot be detected in ~60% of fresh ATL cases due to epigenetic modifications or deletion of the 5'LTR [5]. In contrast, the *HTLV-1 bZIP factor* (HBZ), which is encoded by the minus strand of the HTLV-1 genome, is expressed in all ATL cases and supports the proliferation of HTLV-1 infected cells [6-8].

HTLV-2, a type of retrovirus which is similar with HTLV-1, encodes an antisense protein (APH-2) using the minus strand of its genome. However, APH-2 does not seem to promote cell proliferation [9,10]. HBZ was reported to repress Tax-mediated transactivation of viral transcription from the HTLV-1 5'LTR [11]. Moreover, HBZ dysregulated multiple cellular signalings including the classical pathway of NF- $\kappa$ B, TGF- $\beta$ , AP-1, and the Wnt pathways, which is likely to contribute to viral persistence and clonal expansion of infected cells [12-15].

The CCAAT/enhancer binding protein (C/EBP) family of proteins represents a critical group of bZIP transcription factors that are key to the regulation of cell proliferation, development, and immune responses [16,17]. Dysregulated C/EBP signaling is intimately associated with tumorigenesis and viral diseases [18]. Furthermore, the ability of C/EBPs to direct cellular fate is thought to depend on the presence of specific collaborating transcription factors, and allows C/EBPs to act as both tumor suppressors and tumor promoters under different conditions [17]. C/EBP $\alpha$ , the

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founding member of this family, has been demonstrated to be important for differentiation of several cell types [19]. On the other hand, C/EBP $\alpha$  emerged as an important negative regulator of cell proliferation [20]. Thus, most tumors have evolved distinct strategies to attenuate C/EBP $\alpha$  function [17,21]. Known mechanisms of C/EBP $\alpha$  suppression in cancer cells include (1) transcriptional downregulation of *CEBPA* expression; (2) point mutations and deletions in C/EBP $\alpha$ ; and (3) inhibition of C/EBP $\alpha$  transcriptional activation through protein-protein interaction. However, normal C/EBP $\alpha$  is overexpressed in B-cell precursor acute lymphoblastic leukemia (BCP-ALL), and inhibits apoptosis by upregulating *bcl-2* and *FLIP* expression [22,23]. It suggested that C/EBP $\alpha$  may exhibit oncogenic as well as tumour suppressor properties in human leukaemogenesis.

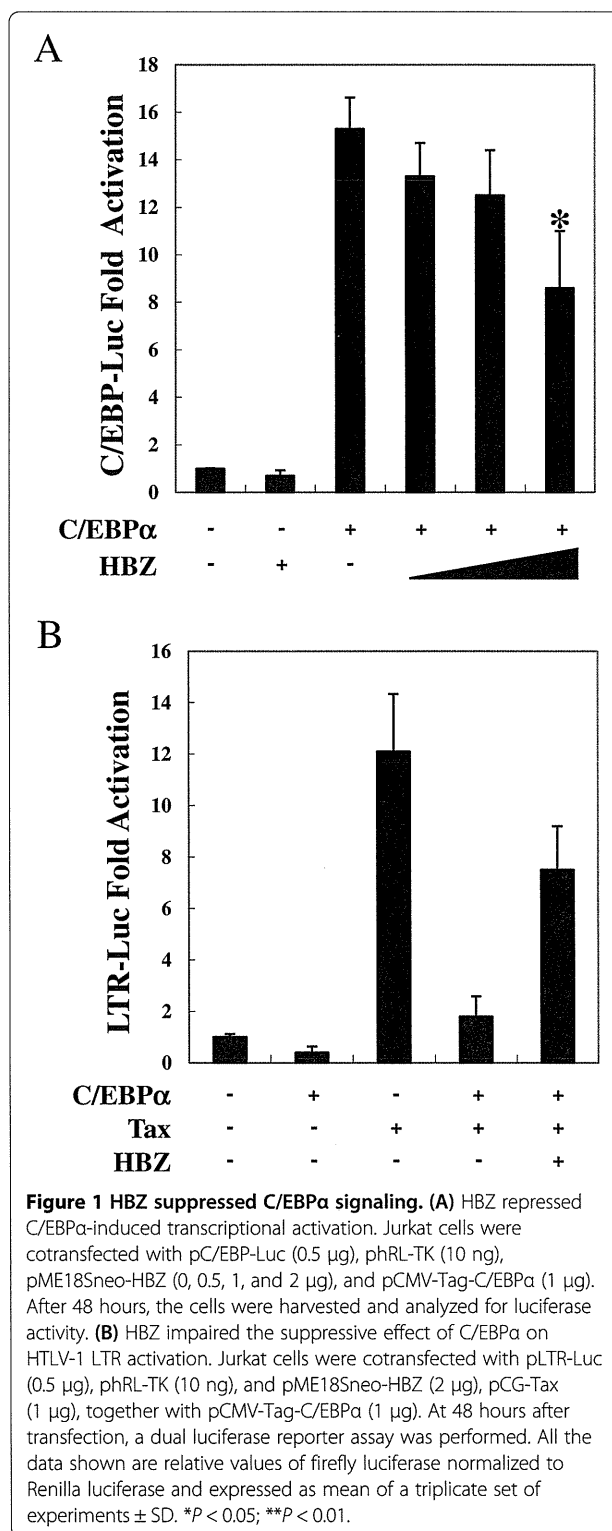
In ATL, Tax has been shown to bind to CCAAT binding proteins such as nuclear factor YB subunit (NF-YB) and C/EBP $\beta$  [24]. Through its association with NF-YB, Tax activates the major histocompatibility complex class II (MHC-II) promoter [24]. Additionally, C/EBP $\beta$  was capable of inhibiting Tax-dependent transactivation of the HTLV-1 LTR, as well as efficiently decreasing Tax synthesis from an infectious HTLV-1 molecular clone [25]. On the other hand, expression of Tax increases binding of C/EBP $\beta$  to and activates the IL-1 $\beta$  promoter [26]. Interestingly, previously published microarray data showed that the *CEBPA* gene was overexpressed in adult T-cell leukemia cells [27,28]. It is thus likely that the dysregulated C/EBP signaling pathway may play a role in ATL.

Although regulation of C/EBP signaling by Tax has been reported, little is known about whether other viral proteins affect C/EBP signaling. In the present study, we found that HBZ suppressed C/EBP signaling by interacting with C/EBP $\alpha$ , resulting in the impairment of C/EBP $\alpha$ -mediated cell growth suppression. This might account for why HBZ supports the proliferation of HTLV-1 infected cells.

## Results

### HBZ suppresses C/EBP $\alpha$ signaling

To investigate the effect of HBZ on the C/EBP signaling pathway, Jurkat cells were cotransfected with expression vectors of C/EBP $\alpha$  and HBZ along with a C/EBP-responsive reporter: C/EBP-Luc. As shown in Figure 1A, C/EBP $\alpha$  enhanced the transcription of luciferase, while HBZ inhibited C/EBP $\alpha$ -mediated C/EBP signaling activation in a dose-dependent manner. It was reported that C/EBP transcription factors dysregulated transcription from long terminal repeat [25]. We therefore analyzed whether HBZ could modulate HTLV-1 promoter activity through C/EBP signaling. Consistent with previous reports, overexpression of C/EBP $\alpha$  inhibited Tax-mediated



HTLV-1 LTR activation [29]. Moreover, HBZ overcame the repression of HTLV-1 viral transcription by C/EBP $\alpha$  (Figure 1B). These results collectively indicate that HBZ impairs the function of C/EBP $\alpha$ .

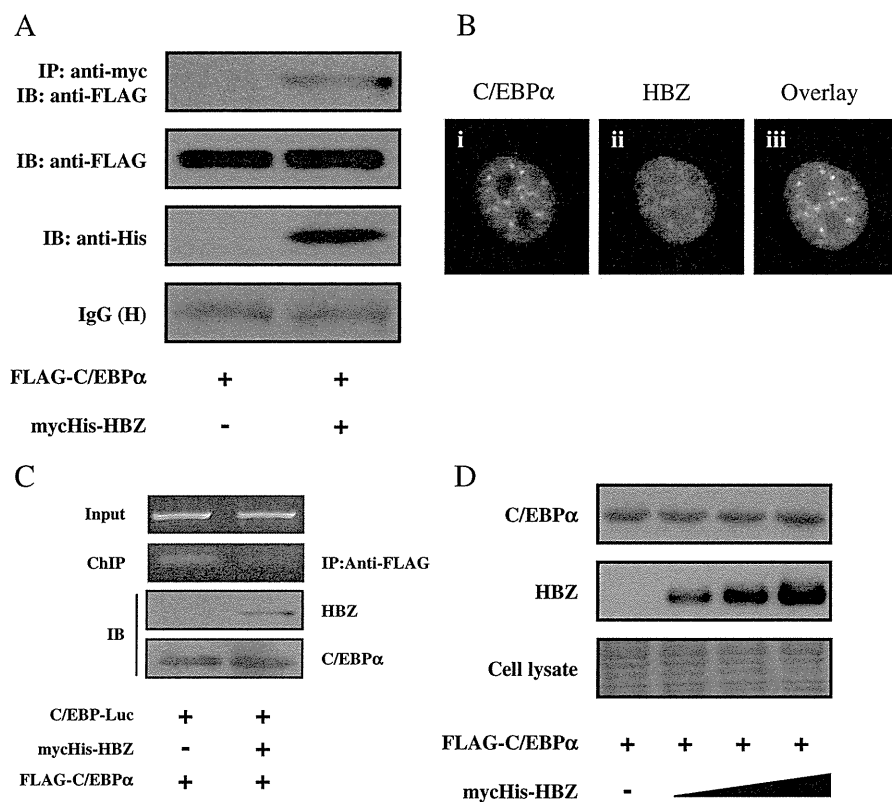
### HBZ interacts with C/EBP $\alpha$

Accumulating evidences show that HBZ dysregulates signaling pathways in ATL by associating with multiple transcriptional factors [8,12-15,30,31]. To clarify the molecular mechanism by which HBZ suppresses the C/EBP $\alpha$  transcriptional response, we investigated whether HBZ can physically interact with C/EBP $\alpha$ . FLAG-tagged C/EBP $\alpha$  and mycHis-tagged HBZ were cotransfected into 293T cells, and an immunoprecipitation assay was performed. Figure 2A illustrates that HBZ interacted with C/EBP $\alpha$ . The HBZ-C/EBP $\alpha$  association was further analyzed by confocal microscopy. Cotransfected cells showed nuclear spots representing co-localization of HBZ and C/EBP $\alpha$  protein (Figure 2B). To investigate whether HBZ influences the ability of C/EBP $\alpha$  to bind its DNA target, we performed a ChIP assay in 293T cells that were cotransfected with C/EBP-Luc reporter together with expression vectors of HBZ and C/EBP $\alpha$ .

The ChIP assay detected the association of C/EBP $\alpha$  with its responsive elements, while HBZ dramatically decreased C/EBP $\alpha$ 's DNA binding capability (Figure 2C). Previous reports showed that HBZ decreased the expression level of its associated proteins [12,13]. Therefore, we analyzed whether HBZ could also affect the expression of C/EBP $\alpha$ . As shown in Figure 2D, HBZ did not induce C/EBP $\alpha$  protein degradation even at high doses. In addition, C/EBP $\alpha$  did not influence HBZ expression (Additional file 1: Figure S1). These observations suggest that HBZ represses C/EBP $\alpha$ -induced transcription through physical association between HBZ and C/EBP $\alpha$ .

### HBZ depends on Smad3 to inhibit C/EBP $\alpha$ -mediated transcription

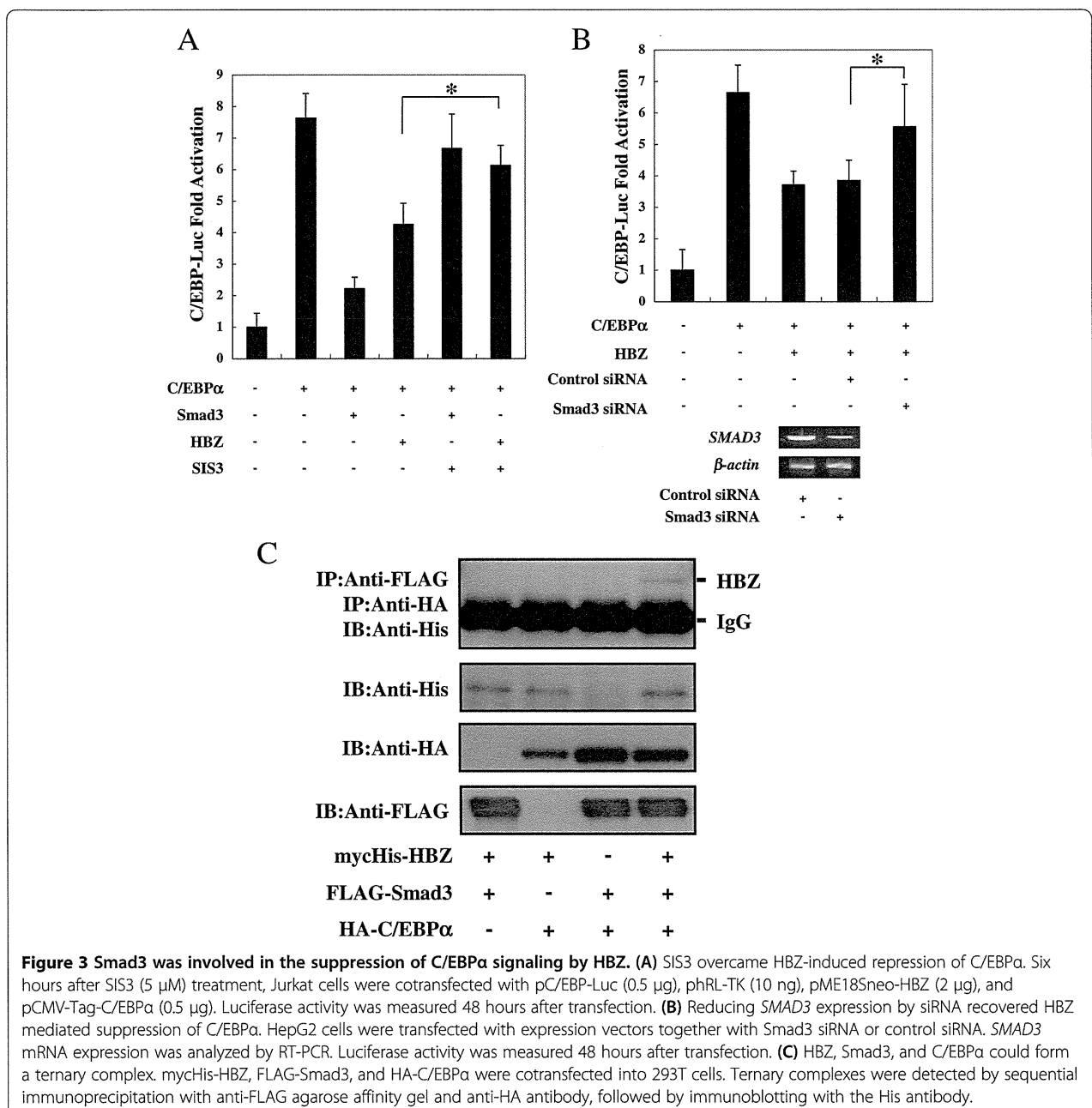
Several reports have indicated that Smad3 interacted with C/EBP and repressed C/EBP transactivation function [32,33]. Moreover, HBZ could enhance the Smad3-



**Figure 2** HBZ interacted with C/EBP $\alpha$  protein. **(A)** HBZ interacted with C/EBP $\alpha$ . 293T cells were cotransfected with mycHis-HBZ together with FLAG-C/EBP $\alpha$ . After 48 hours, cell lysates were subjected to immunoprecipitation using anti-c-Myc followed by immunoblotting using anti-FLAG. **(B)** HBZ co-localized with C/EBP $\alpha$ . HeLa cells were transfected with mycHis-HBZ and FLAG-C/EBP $\alpha$ . HBZ was detected using anti-MYC Cy3 antibody (i). C/EBP $\alpha$  was detected using anti-Flag-biotin and secondary Streptavidin-Alexa 488 antibody (ii). The overlay of HBZ and C/EBP $\alpha$  is shown (iii). **(C)** HBZ decreased C/EBP $\alpha$ 's DNA binding capability. After transfection with mycHis-HBZ, FLAG-C/EBP $\alpha$ , and pC/EBP $\alpha$ -Luc for 48 hours, 293T cells were chromatin immunoprecipitated by anti-FLAG antibody. The precipitated DNAs and 1% of the input cell lysates were amplified by the pC/EBP-Luc specific primers. Expression of HBZ and C/EBP $\alpha$  was detected by Western blot (bottom panel). **(D)** HBZ could not repress the level of C/EBP $\alpha$ . 293T cells were transfected with expression vector of C/EBP $\alpha$  and various amounts of mycHis-HBZ. After 48 hours, the cell lysates were subjected to immunoblotting.

mediated TGF- $\beta$  pathway [14]. To determine whether Smad3 is required for HBZ to suppress C/EBP $\alpha$ , we analyzed the effect of SIS3, an inhibitor of Smad3, on the ability of HBZ to inhibit C/EBP $\alpha$  transcriptional activity. Figure 3A demonstrates that SIS3 impaired the ability of HBZ to suppress transcriptional activity through C/EBP-responsive elements. In addition, when Smad3 expression was inhibited by siRNA, the HBZ-mediated suppression of C/EBP $\alpha$  activity was partially restored, indicating that Smad3 functions to suppress

C/EBP $\alpha$  signaling along with HBZ (Figure 3B). We next explored whether HBZ, C/EBP $\alpha$ , and Smad3 could form a ternary complex. Vectors expressing mycHis-HBZ, FLAG-Smad3, and HA-C/EBP $\alpha$  were cotransfected into 293T cells, and a serial immunoprecipitation assay was performed. As shown in Figure 3C, and Additional file 2: Figure S2, we detected a specific ternary complex only when the three components were coexpressed. These results together suggest that HBZ inhibits C/EBP $\alpha$  signaling by forming complexes of HBZ-Smad3-C/EBP $\alpha$ .

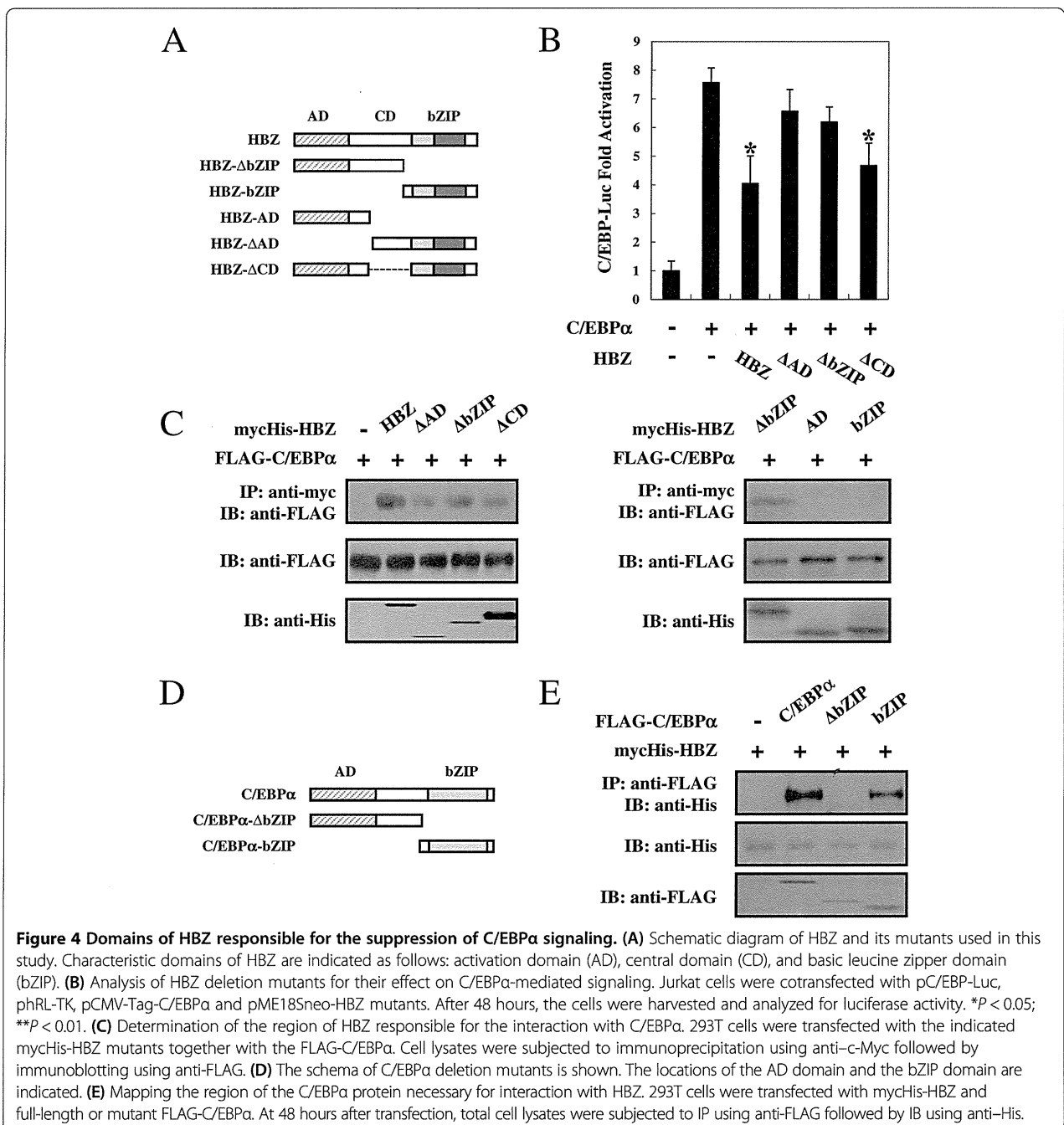


**Figure 3 Smad3 was involved in the suppression of C/EBP $\alpha$  signaling by HBZ. (A)** SIS3 overcame HBZ-induced repression of C/EBP $\alpha$ . Six hours after SIS3 (5  $\mu$ M) treatment, Jurkat cells were cotransfected with pC/EBP-Luc (0.5  $\mu$ g), pRL-TK (10 ng), pME18Sneo-HBZ (2  $\mu$ g), and pCMV-Tag-C/EBP $\alpha$  (0.5  $\mu$ g). Luciferase activity was measured 48 hours after transfection. **(B)** Reducing SMAD3 expression by siRNA recovered HBZ mediated suppression of C/EBP $\alpha$ . HepG2 cells were transfected with expression vectors together with Smad3 siRNA or control siRNA. SMAD3 mRNA expression was analyzed by RT-PCR. Luciferase activity was measured 48 hours after transfection. **(C)** HBZ, Smad3, and C/EBP $\alpha$  could form a ternary complex. mycHis-HBZ, FLAG-Smad3, and HA-C/EBP $\alpha$  were cotransfected into 293T cells. Ternary complexes were detected by sequential immunoprecipitation with anti-FLAG agarose affinity gel and anti-HA antibody, followed by immunoblotting with the His antibody.

**Domains of HBZ responsible for suppression of C/EBP $\alpha$**

Next, we evaluated the region of HBZ responsible for the inhibition of C/EBP signaling. To this end, we tested the HBZ deletion mutants shown in Figure 4A. Figure 4B demonstrated that wild-type HBZ down-regulated C/EBP $\alpha$ -mediated transcriptional responses. Compared with other mutants, only the HBZ  $\Delta$ CD mutant exhibited suppressive activity. We mapped the region of HBZ interacting with C/EBP $\alpha$  in detail. As shown in Figure 4C, full-length HBZ and three of its deletion mutants (HBZ- $\Delta$ AD,

HBZ- $\Delta$ bZIP, and HBZ- $\Delta$ CD) associated with C/EBP $\alpha$ , while HBZ-AD and HBZ-bZIP have no binding capability. These results collectively indicate that both the AD and bZIP domains in HBZ were necessary for suppression of the C/EBP $\alpha$  pathway. To define which part of C/EBP $\alpha$  binds HBZ, we performed a co-immunoprecipitation assay with C/EBP $\alpha$  mutants (Figure 4D). The C/EBP $\alpha$ - $\Delta$ bZIP mutant, which did not contain the bZIP domain, was incapable of interacting with HBZ. However, the mutant containing only the bZIP domain of C/EBP $\alpha$  still



interacted efficiently with HBZ protein. Thus, the interaction with HBZ is mediated by the bZIP segment of C/EBP $\alpha$  (Figure 4E).

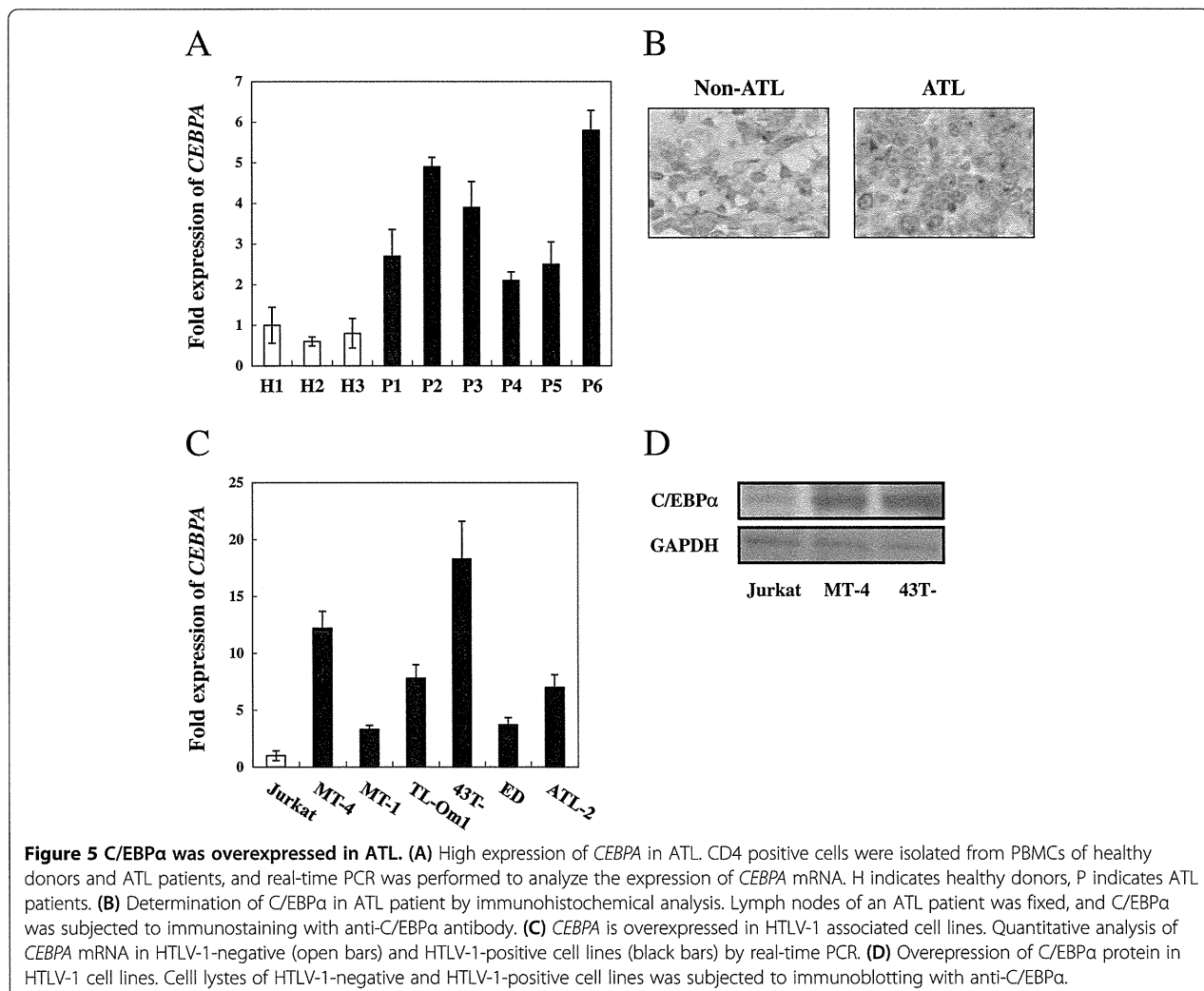
#### C/EBP $\alpha$ is overexpressed in ATL

We next checked the expression level of *CEBPA* mRNA and protein in ATL. Three healthy donors and six ATL patients with different age and disease status were included in this study. CD4 positive cells were isolated from PBMCs of the clinical samples, and real-time PCR was performed to analyze the expression of *CEBPA* mRNA. Compared with normal T cells, all ATL patients constitutively expressed *CEBPA* transcript. Noticeably, the three youngest patients who suffered from acute ATL expressed higher levels of *CEBPA* compared with the other three patients (Figure 5A). Immunohistochemical analysis of lymph nodes of ATL patients showed that lymphoma cells indeed expressed C/EBP $\alpha$  (Figure 5B). Quantitative analyses revealed increased expression of

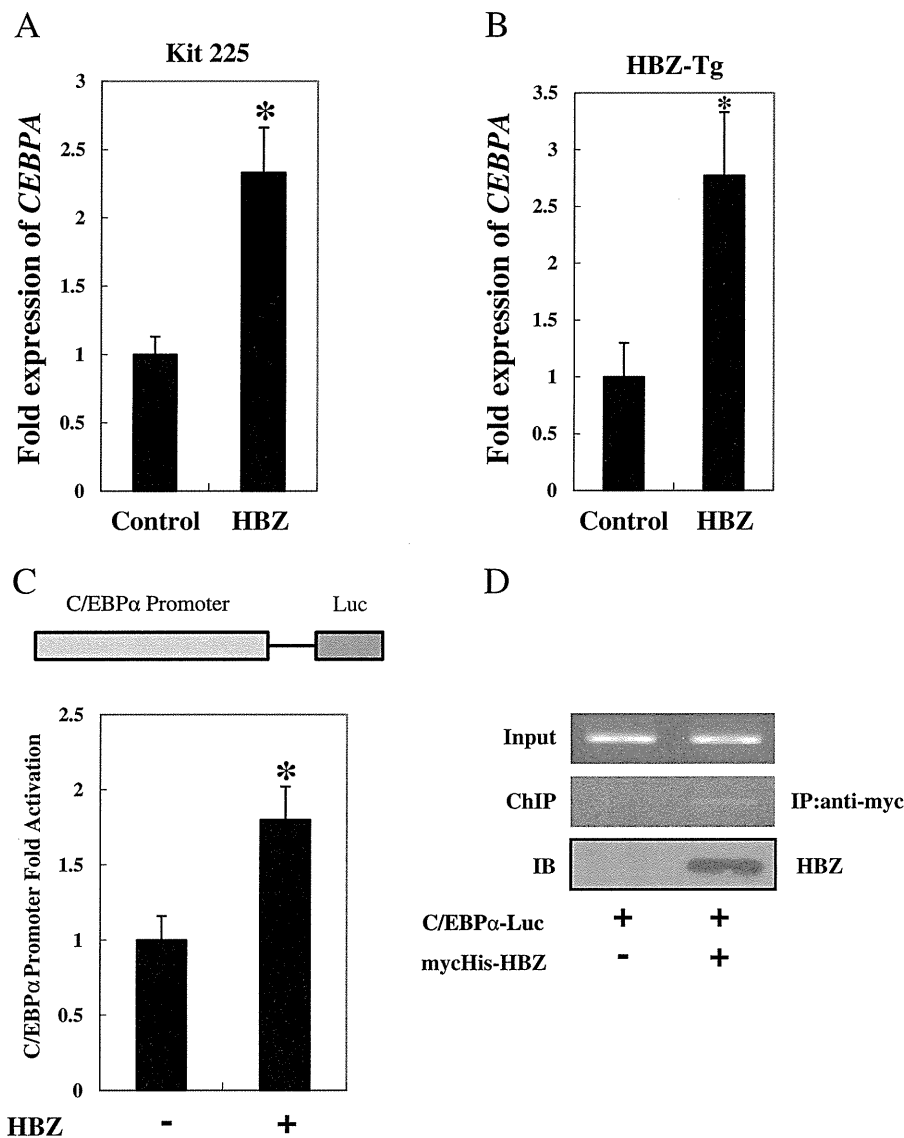
*CEBPA* in HTLV-1-infected cell lines compared with noninfected ones (Figure 5C). Moreover, high levels of C/EBP $\alpha$  protein were detected in ATL cell lines (Figure 5D).

#### C/EBP $\alpha$ expression is induced by HBZ

It is well established that HBZ is the only viral gene that remains intact and is constitutively expressed in all ATL cases [34]. Considering that the level of C/EBP $\alpha$  is elevated in ATL and HTLV-1 associated cell lines, we evaluated whether HBZ controlled the excess expression of C/EBP $\alpha$ . As shown in Figure 6A, the *CEBPA* gene was upregulated in Kit 225 cells, which stably express HBZ. To investigate HBZ-mediated enhancement of *CEBPA* expression *in vivo*, we studied the level of *CEBPA* in splenic CD4<sup>+</sup> T cells from HBZ transgenic mice. Consistently, expression of *CEBPA* was upregulated in HBZ transgenic mice as observed *in vitro* (Figure 6B). We further analyzed the mechanism by which HBZ induced



**Figure 5 C/EBP $\alpha$  was overexpressed in ATL.** (A) High expression of *CEBPA* in ATL. CD4 positive cells were isolated from PBMCs of healthy donors and ATL patients, and real-time PCR was performed to analyze the expression of *CEBPA* mRNA. H indicates healthy donors, P indicates ATL patients. (B) Determination of C/EBP $\alpha$  in ATL patient by immunohistochemical analysis. Lymph nodes of an ATL patient was fixed, and C/EBP $\alpha$  was subjected to immunostaining with anti-C/EBP $\alpha$  antibody. (C) *CEBPA* is overexpressed in HTLV-1 associated cell lines. Quantitative analysis of *CEBPA* mRNA in HTLV-1-negative (open bars) and HTLV-1-positive cell lines (black bars) by real-time PCR. (D) Overexpression of C/EBP $\alpha$  protein in HTLV-1 cell lines. Cell lysates of HTLV-1-negative and HTLV-1-positive cell lines was subjected to immunoblotting with anti-C/EBP $\alpha$ .

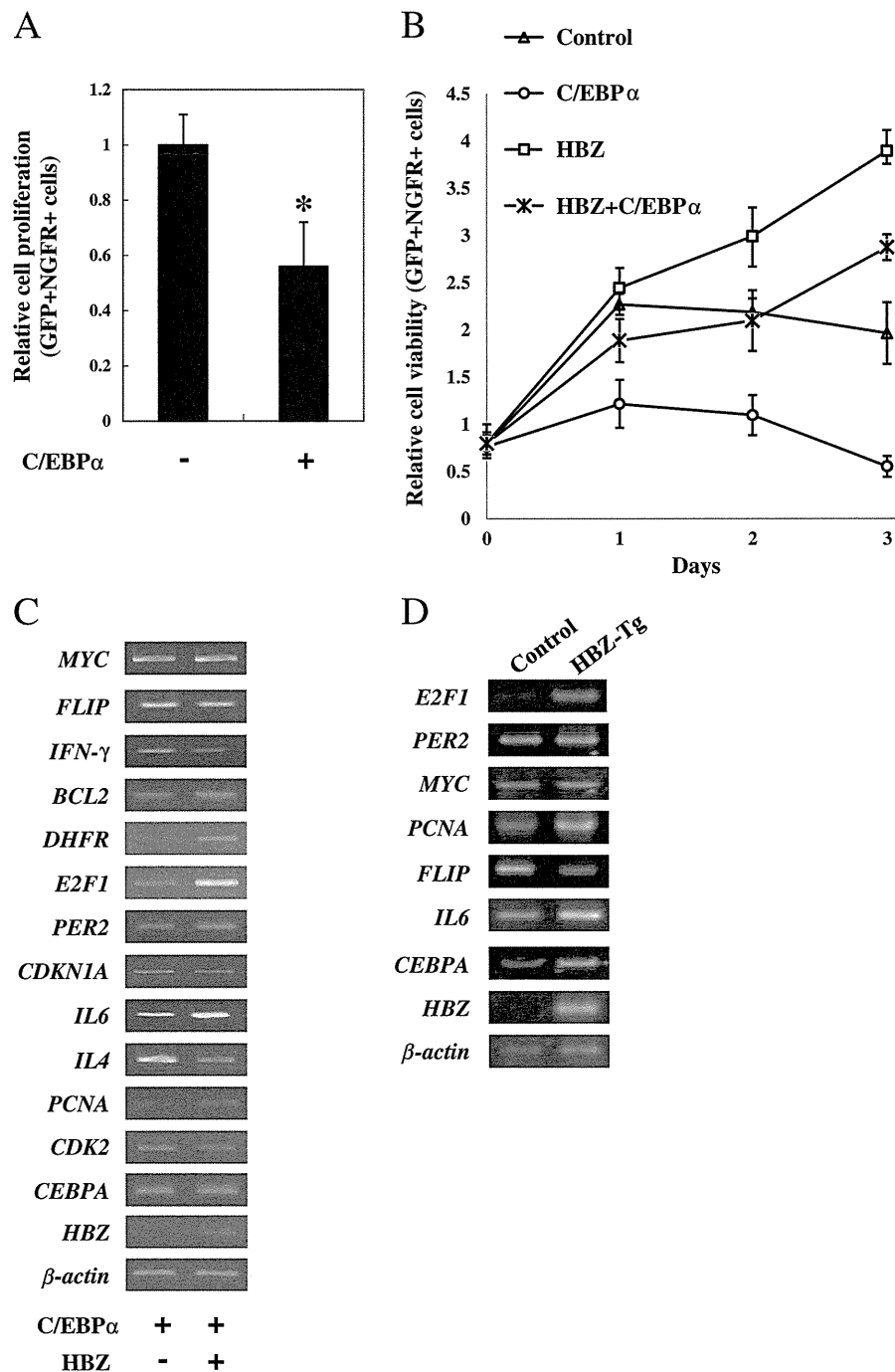


**Figure 6 HBZ induced C/EBPα expression.** Total RNA was extracted from control or HBZ-expressing Kit 225 (A) and CD4<sup>+</sup> cells of HBZ transgenic mice (B). Real-time PCR was performed to analyze the expression of *CEBPA* mRNA. (C) HBZ activated transcription of the *CEBPA* promoter. 293T cells were transfected with the *C/EBPα* reporter plasmid with or without the HBZ-expressing plasmid. Luciferase activity was measured 48 hours after transfection. (D) HBZ binds to the *C/EBPα* promoter. After transfection with mycHis-HBZ and *C/EBPα* reporter vector for 48 hours, 293T cells were chromatin immunoprecipitated by anti-c-Myc antibody. The precipitated DNAs and 1% of the input cell lysates were amplified by the specific primers for *CEBPA* promoter.

*C/EBPα* expression. The 2-kb fragment of the *CEBPA* promoter region was cloned into the pGL4.10 reporter vector and a luciferase assay was performed. As shown in Figure 6C, HBZ enhanced transcription from the *CEBPA* promoter. In addition, a chromatin immunoprecipitation assay detected HBZ bound to the *CEBPA* promoter (Figure 6D). These results collectively indicate that the enhanced induction of *CEBPA* expression by HBZ can be attributed, at least in part, to the association of HBZ with the *CEBPA* promoter.

#### HBZ overcomes *C/EBPα*-mediated suppression of T-cell proliferation

Previous studies have shown that *C/EBPα* inhibits cell proliferation and induces cell cycle arrest [17]. We confirmed that the growth of mouse CD4<sup>+</sup> T cells was inhibited by enforced expression of *C/EBPα* (Figure 7A). To address whether HBZ could affect cell proliferation by suppressing *C/EBPα* signaling, we overexpressed HBZ and *C/EBPα* in primary mouse CD4<sup>+</sup> T cells. Figure 7B demonstrated that *C/EBPα* repressed T cell



**Figure 7** HBZ overcame the C/EBPα-mediated growth suppression. **(A)** Mouse CD4<sup>+</sup>CD25<sup>-</sup> T cells were transduced with pGCDNsaml/GFP vector encoding C/EBPα, or with empty vector. At three days after infection, cell proliferation was analyzed by flow cytometry. **(B)** Mouse CD4<sup>+</sup>CD25<sup>-</sup> T cells were transduced with pGCDNsaml/NGFR vector encoding HBZ together with pGCDNsaml/GFP-C/EBPα. Cells were stained with specific antibody at the time points indicated, and cell growth was detected by flow cytometry. Representative data from three independent experiments are shown. **(C)** HBZ modulated the expression of selected C/EBPα target genes. Total RNA was extracted from samples from the experiment of Figure 7B. The level of MYC, FLIP, IFN-γ, BCL2, DHFR, E2F1, PER2, CDKN1A, IL6, IL4, PCNA, CDK2, β-actin, CEBPA, and HBZ mRNA were analyzed by semiquantitative RT-PCR. **(D)** Transcriptional changes of selected C/EBPα target genes in CD4<sup>+</sup> thymocytes from HBZ transgenic mice. After stimulating the cells with PMA plus ionomycin, the levels of MYC, FLIP, E2F1, PER2, IL6, PCNA, β-actin, CEBPA, and HBZ mRNA were analyzed by semiquantitative RT-PCR.

proliferation, whereas HBZ-expressing cells proliferated regardless of C/EBP $\alpha$ . We next studied the effect of HBZ on transcription of C/EBP $\alpha$ -specific target genes using mouse naïve T cells expressing HBZ. Previous reports showed that C/EBP $\alpha$  suppressed cell proliferation by inhibiting the expression of *E2F1*, *DHFR*, and *PCNA*. When co-expressed with C/EBP $\alpha$ , HBZ enhanced *E2F1*, *DHFR*, *PCNA*, *FLIP*, *BCL2*, *IL6*, and suppressed *IL4* and *IFN- $\gamma$*  (Figure 7C). This indicated that HBZ overcame the suppressive effect of C/EBP $\alpha$  on its target genes, leading to the cell growth. To investigate HBZ-mediated suppression of C/EBP $\alpha$  signaling *in vivo*, we studied the expression of C/EBP $\alpha$ -specific target genes in thymus CD4<sup>+</sup> cells from HBZ transgenic mice. As shown in Figure 7D, expression of HBZ was associated with enhanced transcription of *CEBPA*, *E2F1*, *PCNA*, and *IL6* genes and suppression of *FLIP* gene; such effects were consistent with the observation in HBZ transfected naïve T cells.

These results together indicate that HBZ supports the proliferation of T cells through dysregulation of C/EBP $\alpha$  signaling as well as selective modulation of transcription of C/EBP $\alpha$  target genes.

## Discussion

After transmission, HTLV-1 increases its viral copy number by clonal proliferation of infected cells and results in the onset of ATL [5,35]. In this strategy, Tax was thought to play a critical role in increasing the number of HTLV-1-infected cells by promoting proliferation and inhibiting apoptosis [36,37]. However, because Tax is the major target of cytotoxic T lymphocytes (CTLs), it is frequently inactivated by genetic and epigenetic modifications [5,38]. Therefore, HTLV-1 has evolved mechanisms to maintain cell survival in a Tax-independent manner. We have reported that HBZ, which is consistently expressed in ATL, promotes the proliferation of T-lymphocytes *in vitro*, and increases splenic CD4<sup>+</sup> T-cells in HBZ transgenic mice, indicating a role for HBZ, like tax, in the proliferation of HTLV-1 infected cells [7,31]. So far, the mechanism by which HBZ promotes proliferation of leukemic cells has not been well elucidated. Accumulating evidence shows that C/EBP $\alpha$  possesses the ability to arrest cell proliferation through upregulation of CDKN1A (p21) as well as direct inhibition of E2F [39]. We firstly present evidence that C/EBP $\alpha$  is highly expressed in ATL. However, C/EBP $\alpha$ 's growth-suppression function is impaired by HBZ, resulting in the proliferation of ATL cells despite C/EBP $\alpha$  expression. It is thus likely that HBZ may support the proliferation of HTLV-1 infected cells, whereas other mechanisms, which include dysregulation of C/EBP $\alpha$  signaling and selectively modulate C/EBP $\alpha$  target gene expression. In support of our hypothesis, we showed in this study that HBZ enhanced the expression of *E2F1*,

*PCNA*, and *DHFR* genes in C/EBP $\alpha$ -expressing cells and did not interfere with *MYC*, *CDKN1A*, and *CDK2* expression, contrary to the effect of C/EBP $\alpha$  alone [7].

Apart from the growth suppression function, C/EBP family proteins have oncogenic properties [17,21]. Consistent with our findings, recent studies reported that overexpression of C/EBP $\alpha$  occurs in cancer, such as B precursor acute lymphoblastic leukemia (ALL) and a subset of human hepatocellular carcinomas (HCCs) [22,40]. Importantly, C/EBP $\alpha$  induces *BCL2* and *FLIP* gene expression in cooperation with NF- $\kappa$ B p50, allowing cancer cells to escape apoptosis [23]. We showed here that C/EBP $\alpha$  was overexpressed in ATL, whereas its growth-suppressive function was impaired by the effect of HBZ. In this regard, it is meaningful to raise the question: why do ATL cells need high levels of C/EBP $\alpha$ ? It has been reported that HBZ suppressed apoptosis of HTLV-1 infected cells, while the underlying mechanism is still unknown. As shown in Figure 7C, HBZ selectively suppressed the level of C/EBP $\alpha$  target genes which related with cell growth, but did not inhibit the C/EBP $\alpha$ -induced expression of anti-apoptotic genes including *BCL2* and *FLIP*, suggesting that HBZ may fulfill its anti-apoptotic function through dysregulation of C/EBP $\alpha$  signaling.

Immunodeficiency in ATL patients is pronounced, and results in frequent opportunistic infections by various pathogens [41,42]. As a mechanism of this immunodeficiency, HBZ has been shown to inhibit CD4 T-cell responses, resulting in impaired host immunity *in vivo* [31,43]. Further study demonstrated that HBZ transgenic mice, which expressed excess amount of C/EBP $\alpha$ , were vulnerable to opportunistic pathogens [31]. It was reported that a population of PD-1+ memory phenotype CD4<sup>+</sup> T cell underlies the global depression of the T cell immune response, and such features are attributable to an unusual expression of C/EBP $\alpha$  [44]. Like C/EBP $\alpha$ , C/EBP $\beta$  acts as a master regulator of the tolerogenic and immunosuppressive environment induced by cancer [45]. Thus, our results now open the possibility that HBZ may induce the expression of C/EBP $\alpha$ , leading to immunodeficiency in ATL, and perhaps to oncogenesis. Further studies on C/EBP signaling in ATL are necessary to clarify its roles.

Many viruses have developed distinct strategies to modulate C/EBP $\alpha$  signaling using their own viral proteins. Examples include hepatitis B virus pX; Epstein-Barr virus BZLF; as well as human immunodeficiency virus TAT and Vpr [46-48]. Like HBZ, the HBV pX and EBV BZLF protein prevent C/EBP-mediated activation by interacting directly with C/EBP family members. Similar upregulation of C/EBP expression has been reported for other viruses, including hepatitis C virus, Kaposi's sarcoma-associated herpes virus, and human



immunodeficiency virus [49-51]. These findings show that dysregulation of C/EBP pathways are common among different viruses, suggesting that these activities are critical for viral persistence and oncogenesis.

Accumulating evidences show that HBZ's oncogenic function can be attributed, at least in part, to its selective regulation of multiple signaling pathways in ATL [13-15,30,31]. For example, HBZ inactivates classical NF- $\kappa$ B signaling without inhibiting the alternative pathway, helping cells to evade senescence and supporting cell proliferation [13,52]. Similarly, the negative effects of transcription factors which include ATF3, Wnt5a, and Smad3, were impeded by HBZ, leaving these factors to elude host immune attack and promote cell proliferation [14,15,30]. In this study, we found that HBZ selectively impaired the growth suppression function of C/EBP $\alpha$ , rendering the immunosuppressive and anti-apoptotic effect of C/EBP $\alpha$  predominant. HTLV-1 might escape from host immune surveillance and induce cell proliferation by thus selectively modulating signaling pathways, promoting viral reproduction, and also ATL.

It has been reported that HBZ is not able to form stable homodimers and is therefore dependent on heterodimerization with other proteins to control gene transcription [53]. Thus, the function of HBZ depends, at least in part, on its binding partner. Indeed, HBZ selectively suppressed the classical NF- $\kappa$ B pathway through inhibiting DNA binding of p65 as well as PDLIM2-dependent p65 degradation. The specificity of PDLIM2 E3 ligase in targeting p65 protein, but not p52 of the alternative pathway, may possibly explain why HBZ selectively inhibits the classical pathway of NF- $\kappa$ B [13]. Similarly, we showed in this study that HBZ inhibited C/EBP $\alpha$  signaling via recruitment of Smad3. Because the association with Smad proteins is crucial for C/EBP $\alpha$  in determining its target genes as well as transcriptional outcome, it is likely that the function of HBZ-Smad3-C/EBP $\alpha$  complexes depends on the capacity of HBZ to recruit Smad3-C/EBP $\alpha$  heterodimers onto the DNA target [32,54].

## Conclusion

We showed that HBZ impaired the growth suppression function of C/EBP signaling by physically interacting with C/EBP $\alpha$ . HTLV-1 may take advantage of this mechanism to allow the infected cells to proliferate *in vivo*.

## Methods

### Cell culture, mice, and clinical samples

293T, HeLa, and HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. HTLV-1 immortalized cell lines (MT-4), ATL cell lines (MT-1, ATL-2, ATL-43T, ED, and TL-Om1), and T-cell lines not infected with HTLV-1 (Jurkat) were cultured in

RPMI 1640 supplemented with 10% FBS and antibiotics. Kit 225 cells stably expressing HBZ were maintained as described previously [7]. C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Transgenic HBZ mice expressing HBZ specifically in CD4<sup>+</sup> cells have been described [55]. Peripheral blood mononuclear cells (PBMCs) were isolated from ATL patients (n = 6), and healthy volunteers (n = 3). Details of clinical samples are shown in Additional file 3: Table S1. The study of clinical samples was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Institutional Review Board of Kyoto University (844 and E-921). All patients provided written informed consent for the collection of samples and subsequent analysis.

### Plasmids

The pC/EBP-Luc construct contains three tandem C/EBP binding sites and was purchased from Stratagene (Heidelberg, Germany). phRL-TK was purchased from Promega (Madison, WI). Reporter vector pLTR-Luc as well as expression plasmids for Tax, Smad3, HBZ, and HBZ deletion mutants were prepared as previously described [7,13,14]. Expression vectors for C/EBP $\alpha$  and its deletion mutants were generated by PCR.

### Luciferase assay

Jurkat cells were plated on 6-well plates at  $3.5 \times 10^5$  cells per well. After 24 hours, cells were transfected with the indicated luciferase plasmid DNA. Forty-eight hours after transfection, a luciferase reporter assay was performed as previously described [13]. For the C/EBP $\alpha$  reporter assay, the *CEBPA* gene promoter was cloned into the pGL4.1 vector. Luciferase values were normalized to renilla luciferase and expressed as the mean of a triplicate set of experiments  $\pm$  SD.

### Immunoprecipitation and immunoblotting

293T cells were transfected with the indicated combinations of expression vectors by *TransIT-LT1* (Mirus, Madison, WI). Tagged proteins were immunoprecipitated by anti-c-Myc (clone 9E10, Sigma-Aldrich, St Louis, MO), anti-HA (12CA5, Roche, Mannheim, Germany) or anti-FLAG M2 (Sigma-Aldrich) antibodies, and analyzed by Western blot. Serial immunoprecipitation was performed as described previously [14]. Other antibodies used were as follows: anti-mouse immunoglobulin G (IgG), and anti-rabbit IgG were from GE Healthcare Life Sciences, and anti-C/EBP $\alpha$  from Santa Cruz Biotechnology (Santa Cruz, CA).

### Immunofluorescence analysis

HeLa cells were transfected with expression vectors using *TransIT-LT1*. Forty-eight hours after transfection, HBZ protein was detected using anti-c-MYC Cy3 (clone 9E10, Sigma-Aldrich). C/EBP $\alpha$  was detected using anti-FLAG-

biotin (Sigma-Aldrich) and secondary Streptavidin-Alexa 488 antibody (Invitrogen, Carlsbad, CA). Fluorescence was observed with a confocal microscope system (Leica, Wetzlar, Germany) as described previously [14].

#### Chromatin immunoprecipitation assay

293T cells were transfected with the HBZ and C/EBP $\alpha$  expression vectors together with pC/EBP-Luc reporter vector. Forty-eight hours after transfection, chromatin immunoprecipitation (ChIP) assay was performed as previously described [14]. Precipitated DNA was amplified by PCR using primers specific for the pC/EBP-Luc plasmid. Sequences for the primer set were 5'-TCACTGCATTCTAGTTG TGG-3' and 5'-CCATCCTCTAGAGGATAGA-3'.

#### Semiquantitative RT-PCR and quantitative 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. In the real-time PCR experiment, cDNA product was quantified with Power SYBR Green PCR Master Mix and StepOnePlus Real Time PCR System (Life technologies). Endogenous  $\beta$ -actin mRNA was quantified to normalize the amount of cDNA load. The specific primers used can be found in Additional file 4: Table S2.

#### Immunohistochemical analyses

The tissue specimens were obtained from human lymph nodes filed at the Department of Pathology at Kurume University. Tissue samples were fixed in 10% formalin in phosphate buffer and then embedded in paraffin and analyzed by immunohistochemical methods to determine C/EBP $\alpha$  expression. Images were captured using a Provis AX80 microscope equipped with an OLYMPUS DP70 digital camera, and detected using a DP manager system (Olympus, Tokyo, Japan). The study of clinical samples was approved by the local research ethics committee of Kurume University.

#### Small interfering RNA (siRNA) transfection

siRNA targeted to human Smad3 was synthesized according to a previous report [56]. HepG2 cells were transfected with expression vectors and siRNA using *TransIT-LT1* according to the manufacturer's instructions. RT-PCR detected *SMAD3* 48 hours after transfection.

#### Retroviral constructs and transduction

pGCDNsami/NGFR-HBZ and pGCDNsami/GFP-C/EBP $\alpha$  retroviral constructs were generated by cloning HBZ and C/EBP $\alpha$  cDNA into the pGCDNsami/NGFR and

pGCDNsami/GFP vectors respectively. Transfection of Plat-E packaging cell line was performed as described [57]. Mouse splenocytes were enriched for CD25<sup>-</sup>CD4<sup>+</sup> cells with a CD4 T lymphocyte enrichment set (BD Biosciences) with the addition of biotinylated anti-CD25 antibody (BD Biosciences), and activated by APCs in the presence of anti-CD3 antibody and human rIL-2 in 12-well plates. After 24 hours, activated T cells were transduced with viral supernatant and polybrene, and centrifuged at 3,000 rpm for 60 minutes. Cells were subsequently cultured in medium supplemented with rIL-2.

#### Flow cytometric analysis

Murine cells were washed with PBS containing 1% FBS. After centrifugation, cells were treated with APC-conjugated anti-human NGFR antibody (BD Biosciences) for 30 minutes. After being washed with PBS, the cells were analyzed with a flow cytometer (BD FACSCanto II, BD Biosciences).

#### Statistical analyses

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

#### Additional files

**Additional file 1: Figure S1.** C/EBP $\alpha$  did not influence HBZ expression. 293T cells were transfected with expression vector of HBZ and increasing amounts of C/EBP $\alpha$ . After 48 hours, the cell lysates were subjected to Western blot.

**Additional file 2: Figure S2.** HBZ, Smad3, and C/EBP $\alpha$  formed a ternary complex. mycHis-HBZ, FLAG-Smad3, and HA-C/EBP $\alpha$  were cotransfected into 293T cells. After 48 hours, cell lysates were subjected to immunoprecipitation using anti-c-Myc or anti-FLAG followed by immunoblotting using anti-FLAG, anti-His, and anti-HA antibody.

**Additional file 3: Table S1.** List of healthy donors and ATL patients. The information of six ATL patients and three healthy volunteers are listed.

**Additional file 4: Table S2.** List of primers for semi-quantitative RT-PCR and quantitative real-time PCR. We performed semi-quantitative RT-PCR and quantitative real-time PCR using the following primers.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

This study was designed by TZ, AC, and MM. Laboratory analysis was performed by TZ, AC, LX, and JY. Data analysis was performed by TZ, AC, LX, JY, and MM. Clinical samples and data were provided by KO. TZ, AC, and MM wrote the paper. All authors read and approved the final manuscript.

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