TRAF6 (20, 24, 41, 48). Future studies are needed to fully understand the specificity and the breadth of redundancy of the various deubiquitinases for their substrates.

In our experiments, USP20 also deubiquitinated Tax and inhibited its activity. To our knowledge, USP20 is the first DUB shown to deubiquitinate Tax. Because the Tax–NF-κB pathway is important for cellular transformation by HTLV-1 and because ubiquitinated Tax has been shown to be necessary for NF-κB activation, our findings suggest that USP20 could be a key regulator of Tax that might influence ATL leukemogenesis. Elsewhere, it has been reported that A20 negatively regulates Epstein-Barr virus (EBV)-encoded LMP1 function and that the activity of LMP1 is important for EBV immortalization of B cells (33). Thus, HTLV-1 and EBV may be two viruses that similarly exploit the cellular ubiquitination-deubiquitination machinery for pathogenesis. Potentially, the ubiquitination-deubiquitination process could be a common focal point that could be targeted to interdict HTLV and EBV infections. Additional investigation will be needed to understand the general importance of ubiquitination and deubiquitination in other viral infections.

The reduced expression of several DUBs is associated with tumorigenesis (31). Thus, mutations in the CYLD gene are known to cause familial tumors of skin appendages called cylindromas (3, 24, 48). The inactivation of A20 by genetic changes has also been reported in malignant lymphomas (6, 16, 21, 38). Here, reduced USP20 activity is shown for HTLV-1-transformed MT1, MT2, MT4, and ATL2 cells (Fig. 6). Moreover it was recently reported that in 5% of adult T cell acute lymphoblastic leukemia (T-ALL) cases, an abnormal fusion transcript, TAF I-NUP214, is expressed from a chromosomal aberration. Intriguingly, in these chimeric fusion cases, the levels of USP20 transcript were significantly reduced (13). This clinical finding is additionally consistent with an association between USP20, NF-κB signaling, and T cell malignancies.

In summary, the salient findings from this study are the identification of USP20 as an inhibitor of NF-κB signaling and as a deubiquitinating enzyme for TRAF6 and Tax. Preliminary data suggest that USP20 overexpression may impede the proliferation of HTLV-I/ATL cells (Fig. 6B); however, this finding will need to be verified further through studying a large series of ATL clinical samples. If the notion can be demonstrated to be correct, then the screening for small-molecule compounds that enhance USP20 deubiquitinase activity may unveil new agents that are useful for treating ATL.

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Molecular mechanisms of HTLV-1 infection and pathogenesis

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Abstract Human T cell leukemia virus type 1 (HTLV-1) is an etiological pathogen of several human diseases, including adult T-cell leukemia (ATL), HTLV-1-associated myelopathy (HAM)/tropical spastic paraparesis (TSP), and inflammatory disorders such as uveitis and dermatitis. HTLV-1 spreads mainly through cell-to-cell transmission, induces clonal proliferation of infected T cells in vivo, and after a long latent period, a subset of HTLV-1 carriers develop ATL. Understanding the molecular mechanisms of infection and oncogenesis is important for the development of new strategies of prophylaxis and molecular-targeted therapies, since ATL has a poor prognosis, despite intensive chemotherapy. In this review, we will summarize recent progress in HTLV-1 research, and especially novel findings on viral transmission and leukemogenic mechanisms by two viral oncogenes, *HBZ* and *tax*.

Keywords Human T-cell leukemia virus type 1 (HTLV-1) · Adult T-cell leukemia (ATL) · HTLV-1 bZIP factor (HBZ) · HBZ transgenic mouse · FoxP3 · Tax

1 Introduction

Human T cell leukemia virus type 1 (HTLV-1) was the first retrovirus to be identified as a causative agent of a cancer in humans. In addition to cancer, adult T-cell leukemia (ATL), HTLV-1 also causes inflammatory diseases, including HTLV-1-associated myelopathy (HAM)/tropical spastic paraparesis (TSP), and HTLV-1 associated uveitis [1, 2]. HTLV-1 transmits to uninfected cells through cell conjugation, as cell-free virions are not efficient in transmission [1]. HTLV-1 increases its chance of transmission by the increase of infected cells, rather than viral replication. Subsequently, HTLV-1-encoded products can induce cellular transformation. In addition to essential retroviral components, such as long terminal repeats (LTR), *gag*, *pol* and *env*, HTLV-1 provirus has a unique region between *env* and the 3′LTR; and this region is named pX [3]. The pX region encodes viral regulatory and accessory proteins Tax, Rex, p8, p12, p13, p30, p21, and HTLV-1 bZIP factor (HBZ) which are implicated in viral infectivity and the proliferation of infected cells [3–6]. Tax is recognized as a potent oncoprotein, since it immortalizes human primary T cells by itself, and Tax transgenic mice form tumors [7–15]. Nevertheless, *tax* transcripts are detected in only ~40% of ATL cases [16, 17]. Recently, a viral factor, HBZ, has been shown to have an oncogenic effect in vivo [18]. Expression of HBZ is conserved in all ATL cells, strongly suggesting that it contributes to leukemogenesis.

2 New insights into the machinery of HTLV-1 infection

It is well known that living infected cells are required for the infection of HTLV-1; and this fact indicates that the mechanism of HTLV-1 transmission is quite different from that of another human retrovirus, human immunodeficiency virus 1 (HIV-1). Novel findings on the machinery used by HTLV-1 in transmission are summarized below.

2.1 Receptors

Since HTLV-1 can infect many types of cells, its receptor is thought to be a commonly expressed molecule [20, 21].

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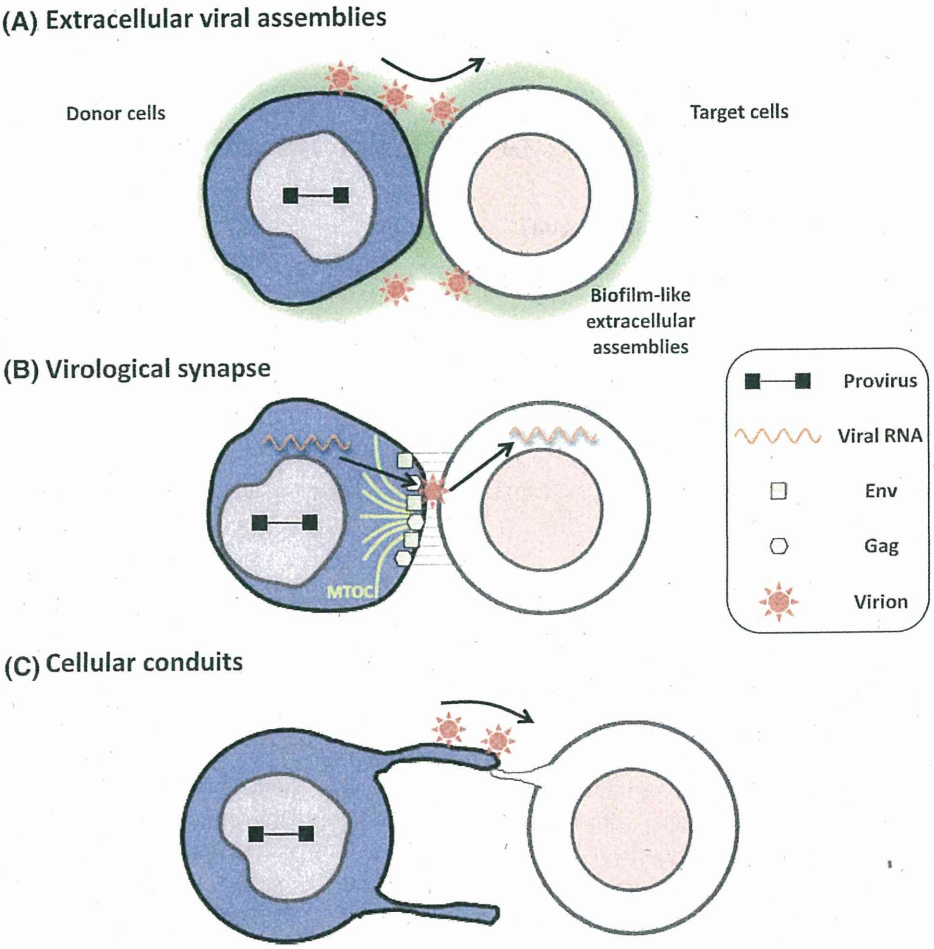

Fig. 1 Mechanisms of cell-to-cell transmission of HTLV-1. **a** HTLV-1 virions are caught in biofilm-like extracellular assemblies surrounding infected cells, and are rapidly transferred to the surface of target cells after conjugation, resulting in infection. **b** An HTLV-1-infected T cell and an uninfected cell form a virological synapse (VS) at the point of contact. HTLV-1 transmits to the target cell through the VS. **c** HTLV-1 viral particles are transferred through conduits to target cells. HTLV-1 p8 protein induces conduit formation

So far, three molecules, a glucose transporter, GLUT1, heparan sulfate proteoglycan (HSPG), and neuropilin-1, are thought to be important for the interaction between the HTLV-1 envelope and the cell membrane, and for entry of the virus to cells. It has been suggested that the virus may first contact HSPG and then form complexes with neuropilin-1, followed by GLUT1 association on the cell surface prior to membrane fusion and entry into the cell [22–24].

2.2 Mechanisms of cell-to-cell transmission (Fig. 1)

HTLV-1 is mainly transmitted by cell-to-cell contact, as cell-free HTLV-1 viral particles are not efficient at infection of the target cells [1, 19]. To date, several distinct mechanisms of cell-to-cell transmission of the virus have been proposed. In 2003, Igakura et al. [25] showed that HTLV-1-infected T cells attach to uninfected cells and form a virological synapse (VS), composed of viral and cellular molecules, at the point of contact. Through this structure, HTLV-1 transmits to the target cells from the donor cells. More recently, another piece of the cell-to-cell infection machinery was demonstrated; after viral budding, HTLV-1-infected cells keep viral particles on their surface, trapped in extracellular viral assemblies composed of collagen, agrin, and linker proteins [26]. When HTLV-1-infected cells covered with these viral assemblies attach to uninfected cells, the extracellular component containing the viral particles is rapidly transferred to the surface of the target cells, resulting in infection.

A third new mechanism of viral transfer has also been recently demonstrated. HTLV-1 encodes a protein, p8, in its pX region. The p8 protein is generated by processing of $p12^{I}$ [4]. By interacting with lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1), p8 enhances T-cell conjugation [27]. Moreover, p8 induces conduit formation among T cells and increases viral transmission through these conduits. Since the replication level of HTLV-1 in infected cells is generally low, HTLV-1 may combine multiple strategies to establish efficient viral transmission directly between cells.

2.3 Integration

After incorporation into target cells, HTLV-1 genomic RNA is reverse transcribed to generate provirus, which is integrated into cellular genomic DNA. It has been shown that integration occurs at random sites in the host genome, whereas proviruses in leukemic cells tend to be integrated near the transcriptional start sites of cellular genes, indicating positive selection of cells with this feature during leukemogenesis [28]. It has long been known that defective proviruses lacking the 5′LTR, or aberrant proviruses containing nonsense mutations in the *tax* gene, are occasionally observed in leukemic cells [29, 30]. Detailed analysis of the sequences of these proviruses reveals two important facts. First, deletion of the 5′LTR in the proviruses occurred before integration in some cases [31]. Since the 5′LTR is the promoter of viral genes encoded in the plus strand of the provirus, cells infected with these proviruses cannot express plus-strand genes, even at the beginning of infection. In contrast, the 3′LTR is maintained in all ATL cases [17]. Second, APOBEC3G generates point mutations in proviral DNA during reverse transcription, resulting in induction of nonsense mutations in all viral genes except for HBZ [32]. Both genetic modifications in the provirus can occur before integration, meaning that some ATL cells are derived from HTLV-1-infected cells that originally lack Tax expression, but possess HBZ.

3 HTLV-1 bZIP factor

HBZ is encoded in the minus strand of the HTLV-1 provirus [5], and constitutively transcribed from the 3′LTR [6]. HBZ is expressed in all ATL cases, whereas transcription of the *tax* gene is frequently inactivated by epigenetic modifications or deletion of the 5′LTR [16, 17, 31, 33]. Sequencing analysis of whole HTLV-1 provirus in 60 ATL cases revealed that only the HBZ coding sequence is preserved in all cases, despite the fact that there are many nonsense mutations or deletions in other regulatory and accessory genes [32]. These findings suggest that HBZ is a critical factor in leukemogenesis.

3.1 HBZ is pathogenic in vivo (Table 1)

We generated transgenic mice expressing HBZ (HBZ-Tg) in CD4+ T cells [6], and recently reported that there are many similarities in symptoms and immunological features between HBZ-Tg mice and HTLV-1-infected individuals [18].

3.1.1 Development of T-cell lymphomas

It is known that ectopic expression of HBZ in human T cells supports cell proliferation [6, 34]. We have observed that CD4+ thymocytes from HBZ-Tg mice are more sensitive to stimulation with anti-CD3 antibody and IL-2 than those of non-Tg mice *ex vivo* [6]. We also found that in vivo proliferation of CD4+ splenocytes in HBZ-Tg was higher than that in non-Tg mice [18]. Most importantly, ~40% of HBZ-Tg mice developed T-cell lymphomas after a long latent period. All lymphomas in HBZ-Tg were CD3+CD4+, and their monoclonal proliferation was proven by the T-cell receptor (TCR) gene rearrangement. Interestingly, most of the lymphomas in HBZ-Tg mice expressed Foxp3, a master molecule of regulatory T cells (Tregs), although the percentage of Foxp3+ cells in each tumor was variable. This heterogeneity of Foxp3 expression in HBZ-Tg lymphomas is also observed in lymphoma tissues from human ATL patients [35]. The development of T-cell lymphomas in HBZ-Tg mice shows oncogenic potential of HBZ, indicating the presence of a cell transformation pathway common to HBZ-Tg mice and ATL cases.

Table 1 Phenotypic and immunological findings in HBZ transgenic mouse

Findings	Refs.
Increased CD4+ cells in spleen	[6]
Enhanced responses of thymocytes to anti-CD3 and IL-2	
T-cell lymphoma (CD4+Foxp3+)	[18]
Dermatitis	
Alveolitis	
Increased CD4+Foxp3+ in spleen	
Increased effector/memory T cells in spleen	
Suppression of Treg fuction	

3.1.2 Inflammatory complications

Most HBZ-Tg mice developed skin inflammation by 18 weeks after birth [18]. Histological examination showed infiltration of CD3+CD4+ T cells into the dermis and epidermis in the lesions. Similar infiltration of lymphocytes was observed in the alveolar septa in the lung. The incidence of these inflammatory diseases correlated with the expression level of HBZ, suggesting a role of HBZ on their pathogenesis. Similar inflammation in skin and lung is known to develop in HTLV-1 carriers [36, 37].

3.1.3 Increased regulatory T cells and effector/memory T cells

It is known that the HTLV-1 provirus is mainly detected in effector/memory CD4+ T cells [38, 39] and Tregs [40]. The number of effector/memory cells increases in HTLV-1 carriers, and correlates with HTLV-1 provirus load [39]. Tregs are CD4+CD25+ T cells, and are also increased in

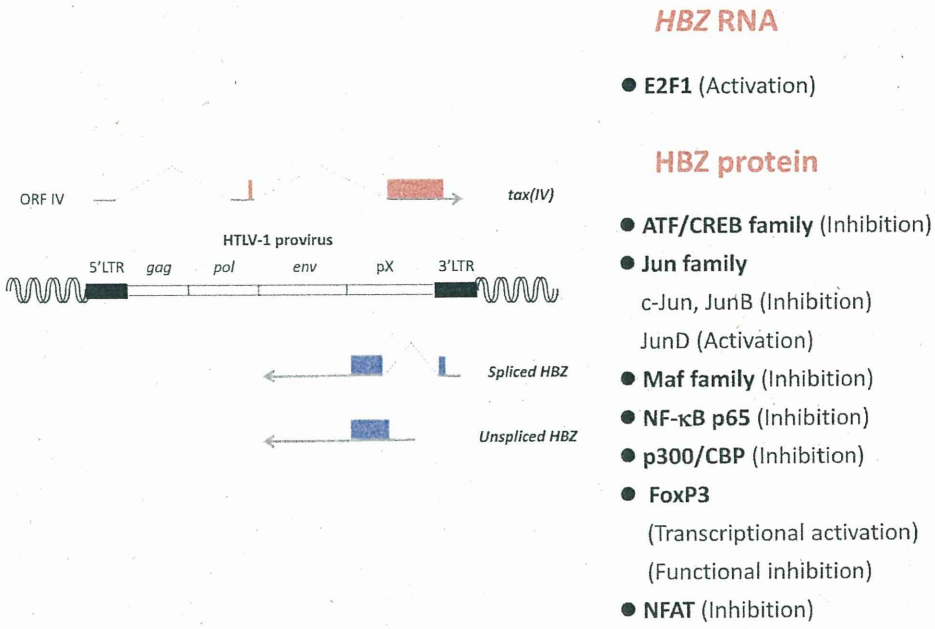

Fig. 2 Function of HBZ RNA and protein. *Left* location of the coding regions of HBZ and Tax in HTLV-1 provirus. *Right* functions of HBZ RNA and protein in the literature

HTLV-1-infected individuals [40]. In HBZ-Tg mice, both CD44highCD62Llow effector/memory CD4+ T cells and CD4+Foxp3+ T cells are increased compared with non-transgenic littermates [18].

3.2 Molecular functions of HBZ (Fig. 2)

3.2.1 Inhibition of the cyclic-AMP responsive element binding protein (CREB) pathway

HBZ was originally identified by yeast two hybrid screening as an interactant of CREB-2 protein in HTLV-1-infected cells [5]. It has been shown that HBZ protein has a basic leucine zipper (bZIP) motif, and forms heterodimers with CREB family proteins [41, 42]. HTLV-1 LTRs contain three 21 bp repeats called Tax-responsive elements (TREs) and CREB recognizes these sites [43]. Tax, by binding to CREB, activates viral transcription by recruiting transcriptional cofactors such as CBP/p300 to the 5'LTR. Meanwhile, HBZ interacts with CREB and CBP/p300, and inhibits Tax-induced viral transcription by dissociating CREB from TREs and inhibiting the binding between Tax and CBP/p300 [44]. Similarly, HBZ perturbs the effect of CREB proteins upon cellular gene transcription. Recently, we reported that HBZ interacts with activating transcription factor 3 (ATF3) and interferes with the activation of p53 by ATF3, suggesting an anti-apoptotic effect of HBZ [42].

3.2.2 Modification of AP-1 activity

Activator protein-1 (AP-1) is a transcription factor complex formed by heterodimers of cellular Jun and Fos proteins. Some AP-1 components, such as c-Jun, JunB, and JunD, have a bZIP domain, and HBZ protein heterodimerizes with them via this motif [45–49]. HBZ suppresses the activity of c-Jun and JunB. There are several mechanisms by which HBZ inhibits c-Jun; HBZ suppresses the DNA-binding activity of c-Jun and tethers it to the proteasome, resulting in ubiquitin-independent degradation of c-Jun [45, 46, 49]. In contrast, HBZ enhances the transcriptional activity of JunD. HBZ–JunD binding induces transcription of telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase [48]. Various malignant tumors, including ATL, are known to express high levels of hTERT, and this telomerase activation is thought to be associated with cellular transformation. Induction of hTERT by HBZ may be implicated in the leukemogenesis of ATL.

3.2.3 Regulation of Foxp3 expression and function

The number of FoxP3+ Tregs is increased in the thymus and spleen of HBZ-Tg mice, and most primary lymphoma tissues developed in HBZ-Tg mice express Foxp3 to varying degrees. Indeed, HBZ directly activates the Foxp3 promoter and induces its transcription [18]. On the other hand, HBZ interferes with the Foxp3 function; association between Foxp3 and NFAT is critical for the transcription of Treg-related genes such as CTLA-4 and GITR, but HBZ physically interacts with both Foxp3 and NFAT, and suppresses the function of Tregs [18], suggesting that HBZ can expand functionally impaired Treg cells and lead them to transformation in vivo.

3.2.4 HBZ inhibits the canonical NF-κB pathway

HBZ suppresses Tax-induced NF-κB activation through inactivation of an NF-κB transcription factor, p65/RelA [50]. HBZ inactivates p65/Rel by two distinct mechanisms: first, it inhibits the DNA binding ability of p65 through physical interaction; and second, it induces p65 degradation by elevating the expression of PDLIM2, an E3 ubiquitin ligase for p65. Importantly, HBZ inhibits the canonical NF-κB pathway, but not the non-canonical pathway, resulting in perturbation of the regulation of NF-κB activities.

3.2.5 HBZ RNA supports T-cell proliferation

Ectopic expression of HBZ enhances cellular growth of T cells, and knocking down HBZ in ATL cell lines attenuates their proliferation, indicating that HBZ is crucial in the continuous expansion of ATL cells [6]. It is suggested that *HBZ* RNA promotes cell proliferation by forming secondary stem-loop structures, like those formed by Epstein-Barr virus non-coding RNAs, EBERs [51]. *HBZ* RNA activates transcription of *E2F1* and its target genes, and increases G1/S transition, but further studies will be required to elucidate the biological properties of *HBZ* RNA in more detail.

4 Tax

Tax is thought to be a potent oncoprotein, as it transforms rodent cells and immortalizes human primary T cells by itself [7–9]. Importantly, Tax transgenic mice develop spontaneous tumors and inflammation [10–15, 52, 53]. Tax enhances viral replication through transactivation of the viral promoter, the 5'LTR, and its pleiotropic functions support cellular proliferation, inhibit apoptosis, impair cell cycle checkpoints, and induce DNA damages [1]. Thus, Tax is thought to play an important role in the leukemogenesis of ATL.

4.1 Activation of the NF-κB pathway

NF-κB is a major survival pathway engaged by HTLV-1. Tax was shown to bind IKKγ, and to activate both the canonical and non-canonical pathways [54].

4.2 Cell cycle progression

Tax also induces significant mitogenic activity, especially at the G1–S-phase transition, by provoking upregulation of G1 D cyclins, activation of cyclin-dependent kinases (CDKs), and downregulation of CDK inhibitors (CKIs) [55].

4.3 Induction of aneuploidy

It has been reported that Tax can induce aneuploidy by several mechanisms [56]. Tax induces multipolar mitoses through interaction with cellular TAX1BP2 and RANBP1 proteins. Tax also impairs the mitotic spindle assembly checkpoint (SAC). Tax can bind to one of the SAC proteins, MAD1 and inactivate its function, thus causing a loss of SAC activity.

4.4 Induction of DNA damage and impairment of DNA repair

Tax can induce direct DNA damage through increased reactive oxygen species [57]. In addition, Tax inactivates p53, CHK1 and CHK2 kinases, and perturbs DNA repair by suppression of base excision repair (BER), and nucleotide excision repair (NER), resulting in accumulation of DNA damage [1].

4.5 Tax-induced cellular transformation in vivo (Table 2)

Several transgenic mice have been generated to analyze Tax function in vivo. In these models, Tax induces neoplasms such as neurofibroma, mesenchymal tumor, large granular lymphocytic leukemia, and pre-T-cell leukemia, and also inflammatory diseases like exocrinopathy, arthritis, and dermatitis in vivo [10–15, 52, 53]. Transgenic mice expressing Tax from the Lck proximal promoter were shown to develop thymus-derived immature T-cell leukemia characterized by tumor cells with hyperlobulated nuclei, immunodeficiency, and constitutive NF-κB activation; these findings resemble features of ATL [14].

Table 2 Phenotypes of Tax transgenic mice

Tax transgenic mice		
Promoter	Phenotype	Refs.
LTR	Neurofibroma	[10]
	Mesenchymal tumor	[11]
	Exocrinopathy	[12]
	Immortalization of CD4 T cell by immunostimulation	[58]
LTR	Arthritis	[52]
Human granzyme B	LGL leukemia	[13]
	Acceleration of tumor formation by immunostimulation	[59]
Tet-off SR*a*	Dermatitis	[53]
Lck proximal	Pre-T-cell leukemia (CD4−CD8−)	[14]
	Immunodeficiency	
Lck distal	T-cell leukemia (CD4+ or CD8+)	[15]

In addition, Tax expression through a Lck distal promoter was shown to induce mature T-cell leukemia/lymphoma in mice [15]. Recently, it has been demonstrated that immune activation enhances Tax expression in the CD4+ T cells of HTLV-1 LTR-Tax transgenic mice, leading to immortalization of these cells [58]. When not immune activated, this strain does not develop any T-cell-associated diseases [11], suggesting that immune activation supports Tax-induced oncogenesis. Another group showed that inflammatory signals, such as TCR stimulation, accelerate tumor promotion by Tax in mice [59]. The link between immune stimulation and oncoproteins such as Tax may be important in the oncogenic process in vivo.

Cancer stem cell (CSC) theory proposes that even a small number of CSCs can generate a tumor, due to their self-renewal properties and potent proliferative potential, and tumors of various tissue types are thought to be initiated from CSCs. It has been reported that Tax-expressing or HTLV-1-infected human hematopoietic stem cells can develop CD4+ T-cell lymphomas after transplantation to immunodeficient mice [60]. Tax-expressing CSCs were also identified in Lck proximal promoter-Tax transgenic mice [61]. These findings suggest the possibility that Tax can target somatic stem cells and utilize their proliferative properties for transformation.

5 Concluding remarks

Intensive studies on Tax since the discovery of HTLV-1 have revealed some molecular strategies used by HTLV-1 for viral replication and cellular transformation [62]. However, the precise mechanisms of viral transmission and leukemogenesis have yet to be clarified. Emerging evidence is highlighting the previously unknown mechanisms of viral pathogenesis. Further studies will be needed to develop new treatment and prophylaxis strategies based on the growing knowledge of HTLV-1 molecular biology.

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HTLV-1 bZIP factor impairs cell-mediated immunity by suppressing production of Th1 cytokines

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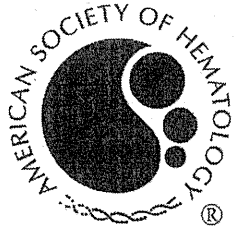

HTLV-1 bZIP factor impairs cell-mediated immunity by suppressing production of Th1 cytokines

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Adult T-cell leukemia (ATL) patients and human T-cell leukemia virus-1 (HTLV-1) infected individuals succumb to opportunistic infections. Cell mediated immunity is impaired, yet the mechanism of this impairment has remained elusive. The *HTLV-1 basic leucine zipper factor* (*HBZ*) gene is encoded in the minus strand of the viral DNA and is constitutively expressed in infected cells and ATL cells. To test the hypothesis that HBZ contributes to HTLV-1–associated immunodeficiency, we challenged transgenic mice that express the *HBZ* gene in CD4 T cells (HBZ-Tg mice) with herpes simplex virus type 2 or *Listeria monocytogenes*, and evaluated cellular immunity to these pathogens. HBZ-Tg mice were more vulnerable to both infections than non-Tg mice. The acquired immune response phase was specifically suppressed, indicating that cellular immunity was impaired in HBZ-Tg mice. In particular, production of IFN-γ by CD4 T cells was suppressed in HBZ-Tg mice. HBZ suppressed transcription from the *IFN-γ* gene promoter in a CD4 T cell–intrinsic manner by inhibiting nuclear factor of activated T cells and the activator protein 1 signaling pathway. This study shows that HBZ inhibits CD4 T-cell responses by directly interfering with the host cell-signaling pathway, resulting in impaired cell-mediated immunity in vivo. (*Blood*. 2012;119(2):434-444)

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that mainly infects CD4 T cells,[1] a critical cell population for the host defense against foreign pathogens. HTLV-1 is known as the causal agent of adult T-cell leukemia (ATL),[2-4] a leukemia derived from CD4 T cells, and chronic inflammatory diseases, including HTLV-1-associated myelopathy/tropical spastic paraparesis,[5,6] alveolitis,[7] and uveitis. It has also been recognized that HTLV-1 infection is complicated by opportunistic infections caused by *Pneumocystis jiroveci*, herpes zoster virus, cytomegalovirus, or *Strongyloides stercoralis*.[8] However, the mechanism by which HTLV-1 causes immune deficiency has remained unknown.

Another human pathogenic retrovirus, HIV, replicates vigorously in vivo and produces a large number of virions. As a result of abundant viral production, HIV-infected CD4 T cells proceed to apoptosis, a phenomenon that eventually results in AIDS. In contrast, HTLV-1 increases its copy number primarily in the form of a provirus, by promoting the clonal proliferation of infected host CD4 T cells.[9,10] Despite this opposite effect on CD4 T-cell homeostasis compared with HIV, HTLV-1 infection and ATL are frequently accompanied by a deficiency of cellular immunity resembling that seen with AIDS.

HTLV-1 encodes several regulatory and accessory genes in the viral genome.[1,11] The viral proteins expressed by the integrated provirus control viral gene transcription and induce host cell proliferation, enabling HTLV-1 to achieve persistent infection. Among the viral genes of HTLV-1, *HTLV-1 bZIP factor* (*HBZ*), which is encoded in the minus strand,[12] is a constitutively expressed viral gene.[13] It has been reported that there are 2 major transcripts of the *HBZ* gene: spliced HBZ (sHBZ) and unspliced HBZ (usHBZ).[14] Based on the findings that sHBZ is more abundantly expressed than usHBZ[15] and that sHBZ has a functionally stronger effect than usHBZ,[16] we focused on sHBZ in this study.

Recently, we have reported that sHBZ expression increases the number of regulatory T cells (Tregs) by inducing transcription of the *Foxp3* gene in transgenic mice that express the *HBZ* gene in CD4 T cells (HBZ-Tg mice).[17] An increase in Tregs might be implicated in the immunodeficiency observed in ATL patients. Furthermore, previous studies have reported that HBZ suppresses host cell-signaling pathways that are critical for T-cell receptor signaling in the immune response, such as the NF-κB[18] and AP-1 pathways.[19] These findings led us to hypothesize that HBZ might have important roles in the dysregulation of cellular immunity associated with HTLV-1 infection.

To verify this hypothesis, we used HBZ-Tg mice that express sHBZ in CD4 T cells and studied well-established infection models of 2 pathogens. The first model involves intravaginal viral infection with herpes simplex virus type-2 (HSV-2). IFN-γ production by CD4 T cells is critical for the exclusion of HSV-2 from the host.[20,21] The other model involves infection with the Gram-positive intracellular bacterium, *Listeria monocytogenes* (LM), which is known as an opportunistic pathogen. In LM infection, CD4 T cells play pivotal roles in the acquired immune response by producing IFN-γ and inducing the activation of macrophages, which eliminate LM

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by phagocytosis and subsequent bactericidal activity.[22,23] Indeed, previous reports have shown that some ATL patients are infected with these 2 pathogens.[24,25] Using these 2 infection models, we demonstrated that sHBZ suppresses cell-mediated immunity. Furthermore, we determined the molecular mechanism of this HBZ-mediated immune suppression.

Methods

Mice

Wild-type C57BL/6J mice were purchased from CREA Japan. Transgenic mice expressing the *sHBZ* gene under control of the CD4 promoter/enhancer/silencer have been described previously.[13] All HBZ-Tg mice were heterozygotes for the transgene. All mice used in this study were maintained in a specific pathogen-free facility and handled according to protocols approved by Kyoto University.

Herpes simplex virus type 2 infection

The HSV-2 wild-type strain UW268 and thymidine kinase (TK)-negative strain UWTK (a gift from T. Suzutani, Fukushima Medical University) used in this study were propagated and titrated on Vero cells.[26] Acyclovir was used for propagation of UWTK to block emergence of TK^+ revertant. To increase their susceptibility to HSV-2, we injected mice subcutaneously with medroxyprogesterone acetate, Depo-provera (Sigma-Aldrich), (2 mg/mouse). Five days after this hormone injection, mice were anesthetized using Avertin (Sigma-Aldrich), preswabbed with a type 2 Calgiswab (Puritan), and inoculated intravaginally with 10^3 or 10^4 plaque-forming units (PFU) of UW268. For studies of secondary infection, mice were first immunized intravaginally with 10^6 PFU of UWTK, and 4 weeks later, they were inoculated intravaginally with 10^5 PFU of UW268. Vaginal secretions were collected by 3 pipettings with 15 μL of PBS, swabbed with a Calgiswab, and added to 955 μL of 5% FCS-DMEM and stored at −80°C. HSV-2 titers were determined by plaque assay on Vero cells. Five days after primary infection, lavage fluid from the vaginal tract was harvested similarly by 3 pipettings with 20 μL of PBS.

At 6 days after infection, the vaginal tissues of infected mice were fixed in 10% formalin in phosphate buffer and embedded in paraffin. H&E staining was performed according to standard procedures. The presence of HSV-2 antigen in tissues was detected using rabbit polyclonal anti–herpes simplex virus type 2 (Dako North America). Images were captured using a Provis AX80 microscope (Olympus) equipped with OLYMPUS DP70 digital camera, and detected using a DP manager system (Olympus; original total magnification ×200).

Splenic CD4 T cells from HSV-2 primary-infected mice were stimulated in a 96-well plate coated with CD3 mAb (1 μg/mL) and CD28 mAb (1 μg/mL) for 24 hours. For antigen specific stimulation, CD4 T cells were cocultured for 48 hours in the presence of irradiated T cell–depleted splenocytes as antigen-presenting cell (APC) and heat-inactivated HSV-2 (heat inactivated at 56°C for 2 hours) at a multiplicity of infection of 1. Supernatant was collected and stored at −20°C until assay.

Evaluation of resistance and immune response to LM in mice

Wild-type LM strain EGD was used in this study. The bacterial suspension was prepared as described previously.[27] For primary infection, mice were inoculated intravenously with 10^3 colony-forming units (CFUs) of LM and the bacterial burden in the spleen was determined on day 2 or 5 after infection.

For studies of secondary infection, mice were immunized intravenously with 10^3 CFUs of LM. From day 3 through day 6.5 after immunization, the drinking water supplemented with ampicillin (2 mg/mL) was given to clear any remaining LM. On day 7, mice were challenged with 10^6 CFUs of LM, and the spleens and sera were harvested after 3 or 12 hours. Spleens were homogenized in PBS, and the number of viable bacteria was determined by plating 10-fold serial dilutions on tryptic soy agar plates and counting the CFUs.

For cytometric assays, immunized mice were re-inoculated with 10^7 CFUs of LM. Splenocytes were harvested after 12 hours, cultured in the presence of protein transport inhibitor for 6 hours, and evaluated by the FACSCanto II (BD Biosciences) for cell surface and intracellular markers.

To determine the functional development of CD4 T cells in immunized mice, we purified splenic CD4 T cells and then stimulated them in a 96-well plate coated with CD3 mAb and CD28 mAb. For LM specific stimulation, CD4 T cells were cocultured with mouse bone marrow–derived macrophages (BMDMs) differentiated in the presence of 100 ng/mL of M-CSF and pulsed with viable LM at a multiplicity of infection of 10. Supernatant after stimulation for 24 hours was collected and stored at −20°C until assay.

Analysis of virus vector-transduced CD4 T cells

Retroviral transduction was performed as described previously.[17] The spliced HBZ gene was cloned into a retroviral vector, pMXs-Ig (a gift from T. Kitamura, The University of Tokyo), to generate pMXs-Ig-HBZ. This plasmid DNA was transfected into the packaging cell line, Plat-E. For retroviral transduction, $CD25^-CD4^+$ cells were enriched by a CD4 enrichment kit (BD Biosciences PharMingen) and were activated by anti-CD3 Ab (0.5 μg/mL) and rIL-2 (50 U/mL) in the presence of T cell–depleted and x-irradiated (20 Gy) C57BL/6J splenocytes as APCs in 12-well plates. After 16 hours, activated T cells were transduced with viral supernatant in the presence of 4 μg/mL polybrene and centrifuged at 1700*g* for 60 minutes. Then, transduced CD4 T cells were stimulated by phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (1 μg/mL) or plate-coated CD3 mAb (1 μg/mL) and CD28 mAb (1 μg/mL) in the presence of protein transport inhibitor and analyzed by a flow cytometry as shown in Figure 3. Dead cells were excluded using forward and side scatter and LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) by flow cytometry. Thereafter, intracellular cytokines were measured.

For generation of the lentivirus vector, sHBZ cDNA was cloned into pCS2-EF-GFP (a gift from H. Miyoshi, RIKEN BioResource Center) as previously described.[13] In brief, 293FT cells were cotransfected with the lentivirus vector, pCMV-Δ8/9 and pVSVG and supernatant containing virus was used for transduction. The lentivirus titer was determined on 293FT cells.

Empty vectors that express only GFP were used as controls for retroviral and lentiviral transductions.

IFN-γ promoter assay

Nucleotides −670 to +64 of the IFN-γ promoter region were amplified by PCR using human genomic DNA as a template, and cloned into pGL4.22 (Promega). The PathDetect pAP-1-Luc and pNFAT-Luc Cis-Reporter Plasmids were purchased from Promega. Transfection and luciferase assay were performed according to supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

ChIP assay

sHBZ-expressing Jurkat cells were stimulated with PMA and ionomycin. ChIP assay was performed as reported previously.[28] ChIP DNA samples were subjected to the StepOnePlus real-time PCR system using Power SYBR Green PCR Master Mix (Applied Biosystems). The sequences of the primers for the human IFN-γ promoter were: 5′-TACCAGGGC-GAAGTGGGGAG-3′ (sense) and 5′-GGTTTTGTGGCATTTGGGTG-3′ (anti-sense).

Statistical analysis

For in vitro and in vivo experiments, multiple data comparisons were performed using the Student unpaired *t* test.

Results

High susceptibility of HBZ-Tg mice to HSV-2 infection

We first evaluated the susceptibility of HBZ-Tg mice to HSV-2 infection. Recently, we reported that HBZ-Tg mice frequently develop T-cell lymphoma and dermatitis after 10 weeks.[17] Therefore, HBZ-Tg mice without skin symptoms at 7 to 10 weeks of age were used in this study. It has been reported that the host immune response against primary HSV-2 infection can be divided into 2 stages: the innate immune response plays a dominant role by day 2 after infection, whereas cellular immunity plays an important role later, after day 5 after infection.[29] IFN-γ production by CD4 T cells is known as a critical factor in the cellular immune response against pathogens.[29] To determine whether cellular immunity is impaired in HBZ-Tg mice, we pretreated HBZ-Tg and non-Tg mice with Depo-provera for efficient infection and inoculated them with HSV-2 through the vaginal route.[30] The viral titer of HSV-2 in the lesion was measured. In this primary infection assay, there was no significant difference in the viral titers between non-Tg and HBZ-Tg mice at day 2 after inoculation (Figure 1A), when innate immunity is responsible for the host defense. In contrast, at day 6 after infection, when acquired immunity becomes important, HBZ-Tg mice showed significantly higher viral titers of HSV-2 than non-Tg mice. (Figure 1A). Immunohistochemical analysis revealed that abundant viral antigens were detected in the vaginal epithelial cells and ganglia of HSV-2 challenged HBZ-Tg mice but not in non-Tg mice (Figure 1B).

To explore the mechanism of this immune deficiency, we examined cytokine production by CD4 T cells stimulated with antibodies to CD3 and CD28 or with heat-inactivated HSV-2 and APC. On day 6 after infection, the production of Th1 effector cytokines, including IFN-γ, IL-2, and TNF-α, was significantly reduced in CD4 T cells from HBZ-Tg mice compared with non-Tg mice (Figure 1C). Furthermore, IFN-γ concentration in vaginal wash fluids at day 5 after infection was significantly suppressed in HBZ-Tg compared with non-Tg mice (Figure 1D). When we challenged mice with a 50% lethal dose of HSV-2, the survival rate of non-Tg mice at day 20 after infection was 53%. In contrast, HBZ-Tg mice could not survive a viral challenge at the same dose (Figure 1E).

To study acquired immunity against HSV-2, we immunized and challenged mice as shown in Figure 1F. First, mice were immunized by TK-negative HSV-2 strain, the attenuated mutant of HSV-2, and then they were challenged with wild-type HSV-2. The vaginal virus titer in HBZ-Tg mice at day 3 after challenge was similar to that in nonimmune non-Tg mice (Figure 1F), whereas HSV-2 was not detected in immune non-Tg mice. The difference in viral titer between non-Tg and HBZ-Tg mice was much more remarkable in these secondary infection experiments than in the previous primary infection experiments, implicating impaired acquired immunity in HBZ-Tg mice. These results demonstrate that expression of sHBZ in CD4 T cells induces a deficiency in the immune response against HSV-2 and impairs the production of IFN-γ, IL-2, and TNF-α.

HBZ-Tg mice have an impaired T cell–dependent immune response to LM

We next evaluated the susceptibility of HBZ-Tg mice to infection with LM via an intravenous route. As with HSV-2 infection, production of IFN-γ by CD4 T cells plays a crucial role in the growth inhibition and elimination of LM in vivo.[31,32] On day 2 or 5 after primary infection with LM, we removed spleens and evaluated the bacterial burdens in the organs. The number of LM recovered from HBZ-Tg spleen on day 2 was comparable to that from non-Tg mice, yet the bacterial burden in HBZ-Tg mice at day 5 was higher than that in non-Tg mice (Figure 2A), suggesting a reduced protection in HBZ-Tg mice against LM, especially when acquired immunity is being established. We next a performed secondary infection experiment to evaluate the T cell–dependent immunity that developed after primary infection. Non-Tg mice immunized with a small dose of LM and later challenged with a high dose exhibited a significant level of bacterial elimination 12 hours after challenge compared with nonimmunized mice (Figure 2B). By contrast, such a significant level of bacterial elimination was not observed in immunized HBZ-Tg mice (Figure 2B), indicating that acquired LM-specific immunity is impaired in HBZ-Tg mice.

Characterization of cytokine production in the LM-infected mice

We next measured the concentration of several cytokines in the sera and homogenized spleen supernatant of HBZ-Tg and non-Tg mice during secondary infection with LM. IFN-γ, TNF-α, IL-2, IL-6, and IL-10 were decreased in HBZ-Tg mice (Figure 2C) compared with non-Tg mice. On the other hand, IL-12, which is mainly secreted by APCs, was increased in HBZ-Tg at 12 hours. To explore whether impaired production of Th1 cytokines by CD4 T cells is responsible for the decrease in levels of IFN-γ, TNF-α, and IL-2 in the serum, we enriched CD4 T cells from the spleens of immunized mice and then stimulated the cells ex vivo nonspecifically (with mAbs to CD3 and CD28) or specifically (with BMDMs pulsed with viable LM). The ability of CD4 T cells from HBZ-Tg mice to produce IFN-γ and IL-2 in response to either kind of stimulation was markedly impaired compared with that of cells from non-Tg mice (Figure 2D). In contrast, a considerable amount of TNF-α production was detected in tests of both HBZ-Tg and non-Tg CD4 T cells after stimulation with LM-pulsed BMDMs. However, this level of TNF-α was almost comparable with that observed in the culture of LM-pulsed BMDMs alone (Figure 2D). Therefore, the TNF-α detected in this experiment was probably produced by the macrophages, not by the CD4 T cells. These results strongly suggest that the ability of CD4 T cells to produce Th1 cytokines is impaired in HBZ-Tg mice.

Because IFN-γ is reported to play a pivotal role in the acquired protection of mice against LM,[22,23] we focused on IFN-γ production by LM-specific CD4 T cells. Splenic cell suspensions were prepared from 2 groups of mice immunized and challenged according to the protocol shown in Figure 2B. Cells were cultured for 6 hours in the presence of protein transport inhibitor and then subjected to flow cytometric analysis for IFN-γ production by intracellular cytokine staining. The number of IFN-γ–producing CD4 T cells in HBZ-Tg mice was remarkably reduced compared with that in non-Tg mice (Figure 3A). In contrast, IFN-γ production by CD8 T cells showed no significant difference between non-Tg and HBZ-Tg mice (Figure 3A). In addition, there were no differences between HBZ-Tg mice and control littermates in both total and $CD4^+$ splenocytes (supplemental Figure 1).

We recently reported that the proportion of $Foxp3^+$ $CD4^+$ T cells is increased in HBZ-Tg mice.[17] A previous study reported that Foxp3 expression inhibits the production of IFN-γ,[33] suggesting that a decreased proportion of effector T cells in HBZ-Tg mice might be responsible for the low number of IFN-γ–producing CD4

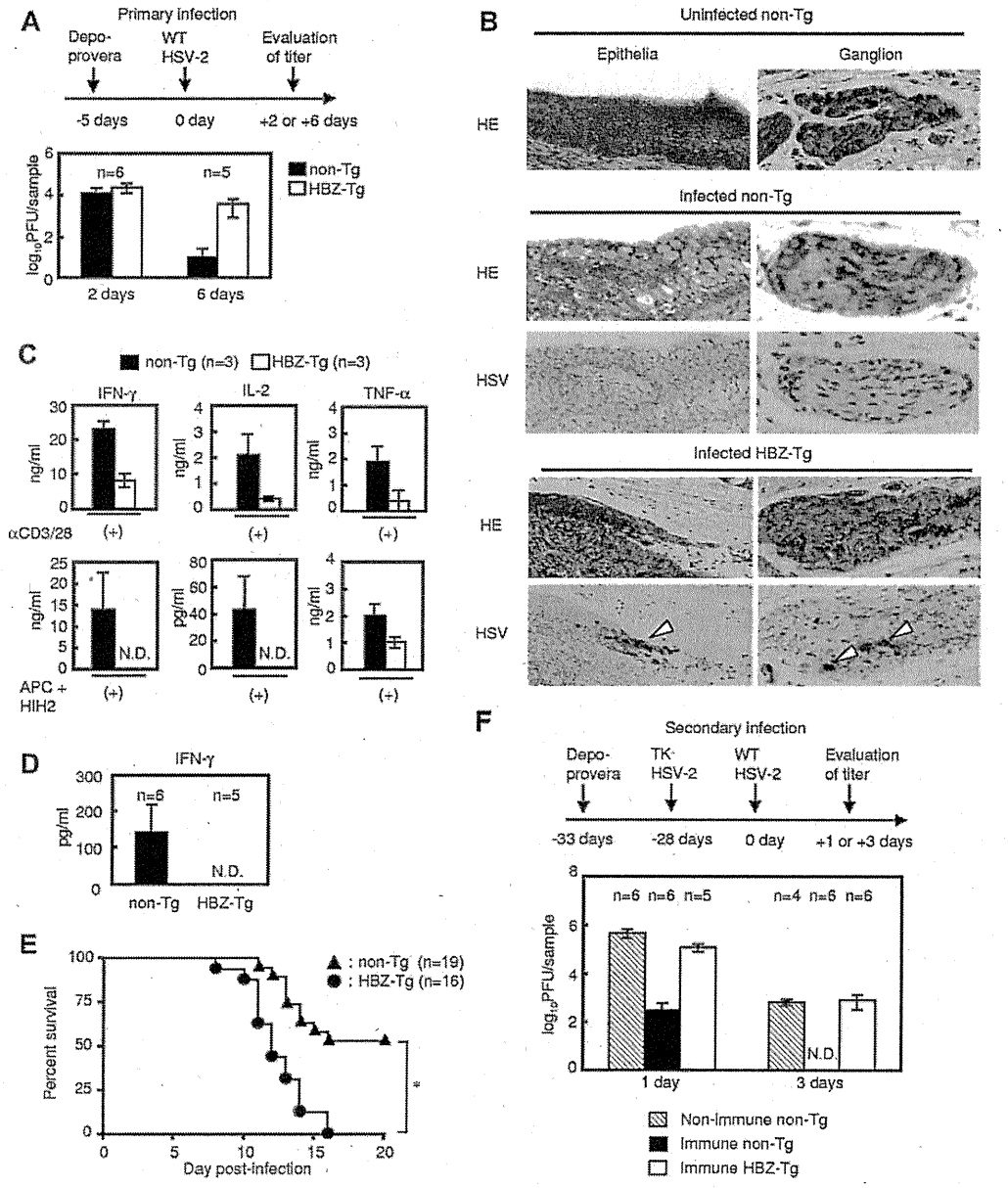

Figure 1. Transgenic mice expressing sHBZ in CD4 T cells are highly susceptible to intravaginal infection with HSV-2. (A) Virus titer in vaginal washes in primary infection. (B) Histologic analysis of epithelia and ganglion in vaginal tissue from mice infected with HSV-2. Uninfected vaginal tissues are presented as controls. HE indicates H&E stain; and HSV, immunohistochemical analysis for the viral antigen. Arrowheads indicate HSV-2–positive cells. (C) Cytokine production by splenic CD4 T cells from mice infected with 10^4 plaque-forming units (PFU) of HSV-2. Cells were stimulated with mAbs to CD3 and CD28 or APC plus heat-inactivated HSV-2 (HIH2) in ex vivo culture. (D) IFN-γ concentration in vaginal wash fluid harvested at day 5 after infection. (E) Survival curve of non-Tg or HBZ-Tg mice infected with 10^3 PFU of HSV-2. $^*P < .05$ (log-rank test). (F) Viral titer in vaginal washes during HSV-2 secondary infection. To evaluate adaptive immunity against HSV-2 infection, mice were immunized and infected with the virus as shown in the upper panel. Bars represent the mean ± SD of all mice per genotype. Two or 3 independent experiments have been performed. N.D. indicates not detected.

T cells. However, the impairment of IFN-γ production was still observed in the Foxp3-negative effector CD4 T-cell population (Figure 3B), indicating that the reduction in IFN-γ production is independent of Foxp3 expression. These results collectively indicate that transgenic expression of sHBZ in CD4 T cells results in a reduction in effector cytokine production by CD4 T cells.

sHBZ directly inhibits IFN-γ production in a CD4 T cell–intrinsic manner

To determine whether sHBZ-mediated IFN-γ suppression was induced by a cell-intrinsic effect of sHBZ in CD4 T cells or by a dysregulated immunologic status in vivo indirectly caused by sHBZ expression, we used a retrovirus vector to express sHBZ in naive CD4 T cells. Wild-type CD4 T cells transduced with sHBZ showed lower IFN-γ production than empty vector-transduced cells (Figure 4A-B), demonstrating that sHBZ directly suppresses IFN-γ production in CD4 T cells. It is noteworthy that sHBZ suppressed IFN-γ production in human CD4 T cells as well as mouse T cells. This suppression was not limited to IFN-γ but was also observed for TNF-α (Figure 4C) and IL-2 (Figure 4D). Expression level of the *HBZ* gene transcript was much higher than that of HBZ-Tg mice (supplemental Figure 2). IL-4 production was

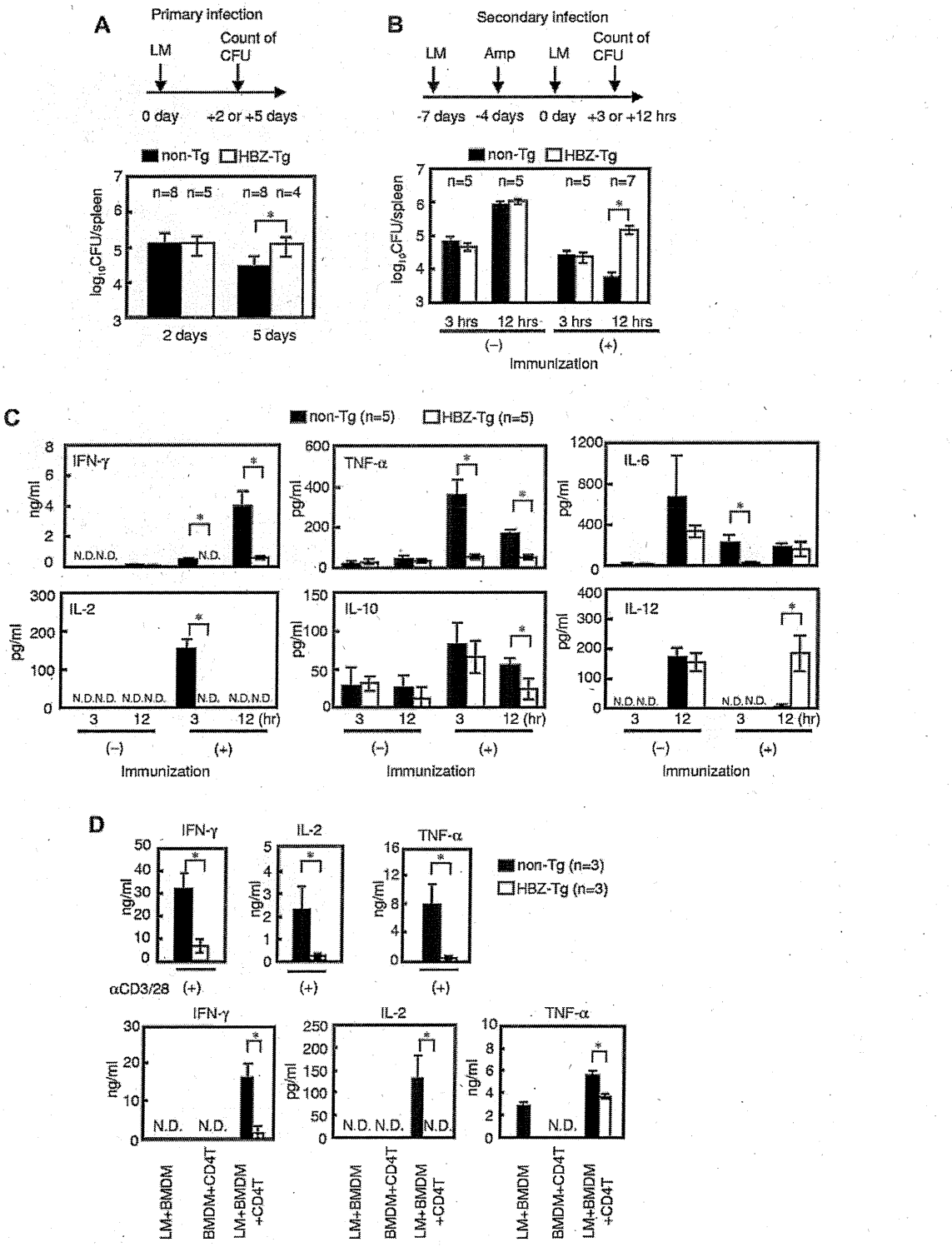

Figure 2. HBZ-Tg mice show decreased immune response to primary and secondary infection with LM. Bacterial loads of spleens from mice challenged with LM in primary (A) and secondary (B) infection are shown. (C) Concentrations of IFN-γ, TNF-α, IL-2, IL-6, and IL-12 in serum and IL-10 in homogenized spleen supernatant from the secondarily infected mice. (D) Cytokine production by CD4 T cells from secondarily infected mice. Mice were immunized as shown in panel B. CD4 T cells were stimulated ex vivo with mAbs to CD3 and CD28 or with LM-infected WT-BMDMs. Bars represent the mean ± SD of all mice per genotype. Two independent experiments have been performed; representative results are shown. *$P < .05$ by Student t test. N.D. indicates not detected.

not detected in CD4 T cells (supplemental Figure 3A). Although production of Th1 cytokines was reduced in sHBZ-expressing CD4 T cells, IL-6 and IL-10 production was not altered by sHBZ expression (supplemental Figure 3B-C). These results collectively suggest that sHBZ expression in HTLV-1–infected CD4 T cells inhibits transcription of the *IFN-γ*, *TNF-α*, and *IL-2* genes, which

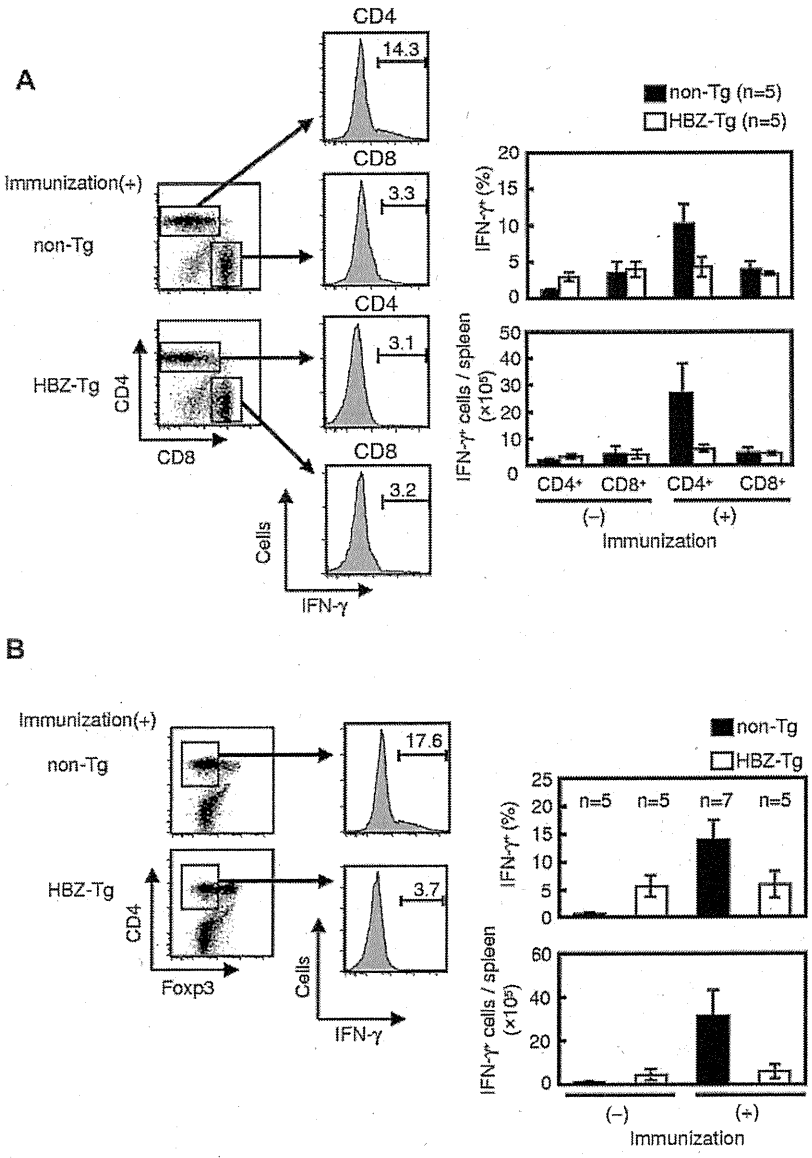

Figure 3. IFN-γ production by CD4 splenocytes from LM secondarily infected HBZ-Tg mice decreases in CD4⁺ Foxp3⁻ T cells. Mice were immunized and challenged as shown at the top of Figure 2B, and their splenocytes were harvested at 12 hours after challenge and analyzed for intracellular IFN-γ production. (A) Splenocytes were gated by CD3 expression, and IFN-γ production was measured in living CD4 or CD8 T cells using FACS. (B) IFN-γ production in CD3⁺ CD4⁺ Foxp3⁻ cells was determined. Bars represent the mean ± SD of all mice per genotype. Two independent experiments have been performed.

play important roles in the immune response against foreign pathogens.

sHBZ suppresses the activity of the IFN-γ promoter by inhibiting the NFAT and AP-1 signaling pathways

To further elucidate the mechanism of sHBZ-mediated IFN-γ inhibition, we performed a promoter assay using a human −670 to +64 IFN-γ promoter construct in the human T-cell line Jurkat. Previous reports have demonstrated that NFAT, AP-1, and NF-κB signaling pathways are involved in the regulation of *IFN-γ* transcription.[34] We found that PMA and ionomycin treatment enhanced IFN-γ promoter activity, and sHBZ suppressed this enhancement in a dose-dependent manner (Figure 5A). In contrast, another viral protein, Tax, enhanced the promoter activity as reported previously (Figure 5B),[35] an observation that is in line with previous findings that Tax is capable of activating the NF-κB and AP-1 signaling pathways.[36] Previous studies have demonstrated that the level of *sHBZ* transcripts in ATL patients and HTLV-1 carriers is approximately 4-fold higher than the level of *tax* transcripts.[15] The activation of the IFN-γ promoter by Tax was inhibited by sHBZ when sHBZ was expressed at levels similar to those in HTLV-1 carriers (Figure 5C), suggesting that sHBZ can have an inhibitory effect on Tax-mediated IFN-γ induction in HTLV-1 infected cells.

To identify the region of the IFN-γ promoter responsible for sHBZ-mediated suppression, we conducted further analyses using serially deleted promoter constructs. The human IFN-γ promoter (−670 to +64) contains NFAT, AP-1, STAT, ATF, and T-bet binding regions, and these transcription factors are reported to be involved in IFN-γ expression. The suppressive effect of sHBZ on the IFN-γ promoter was reduced by the deletion between dM2 and dM3 ($P < .001$; Figure 5D: a deletion, which removes 2 NFAT sites, an AP-1 site, and a STAT binding site). Because HBZ has a suppressive effect on the NFAT and AP-1 signaling pathways,[17,19] these binding sites might be associated with the suppressive effect of sHBZ. To further explore this possibility, we generated the promoter constructs with point mutation for each NFAT or AP-1 sites, and performed the promoter assay. The point mutation for

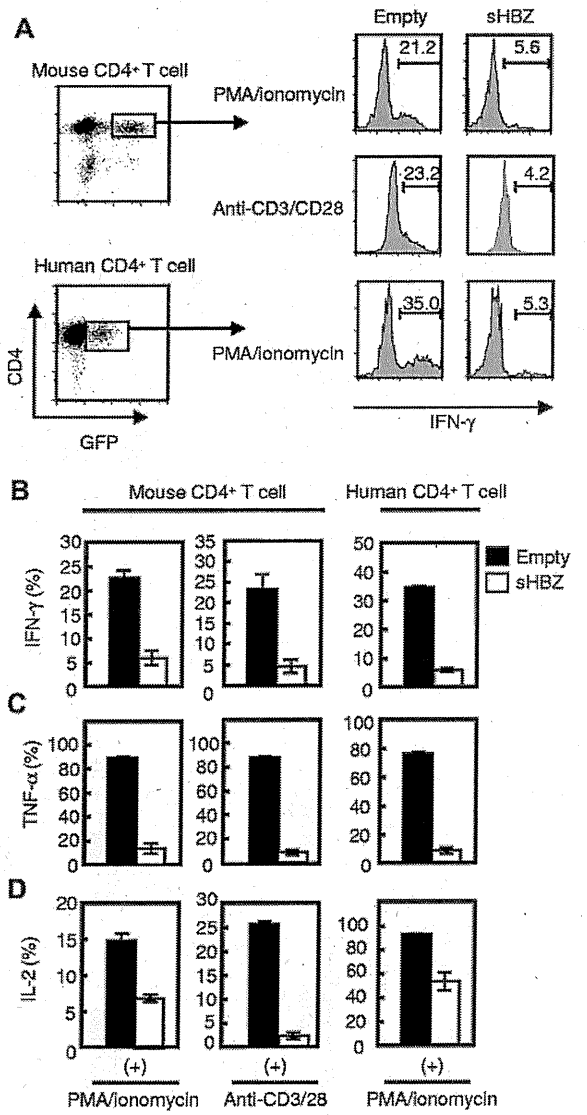

Figure 4. sHBZ directly inhibits IFN-γ production in both human and mouse CD4 T cells. Mouse and human CD4 T cells were transduced with recombinant retroviruses or lentiviruses, respectively, expressing sHBZ, and stimulated with PMA and ionomycin or antibodies to CD3 and CD28. Then, intracellular cytokines in living HBZ-expressing CD4 T cells were measured using FACS. (A) GFP$^+$ and CD4$^+$ cells were gated as shown in the left panel and evaluated for intracellular production of IFN-γ, TNF-α, or IL-2 by flow cytometry. Representative histograms of IFN-γ are shown. (B-D) Percentages of IFN-γ$^+$ (B), TNF-α$^+$ (C), or IL-2$^+$ (D) cells in mouse and human CD4 T cells. Representative data from 2 independent experiments in triplicate (mean ± SD) are shown.

−163 to −153 (P = .025) but not −279 to −269 (P = .057) NFAT binding site remarkably reduced suppressive effect of promoter activity by HBZ (Figure 5E). We next characterized effect of sHBZ on AP-1 binding sites in the IFN-γ promoter. The point mutation for −193 to −183 AP-1 binding site partially impaired the inhibitory effect (P = .042; Figure 5F). Three point mutations of all AP-1 binding sites much more reduced the HBZ-mediated suppressive effect on the promoter (P = .001; Figure 5F). These results indicate that NFAT and AP-1 binding sites are involved in the suppressive effect of HBZ on this promoter.

To further elucidate the involvement of the AP-1 or NFAT signaling pathway in the sHBZ-induced impairment of IFN-γ production, we used sHBZ mutants, which are unable to exert an inhibitory effect on NFAT or AP-1 signaling. We have reported that activation and central domains of HBZ interacted with NFAT.[17] We constructed deletion mutants and 7 amino-acid substitution mutants of sHBZ central domain and assessed their abilities to function in the NFAT or AP-1 signaling pathway (Figure 6A-B; supplemental Figure 4A-C). We found 2 mutants of interest: sHBZ-CDm7 and sHBZ-ΔAD. sHBZ-CDm7 contained amino acid substitutions in the central domain of sHBZ, and these mutations abrogated the inhibitory effect of sHBZ on the activity of an NFAT reporter plasmid (Figure 6A). In contrast, sHBZ-ΔAD, which contains a deletion of the activation domain of sHBZ, did not have suppressive activity on the AP-1 signaling pathway (Figure 6B). We confirmed that expression levels of the sHBZ mutants were comparable with that of WT-sHBZ (supplemental Figure 4D). Consistent with the findings of the reporter assay with the deleted promoters, sHBZ-CDm7 and sHBZ-ΔAD showed remarkable reduction in the inhibitory effect on the IFN-γ promoter (Figure 6C). Furthermore, we generated retrovirus vectors that express these sHBZ mutants, transduced them to mouse CD4 T cells, and evaluated their effect on IFN-γ production. We found that these 2 sHBZ mutants lost their inhibitory effect on IFN-γ production compared with WT-sHBZ (Figure 6D). Previous reports have shown that bZIP domain of HBZ plays a role in suppression for transcriptional activity of AP-1 family, including c-Jun and JunB.[19,37] In this study, deletion mutant of bZIP domain in sHBZ did not influence NFAT and AP-1 pathway in Jurkat cell (Figure 6A-B) and IFN-γ production in mouse CD4$^+$ T cell (supplemental Figure 5A), indicating that not bZIP domain but activation domain of HBZ is essential for suppression of AP-1 pathway in this study.

In addition, we performed a ChIP assay to explore recruitment of the transcription factors NFAT and AP-1 to the IFN-γ promoter in the presence of sHBZ. This experiment showed that sHBZ inhibited recruitment of NFATc2 and c-Jun to the IFN-γ promoter containing 2 NFAT sites and one AP-1 binding site (Figure 6E). These results suggest that sHBZ physically inhibits DNA binding of c-Jun and NFATc2 and suppresses the NFAT and/or AP-1 signaling pathways, which are critical for IFN-γ production in CD4 T cells.

Impaired production of IFN-γ in primary ATL cells

Jurkat T cells express *IFN-γ* gene transcripts after stimulation with PMA and ionomycin. sHBZ expression in Jurkat cells remarkably reduced the level of *IFN-γ* mRNA (Figure 7A). It is critical to study IFN-γ expression in naturally HTLV-1–infected T cells. Therefore, we examined IFN-γ production in PBMCs from ATL patients (supplemental Table 1). PBMCs were stimulated by PMA and ionomycin for 5 hours, and intracellular IFN-γ was stained. We found that IFN-γ production by CD4 T cells was remarkably decreased in ATL patients compared with healthy donors (Figure 7B). In addition, TNF-α and IL-2 production also was suppressed in CD4 T cells from ATL patients. These data suggest that impaired production of IFN-γ is observed not only in HBZ-Tg or ectopically transfected cells but also in primary CD4 T cells from ATL patients.

Discussion

Viruses that cause chronic infections, including hepatitis C virus, HIV, Epstein-Barr virus, and HTLV-1, have strategies to evade the host immune system and to replicate in vivo despite detectable immune responses.[38] For HTLV-1, it has been reported that p12 binds to free human major histocompatibility complex class

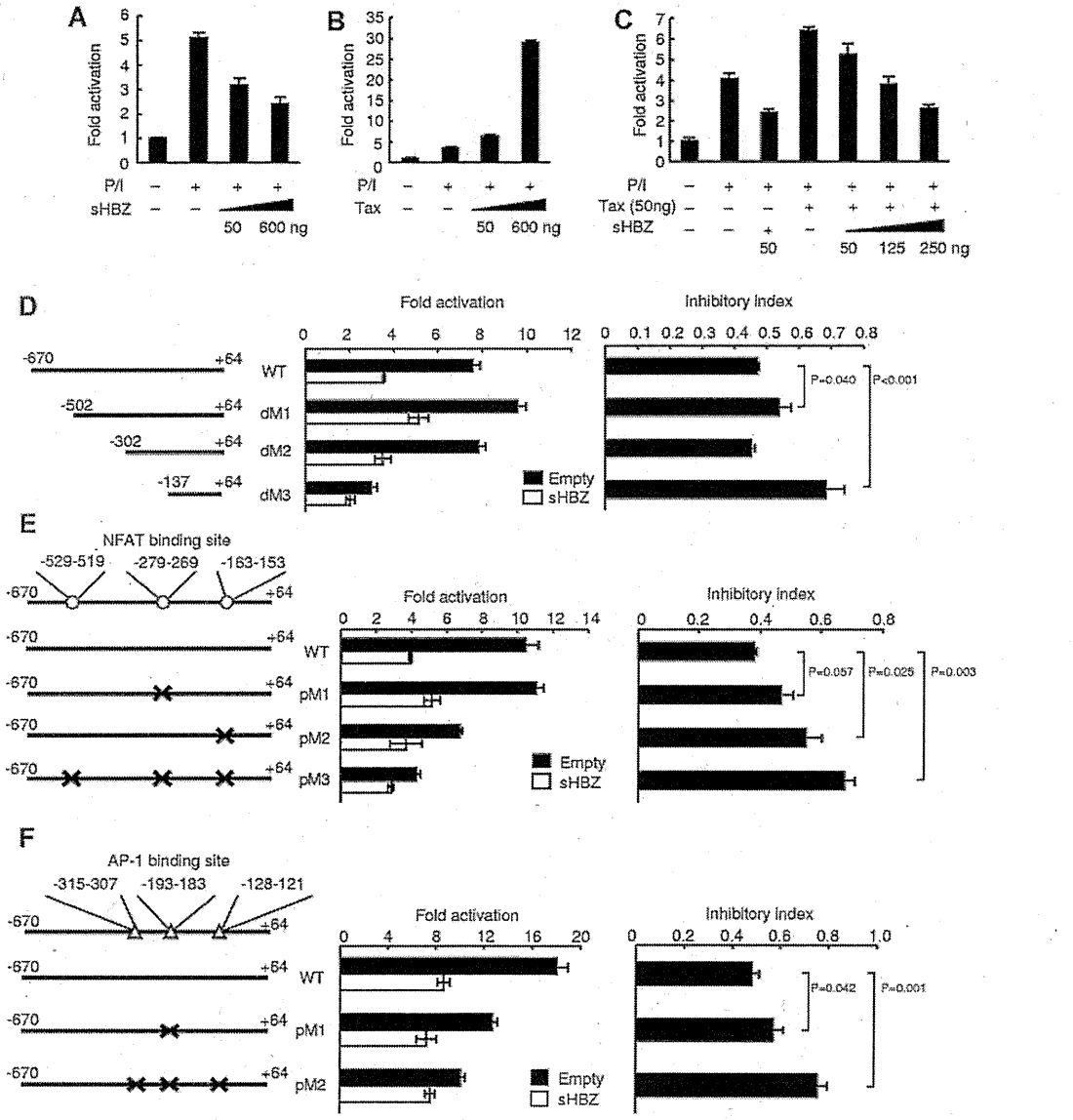

Figure 5. sHBZ suppresses IFN-γ promoter activity. Luciferase assay of the IFN-γ promoter reporter constructs (−670 to +64) cotransfected with an expression plasmid for sHBZ (A), Tax (B), or both (C) is performed in Jurkat cells, which were stimulated with PMA and ionomycin. Luciferase assays of reporter plasmids containing deletions (D) or point mutations in the NFAT (E) or AP-1 (F) consensus-binding region of IFN-γ promoter are performed. The positions of the deleted or mutated regions are indicated in the left of each graph. Consensus sequences for NFAT and AP-1 binding sites were mutated. Inhibitory index is represented as a ratio of fold activation with empty vector or HBZ expression vector. Representative data (mean ± SD) from 2 independent experiments in triplicate are shown.

I heavy chains and inhibits its expression, which results in escape of infected cells from host immune system.[39] A number of viruses evade the host immune response by perturbing the production of cytokines. It has been reported that the core protein of HCV decreases IL-2 production via suppression of mitogen-activated protein kinase.[40] The vaccinia virus double-strand RNA binding protein E3 inhibits the PKR, NF-κB, and IRF3 pathways, thus suppressing IFN-β, TNF-α, and TGF-β production.[41] The HIV-1 Tat protein perturbs signal transduction by IFN-γ.[42] However, it has not been known precisely how HTLV-1 evades the host immune system. In this study, we show that sHBZ inhibits the effector function of CD4 T cells via interaction with NFAT and AP-1, leading to a suppressive effect on the production of Th1 cytokines, such as IFN-γ. This is probably a mechanism of the cellular immune deficiency observed in HTLV-1 infection.

It is well known that NF-κB, AP-1, and NFAT are involved in T-cell receptor signaling pathways.[43] Tax is broadly recognized to play a crucial role in the pathogenesis of HTLV-1, including oncogenesis and inflammation. Previous studies showed that Tax could activate cellular signaling pathways, including NF-κB, and AP-1.[36] Thus, Tax has an enhancing effect, not a suppressive effect, on the immune response of infected cells. On the other hand, HBZ is constitutively transcribed in infected cells and suppresses cellular signaling pathways, including the CREB, AP-1, and canonical NF-κB pathways.[44] These findings suggest that HBZ, rather than Tax, is probably responsible for the immune deficiency in HTLV-1 infection and may act through the impairment of effector cytokine production. Indeed, this study shows that sHBZ suppresses the *IFN-γ* transcription through interaction with NFAT and c-Jun.

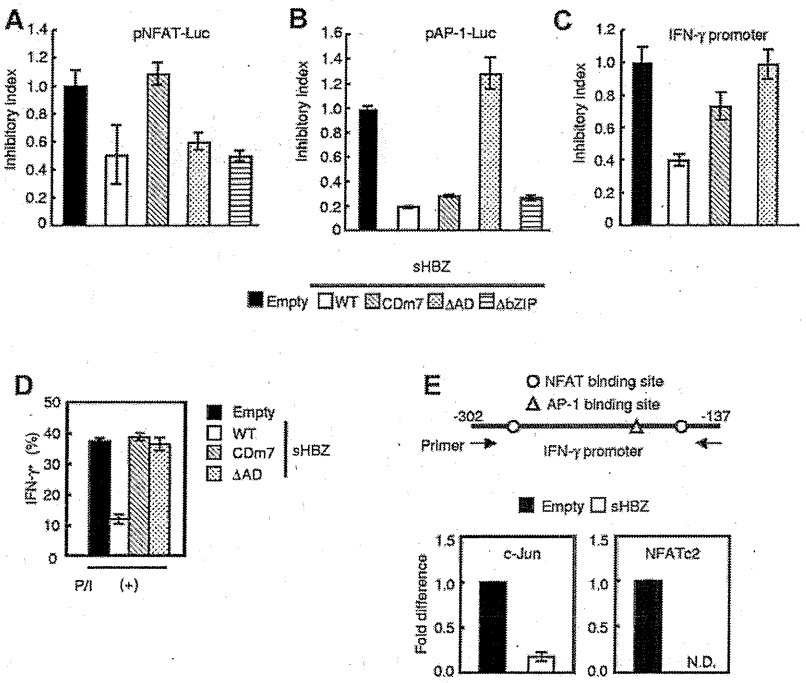

Figure 6. NFAT and AP-1 signaling pathways are responsible for HBZ-mediated inhibition of IFN-γ production. (A-C) Effects of wild-type and mutant sHBZ on (A) an NFAT-Luc reporter, (B) an AP-1-Luc reporter, and (C) the IFN-γ promoter. (D) The suppressive effect of sHBZ mutants on IFN-γ production from primary mouse CD4 T cells. Retroviruses expressing wild-type and mutated HBZ were transduced to primary mouse CD4 T cells, stimulated with PMA and ionomycin, and stained. (E) ChIP assay of the NFAT and AP-1 binding sites of IFN-γ promoter. sHBZ-expressing Jurkat cells were stimulated with PMA and ionomycin, and ChIP assay was performed using anti-NFATc2 or anti–c-Jun antibodies. The IFN-γ promoter (−302 to −137) was amplified by real-time PCR. The data from stimulated empty-transfected Jurkat cells were used as a reference. Representative data (mean ± SD) from 2 or 3 independent experiments are shown. N.D. indicates not detected.

We have recently reported that the HBZ-Tg mice used in this study harbor increased numbers of $CD4^+$ $Foxp3^+$ Tregs compared with non-Tg mice.[17] Tregs are known as negative regulators of the host immune response to pathogens[45]; hence, an increase in the number of Tregs might contribute to the suppression of effector T-cell responses against HSV-2 or LM in vivo. Tregs suppress the memory CD8 T-cell response.[46] However, we found that the production of IFN-γ was impaired in sHBZ-expressing CD4 T cells but not in CD8 T cells (Figure 3A). IFN-γ production was impaired in a CD4 T cell–intrinsic manner. In addition, the suppressive effect of Tregs on IFN-γ production by effector CD4 T cells was not observed in mice immunized with LM (supplemental Figure 6). Taken together, these data imply that the increased number of Tregs is not the main cause of the CD4 T-cell specific reduction of IFN-γ production; rather, sHBZ expression in CD4 T cells may lead directly to suppressed production of IFN-γ.

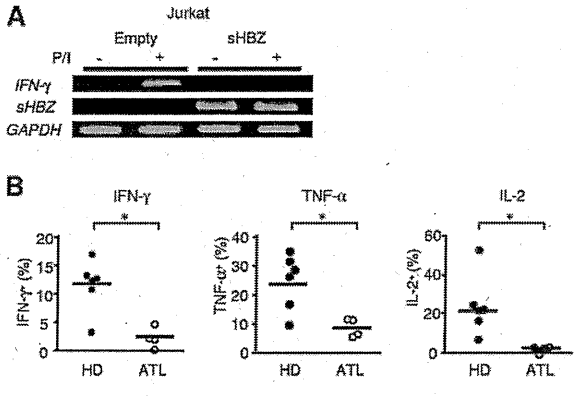

Figure 7. IFN-γ production is suppressed in sHBZ-expressing Jurkat cells and PBMCs of ATL patients. (A) sHBZ inhibits *IFN-γ* gene transcription after stimulation with PMA and ionomycin. Transcripts of the *IFN-γ* and *sHBZ* genes were analyzed by RT-PCR. (B) IFN-γ, TNF-α, and IL-2 production by CD4 T cells in PBMCs from healthy donors (HD; n = 6) and ATL patients (n = 4). PBMCs were separated from the peripheral blood and then stimulated with PMA and ionomycin for 5 hours. Thereafter, intracellular production of Th1 cytokines in living cells was measured by flow cytometry. The y-axis indicates the percentages of cytokine-producing cells in CD4 T cells. *$P < .05$ by Student *t* test.

In this study, we evaluated the cell-mediated immunity of HBZ-Tg mice against HSV-2 and LM. The protective immune response to these pathogens is mediated by IFN-γ production by NK cells, CTLs, and/or Th1 cells.[47] IFN-γ up-regulates major histocompatibility complex molecules, and inducible nitric oxide synthase, activates NK cells and macrophages, and induces Th1 development,[47] thus leading to the elimination of HSV-2 and LM. Lack of IFN-γ function (because of mutation of IFN-γ or its receptor, or because of the presence of IFN-γ specific antibody) in vivo increases susceptibility to many pathogens, including lymphocytic choriomeningitis virus, *Mycobacterium tuberculosis*, and *Leishmania major*.[47] Of particular interest is the fact that protection against infection with *Cryptosporidium parvum*,[48] or *Candida albicans*,[49] which cause opportunistic infections in immune compromised hosts, depends on IFN-γ production from CD4 T cells. In addition, previous reports have shown that a lack of CD4 T-cell help during primary infection results in an incomplete memory immune response in which CTL activity and antibody production by plasma cells are impaired.[50] Our current results, therefore, indicate that the reduced production of helper cytokine caused by sHBZ expression in CD4 T cells may contribute to the immunodeficiency observed in HTLV-1–infected persons and in HBZ-Tg mice.

Previous studies reported that activation and bZIP domains of HBZ played important roles in suppressive effects on the AP-1 pathway.[19,37] However, this study showed that only activation domain was critical in T cells when stimulated by PMA and ionomycin. Deletion of bZIP domain partially impaired AP-1 activation by Tax (supplemental Figure 5B). Previous studies used 293T cells and stimulated them by expression of c-Jun or Tax to analyze suppressive function of HBZ for the AP-1 pathway.[19,37] Therefore, this difference might be because of not only cell type, but also stimulator. HTLV-1 infects CD4 T cells and IFN-γ is