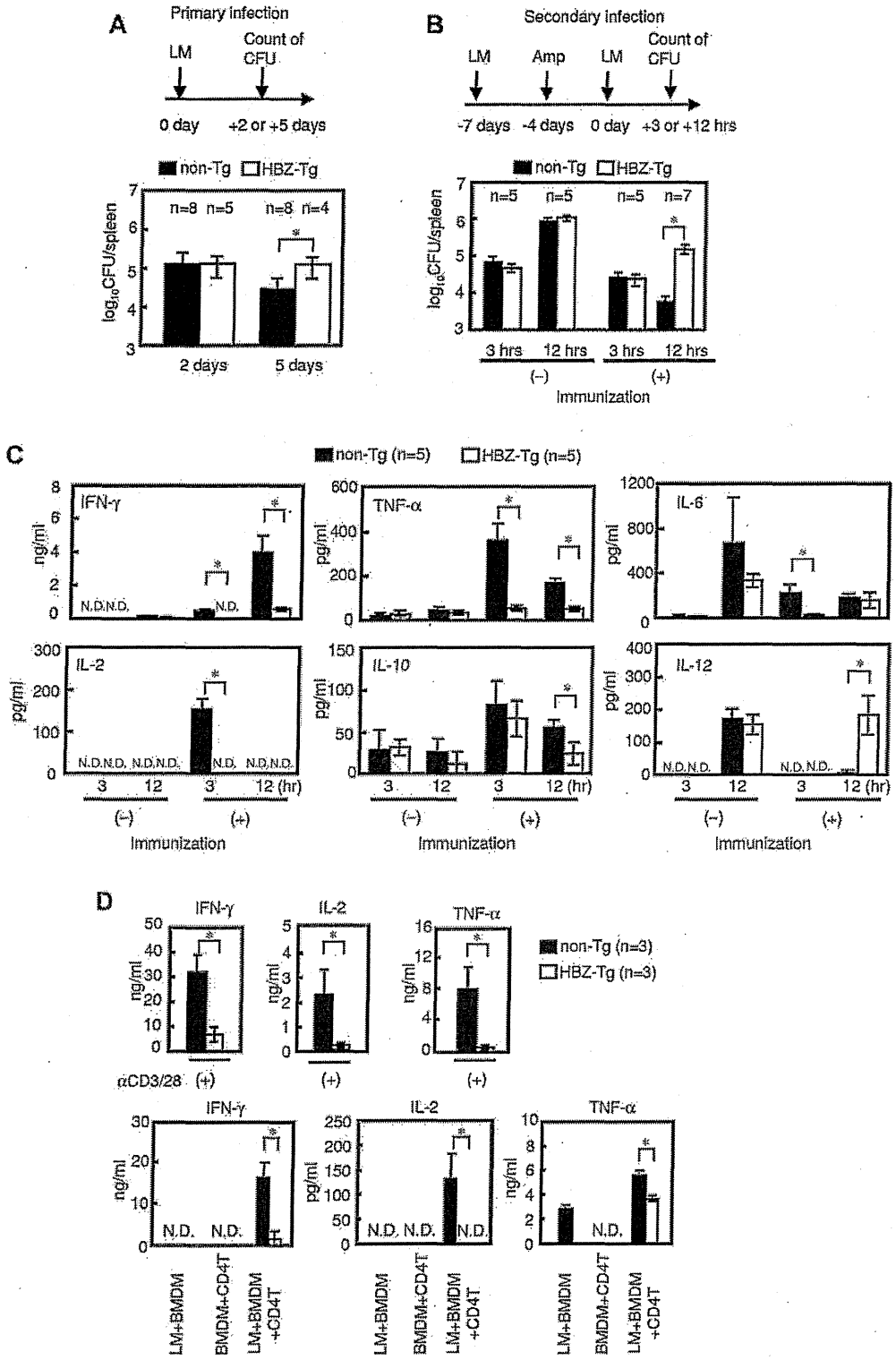

Figure 2. HBZ-Tg mice show decreased immune response to primary and secondary infection with LM. Bacterial loads of spleens from mice challenged with LM in primary (A) and secondary (B) infection are shown. (C) Concentrations of IFN-γ, TNF-α, IL-2, IL-6, and IL-12 in serum and IL-10 in homogenized spleen 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

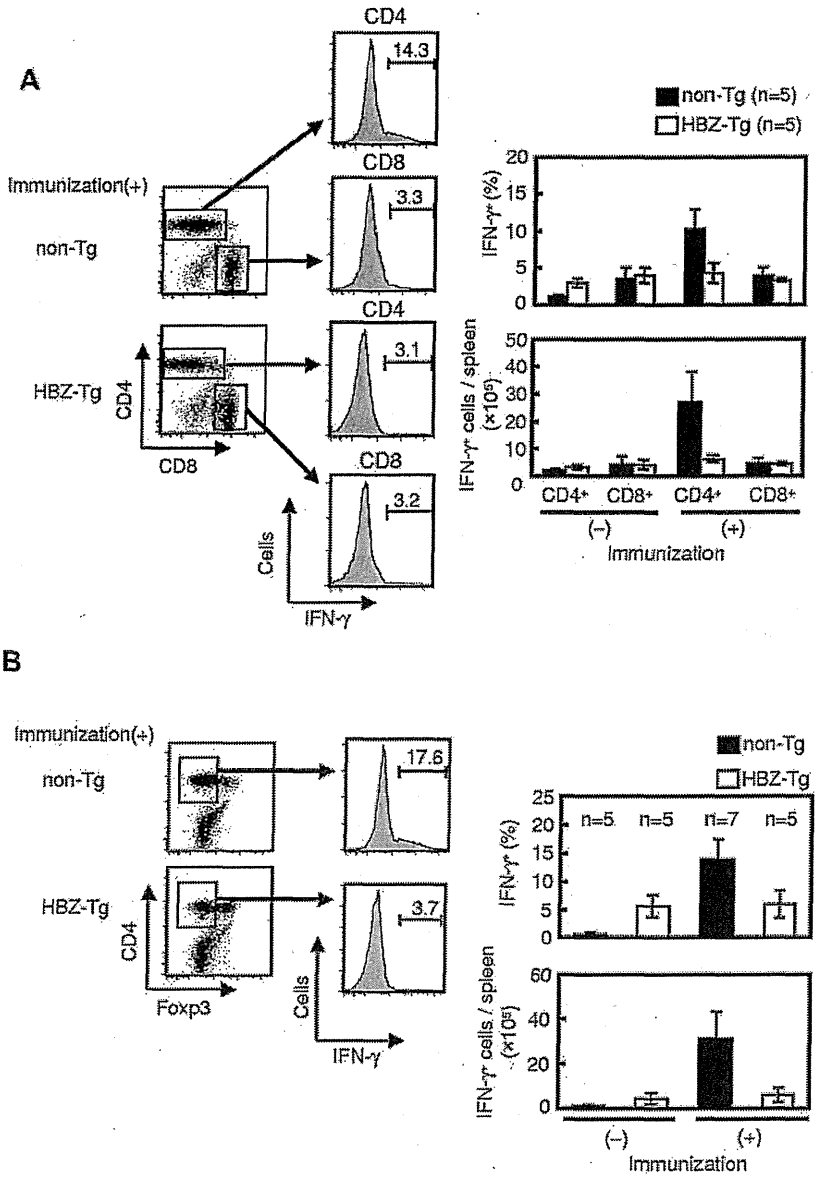

Figure 3. IFN-γ production by CD4 splenocytes from LM secondarily infected HBZ-Tg mice decreases in CD4⁺ Foxp3⁻ T cells. Mice were immunized and challenged as shown at the top of Figure 2B, and their splenocytes were harvested at 12 hours after challenge and analyzed for intracellular IFN-γ production. (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

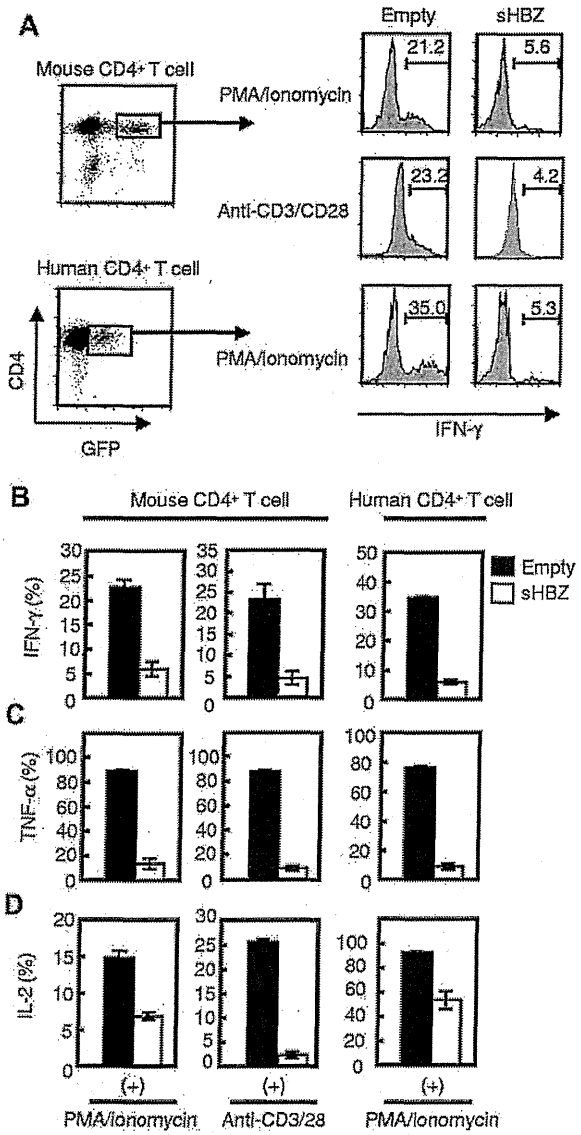

Figure 4. sHBZ directly inhibits IFN-γ production in both human and mouse CD4 T cells. Mouse and human CD4 T cells were transduced with recombinant retroviruses or lentiviruses, respectively, expressing sHBZ, and stimulated with PMA and ionomycin or antibodies to CD3 and CD28. Then, intracellular cytokines 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

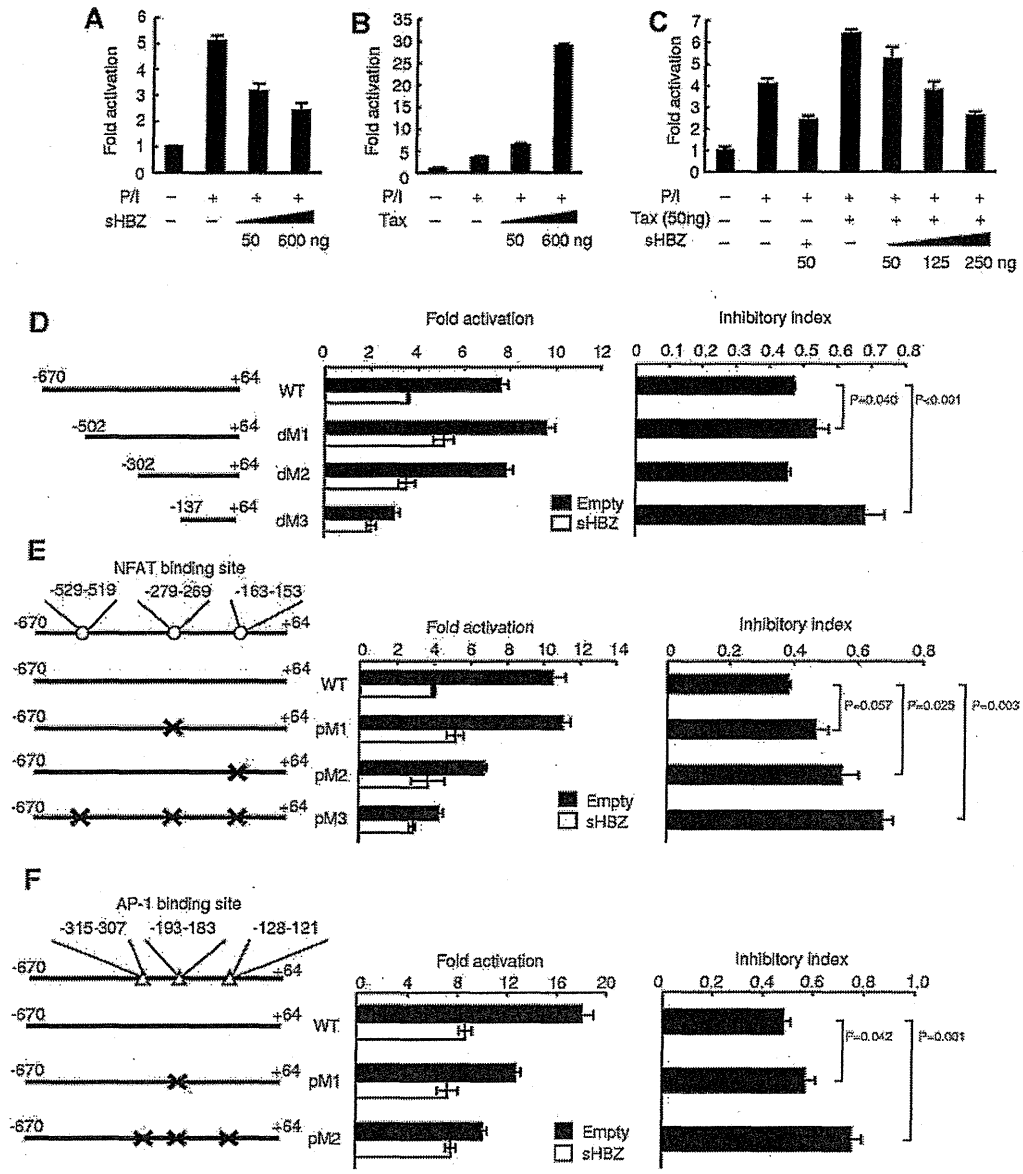

Figure 5. sHBZ suppresses IFN-γ promoter activity. Luciferase assay of the IFN-γ promoter reporter constructs (−670 to +64) cotransfected with an expression plasmid for sHBZ (A), Tax (B), or both (C) is performed in Jurkat cells, which were stimulated with PMA and ionomycin. Luciferase assays of reporter 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.

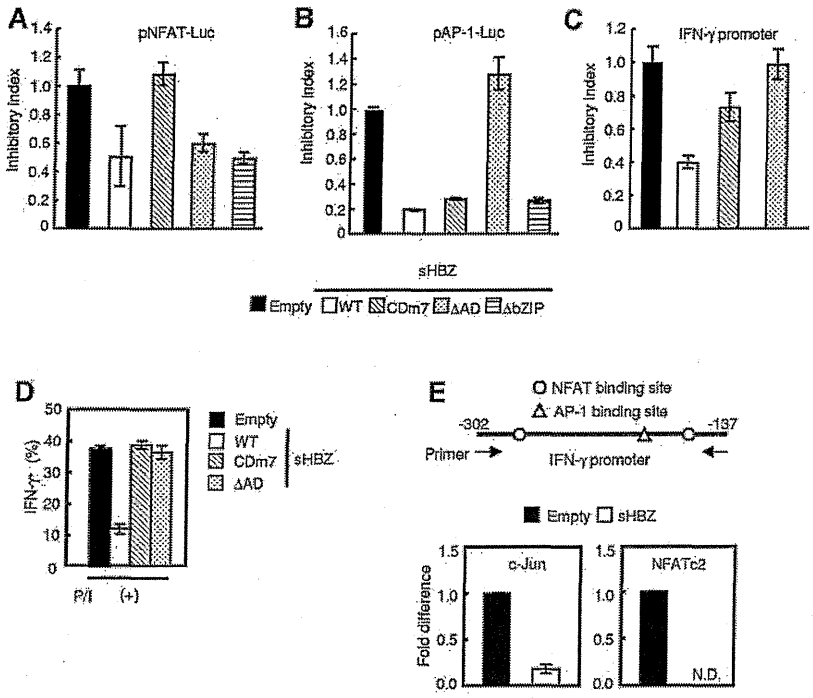

Figure 6. NFAT and AP-1 signaling pathways are responsible for HBZ-mediated inhibition of IFN-γ production. (A-C) Effects of wild-type and mutant sHBZ on (A) an NFAT-Luc reporter, (B) an AP-1-Luc reporter, and (C) the IFN-γ promoter. (D) The suppressive effect of sHBZ mutants on IFN-γ production from 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.

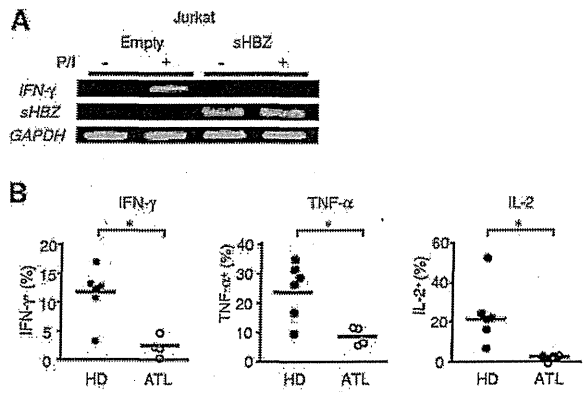

Figure 7. IFN-γ production is suppressed in sHBZ-expressing Jurkat cells and PBMCs of ATL patients. (A) sHBZ inhibits *IFN-γ* gene transcription after stimulation with PMA and ionomycin. Transcripts of the *IFN-γ* and *sHBZ* genes were analyzed by RT-PCR. (B) IFN-γ, TNF-α, and IL-2 production by CD4 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

produced by stimulation of T cells, indicating that activation domain of HBZ plays an important role in suppression of AP-1 signaling.

The immune deficiency observed in ATL patients is one of the major factors in their poor prognosis. The mechanisms of HTLV-1–associated oncogenesis have been extensively investigated, yet there are only a limited number of reports regarding HTLV-1-related immune deficiency. Our results contribute to the understanding of this phenomenon by identifying a new mechanism of HTLV-1–induced immunodeficiency.

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Authorship

Contribution: K.S., Y.S., J.Y., H.H., M. Mitsuyama, and M. Matsuoka conceived and designed the experiments; K.S., Y.S., and K.O. performed the experiments; K.S., Y.S., J.Y., H.H., K.O., M. Mitsuyama, and M. Matsuoka analyzed the data; A.U. and M. Mitsuyama contributed reagents/materials/analysis tools; and K.S., Y.S., J.Y., M. Mitsuyama, and M. Matsuoka wrote the paper.

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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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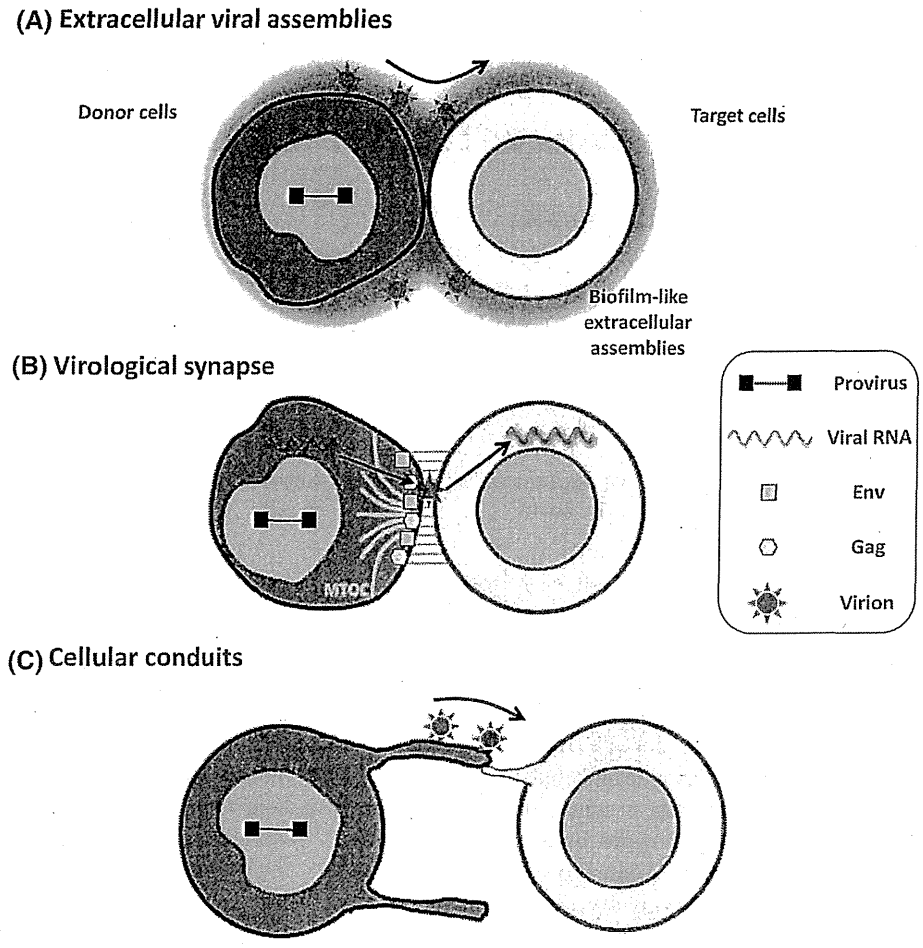

Fig. 1 Mechanisms of cell-to-cell transmission of HTLV-1. **a** HTLV-1 virions are caught in biofilm-like extracellular assemblies surrounding infected cells, and are rapidly transferred to the surface of target cells after conjugation, resulting in infection. **b** An HTLV-1-infected T cell and an uninfected 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

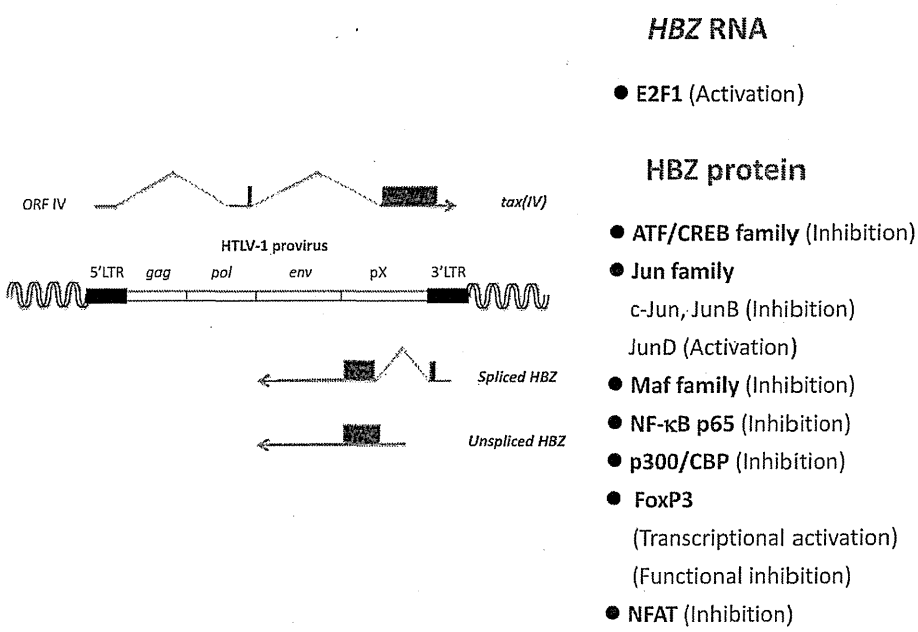

Fig. 2 Function of HBZ RNA and protein. *Left* location of the coding regions of HBZ and Tax in HTLV-1 provirus. *Right* functions of HBZ RNA and protein in the literature

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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Ubiquitin-Specific Peptidase 20 Targets TRAF6 and Human T Cell Leukemia Virus Type 1 Tax To Negatively Regulate NF-κB Signaling[▽]

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NF-κB plays a key role in innate and acquired immunity. Its activity is regulated through intricate signaling networks. Persistent or excessive activation of NF-κB induces diseases, such as autoimmune disorders and malignant neoplasms. Infection by human T cell leukemia virus type 1 (HTLV-1) causes a fatal hematopoietic malignancy termed adult T cell leukemia (ATL). The HTLV-1 viral oncoprotein Tax functions pivotally in leukemogenesis through its potent activation of NF-κB. Recent findings suggest that protein ubiquitination is crucial for proper regulation of NF-κB signaling and for Tax activity. Here, we report that ubiquitin-specific peptidase USP20 deubiquitinates TRAF6 and Tax and suppresses interleukin 1β (IL-1β)- and Tax-induced NF-κB activation. Our results point to USP20 as a key negative regulator of Tax-induced NF-κB signaling.

Protein ubiquitination is an essential posttranslational modification that is implicated in many biological processes (14, 52). Ubiquitin is a small protein composed of 76 amino acids. It contains 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63). Multiple ubiquitin monomers can become covalently linked, and polyubiquitin molecules linked through the lysine 48 residue (K48) are known to modulate protein degradation. In contrast, polyubiquitin molecules linked through the lysine 63 (K63) residue do not induce degradation but influence protein localization, protein-protein interaction, protein functional activation, and other activities (14, 44). The addition of ubiquitin to or the removal of ubiquitin from protein substrates can reversibly and dynamically change protein functions, and these reactions are executed by ubiquitin ligases and deubiquitinases (44). Accordingly, the ubiquitination process is mediated by the serial actions of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase (14, 52), and deubiquitination is through deubiquitining enzymes (DUBs) that directly remove ubiquitin molecules from their substrates (32). Based on sequence analyses, approximately 90 DUB genes have been identified in the human genome. These DUBs are divided into 5 subclasses according to their protein sequences: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado-Joseph disease protein domain proteases (MJDs), ovarian tumor proteases (OTUs), and JAMM motif proteases (32). The specificities and actions of these various deubiquitinases remain to be fully characterized.

In mammals, NF-κB signaling plays key roles in inflammation, cell proliferation, and apoptosis (36). Like phosphorylation, ubiquitination is an important posttranslational modification that can regulate NF-κB activity (43). For example, the ubiquitination of a regulatory subunit in the IKK complex, IKKγ which is also known as NEMO, has a central role in signal transduction. It has been shown that K63-linked linear conjugation of ubiquitin to IKKγ positively regulates NF-κB signaling (47, 53). In addition, the ubiquitination of tumor necrosis factor receptor-associated factors (TRAFs) is also important for IKK activation. TRAF6 is an E3 ubiquitin ligase, and it performs self-ubiquitination through K63-linked chains upon cellular activation through Toll-like receptors (TLRs) and cytokine receptors (8). Moreover, in the canonical NF-κB pathway, IκBα, which sequesters NF-κB proteins in the cytoplasm in an inactive state, is conjugated with K48-linked polyubiquitin chains and is proteasomally degraded when cells are stimulated to activate NF-κB (4). Similarly, cellular activation induces the ubiquitination and proteosomal processing of NF-κB2 p100 to p52, allowing NF-κB RelB/p52 dimers to translocate into the nucleus.

The role of DUBs in the NF-κB pathway has also been studied. For example, the familial cylindromatosis tumor suppressor CYLD is one of the DUBs that have been found to suppress NF-κB activity. CYLD has been shown to bind IKKγ and to reduce the ubiquitination of TRAF2, TRAF6, and IKKγ (3, 24, 48). A20 is a second well-studied DUB that negatively regulates NF-κB activation by reducing the ubiquitination of TRAF2, TRAF6, and RIP1 (17, 20, 41). A20 has dual activities in ubiquitination and deubiquitination. Hence, A20 with TAX1BP1 as a cofactor promotes the cleavage of K63-linked polyubiquitin chains on RIP1, and A20 with E3 ligase Itch can conjugate K48-linked chains on RIP for proteosomal degradation (39, 51). Recently, it was also found that A20 can inhibit the ubiquitination of TRAF2 and TRAF6 by dissociating complexes composed of TRAFs and E2 ubiquitin-conjugating enzymes (41).

Infection by human T cell leukemia virus type 1 (HTLV-1) causes a fatal hematopoietic malignancy, adult T cell leukemia

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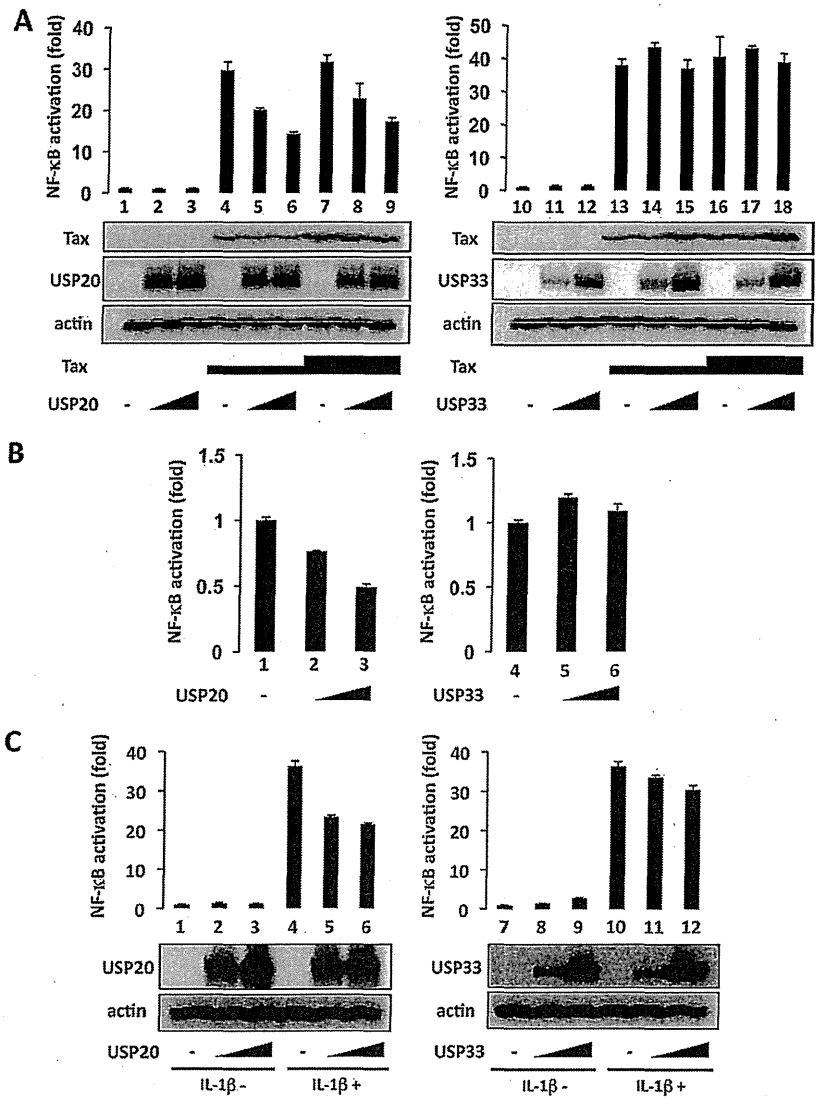

FIG. 1. Expression of USP20 suppresses HTLV-1 Tax- and IL-1β-induced NF-κB activation. (A) HEK293T cells were transfected with a Tax-expressing plasmid, Hpx-Tax (0.05 μg in lanes 4 to 6 and 13 to 15; 0.15 μg in lanes 7 to 9 and 16 to 18), and κB-luc reporter (0.05 μg) with pcDNA3-USP20 (0.5 μg in lanes 2, 5, and 8; 1 μg in lanes 3, 6, and 9) or pCMV-USP33 (0.5 μg in lanes 11, 14, and 17; 1 μg in lanes 12, 15, and 18). In each case, a pCMV-β-Gal plasmid (0.01 μg) was included, and β-Gal values were used to normalize for transfection efficiency. Immunoblotting (lower panels) was done to confirm the expression of transfected and control (actin) protein using the indicated antibodies. (B) ATL2 cells were transfected with κB-luc reporter (1 μg) with pcDNA3-USP20 (2 μg in lane 2; 4 μg in lane 3) or pCMV-USP33 (2 μg in lane 5; 4 μg in lane 6). pCMV-β-Gal (0.2 μg) was included in each transfection as an internal normalization control. (C) HEK293T cells were transfected with κB-luc reporter (0.05 μg) with pcDNA3-USP20 (0.5 μg in lanes 2 and 5; 1 μg in lanes 3 and 6) or pCMV-USP33 (0.5 μg in lanes 8 and 11; 1 μg in lanes 9 and 12) and were treated with recombinant IL-1β (10 ng/ml) for 8 h (lanes 4 to 6 and 10 to 12). pCMV-β-Gal (0.01 μg) was included in each transfection as an internal control. Immunoblotting was performed to confirm the expression of transfected and control (actin) protein using the indicated antibodies. Total amounts of transfected DNA were equalized in each sample by the addition of vector DNA. Cell lysates were assayed for luciferase. The results from three independent experiments are shown as average values ± standard deviations (SD).

(ATL) (29), and one of its key regulatory proteins, Tax, plays important roles in viral pathogenesis (2, 10, 11, 26, 37). Tax (29) can potently activate NF-κB through both the canonical and noncanonical pathways (15, 42, 45). Tax was recently found to inhibit A20 function by disrupting its interaction with TAX1BP1 and Itch (39). Currently, how other DUBs may contribute to Tax-induced NF-κB signaling has not been studied. Here, we report on the characterization of USP20 for its regulation of Tax- and TRAF6-mediated activation of NF-κB.

MATERIALS AND METHODS

Cells and reagents. Human embryonic kidney cell line HEK293T and human cervical cancer cell line HeLa were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics. Human T cell lines MT1, MT2, MT4, ATL2, Jurkat, CEM, and H9 were maintained in RPMI 1640 with 10% FBS. Recombinant human interleukin-1β (IL-1β) (Peprotech) was used.

Plasmids and siRNAs. The κB-luc reporter plasmid was described previously (19). Human USP20 coding sequences were amplified by PCR and subcloned into the pcDNA3 (Invitrogen) and the pCAG-HA (50) expression vectors. A

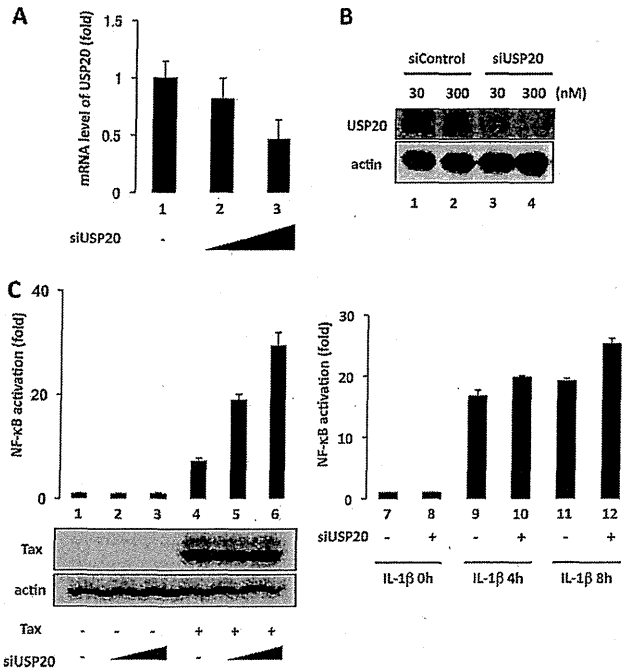

FIG. 2. Knockdown of cell-endogenous USP20 enhances HTLV-1 Tax- and IL-1β-induced NF-κB activation. (A) Sequence-specific siRNA was used to knock down USP20. The knockdown of cell-endogenous USP20 by siRNA was confirmed with real-time RT-PCR. USP20-specific siRNAs at 20 nM (bar 2) or 40 nM (bar 3) were transfected into HEK293T cells, from which RNA was extracted. USP20 mRNA levels were quantified by real time RT-PCR. (B) The competent knockdown of cell-endogenous USP20 by siRNA was checked by immunoblotting. USP20-specific siRNAs at 30 nM (lane 3) or 300 nM (lane 4) were transfected into Jurkat cells, and cell-endogenous USP20 protein was assayed by immunoblotting. (C) HEK293T cells were transfected with Hpx-Tax (bars 4 to 6) or treated with IL-1β (50 ng/ml) at the indicated times (bars 9 to 12) with or without the cotransfection of siRNA for USP20 (20 nM in lanes 2 and 5; 40 nM in lanes 3, 6, 8, 10, and 12) (0 h of IL-1β means that no treatment occurred). Total amounts of transfected DNAs and siRNAs were equalized by the addition of either vector DNA or control siRNA, respectively. Cell lysates were assayed for luciferase. Immunoblotting was done to confirm the expression of transfected and control (actin) protein using the indicated antibodies. The results from three independent experiments are shown as average values ± SD.

human USP33 cDNA clone (I.M.A.G.E. 3874822) was purchased from ATCC, and the coding region was inserted into pCAG-HA. For ectopic expression of Tax, Hpx-Tax (18) and pCAG-Flag-Tax were used. Flag-TRAF6 has been described elsewhere (20). The pcDNA-HA-ubiquitin expression vector was used in immunoblotting experiments to detect ubiquitinated Tax and TRAF6. For the knockdown of USP20, synthesized small interfering RNAs (siRNAs) (Qiagen) were used. The target sequence of USP20 is 5′-TCGAGTGACACGGATGAG AAA-3′. Sequence-nonspecific siRNA was purchased from Qiagen and used as a negative control.

Antibodies. Mouse monoclonal anti-Tax (NIH AIDS Research and Reference Reagent Program) was used to detect Tax protein in immunoblotting. Anti-Flag monoclonal antibody (M2; Sigma), anti-Flag polyclonal antibody (Sigma), anti-hemagglutinin (anti-HA) monoclonal antibody (HA-7; Sigma), anti-HA polyclonal antibody (Sigma), anti-USP20 polyclonal antibody (Bethyl Laboratories), anti-USP33 polyclonal antibody (Bethyl Laboratories), anti-tubulin monoclonal antibody (DM1A; Sigma), and anti-actin monoclonal antibody (AC-40; Sigma) were purchased.

Luciferase assay. Expression plasmids or siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen). Cells were transfected with Tax and USP expression vectors or were transfected with siRNAs first, followed 48 h later with κB-Luc and pCMV-β-galactosidase (pCMV-β-Gal) plasmids. At 24 h after transfection of the reporters, cell lysates were subjected to luciferase assay. Total amounts of DNA and RNA to be transfected were adjusted by the addition of empty vectors or nonspecific siRNAs. To detect luciferase and β-Gal activity, luciferase substrate (Promega) and the Galacto-Star assay system (Applied Biosystems) were used. Relative values of luciferase activity were calculated using β-Gal activity as an internal control for transfection.

Coimmunoprecipitation assay. At 48 h after transfection, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES, 0.3% NP-40, 150 mM NaCl, 2 mM EDTA, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 0.5 mM dithiothreitol [DTT], and 1× protease inhibitor cocktail from Roche). Cell extracts were subjected to immunoprecipitation with anti-Flag or anti-HA antibodies, and the captured proteins were detected by immunoblotting using the indicated antibodies.

Immunofluorescence. Cells were cultured on glass coverslips, and fixed in 4% paraformaldehyde at 24 h after transfection. After blocking of nonspecific reactions with 1% bovine serum albumin (BSA), cells were then incubated with the indicated primary antibodies, followed by a subsequent incubation with the secondary antibodies conjugated with Alexa Fluor 488 or 594 (Molecular Probes). DNA was counterstained with 0.1 μg/ml Hoechst 33342. Coverslips were mounted in Prolong Antifade (Molecular Probes), and cells were visualized with a Leica TCS SP2 confocal microscope.

Real-time PCR. USP20 transcripts were quantified by real-time PCR. The sequences of the primers for USP20 were 5′-TCACAGAAGTCCACGAGAC G-3′ (sense) and 5′-TTGTCCTTCCCCTTGACGAA-3′ (antisense). To compare the relative expression levels between samples, we quantified mRNA levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal control using the primers 5′-GCTCACTGGCATGGCCTTCCGTGT-3′ (sense) and 5′-TGGAGGAGTGGGTGTCGCTGTTGA-3′ (antisense). Power SYBR green PCR Master Mix (Applied Biosystems) was used for preparation of the PCR mixtures, and the reaction was carried out with an Applied Biosystems 7500 real-time PCR system. All samples were measured in triplicate and analyzed with $\Delta\Delta CT$ method.

Cell proliferation assay. ATL2 cells were transfected with USP20, USP33, or control expression vector using Amaxa Nucleofector II (Human T Cell Nucleofector kit, protocol X-001). Using a green fluorescent protein (GFP)-expressing plasmid, we analyzed the transfection efficiency of ATL2 cells with this method and found that routinely ~60% of cells were visibly GFP positive at 48 h after transfection. Cell proliferation was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

RESULTS

USP20 suppresses HTLV-1 Tax- and cytokine-induced NF-κB activation. Activation of NF-κB contributes to cellular transformation by HTLV-1, and Tax is a potent activator of NF-κB (15, 29, 42). Protein ubiquitination plays an important role in signal transduction; indeed, individual NF-κB pathway constituents are frequently ubiquitinated when cells are stimulated (4, 14, 36). We reasoned that to maintain homeostasis, *de novo*-ubiquitinated proteins must eventually be deubiquitinated. To identify the cellular DUBs that might affect Tax-induced NF-κB activation, we tested 12 discrete DUBs and found that USP20 can suppress Tax-induced luciferase expression in a dose-dependent manner (Fig. 1A, lanes 1 to 9). By comparison, a related DUB, USP33 (and 10 other DUBs [data not shown]), failed to influence NF-κB activation by Tax (Fig. 1A, lanes 10 to 18). Next, we checked the activities of USP20 and USP33 in HTLV-1 transformed ATL2 leukemic cells which were directly derived from the leukemic cells of an ATL patient (46). We found that USP20, but not USP33, inhibited NF-κB activity in ATL2 cells (Fig. 1B). Collectively, the results suggest that the NF-κB activating pathways in HEK293T (Fig. 1A) and ATL2 (Fig. 1B) cells are both negatively regulated by USP20.

In order to characterize the roles of USP20 and USP33 in regard to other activators of NF-κB, we investigated their

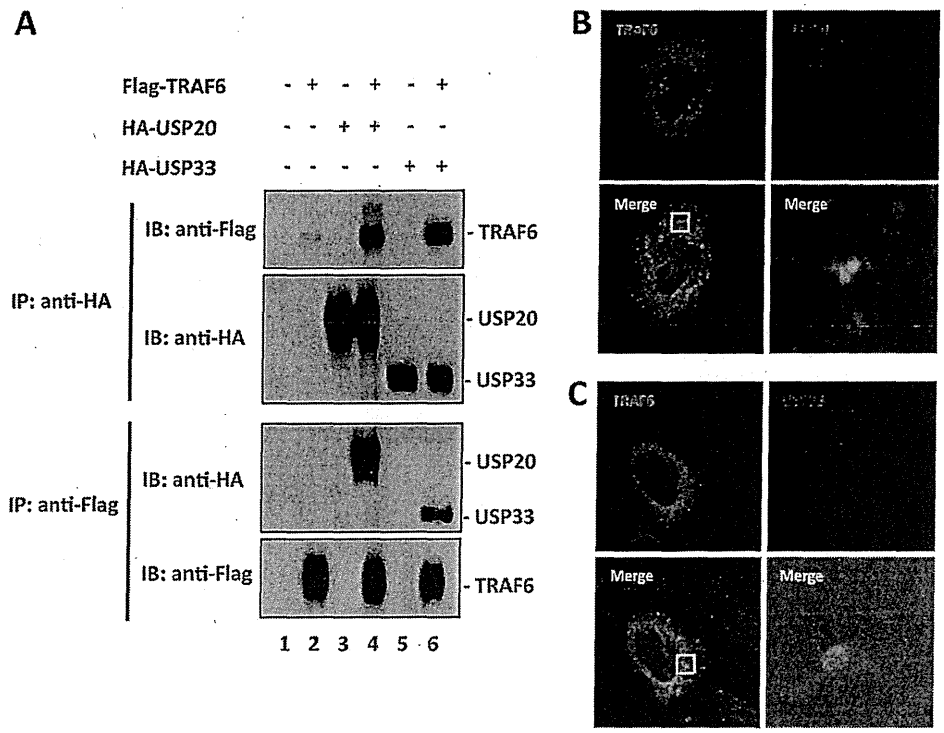

FIG. 3. TRAF6 interacts with USP20 and USP33. (A) HEK293T cells were transfected with the indicated plasmids, and immunoprecipitation was done with either anti-Flag or anti-HA antibody as indicated. The immunoprecipitates and total cell extracts were resolved by SDS-PAGE and immunoblotted. The blots were probed with either anti-Flag or anti-HA. (B and C) Flag-TRAF6 plasmid with either HA-USP20 or HA-USP33 was cotransfected into HeLa cells. Subcellular localizations of TRAF6, USP20, or USP33 were visualized with anti-Flag (green) or anti-HA (red). The nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) (blue). The higher magnification of the "Merge" is shown in the lower right panels of panels B and C.

effects on IL-1β-mediated activation. As shown in Fig. 1C, NF-κB activation by IL-1β was suppressed when either USP20 (Fig. 1C, lanes 5 and 6) or USP33 (Fig. 1C, lanes 11 and 12) was expressed in HEK293T cells. These findings suggest that both USP20 and USP33 can negatively regulate IL-1β-mediated NF-κB signaling, while USP20, but not USP33, can modulate Tax-induced NF-κB activation (Fig. 1A).

To check for physiological relevance and to rule out that the results were simply from nonphysiological overexpression, we knocked down cell-endogenous USP20 in HEK293T cells using siRNAs. Using real-time reverse transcription-PCR (RT-PCR), we documented an siRNA dose-dependent knockdown of USP20 mRNA in HEK293T (Fig. 2A, bars 2 and 3). To check the knockdown effect of the siRNA at the protein level, we transfected the siRNA into Jurkat cells and confirmed by immunoblotting that USP20 expression was reduced as expected (Fig. 2B). Next, at 48 h after USP20 siRNA transfection into HEK293T cells, we transfected a κB-luc reporter plasmid and checked for its induction by Tax or IL-1β. With either Tax (Fig. 2C, compare bar 4 to bars 5 and 6) or IL-1β (Fig. 2C, compare bar 9 to 10 and bar 11 to 12), the activity of the κB-luc reporter was higher in USP20-knocked-down cells than in control siRNA-treated cells. Collectively, the findings in Fig. 1 and 2 are consistent with USP20 serving to negatively modulate cellular NF-κB activity.

TRAF6 interacts with USP20 and USP33. To our knowledge, the above observations on USP20 and USP33 represent the first demonstration of their effects on NF-κB signaling. We were thus interested in searching for potential USP20/33 cellular substrates among known NF-κB-signaling proteins. TRAF6 is an NF-κB-signaling protein that has been reported to be regulated by ubiquitination (8). We next asked if TRAF6 would interact with USP20 or USP33 in coimmunoprecipitations. We transfected Flag-tagged TRAF6 and either HA-tagged USP20 or HA-tagged USP33 into HEK293T cells. Cell lysates were immunoprecipitated using anti-Flag or anti-HA, and the presence of HA- or Flag-tagged proteins in the respective immunoprecipitates was assessed by immunoblotting. As shown in Fig. 3A, TRAF6 bound USP20 and USP33. As a control, we checked if another NF-κB-signaling protein also known to be ubiquitinated, IKKγ (47, 53), would interact with USP20 or USP33; however, the latter interaction was not observed (data not shown).

USP20 and USP33 have been reported to localize in perinuclear regions and in small vesicles throughout the cytoplasm (1, 7), and TRAF6 has been shown to locate diffusely in the cytoplasm (49). Next, we immunostained HeLa cells transfected with Flag-TRAF6 and either HA-USP20 or HA-USP33 (Fig. 3B and C). Although not all TRAF6 and USP20/USP33 colocalized together, we did observe partial colocalization of these proteins in the cytoplasm (see yellow costaining in magnified views of the "Merge" panels in Fig. 3B and C), consistent with the potential for intracellular protein-protein interaction.