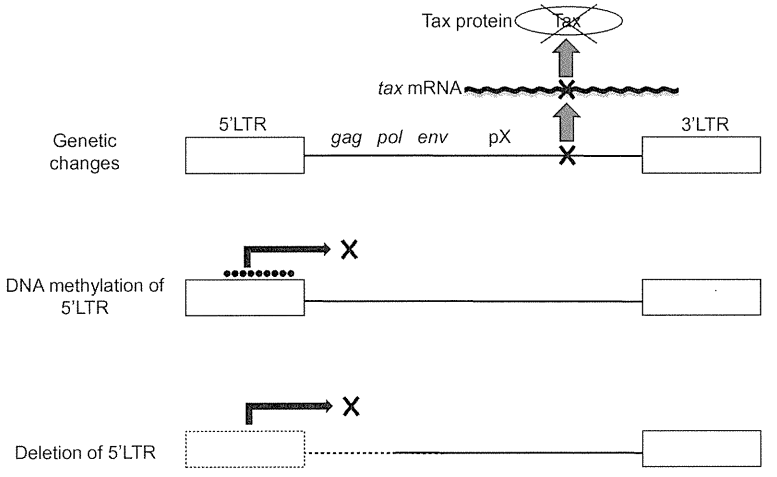


Fig. 2. Three mechanisms to inactivate Tax expression in ATL cells. (1) Genetic changes of the *tax* gene (nonsense mutation, deletion and insertion); (2) DNA methylation of 5′LTR silences transcription of the *tax* gene; and (3) deletion of 5′LTR, which is the promoter/enhancer of the *tax* gene transcription.

increased and stabilized, while other viral genes were at or below the limit of detection (Li et al., 2009). This finding supports a correlation between s*HBZ* gene expression, provirus load, and survival of HTLV-1 infected cells.

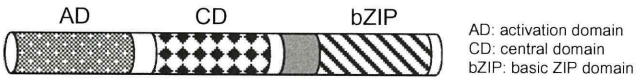
It is noteworthy that the *HBZ* mRNA was well correlated with disease severity in HAM/TSP patients. In addition, neopterin in the cerebrospinal cord fluid, which reflects cellular immune responses, was positively correlated with the level of *HBZ* mRNA, but not with *tax* mRNA. Taken together, these results show that the *HBZ* gene expression is closely linked with disease severity of HAM/TSP, suggesting that the *HBZ* gene expression plays critical roles in proliferation of HTLV-1 infected cells and pathogenesis.

3. The *HBZ* gene in ATL cells

HTLV-1 provirus is the only evidence of HTLV-1 infection in ATL cells, and analysis of provirus is expected to provide clues on leukemogenesis, and on which viral gene is critical for ATL cells or preferential integration sites of HTLV-1 provirus in leukemic cells. It has been thought that Tax is critical for leukemogenesis due to the abundance of reports on its potent effect on cell proliferation, genetic instability, and dysregulation of the cell cycle (Grassmann et al., 2005). Tax expression in transgenic animals induces cancers depending on the promoter used (Lairmore et al., 2005). For example, transgenic mice that expressed Tax under control of the human granzyme B gene promoter developed natural killer cell leukemia while salivary and mammary adenomas were found in the transgenic mice expressing Tax under the CD3-epsilon promoter–enhancer. *In vitro* expression of Tax could immortalize human T-lymphocytes although Tax expression could not transform T-lymphocytes (Akagi et al., 1995).

However, Tax expression was not detected in about 60% of freshly isolated samples from ATL cases (Takeda et al., 2004), indicating that Tax expression is not always necessary for ATL. Detailed analyses of HTLV-1 provirus showed three mechanisms to disrupt Tax expression in ATL cells (Fig. 2). First, the 5′LTR, which is a promoter/enhancer of viral gene transcription of the plus strand, was deleted (Miyazaki et al., 2007; Tamiya et al., 1996). Second, CpG sites of the 5′LTR were hypermethylated, which lead to silencing of plus strand viral gene transcription (Koiwa et al., 2002; Takeda et al., 2004; Taniguchi et al., 2005). Finally, genetic changes (nonsense mutations, deletions, and insertions) were found in the *tax* gene itself (Furukawa et al., 2001; Takeda et al., 2004). These findings suggest that Tax expression is not necessary in the late steps of the leukemogenic process although its expression is required at an early stage of ATL or in the carrier state. However, an analysis of defective provirus without a 5′LTR showed that 8 of 12 cases retained 6-bp short repeats at both ends of the LTR, while four cases lacked these sequences. Since these short repeats are generated during integration, the defective provirus with the 6-bp short repeat is formed before integration. Importantly, defective proviruses in two of eight cases lacked the second exon of the *tax* gene (Miyazaki et al., 2007), indicating that Tax was not expressed in these cases prior to development of leukemia. Nonsense mutations in the *tax* gene by APOBEC3G were found in 7 of 60 ATL cases (Fan et al., 2010). Similar nonsense mutations of the *tax* gene were also found in the carrier state. Thus, Tax expression is not necessary at least for these cases. Conversely, nonsense mutations were not found in the *HBZ* gene in both ATL cases and HTLV-1 carriers, suggesting that HBZ expression is indispensable for proliferation and survival of ATL cells and HTLV-1 infected cells.

As described, three mechanisms prevent Tax expression in ATL cells. However, the 3′LTR remained unmethylated and intact in all of the ATL cases, indicating that the 3′LTR was critical for ATL cells. Since viral genes other than the *HBZ* gene are transcribed from the 5′LTR that is frequently silenced or lost, we expected *HBZ* to be the only viral gene that is consistently expressed in ATL cells. In fact, the *HBZ* gene was transcribed in all ATL cases while transcripts of the *tax* gene were detected in



AD	*Binding with 26S proteasome (usHBZ) Degradation of c-Jun *Interaction with p300
CD	*Nuclear localization
AD+bZIP	*Binding with p65, inhibition of classical NF-κB pathway *Increase of hTERT promoter activity *Activation of JunD
bZIP	*Interaction and inhibition of c-Jun, Jun B, CREB, CREB2

Fig. 3. Functional domains of HBZ. HBZ has three domains: activation, central, and bZIP. Functions of each domain are summarized.

only a limited number of cases (Satou et al., 2006). Suppressing expression of the *HBZ* gene inhibited proliferation of ATL cells (Satou et al., 2006), indicating that the *HBZ* gene has growth-promoting activity in ATL.

4. Property and function of HBZ protein

The spliced transcript of HBZ is translated into a polypeptide of 206 amino acids, while the protein product of unspliced HBZ is a polypeptide of 209 amino acids. HBZ has three domains; the activation, central, and bZIP domains (Fig. 3). HBZ protein is localized in the nucleus with a speckled pattern. Three regions are associated with nuclear localization: two regions rich in basic amino acids and a DNA binding domain (Fig. 3) (Hivin et al., 2005). In addition, the integrity of the HBZ amino acid sequence is necessary for the speckled distribution in the nucleus. HBZ is localized in heterochromatin, consistent with its association with transcriptional inhibition (Hivin et al., 2005). Furthermore, HBZ has been shown to sequester JunB to nuclear bodies, thus suppressing JunB-dependent transcriptional activity (Hivin et al., 2007).

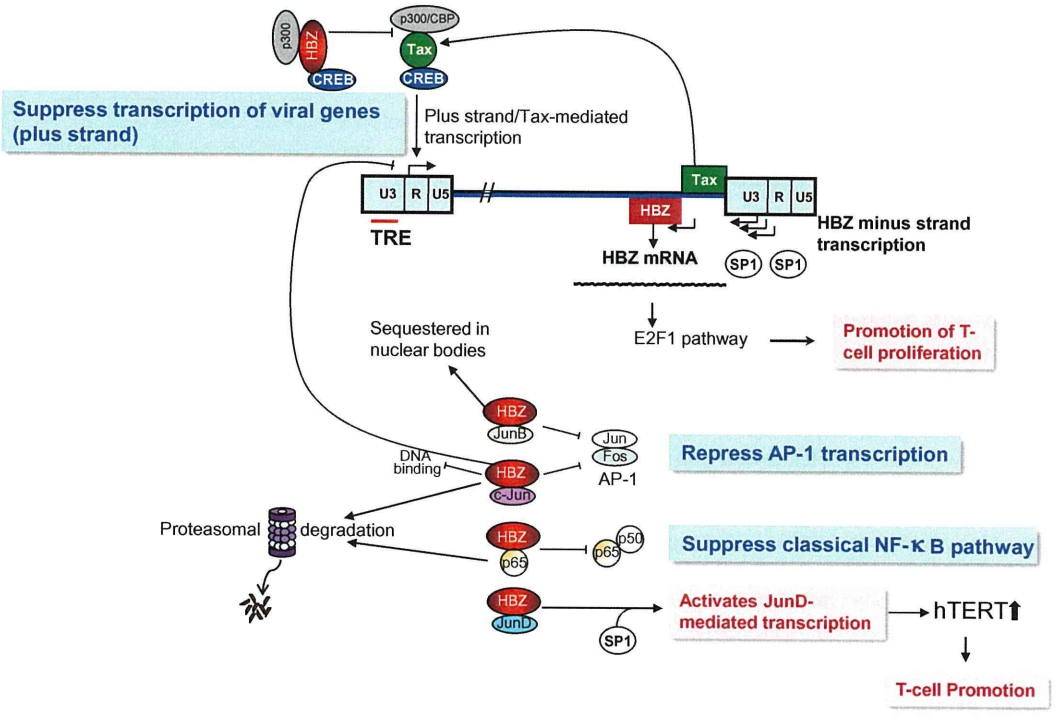


Fig. 4. Functions of HBZ. HBZ protein interacts with c-Jun or CREB and suppresses viral transcription from the 5′LTR. *HBZ* mRNA promotes T-cell proliferation. A transcription factor, SP1, plays a critical role in the *HBZ* gene transcription. Detailed description can be found in the text.

HBZ is not necessary for *in vitro* transformation of T-cells although mutation of the *HBZ* gene resulted in decreased proliferation of infected cells *in vivo* (Arnold et al., 2006), implicating HBZ in infectivity and viral persistence.

4.1. Suppression of Tax mediated viral gene transcription

HBZ was first found as a viral protein that binds to CREB-2 (ATF-4) (Gaudray et al., 2002). Through bZIP domain interaction, HBZ abolished the ability of CREB-2 to bind to the Tax responsive element (TxRE) in the HTLV-1 LTR, resulting in the suppression of transcription from the 5′LTR by Tax (Fig. 4). Moreover, HBZ interacts with cellular coactivators CBP/p300, via LXXLL-like motifs in its N-terminal region, leading to suppression of viral transcription by inhibiting the recruitment of CBP/p300 to the HTLV-1 promoter (Clerc et al., 2008). So far, there is no report that HBZ has the capacity to associate with DNA directly. It is apparent that HBZ exerts its suppressive effect on HTLV-1 transcription mainly by interacting with cellular proteins on the HTLV-1 promoter.

4.2. Interaction of HBZ with cellular factors with bZIP domains

The identification of other cellular factors that interact with HBZ was undertaken on an individual basis after analyses of the factors suspected to be involved in the signal pathway of ATL. HBZ, via its bZIP domain, reportedly forms heterodimers with several AP-1 transcriptional family members, such as c-Jun, JunB, and JunD, but not c-Fos, and modulates their activity (Fig. 4) (Basbous et al., 2003; Thebault et al., 2004). Analysis revealed that HBZ decreases the DNA binding capability of c-Jun and JunB, as is the case for ATF-4 and CREB. JunB was sequestrated into nuclear bodies through interaction with HBZ (Hivin et al., 2007). For c-Jun, HBZ enhances degradation of c-Jun, as described later, and inhibits its DNA binding (Fig. 4). Interactions between HBZ and other bZIP factors, such as ATF1 and ATF2, have also been shown (Isono et al., 2008; Lemasson et al., 2007). Recent study using coiled-coil arrays revealed binding of factors containing bZIP domains with HBZ (Reinke et al., 2010). The function of such associations remains to be elucidated.

4.3. Activation of transcription by interacting with JunD

HBZ suppresses transcription mediated by c-Jun and JunB. However, HBZ can also activate transcription mediated by JunD, another member of the AP-1 family. HBZ forms heterodimers with JunD via its bZIP domain, and the activation domain of HBZ is necessary for this activation (Thebault et al., 2004). In addition to this activity, HBZ–JunD heterodimers interact with Sp1 and activate transcription of the human telomerase catalytic subunit (hTERT) (Fig. 4) (Kuhlmann et al., 2007).

4.4. Inhibition of the classical NF-κB pathway

NF-κB is activated in HTLV-1 infected cells and ATL cells. Activated NF-κB plays important roles in the proliferation of ATL cells and inhibition of apoptosis (Sun and Yamaoka, 2005). Tax can activate both the classical and alternative pathways of NF-κB. Tax binds to IκB, resulting in dissociation of the IκB/NF-κB complex and promoting nuclear translocation of NF-κB (Suzuki et al., 1995). A further mechanism is Tax enhancing IκBα degradation by interacting with IKKγ, leading to subsequent activation of NF-κB (Jin et al., 1999). In contrast, HBZ inhibits only the classical NF-κB pathway, doing so by two mechanisms (Fig. 4). One mechanism is that HBZ inhibits DNA binding of p65. Another is that HBZ increases the expression of PDLIM2, the E3 ubiquitin ligase of p65, leading to increased ubiquitination and enhanced degradation of p65 (Zhao et al., 2009).

Many viruses have developed strategies to manipulate NF-κB signaling through the use of multifunctional viral proteins. HIV-1 Nef induces the expression of the NF-κB inhibitor IκBα, to suppress this pathway (Qiao et al., 2006). In Epstein–Barr virus, the LMP-1 viral protein activates the NF-κB pathway by recruiting cellular adaptor proteins, TNF receptor-associated factor families and TNF receptor-associated death domain, to its C-terminal domain. Like HBZ, the EBV bZIP protein, BZLF1, suppresses the classical NF-κB pathway by inhibiting DNA binding of p65 (Morrison and Kenney, 2004). Similar suppression of NF-κB has been reported for other viruses, including African swine fever virus, and human herpesvirus-8 (HHV-8). In the case of HHV-8, vFLIP activates both the classical and alternative pathways while viral interferon regulatory factor 3 (vIRF3: LANA2) inhibits NF-κB by binding to IKKβ. These findings show that NF-κB suppressive activities are common among different viruses, suggesting that these suppressive activities are important for viral infection. A virus might escape from the host immune system by suppressing the classical NF-κB pathway.

5. The proteasome and HBZ

One mechanism by which HBZ inhibits transcriptional activation by c-Jun and JunB (Basbous et al., 2003) is by forming heterodimers, and thereby inhibiting their DNA binding. HBZ further inhibits c-Jun by promoting the degradation of its protein product (Fig. 4) (Matsumoto et al., 2005). Since it can be blocked by a proteasome inhibitor, this effect relies on a proteasome-mediated pathway. Degradation of c-Jun does not, however, depend on ubiquitination (Isono et al., 2008). usHBZ protein directly interacts with both the 26S proteasome and c-Jun, which results in the delivery of c-Jun to the proteasome.

Thus, HBZ suppresses c-Jun by three mechanisms; (1) inhibition of DNA binding, (2) ubiquitin-independent degradation of c-Jun, and (3) sequestration of c-Jun in nuclear bodies.

It has been reported that degradation of c-Jun by sHBZ is much weaker than that by usHBZ (Isono et al., 2008). However, inhibition of AP-1 mediated transcription by sHBZ was much stronger than that of usHBZ (Yoshida et al., 2008). For the sHBZ protein, the inhibition of DNA binding by c-Jun or sequestration in nuclear bodies might be the predominant mechanism of transcriptional suppression. Further, the expression level of sHBZ RNA is much higher than that of usHBZ RNA, and the half-life of sHBZ protein is much longer than that of usHBZ. Only one HBZ protein was detected in ATL cell lines (Arnold et al., 2008). Taken together, sHBZ expression is much higher than that of usHBZ, and only sHBZ RNA has growth-promoting activity as described later, indicating that sHBZ is more important than usHBZ for HTLV-1 infected cells and ATL cells.

6. Function of the *HBZ* gene as RNA

Suppressed expression of the *HBZ* gene by shRNA leads to decreased proliferation of ATL cell lines (Arnold et al., 2008; Satou et al., 2006). Expression of HBZ in transgenic mice increases the number of T-cells (Satou et al., 2006), and tumor formation and infiltration of ATL cells is decreased by suppressed HBZ expression (Arnold et al., 2008). Thus, HBZ expression is associated with proliferation of ATL cells *in vivo* and *in vitro*. Mutation analyses of the *HBZ* gene showed that HBZ RNA, rather than HBZ protein, has a growth-promoting effect on T-cells (Fig. 4) (Satou et al., 2006). The coding sequence of the *HBZ* gene was replaced with silent mutations, which could produce the same protein while the RNA structure was completely altered. This mutant did not have a growth-promoting activity. Analysis of the transcription profile using DNAchip demonstrated that expression of the *HBZ* gene upregulates *E2F1* gene transcription. Only sHBZ RNA, not usHBZ RNA, promotes proliferation of T-cells, indicating that the first exon of the sHBZ transcript is critical for this activity (Yoshida et al., 2008). This exon overlaps with the Rex responsive element (RxRE) in the R region of 3′LTR. The RxRE region forms a stem-loop structure that binds to Rex. Rex promotes the export of viral RNA with a RxRE region, Thus, sHBZ RNA functions to promote proliferation of T-cells by a region containing the first exon, which can form a strong stem-loop structure. Further details of how sHBZ RNA promotes proliferation remain to be elucidated.

7. Anti-sense transcript of HTLV-2

HTLV-2 is similar to HTLV-1: both retroviruses target T-lymphocytes, and induce their proliferation. However, HTLV-2 tends to infect $CD8^+$ T-cells instead of $CD4^+$ T-cells (Kwaan et al., 2006), and induces cancer in humans only in very rare cases. Following the discovery of HBZ, an anti-sense transcript was identified in HTLV-2. Its product was named anti-sense protein of HTLV-2 (APH-2) (Halin et al., 2009). APH-2 mRNA is transcribed from the 3′LTR, and spliced and polyadenylated like the *sHBZ* gene. Although APH-2 does not have a basic leucine zipper domain, it interacts with CREB and suppresses Tax2-mediated transcription. Thus, APH-2 has similar structure and functions to sHBZ. However, it remains to be elucidated whether APH-2 induces proliferation of infected lymphocytes *in vivo*.

8. *HBZ* gene transcript and diseases

The *HBZ* gene is the only viral gene that is consistently expressed in HTLV-1 infected cells and ATL cells, indicating that HBZ plays a critical role in both infected cells and leukemic cells. For ATL cells, HBZ RNA has growth-promoting activity. ATL cells frequently express Foxp3, a master factor of regulatory T-cells. HBZ expression is associated with a phenotype of regulatory T-cells (our unpublished data). Thus, HBZ not only promotes proliferation of ATL cells, but also modulates the phenotype of HTLV-1 infected cells.

The expression level of *HBZ* transcripts is closely correlated with provirus load and the disease severity of HAM/TSP (Saito et al., 2009). Increased infected cells are also implicated in the pathogenesis of HAM/TSP. There are two scenarios for the role of HBZ: (1) HBZ is important for the survival of HTLV-1 infected cells *in vivo*, (2) HBZ expression confers infiltrative phenotypes on infected cells, and causes dysregulation of cytokine production. The role of *HBZ* gene in HAM/TSP should be studied in the future.

9. Perspectives

A limited number of viral genes are implicated in virus-induced oncogenesis. Both E6 and E7 are critical for human papilloma virus (HPV)-induced cervical cancer. In hepatitis B virus-induced hepatoma, HBx protein is a critical viral factor for oncogenesis. The pleiotropic functions of Tax are thought to play important roles in HTLV-1, however, this does not explain the frequent loss of Tax expression from ATL. It is hypothesized that Tax expression is necessary in the early stages of leukemia, but not required in late stages. However, HBZ is expressed in all ATL cases, and promotes proliferation of ATL cells. This indicates that HBZ is a critical viral gene in oncogenesis by HTLV-1. A few ATL cases had HTLV-1 provirus that could not produce Tax before its integration (Miyazaki et al., 2007; Tamiya et al., 1996). In addition, nonsense mutations of viral genes except the *HBZ* gene are generated by APOBEC3G before integration of provirus (Fan et al., 2010). These findings suggest that the *HBZ* gene is responsible for leukemogenesis by HTLV-1. Like other viral proteins of oncogenic viruses, recent studies

show the multiple functions of HBZ. Further studies of *HBZ* gene will shed light on leukemogenesis by HTLV-1. Further, HBZ might be a novel target for prevention of, and therapies for, HTLV-1-associated diseases.

References

Akagi, T., Ono, H., Shimotohno, K., 1995. Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1. Blood 86, 4243–4249.

Arnold, J., Yamamoto, B., Li, M., Phipps, A.J., Younis, I., Lairmore, M.D., Green, P.L., 2006. Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1. Blood 107, 3976–3982.

Arnold, J., Zimmerman, B., Li, M., Lairmore, M.D., Green, P.L., 2008. Human T-cell leukemia virus type-1 antisense-encoded gene, Hbz, promotes T-lymphocyte proliferation. Blood 112, 3788–3797.

Basbous, J., Arpin, C., Gaudray, G., Piechaczyk, M., Devaux, C., Mesnard, J.M., 2003. The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity. J. Biol. Chem. 278, 43620–43627.

Boam, D.S., Davidson, I., Chambon, P., 1995. A TATA-less promoter containing binding sites for ubiquitous transcription factors mediates cell type-specific regulation of the gene for transcription enhancer factor-1 (TEF-1). J. Biol. Chem. 270, 19487–19494.

Cavanagh, M.H., Landry, S., Audet, B., Arpin-Andre, C., Hivin, P., Pare, M.E., Thete, J., Wattel, E., Marriott, S.J., Mesnard, J.M., et al, 2006. HTLV-I antisense transcripts initiating in the 3′ LTR are alternatively spliced and polyadenylated. Retrovirology 3, 15.

Clerc, I., Polakowski, N., Andre-Arpin, C., Cook, P., Barbeau, B., Mesnard, J.M., Lemasson, I., 2008. An interaction between the human T cell leukemia virus type 1 basic leucine zipper factor (HBZ) and the KIX domain of p300/CBP contributes to the down-regulation of tax-dependent viral transcription by HBZ. J. Biol. Chem. 283, 23903–23913.

Fan, J., Ma, G., Nosaka, K., Tanabe, J., Satou, Y., Koito, A., Wain-Hobson, S., Vartanian, J.P., Matsuoka, M., 2010. APOBEC3G generates nonsense mutations in HTLV-1 proviral genomes in vivo. J. Virol. 84, 7278–7287.

Furukawa, Y., Kubota, R., Tara, M., Izumo, S., Osame, M., 2001. Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. Blood 97, 987–993.

Gaudray, G., Gachon, F., Basbous, J., Biard-Piechaczyk, M., Devaux, C., Mesnard, J.M., 2002. The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. J. Virol. 76, 12813–12822.

Grassmann, R., Aboud, M., Jeang, K.T., 2005. Molecular mechanisms of cellular transformation by HTLV-1 Tax. Oncogene 24, 5976–5985.

Halin, M., Douceron, E., Clerc, I., Journo, C., Ko, N.L., Landry, S., Murphy, E.L., Gessain, A., Lemasson, I., Mesnard, J.M., et al, 2009. Human T-cell leukemia virus type 2 produces a spliced antisense transcript encoding a protein that lacks a classic bZIP domain but still inhibits Tax2-mediated transcription. Blood 114, 2427–2438.

Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K.I., Shirakawa, S., Miyoshi, I., 1981. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc. Natl. Acad. Sci. USA 78, 6476–6480.

Hivin, P., Basbous, J., Raymond, F., Henaff, D., Arpin-Andre, C., Robert-Hebmann, V., Barbeau, B., Mesnard, J.M., 2007. The HBZ-SP1 isoform of human T-cell leukemia virus type I represses JunB activity by sequestration into nuclear bodies. Retrovirology 4, 14.

Hivin, P., Frederic, M., Arpin-Andre, C., Basbous, J., Gay, B., Thebault, S., Mesnard, J.M., 2005. Nuclear localization of HTLV-I bZIP factor (HBZ) is mediated by three distinct motifs. J. Cell Sci. 118, 1355–1362.

Isono, O., Ohshima, T., Saeki, Y., Matsumoto, J., Hijikata, M., Tanaka, K., Shimotohno, K., 2008. Human T-cell leukemia virus type 1 HBZ protein bypasses the targeting function of ubiquitination. J. Biol. Chem. 283, 34273–34282.

Jin, D.Y., Giordano, V., Kibler, K.V., Nakano, H., Jeang, K.T., 1999. Role of adapter function in oncoprotein-mediated activation of NF-kappaB. Human T-cell leukemia virus type I Tax interacts directly with IkappaB kinase gamma. J. Biol. Chem. 274, 17402–17405.

Koiwa, T., Hamano-Usami, A., Ishida, T., Okayama, A., Yamaguchi, K., Kamihira, S., Watanabe, T., 2002. 5′-Long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo. J. Virol. 76, 9389–9397.

Kuhlmann, A.S., Villaudy, J., Gazzolo, L., Castellazzi, M., Mesnard, J.M., Duc Dodon, M., 2007. HTLV-1 HBZ cooperates with JunD to enhance transcription of the human telomerase reverse transcriptase gene (hTERT). Retrovirology 4, 92.

Kwaan, N., Lee, T.H., Chafets, D.M., Nass, C., Newman, B., Smith, J., Garratty, G., Murphy, E.L., 2006. Long-term variations in human T lymphotropic virus (HTLV)-I and HTLV-II proviral loads and association with clinical data. J. Infect. Dis. 194, 1557–1564.

Lairmore, M.D., Silverman, L., Ratner, L., 2005. Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation. Oncogene 24, 6005–6015.

Landry, S., Halin, M., Vargas, A., Lemasson, I., Mesnard, J.M., Barbeau, B., 2009. Upregulation of human T-cell leukemia virus type 1 antisense transcription by the viral tax protein. J. Virol. 83, 2048–2054.

Larocca, D., Chao, L.A., Seto, M.H., Brunck, T.K., 1989. Human T-cell leukemia virus minus strand transcription in infected T-cells. Biochem. Biophys. Res. Commun. 163, 1006–1013.

Lemasson, I., Lewis, M.R., Polakowski, N., Hivin, P., Cavanagh, M.H., Thebault, S., Barbeau, B., Nyborg, J.K., Mesnard, J.M., 2007. Human T-cell leukemia virus type 1 (HTLV-1) bZIP protein interacts with the cellular transcription factor CREB to inhibit HTLV-1 transcription. J. Virol. 81, 1543–1553.

Li, M., Kesic, M., Yin, H., Yu, L., Green, P.L., 2009. Kinetic analysis of human T-cell leukemia virus type 1 gene expression in cell culture and infected animals. J. Virol. 83, 3788–3797.

Liu, S., Cowell, J.K., 2000. Cloning and characterization of the TATA-less promoter from the human GFI1 proto-oncogene. Ann. Hum. Genet. 64, 83–86.

Matsumoto, J., Ohshima, T., Isono, O., Shimotohno, K., 2005. HTLV-1 HBZ suppresses AP-1 activity by impairing both the DNA-binding ability and the stability of c-Jun protein. Oncogene 24, 1001–1010.

Matsuoka, M., Jeang, K.T., 2007. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat. Rev. 7, 270–280.

Miyazaki, M., Yasunaga, J., Taniguchi, Y., Tamiya, S., Nakahata, T., Matsuoka, M., 2007. Preferential selection of human T-cell leukemia virus type 1 provirus lacking the 5′ long terminal repeat during oncogenesis. J. Virol. 81, 5714–5723.

Morrison, T.E., Kenney, S.C., 2004. BZLF1, an Epstein–Barr virus immediate-early protein, induces p65 nuclear translocation while inhibiting p65 transcriptional function. Virology 328, 219–232.

Murata, K., Hayashibara, T., Sugahara, K., Uemura, A., Yamaguchi, T., Harasawa, H., Hasegawa, H., Tsuruda, K., Okazaki, T., Koji, T., et al, 2006. A novel alternative splicing isoform of human T-cell leukemia virus type 1 bZIP factor (HBZ-SI) targets distinct subnuclear localization. J. Virol. 80, 2495–2505.

Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.A., Minna, J.D., Gallo, R.C., 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA 77, 7415–7419.

Qiao, X., He, B., Chiu, A., Knowles, D.M., Chadburn, A., Cerutti, A., 2006. Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells. Nat. Immunol. 7, 302–310.

Reinke, A.W., Grigoryan, G., Keating, A.E., 2010. Identification of bZIP interaction partners of viral proteins HBZ, MEQ, BZLF1, and K-bZIP using coiled-coil arrays. Biochemistry 49, 1985–1997.

Saito, M., Matsuzaki, T., Satou, Y., Yasunaga, J., Saito, K., Arimura, K., Matsuoka, M., Ohara, Y., 2009. In vivo expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). Retrovirology 6, 19.

Satou, Y., Yasunaga, J., Yoshida, M., Matsuoka, M., 2006. HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. Proc. Natl. Acad. Sci. USA 103, 720–725.

Seiki, M., Hattori, S., Hirayama, Y., Yoshida, M., 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. USA 80, 3618–3622.

Sun, S.C., Yamaoka, S., 2005. Activation of NF-kappaB by HTLV-I and implications for cell transformation. Oncogene 24, 5952–5964.
Suzuki, T., Hirai, H., Murakami, T., Yoshida, M., 1995. Tax protein of HTLV-1 destabilizes the complexes of NF-kappa B and I kappa B-alpha and induces nuclear translocation of NF-kappa B for transcriptional activation. Oncogene 10, 1199–1207.
Takeda, S., Maeda, M., Morikawa, S., Taniguchi, Y., Yasunaga, J., Nosaka, K., Tanaka, Y., Matsuoka, M., 2004. Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. Int. J. Cancer 109, 559–567.
Tamiya, S., Matsuoka, M., Etoh, K., Watanabe, T., Kamihira, S., Yamaguchi, K., Takatsuki, K., 1996. Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia. Blood 88, 3065–3073.
Taniguchi, Y., Nosaka, K., Yasunaga, J., Maeda, M., Mueller, N., Okayama, A., Matsuoka, M., 2005. Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. Retrovirology 2, 64.
Thebault, S., Basbous, J., Hivin, P., Devaux, C., Mesnard, J.M., 2004. HBZ interacts with JunD and stimulates its transcriptional activity. FEBS Lett. 562, 165–170.
Usui, T., Yanagihara, K., Tsukasaki, K., Murata, K., Hasegawa, H., Yamada, Y., Kamihira, S., 2008. Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells. Retrovirology 5, 34.
Yamano, Y., Nagai, M., Brennan, M., Mora, C.A., Soldan, S.S., Tomaru, U., Takenouchi, N., Izumo, S., Osame, M., Jacobson, S., 2002. Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). Blood 99, 88–94.
Yoshida, M., Satou, Y., Yasunaga, J., Fujisawa, J., Matsuoka, M., 2008. Transcriptional control of spliced and unspliced human T-cell leukemia virus type 1 bZIP factor (HBZ) gene. J. Virol. 82, 9359–9368.
Zhao, T., Yasunaga, J., Satou, Y., Nakao, M., Takahashi, M., Fujii, M., Matsuoka, M., 2009. Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. Blood 113, 2755–2764.

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

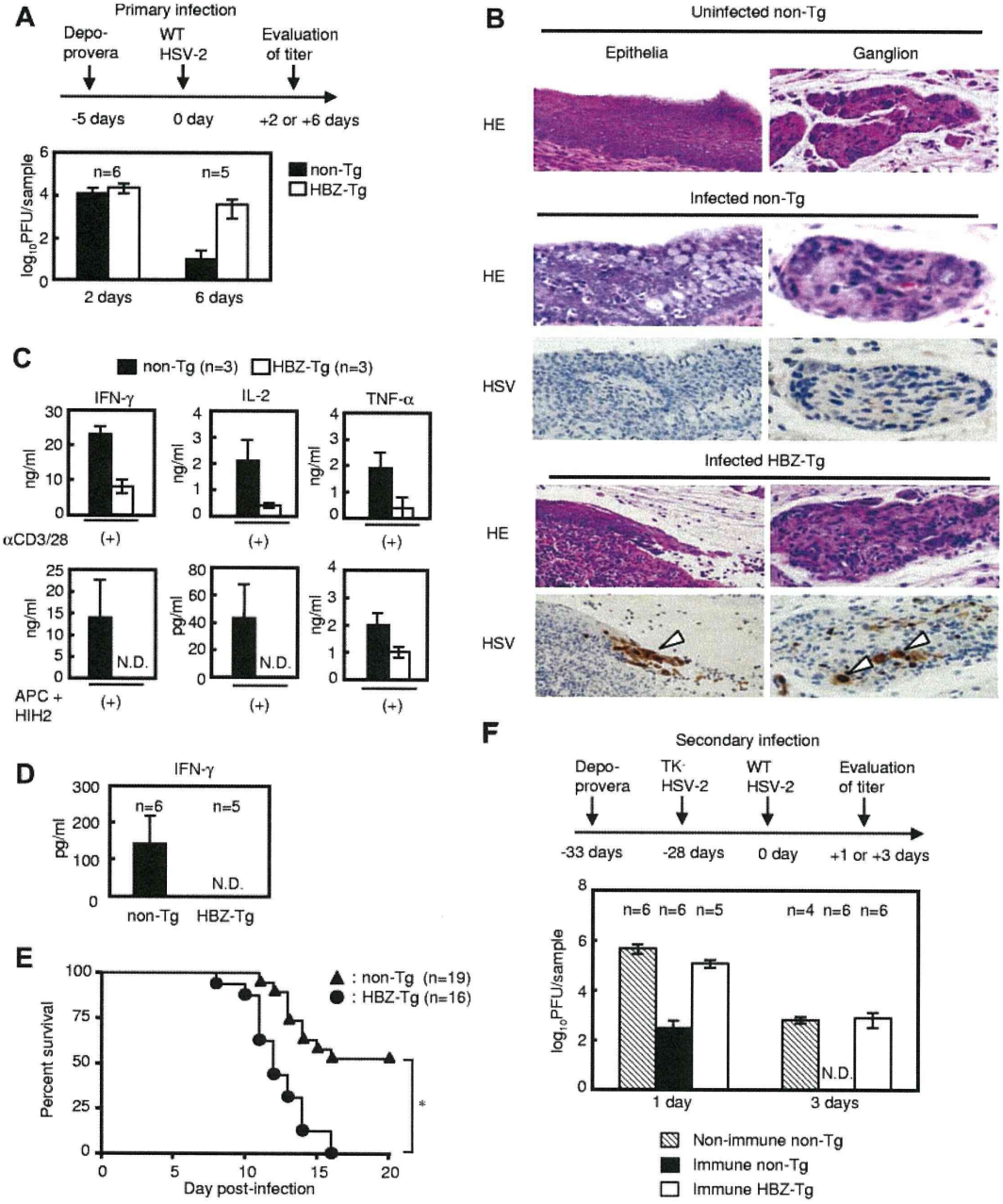

Figure 1. Transgenic mice expressing sHBZ in CD4 T cells are highly susceptible to intravaginal infection with HSV-2. (A) Virus titer in vaginal washes in primary infection. (B) Histologic analysis of epithelia and ganglion in vaginal tissue from mice infected with HSV-2. Uninfected vaginal tissues are 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

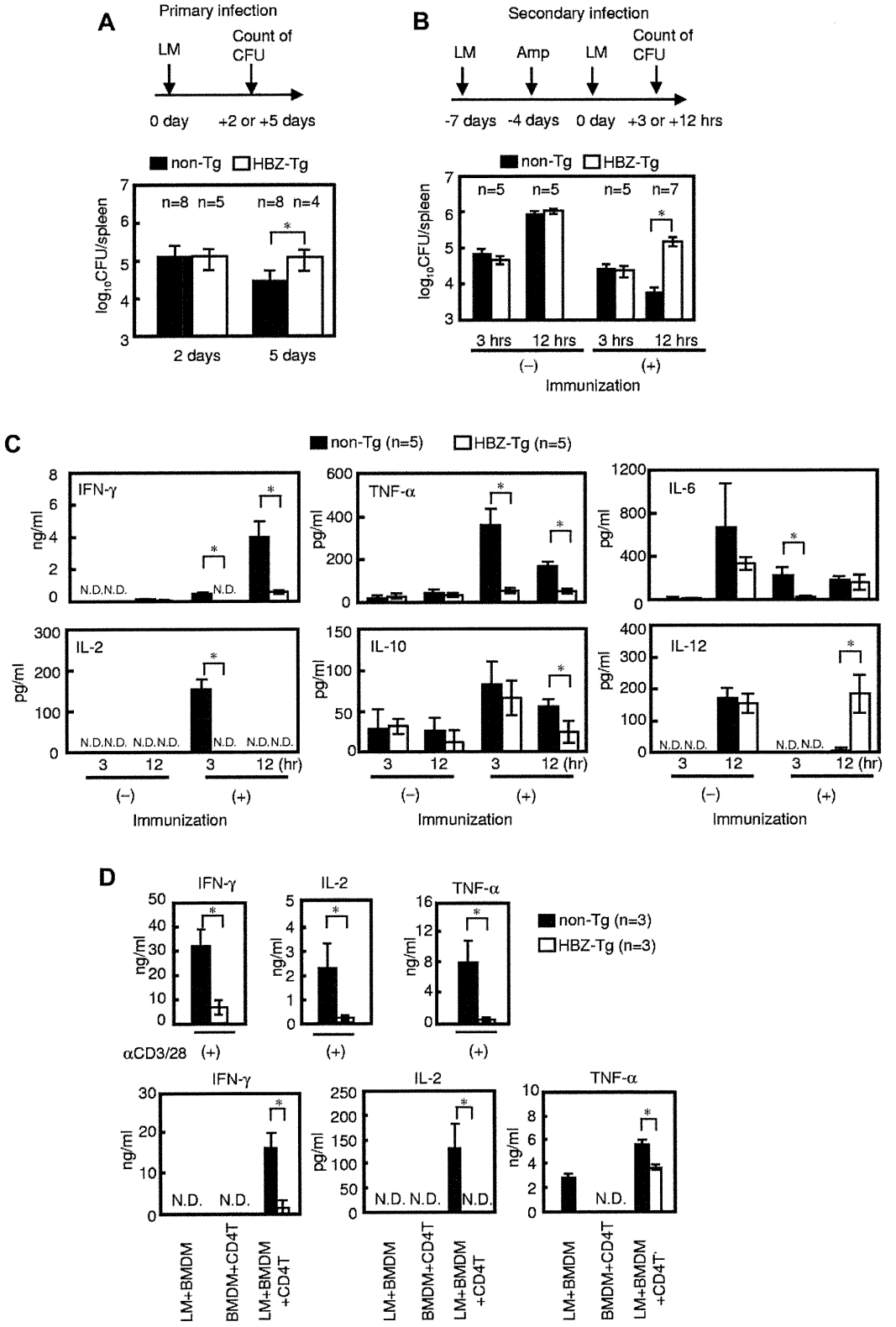

Figure 2. HBZ-Tg mice show decreased immune response to primary and secondary infection with LM. Bacterial loads of spleens from mice challenged with LM in primary (A) and secondary (B) infection are shown. (C) Concentrations of IFN-γ, TNF-α, IL-2, IL-6, and IL-12 in serum and IL-10 in homogenized spleen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

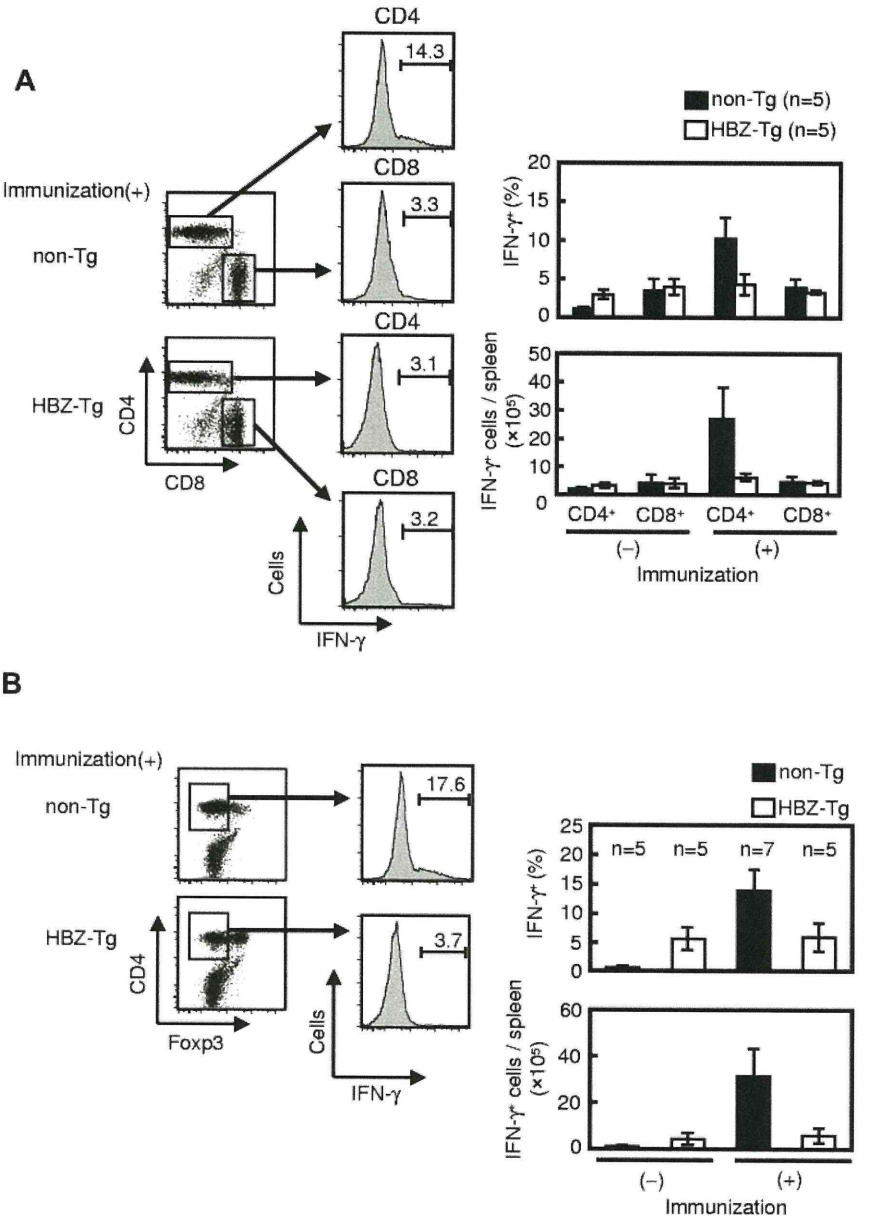

Figure 3. IFN-γ production by CD4 splenocytes from LM secondarily infected HBZ-Tg mice decreases in CD4$^+$ Foxp3$^-$ T cells. Mice were immunized and challenged as shown at the top of Figure 2B, and their splenocytes were harvested at 12 hours after challenge and analyzed for intracellular IFN-γ production.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

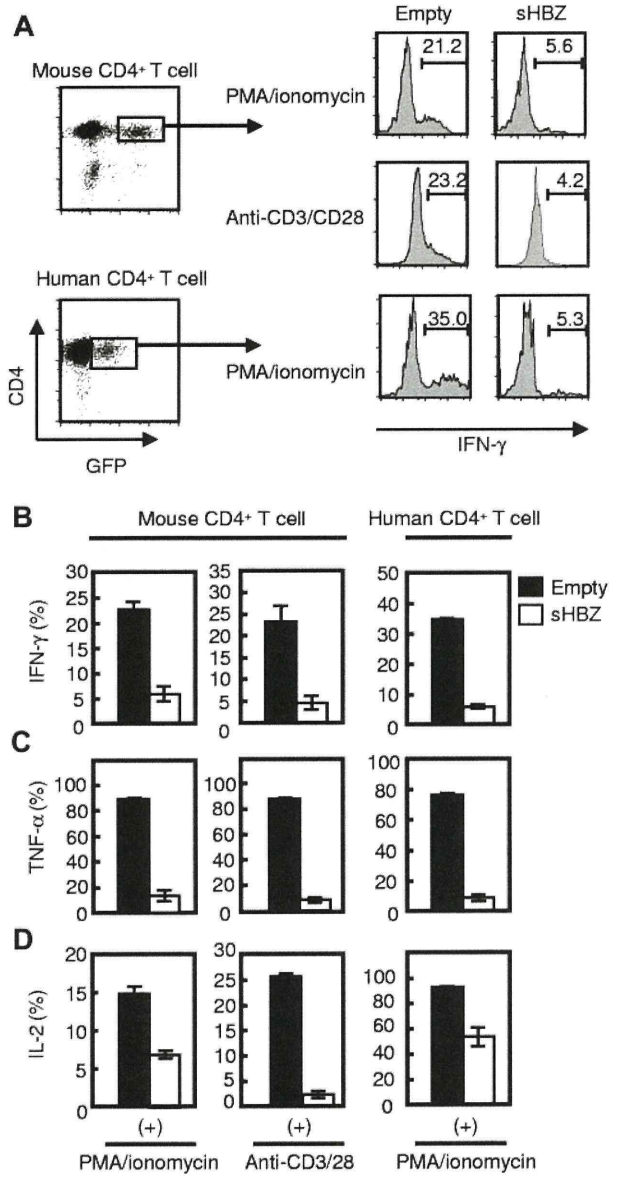

Figure 4. sHBZ directly inhibits IFN-γ production in both human and mouse CD4 T cells. Mouse and human CD4 T cells were transduced with recombinant retroviruses or lentiviruses, respectively, expressing sHBZ, and stimulated with PMA and ionomycin or antibodies to CD3 and CD28. Then, intracellular cytokines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

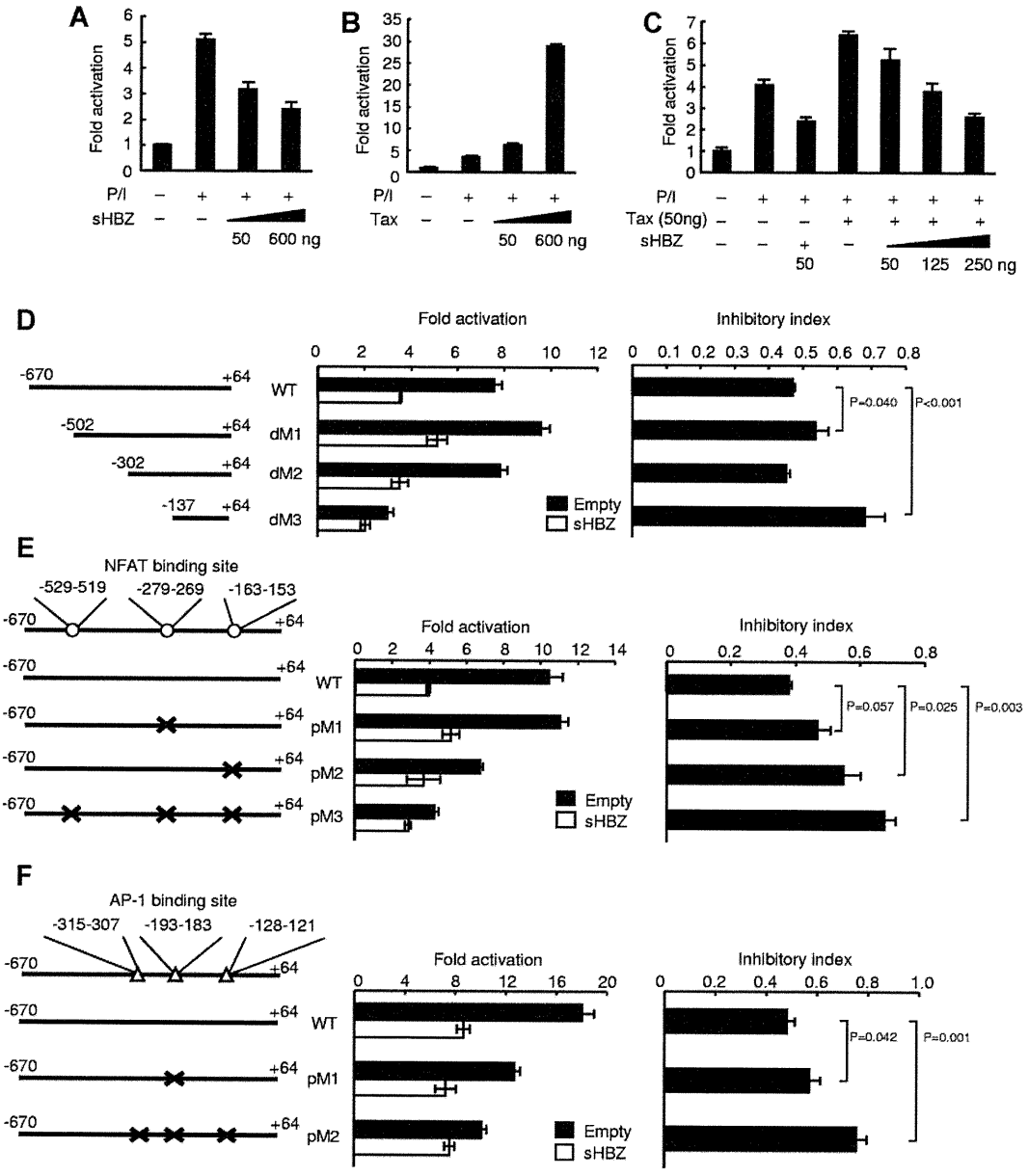

Figure 5. sHBZ suppresses IFN-γ promoter activity. Luciferase assay of the IFN-γ promoter reporter constructs (−670 to +64) cotransfected with an expression plasmid for sHBZ (A), Tax (B), or both (C) is performed in Jurkat cells, which were stimulated with PMA and ionomycin. Luciferase assays of reporter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

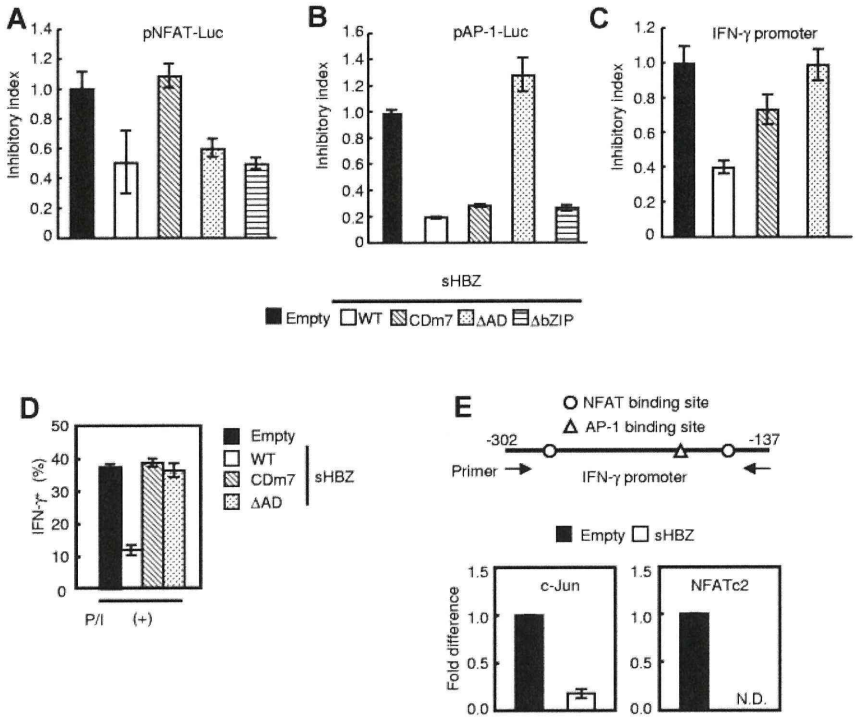

Figure 6. NFAT and AP-1 signaling pathways are responsible for HBZ-mediated inhibition of IFN-γ production. (A-C) Effects of wild-type and mutant sHBZ on (A) an NFAT-Luc reporter, (B) an AP-1-Luc reporter, and (C) the IFN-γ promoter. (D) The suppressive effect of sHBZ mutants on IFN-γ production from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

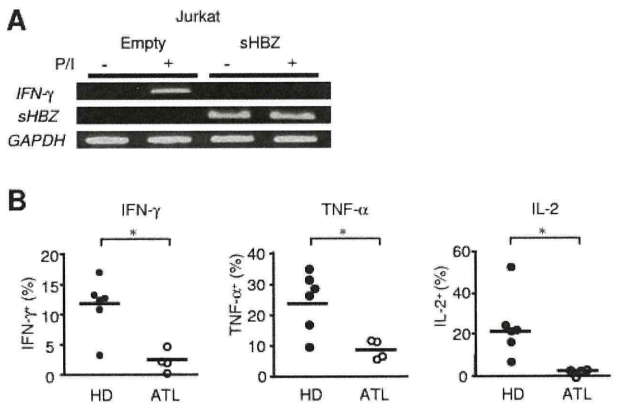

Figure 7. IFN-γ production is suppressed in sHBZ-expressing Jurkat cells and PBMCs of ATL patients. (A) sHBZ inhibits *IFN-γ* gene transcription after stimulation with PMA and ionomycin. Transcripts of the *IFN-γ* and *sHBZ* genes were analyzed by RT-PCR. (B) IFN-γ, TNF-α, and IL-2 production by CD4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

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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References

1. Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer.* 2007;7(4):270-280.
2. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood.* 1977; 50(3):481-492.
3. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A.* 1980; 77(12):7415-7419.
4. Hinuma Y, Nagata K, Hanaoka M, et al. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc Natl Acad Sci U S A.* 1981;78(10): 6476-6480.
5. Gessain A, Barin F, Vernant JC, et al. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet.* 1985; 2(8452):407-410.
6. Osame M, Usuku K, Izumo S, et al. HTLV-I associated myelopathy, a new clinical entity. *Lancet.* 1986;1(8488):1031-1032.
7. Sugimoto M, Nakashima H, Watanabe S, et al. T-lymphocyte alveolitis in HTLV-I-associated myelopathy. *Lancet.* 1987;2(8569):1220.
8. Takatsuki K, Matsuoka M, Yamaguchi K. ATL and HTLV-I-related diseases. In: Takatsuki K, ed. *Adult T-Cell Leukemia.* New York: Oxford University Press; 1994:1-27.
9. Cavrois M, Leclercq I, Gout O, Gessain A, Wain-Hobson S, Wattel E. Persistent oligoclonal expansion of human T-cell leukemia virus type 1-infected circulating cells in patients with tropical spastic paraparesis/HTLV-1-associated myelopathy. *Oncogene.* 1998;17(1):77-82.
10. Etoh K, Tamiya S, Yamaguchi K, et al. Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells in vivo. *Cancer Res.* 1997; 57(21):4862-4867.
11. Nicot C, Harrod RL, Ciminale V, Franchini G. Human T-cell leukemia/lymphoma virus type 1 nonstructural genes and their functions. *Oncogene.* 2005;24(39):6026-6034.
12. Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM. The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. *J Virol.* 2002;76(24):12813-12822.
13. Satou Y, Yasunaga J, Yoshida M, Matsuoka M. HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc Natl Acad Sci U S A.* 2006;103(3):720-725.
14. Cavanagh MH, Landry S, Audet B, et al. HTLV-I antisense transcripts initiating in the 3′LTR are alternatively spliced and polyadenylated. *Retrovirology.* 2006;3:15.
15. Usui T, Yanagihara K, Tsukasaki K, et al. Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells. *Retrovirology.* 2008;5:34.
16. Yoshida M, Satou Y, Yasunaga J, Fujisawa J, Matsuoka M. Transcriptional control of spliced and unspliced human T-cell leukemia virus type 1 bZIP factor (HBZ) gene. *J Virol.* 2008;82(19): 9359-9368.
17. Satou Y, Yasunaga J, Zhao T, et al. HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation in vivo. *PLoS Pathog.* 2011;7(2): e1001274.
18. Zhao T, Yasunaga J, Satou Y, et al. Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. *Blood.* 2009;113(12):2755-2764.
19. Basbous J, Arpin C, Gaudray G, Piechaczyk M, Devaux C, Mesnard JM. The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity. *J Biol Chem.* 2003;278(44):43620-43627.
20. Milligan GN, Bernstein DI, Bourne N. T lymphocytes are required for protection of the vaginal mucosae and sensory ganglia of immune mice against reinfection with herpes simplex virus type 2. *J Immunol.* 1998;160(12):6093-6100.
21. Iijima N, Linehan MM, Zamora M, et al. Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. *J Exp Med.* 2008;205(13):3041-3052.
22. Magee DM, Wing EJ. Cloned L3T4+ T lymphocytes protect mice against *Listeria monocytogenes* by secreting IFN-gamma. *J Immunol.* 1988;141(9):3203-3207.
23. Yang J, Kawamura I, Mitsuyama M. Requirement of the initial production of gamma interferon in the generation of protective immunity of mice against *Listeria monocytogenes*. *Infect Immun.* 1997; 65(1):72-77.
24. Iwamasa T, Utsumi Y, Sakuda H, et al. Two cases of necrotizing myelopathy associated with malignancy caused by herpes simplex virus type 2. *Acta Neuropathol.* 1989;78(3):252-257.
25. Ikehara O, Endo K, Hakamada K. Listeriosis in hematological malignancies: report of two cases. *Jpn J Clin Oncol.* 1989;19(2):159-162.
26. Suzutani T, Machida H, Sakuma T, Azuma M. Effects of various nucleosides on antiviral activity and metabolism of 1-beta-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil against herpes simplex virus types 1 and 2. *Antimicrob Agents Chemother.* 1988;32(10):1547-1551.
27. Hara H, Kawamura I, Nomura T, Tominaga T, Tsuchiya K, Mitsuyama M. Cytolysin-dependent escape of the bacterium from the phagosome is required but not sufficient for induction of the Th1 immune response against *Listeria monocytogenes* infection: distinct role of Listeriolysin O determined by cytolysin gene replacement. *Infect Immun.* 2007;75(8):3791-3801.
28. Fan J, Kodama E, Koh Y, Nakao M, Matsuoka M. Halogenated thymidine analogues restore the expression of silenced genes without demethylation. *Cancer Res.* 2005;65(15):6927-6933.
29. Iwasaki A. Antiviral immune responses in the genital tract: clues for vaccines. *Nat Rev Immunol.* 2010;10(10):699-711.
30. Kaushic C, Ashkar AA, Reid LA, Rosenthal KL. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. *J Virol.* 2003;77(8):4558-4565.
31. Unanue ER. Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response. *Immunol Rev.* 1997;158:11-25.
32. Ladel CH, Flesch IE, Arnoldi J, Kaufmann SH. Studies with MHC-deficient knock-out mice reveal impact of both MHC I- and MHC II-dependent T cell responses on *Listeria monocytogenes* infection. *J Immunol.* 1994;153(7):3116-3122.
33. Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci U S A.* 2005;102(14):5138-5143.
34. Nakanishi K. Innate and acquired activation pathways in T cells. *Nat Immunol.* 2001;2(2):140-142.
35. Brown DA, Nelson FB, Reinherz EL, Diamond DJ. The human interferon-gamma gene contains an inducible promoter that can be transactivated by tax I and II. *Eur J Immunol.* 1991;21(8):1879-1885.
36. Hall WW, Fujii M. Deregulation of cell-signaling pathways in HTLV-1 infection. *Oncogene.* 2005; 24(39):5965-5975.
37. Matsumoto J, Ohshima T, Isono O, Shimotohno K. HTLV-1 HBZ suppresses AP-1 activity by impairing

both the DNA-binding ability and the stability of c-Jun protein. *Oncogene.* 2005;24(6):1001-1010.

38. Rouse BT, Sehrawat S. Immunity and immunopathology to viruses: what decides the outcome? *Nat Rev Immunol.* 2010;10(7):514-526.
39. Johnson JM, Nicot C, Fullen J, et al. Free major histocompatibility complex class I heavy chain is preferentially targeted for degradation by human T-cell leukemia/lymphotropic virus type 1 p12(I) protein. *J Virol.* 2001;75(13):6086-6094.
40. Sundstrom S, Ota S, Dimberg LY, Masucci MG, Bergqvist A. Hepatitis C virus core protein induces an anergic state characterized by decreased interleukin-2 production and perturbation of mitogen-activated protein kinase responses. *J Virol.* 2005;79(4):2230-2239.
41. Myskiw C, Arsenio J, van Bruggen R, Deschambault Y, Cao J. Vaccinia virus E3 suppresses expression of diverse cytokines through inhibition of the PKR, NF-kappaB, and IRF3 pathways. *J Virol.* 2009;83(13):6757-6768.
42. Li JC, Au KY, Fang JW, et al. HIV-1 trans-activator protein dysregulates IFN-gamma signaling and contributes to the suppression of autophagy induction. *AIDS.* 2011;25(1):15-25.
43. Li-Weber M, Krammer PH. Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat Rev Immunol.* 2003;3(7):534-543.
44. Matsuoka M. HTLV-1 bZIP factor gene: its roles in HTLV-1 pathogenesis. *Mol Aspects Med.* 2010; 31(5):359-366.
45. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature.* 2002;420(6915):502-507.
46. Kursar M, Bonhagen K, Fensterle J, et al. Regulatory CD4+CD25+ T cells restrict memory CD8+ T cell responses. *J Exp Med.* 2002; 196(12):1585-1592.
47. Billiau A, Matthys P. Interferon-gamma: a historical perspective. *Cytokine Growth Factor Rev.* 2009;20(2):97-113.
48. Ungar BL, Kao TC, Burris JA, Finkelman FD. Cryptosporidium infection in an adult mouse model. Independent roles for IFN-gamma and CD4+ T lymphocytes in protective immunity. *J Immunol.* 1991;147(3):1014-1022.
49. Cenci E, Mencacci A, Del Sero G, et al. IFN-gamma is required for IL-12 responsiveness in mice with *Candida albicans* infection. *J Immunol.* 1998;161(7):3543-3550.
50. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science.* 2003;300(5617):339-342.

HTLV-1 bZIP factor enhances TGF-β signaling through p300 coactivator

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Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus that is etiologically associated with adult T-cell leukemia. The HTLV-1 bZIP factor (HBZ), which is encoded by the minus strand of the provirus, is involved in both regulation of viral gene transcription and T-cell proliferation. We showed in this report that HBZ interacted with Smad2/3, and enhanced transforming growth factor-β (TGF-β)/Smad transcriptional responses in a p300-dependent manner. The N-terminal LXXLL motif of HBZ was responsible for HBZ-mediated TGF-β signaling activation. In a serial immunoprecipitation assay, HBZ, Smad3, and p300 formed a ternary complex, and the association between Smad3 and p300 was markedly enhanced in the presence of HBZ. In addition, HBZ could overcome the repression of the TGF-β response by Tax. Finally, HBZ expression resulted in enhanced transcription of *Pdgfb*, *Sox4*, *Ctgf*, *Foxp3*, *Runx1*, and *Tsc22d1* genes and suppression of the *Id2* gene; such effects were similar to those by TGF-β. In particular, HBZ induced Foxp3 expression in naive T cells through Smad3-dependent TGF-β signaling. Our results suggest that HBZ, by enhancing TGF-β signaling and Foxp3 expression, enables HTLV-1 to convert infected T cells into regulatory T cells, which is thought to be a critical strategy for virus persistence. (*Blood*. 2011;118(7):1865-1876)

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia (ATL) and HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1,2] The unique sequence of HTLV-1 between the *env* region and 3′ long terminal repeat, denoted the pX region, encodes regulatory (*tax* and *rex*) and accessory (*p12*, *p13*, and *p30*) genes in the plus strand.[3] The pleiotropic actions of Tax are thought to play a central role in the early stage of leukemogenesis.[4] However, approximately 60% of fresh ATL cells lack Tax expression because of genetic and epigenetic changes in the HTLV-1 provirus, suggesting that Tax may not be essential for the maintenance of ATL cells in vivo.[5] The *HTLV-1 bZIP factor* (*HBZ*), which is encoded by the complementary strand of the HTLV-1 genome, is expressed in all ATL cases and supports the proliferation of ATL cells.[6,7] In addition, HBZ was found to inhibit Tax-mediated activation of viral transcription from the 5′-long terminal repeat by heterodimerizing with c-Jun, CREB2, and to selectively suppress the classic pathway of nuclear factor-κB by 2 distinct mechanisms.[8-11] Thus, HBZ has multifunctional roles in cellular signaling and proliferation. Recently, we reported that nonsense mutations in all HTLV-1 genes except *HBZ* were generated by APOBEC3G before integration of the provirus in ATL cases and HTLV-1 infected persons, indicating that the *HBZ* gene is essential for leukemogenesis.[12]

Transforming growth factor-β (TGF-β) controls a variety of biologic processes, including cell growth, differentiation, apoptosis, development, and immune homeostasis.[13] The Smad proteins, which are mediators of TGF-β signaling, transduce the TGF-β signal at the cell surface into gene regulation in the nucleus. Receptor-regulated Smad, R-Smads (Smad1, 2, 3, 5, and 8), are phosphorylated by the activated TGF-β receptor, form complexes with Comediator Smad, Co-Smad (Smad 4), and together accumulate in the nucleus to regulate transcription of target genes. Smads can regulate gene expression positively by recruiting coactivators, such as CBP/p300,[14] or negatively by direct recruitment of histone deacetylases or corepressors, such as c-Ski and SnoN.[15] In ATL cells, constitutively activated AP-1 leads to the production of TGF-β1,[16] which can be readily detected in the serum of infected persons.[17] Subsequent studies reported that HTLV-1–infected T cells were resistant to TGF-β–induced growth inhibition and that resistance was related to Tax expression. Three distinct mechanisms by which Tax suppressed TGF-β–mediated signaling were reported: (1) inhibition of Smad3-Smad4 complex formation and DNA binding; (2) prevention of the recruitment of CBP/p300 to the Smad transcription complex on TGF-β response elements; and (3) inhibition of Smad3 DNA binding through activation of the JNK/c-Jun pathway.[18-20]

TGF-β signaling is critical for the development of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs).[21] ATL cells possess a $CD4^+CD25^+$ phenotype, similar to that of Tregs. The forkhead box P3 (FoxP3) is critical for the function of Tregs.[22] Expression of FoxP3 was detected in two-thirds of ATL cases,[23] indicating that ATL cells are derived from Tregs, and a recent study reported a higher proportion of $FoxP3^+$ Tregs among the HTLV-1–infected cells than among the HTLV-1–negative $CD4^+$ cells.[24] Although Tax has been reported to suppress FoxP3 expression in T cells,[25] we recently found that HBZ expression-induced Foxp3

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expression and increased the number of Tregs.[26] Therefore, we set out to determine how HBZ and TGF-β cooperate to induce FoxP3 in HTLV-1–infected T cells.

In the present study, we found that HBZ enhanced TGF-β signaling by interacting with Smad3 and p300, resulting in enhanced Foxp3 expression. This might account for why HTLV-1 infection increases Tregs in vivo.

Methods

Cell culture and mice

HTLV-1 immortalized cell lines (MT-2, MT-4), ATL cell lines (MT-1, ATL-2, ATL-T, ATL-43T, ATL-55T, ED, and TL-Om1), T-cell lines not infected with HTLV-1 (Jurkat, Hut78, CEM, and Kit 225), and mouse T-cell line (CTLL-2) were maintained as described previously.[27] HepG2, 293T, and COS7 cells were grown in Dulbecco modified Eagle medium supplemented with 10% FBS and antibiotics. Plat-E cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FBS containing 10 μg/mL blasticidin and 1 μg/mL puromycin. EL4 cells were cultured with RPMI 1640 containing 10% FCS, antibiotics, and 50μM 2-mercaptoethanol. C57BL/6J mice were purchased from CLEA Japan. Peripheral blood mononuclear cells were isolated from healthy volunteers under an institutional review board-approved protocol. All animals used in this study were maintained and handled according to protocols approved by Kyoto University.

Plasmids

The 3TP-Lux construct contains the phorbol myristate acetate-response elements along with the Smad3/4 binding sites of the PAI-1 promoter. A total of 9 × CAGA-Luc contains 9 tandem Smad3/4 binding sites. pTARE-Luc was purchased from Stratagene. phRL-TK was purchased from Promega. Expression plasmids for Tax, spliced HBZ (sHBZ), unspliced HBZ (usHBZ), c-Ski, Smads, and their deletion mutants were prepared as previously described.[11,28] Expression vectors for sHBZ-ΔbZIP deletion mutants were generated by polymerase chain reaction (PCR). The coding region of c-Fos was amplified by reverse-transcribed (RT)-PCR from total RNA derived from primary peripheral blood mononuclear cells and cloned into the vector pCDNA3. Expression plasmids for E1A and its mutants were a gift from Dr Akiyoshi Fukamizu of University of Tsukuba, and pEF-HA-p300 was from Dr Takashi Fujita of Kyoto University. The STAT5-responsive Jγ1 promoter reporter plasmid (pGL4-Jγ1), pCAGGS-WT-STAT5a (wild-type), and pCAGGS-CA-STAT5a (constitutively active) were provided by Dr Koichi Ikuta of Kyoto University.

Luciferase assay

HepG2 cells were plated on 12-well plates at 2.5×10^5 cells per well. After 24 hours, cells were transfected with the indicated plasmid. At 48 hours after transfection, a luciferase reporter assay was performed as previously described.[11] For CTLL-2 cells, vectors were transfected with a Gene Pulser II electroporation system (Bio-Rad). The transfected cells were treated with or without 10 ng/mL TGF-β (RD Systems) for 24 hours before being harvested for the reporter assay. For the Foxp3 reporter assay, the *Foxp3* gene promoter and enhancer were cloned into the pGL4.1 basic vector as previously reported.[29] Mutations of the Smad binding site in the enhancer fragment were generated by PCR. Foxp3 reporter assays were performed in EL4 cells as described.[29] Each experiment was performed in triplicate, and the data represent the mean plus or minus SD of 3 independent experiments, each normalized to *Renilla* activity.

Immunoprecipitation and immunoblotting

HepG2 and COS7 cells were transfected with the indicated plasmids by *Trans*IT-LT1 (Mirus). Tagged proteins were isolated from transfected COS7 cells by immunoprecipitation with anti–c-Myc (clone 9E10, Sigma-Aldrich), anti-HA (12CA5, Roche Diagnostics) or anti-FLAG M2 (Sigma-Aldrich) antibodies, and analyzed by Western blot as described previously.[11]

For sequential immunoprecipitation, transfected COS7 cells were lysed in radioimmunoprecipitation assay buffer and immunoprecipitated with anti-FLAG M2 agarose affinity gel. The precipitates were eluted with FLAG peptide and the eluate diluted with radioimmunoprecipitation assay buffer, immunoprecipitated with anti-HA antibody, and subjected to anti-His (MBL) immunoblotting. Membranes were developed by enhanced chemiluminescence (GE Healthcare Life Sciences). Other antibodies used were as follows: anti–mouse immunoglobulin G (IgG), and anti–rabbit IgG were from GE Healthcare Life Sciences; anti-Smad3 was from ZYMED Laboratories (Zymed); and anti-HBZ was used as previously described.[30]

Immunofluorescence analysis

COS7 cells were transfected with expression vectors using *Trans*IT-LT1 and treated with TGF-β (5 ng/mL) for 2 hours. At 48 hours after transfection, sHBZ protein was detected using anti–c-MYC Cy3 (clone 9E10, Sigma-Aldrich). Smad3 was detected using anti–FLAG-biotin (Sigma-Aldrich) and secondary streptavidin-Alexa-488 antibody (Invitrogen). Fluorescence was observed with a 63×/1.4-0.60 HCX PL APO objective on a DMIRE2-TCS AOBS confocal microscope system (Leica). Images were acquired and analyzed using LCS 2.61 (Leica) and processed using Photoshop CS2 (Adobe Systems).

ChIP assay

HepG2 cells were transfected with the indicated expression vectors together with 3TP-Lux reporter plasmid. At 24 hours after transfection, cells were treated with TGF-β (5 ng/mL). At 48 hours after transfection, ChIP assay was done according to the protocol recommended by Upstate Biotechnology. Precipitated DNA was amplified by PCR using primers specific for 3TP-Lux vector. Sequences for the primer set were 5′-CCCCCTGAACCTGAAACATA-3′ and 5′-CCTGAGGGCTCTCTTGTGTC-3′. For the endogenous *FoxP3* enhancer, chromatin samples prepared from MT-2 cells treated with 5 ng/mL of TGF-β for 2 hours were subjected to ChIP analysis using the following antibodies: anti-HBZ,[31] anti-Smad3 (BD Biosciences), anti-p300 (Santa Cruz Biotechnology), and normal mouse or rabbit immunoglobulin G (Santa Cruz Biotechnology). Primers used were 5′-CCTATGTTGGCTTCTAGTCTCTTTTATGG-3′ and 5′-TATGGAGAGGTTAAGTGCCTGGCTA-3′.

Synthesis of cDNA and semiquantitative RT-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. We reverse-transcribed 1 μg of total RNA into single-stranded cDNA with SuperScript II reverse transcriptase (Invitrogen). Using forward (F) and reverse (R) primers specific to the target genes, the cDNA was amplified by increasing PCR cycles. The specific primers used can be found in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Transfection and cell proliferation assay

Expression and control vectors were transfected into CTLL-2 cells by electroporation, and those cells were selected by G418 (0.5 mg/mL). Two transfectants, CTLL-2/pME18Sneo and CTLL-2/sHBZ, were established. For cell proliferation studies, sHBZ-expressing CTLL-2 and the control cells were plated at a density of 1×10^4 cells/well in 96-well plates. Cells were treated with increasing concentrations of TGF-β for 72 hours and assayed for cell growth by the methyl thiazolyl tetrazolium assay. Each experiment was performed 3 times, and representative results are presented.

Retroviral constructs and transduction

sHBZ cDNA was cloned into the retroviral vectors, pGCDNsamI/N and pMXs-IG, to generate pGCDNsamI/N-sHBZ and pMXs-sHBZ-IG. Transfection of the packaging cell line, Plat-E, was performed as described.[32] Mouse splenocytes were enriched for $CD25^-CD4^+$ cells with a CD4 T lymphocyte enrichment set (BD Biosciences) with the addition of

biotinylated anti-CD25 antibody (BD Biosciences), and activated by antigen-presenting cells (T cell-depleted and X-irradiated (20 Gy) C57BL/6J splenocytes) in the presence of 0.5 μg/mL anti-CD3 antibody and 50 U/mL human recombinant IL-2 in 12-well plates. After 24 hours, activated T cells were transduced with viral supernatant and 4 μg/mL polybrene, and centrifuged at 1700*g* for 60 minutes. Cells were subsequently cultured in medium supplemented with 50 U/mL recombinant IL-2.

Lentiviral vector construction and transfection of recombinant lentivirus

We cloned sHBZ cDNA into the *Eco*RI site of a lentiviral vector, pCSII-EF-MCS. The IRES-NGFR cassette was inserted into the *Xba*I site, which is located downstream of the sHBZ gene. Lentiviral vectors were produced as described.[7] Human peripheral blood mononuclear cells were enriched for $CD4^+CD25^-$ cells with a human naive CD4 T-cell enrichment set (BD Biosciences) and activated by anti-CD3/28–coated beads (Invitrogen). After 24 hours, cells were incubated with concentrated vector stocks in the presence of 4 μg/mL polybrene.

Flow cytometric analysis

Cells were washed with phosphate-buffered saline containing 1% FBS. After centrifugation, cells were treated with AlexaFluor-467–conjugated anti–human NGFR antibody (BD Biosciences) for 30 minutes. Cells were fixed and permeabilized at 4°C overnight and then treated with eFluor 450 conjugated anti-Foxp3 antibody (eBioscience) at 4°C for 30 minutes. After being washed with PBS, the cells were analyzed with a flow cytometer (BD FACSCanto II, BD Biosciences).

Cell sorting

Mouse naive T cells transduced with viral supernatant derived from the pMXs-sHBZ-IG and pMXs-IG vectors were sorted for the expression of green fluorescent protein using a FACSAria with Diva software (BD Biosciences PharMingen).

Statistical analyses

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

Results

HBZ protein enhances TGF-β/Smad–mediated signaling

To analyze the effect of HBZ on TGF-β–mediated transcriptional responses, HepG2 cells were cotransfected with an HBZ expression vector along with different TGF-β responsive reporters: TARE-Luc, 3TP-Lux, and 9 × CAGA-Luc. As shown in Figure 1A-C, sHBZ enhanced the transcription of luciferase in each of the reporter plasmid DNAs. sHBZ also up-regulated TGF-β transcription in a mouse T-cell line, CTLL-2 (Figure 1D). TGF-β signaling was increasingly activated when less than 200 ng of sHBZ expression vector, pcDNA3.1-mycHis-sHBZ, was added to each well. At higher levels (> 200 ng) of sHBZ plasmid DNA, however, activation of TGF-β signaling partially decreased (Figure 1E). In addition, pME18Sneo-based sHBZ vector activated TGF-β signaling with the same tendency as pcDNA3.1-mycHis-sHBZ did, whereas 20 ng of pME18Sneo-sHBZ plasmid was sufficient for maximal activation (supplemental Figure 1).

HBZ interacts with Smads

To clarify the molecular mechanism by which HBZ enhances the TGF-β transcriptional response, FLAG-tagged Smad2, Smad3, Smad4, or Smad7 and mycHis-tagged sHBZ were cotransfected into COS7 cells. Figure 2A demonstrates that sHBZ interacted with Smad2/3 and, to a lesser extent, with Smad4. TGF-β treatment had little effect on these interactions. Because a high degree of homology exists between Smad2 and Smad3, our subsequent analyses focused on the Smad3-HBZ interaction. We next examined the subcellular localization of sHBZ and Smad3 by confocal microscopy. After stimulating with TGF-β, cotransfected cells showed nuclear spots representing colocalization of sHBZ and Smad3 (Figure 2B). Activated Smad3 forms heteromeric complexes with the Co-Smad, Smad4, and translocates to the nucleus where it directly regulates transcription of target genes.[15] We therefore investigated whether HBZ influenced Smad3/Smad4 complex formation. COS7 cells were transfected with 6myc-Smad4 and FLAG-Smad3 with or without sHBZ. As shown in Figure 2C, sHBZ did not influence interaction between Smad3 and Smad4.

HBZ depends on the coactivator p300 to enhance TGF-β–mediated transcription

To address whether p300 is functionally required for the enhancement of TGF-β–mediated transcription by HBZ, we evaluated the effect of the adenovirus E1A oncoprotein,[33] an inhibitor of p300 activity, on the ability of HBZ to enhance TGF-β transcriptional activity. As shown in Figure 3A, wild-type E1A significantly suppressed the ability of TGF-β and sHBZ to enhance transcriptional activity through Smad-responsive elements, whereas a mutant form of E1A, E1A-ΔNT, which is defective for p300/CBP binding, had no effect on these responses. Moreover, in the presence of p300, sHBZ dramatically up-regulated Smad3-mediated TGF-β activation, whereas it had a less obvious effect without p300. This synergistic effect of HBZ and p300 on TGF-β activation was augmented with TGF-β treatment (Figure 3B).

As HBZ enhanced p300 and Smad3's activation of TGF-β signaling, we next explored whether HBZ, Smad3, and p300 could form a ternary complex. The plasmid DNAs, mycHis-sHBZ, FLAG-Smad3, and HA-p300 were cotransfected into COS7 cells that were then treated with or without TGF-β. FLAG-Smad3 and its associated proteins were immunoprecipitated using anti-FLAG, eluted with FLAG peptide, and subjected to a second immunoprecipitation with anti-HA antibody. The anti-HA immunoprecipitates were then subjected to Western blot analysis with anti-His antibody. As shown in Figure 3C, we detected a specific ternary complex only when the 3 components were coexpressed, which was enhanced in the presence of TGF-β. Furthermore, sHBZ protein greatly enhanced the interaction between Smad3 and p300 (Figure 3D). To investigate the binding of sHBZ/Smad3/p300 to DNA, we performed ChIP assay in HepG2 cells that were cotransfected with 3TP-Lux reporter along with expression vectors of sHBZ, Smad3, and p300. The ChIP assay detected the association of each of 3 proteins with Smad responsive elements, which indicated that sHBZ was recruited to 3TP promoter through forming complex with Smad3/p300. Akiyoshi et al reported that c-Ski counteracted p300 and acted as a transcriptional corepressor for Smads in the TGF-β signaling pathway.[34] Therefore, we studied whether HBZ could affect the interaction between Smad3 and c-Ski and found that sHBZ did not interfere with the formation of Smad3/c-Ski complex (supplemental Figure 2). These results together suggest that HBZ augments the interaction between Smad3 and p300, thereby potentiating TGF-β signaling.