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Supplementary Information for

# HTLV-1 induces T-cell malignancy and inflammation by viral antisense factor-mediated modulation of the cytokine signaling

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**This PDF file includes:**

## Supplementary Methods

### Plasmids

The expression vectors for HBZ and its deletion mutants, pME18Sneo-HBZ, pcDNA3.1-HBZ-mycHis, pcDNA3.1-HBZ-ΔbZIP-mycHis, pcDNA3.1-HBZ-AD+bZIP-mycHis and pcDNA3.1-HBZ-ΔAD-mycHis were described previously (1-3). Expression vectors for mouse STAT1 and mouse STAT3 were subcloned from pMX-mouse STAT1 and pEFBOS-mouse STAT3 provided by Dr. Kouichi Ikuta of Kyoto University, and human STAT1 was subcloned from pBS-HA-hSTAT1 provided by Dr. Koichi Nakajima of Osaka City University. The coding regions of human STAT3 and IL-10R1 were amplified by RT-PCR from total RNA derived from the HEK293T cell line and normal human PBMCs. pNL[NlucP/ISRE/Hygro], pNL[NlucP/GAS-RE/Hygro], pRL-CMV, pGL4.10[luc2] and pNL3.2[NlucP/minP] were purchased from Promega. The plasmid expressing the luciferase reporter gene *luc2* (*Photinus pyralis*) through the CMV promoter was generated by inserting the CMV promoter of pRL-CMV into pGL4.10[luc2], and the resulting plasmid was used as a reference vector in the luciferase assay. Five tandem sis-inducible element sequences (5 x SIE) were cloned into pNL3.2[NlucP/minP].

### Flow cytometric analysis

Splenocytes from mice were stained with the following antibodies for flow cytometric analysis. Anti-CD3 (PE/Cy7, 145-2C11), anti-CD4 (PerCP/Cy5.5, GK1.5), anti-CD8a (FITC, 53-6.7), anti-CD62L (APC, MEL-14), and anti-Foxp3 (BV421, MF-14) antibodies were purchased from BioLegend. Anti-CD44 (V500, IM7), anti-IFN-γ (PE, XMG1.2), anti-IL-10 (PE, JES5-16E3), and anti-IL-17A (PE, TC11-18H10) antibodies were from BD Biosciences. Anti-IL-4 (PE, 11B11) and anti-GITR (FITC, DTA-1) antibodies were from eBioscience. Rat IgG1κ (PE, R3-34, BD Biosciences), rat IgG2aκ (APC, eBR2a, eBioscience) and rat IgG2bκ (FITC, BV421, and RTK4530 from BioLegend; PE, A95-1 from BD Biosciences) were used as isotype controls. For cytokine staining, splenocytes were cultured with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Nacalai Tesque) and 1 μg/mL ionomycin (Nacalai Tesque) in the presence of

protein transport inhibitor, BD GolgiPlug or BD GolgiStop (BD Biosciences), for 5 hours.

Human PBMCs were stained with the following antibodies. Anti-CD4 (PerCP/Cy5.5, RPA-T4), anti-Foxp3 (Pacific Blue, 259D), and anti-IL-10R (PE, 3F9) antibodies were purchased from BioLegend. Anti-CADM1 (FITC, 3E1) antibody was from MBL. Mouse IgG1κ (Pacific Blue, MOPC-21 from BioLegend) and Chicken IgY (FITC, polyclonal from MBL) were used as isotype controls. For intracellular staining, cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), and intracellular antigens were stained. Dead cells were excluded using the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen). Flow cytometric analysis was carried out using a FACSVerse with FACSuite software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

**Immunohistochemical analysis**

Formalin-fixed paraffin-embedded tissue samples were sectioned into 2.5 μm slices. The specimens were deparaffinized and antigen was retrieved in Tris-EDTA buffer pH 9.0 (Dako, Tokyo, Japan). Monoclonal rat anti-mouse Foxp3 (1:50, FJK-16s, eBioscience, San Diego, California, USA) was used as the primary antibody for immunohistochemical staining. The slides were incubated with anti-Foxp3 for 60 min at room temperature, and then with secondary antibody using the anti-rat IgG Polymer Detection Kit (VECTOR, UK) for 30 min at room temperature. The signals were detected using 3,3'-diaminobenzidine chromogen (Dako, Tokyo, Japan), and the samples were counterstained with haematoxylin.

**Cell Proliferation Assay**

Splenic $CD4^+$ T cells were enriched by negative selection using the Mouse CD4 T Lymphocyte Enrichment Set (BD Biosciences), then cultured in RPMI 1640 medium supplemented with 10% FBS and 55 μM 2-mercaptoethanol, and labeled with 5 μM CellTrace Violet. Cells were plated at a density of $2 \times 10^5$ cells/well in 96-well round-bottom plates and treated by immobilized anti-CD3 antibodies with or without

recombinant IL-10. 48 hours after stimulation, the intensity of CellTrace was measured by flow cytometry.

**Immunoprecipitation and immunoblotting**

HEK293T cells were transfected with the indicated plasmids using TransIT-LT1 (Mirus) according to the manufacturer's instructions. After 48 hours, cells were lysed in RIPA buffer with protease inhibitor for 30 min on ice. The lysates were immunoprecipitated with antibody-conjugated SureBeads Protein G Magnetic Beads (BioRad). ATL-43T(-) cells were treated with or without a selective JAK1/2 inhibitor Ruxolitinib for 24 hours and lysed in RIPA buffer with protease inhibitor and phosphatase inhibitor for 30 min on ice. For immunoprecipitation and immunoblotting, the following antibodies were used: Mouse anti-c-Myc (9E10) antibody was purchased from Sigma-Aldrich and Wako. Rabbit and mouse anti-FLAG, and mouse anti-α-Tubulin antibodies were purchased from Sigma-Aldrich. Rabbit anti-c-Myc antibody was purchased from MBL. Rabbit anti-Stat1, rabbit anti-phospho Stat1 (58D6), rabbit anti-Stat3 (79D7), rabbit anti-phospho-Stat3, rabbit anti-STAT5 (D2O6Y), and rabbit anti-STAT6 (D3H4) antibodies were purchased from Cell Signaling Technology. Mouse anti-HBZ monoclonal antibody (1A10) was generated by immunizing C57BL/6 mice with keyhole limpet hemocyanin (KLH)-conjugated HBZ peptide 97-133 (CKQIAEYLKRKEEEKARRRRRAEKKAADVARRKQEEQE). Rabbit anti-HBZ polyclonal antibody was generated by immunizing with HBZ-peptides (CRGPPGEKAPPRGETH and QERRERKWRQGAEKC) (Medical Biological Laboratories). Normal mouse IgG (Santa Cruz Biotechnology) was used as a control. Anti-mouse IgG-HRP was from Thermo Fisher Scientific and anti-rabbit IgG-HRP was from GE Healthcare Life Sciences. Immunoblot data were collected using ImageQuant LAS 4000 mini (GE Healthcare).

**Immunofluorescence**

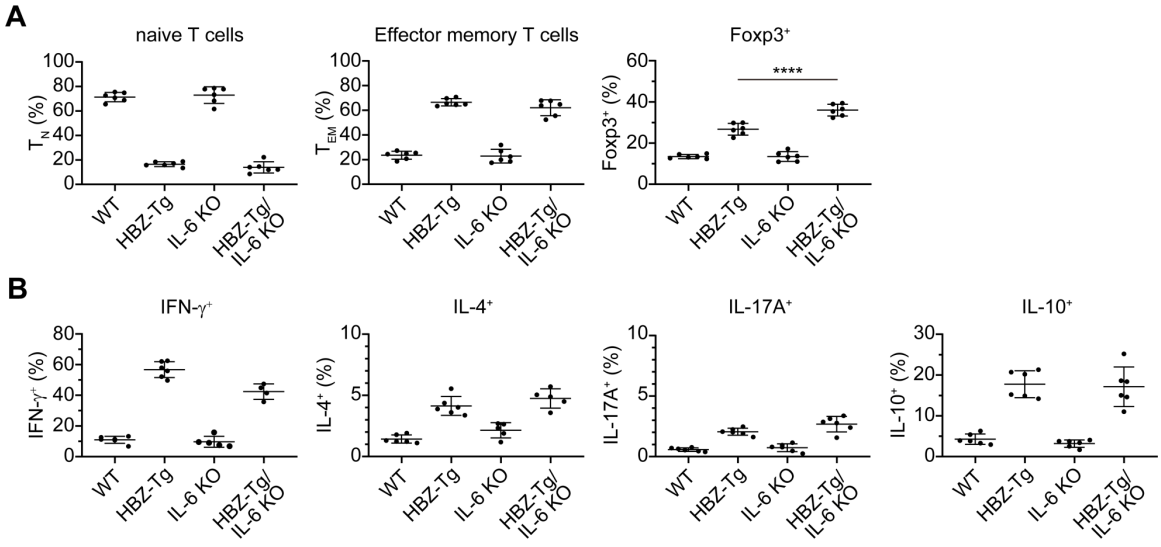
HeLa cells were cultured on chamber slides (Matsunami glass) and transfected with pcDNA3.1(-)-HBZ-myc-His and pcDNA3.1(-)-STAT1 or STAT3-3xFLAG using TransIT-LT1 (Mirus). Jurkat cells were transfected by using Neon transfection system

(Invitrogen) and centrifuged onto APS-coated glass slides (Matsunami glass) by Cytospin (Shandon). These cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 15 min, and blocked by incubation in 5% donkey serum (Jackson ImmunoResearch). For immunostaining, cells were incubated with mouse anti-Myc (9E10, Wako) and rabbit anti-FLAG (Sigma-Aldrich) antibodies for 60 min, followed by incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG (Invitrogen) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG antibodies (Invitrogen) for 60min. The stained cells were mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes), imaged using C2 confocal microscope (Nikon), and analyzed with ImageJ.

**Luciferase assay**

On day 1, HEK293-IL-10R cells were seeded into a 24-well plate at 1 x $10^5$ per well. After 24 hours, cells were transfected with the indicated plasmids. At 24 hours after transfection, cells were treated with or without 100 ng/mL IL-10 (PeproTech). For luciferase assay, the transfected cells were lysed in Passive Lysis Buffer (Promega) and luciferase activities were measured using the Nano-Glo Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activities of NanoLuc to Firefly were calculated. For immunoblotting, cells were lysed in RIPA buffer with protease inhibitor and phosphatase inhibitor. Luciferase activities were determined by TriStar LB941 and MikroWin 2000 version 4.41 (Berthold Technologies).

**Quantitative RT-PCR.** Total RNA was extracted using Trizol Reagent (Invitrogen). cDNAs were synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) according to the manufacturer's instructions. The transcription level of each gene was analyzed by real-time PCR using FastStart Universal SYBR Green Master (Roche) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Primers used in this study are shown in Table S1.




C

| % in CD4+ T cells | | Tn | Tem | Foxp3+ | IFN-γ+ | IL-4+ | IL-17A+ | IL-10+ |
|---|---|---|---|---|---|---|---|---|
| 4 weeks old | WT | 80.5+/-3.3 | 15.4+/-2.9 | 10.9+/-1.1 | 9.0+/-2.9 | 2.0+/-0.3 | 1.1+/-0.4 | 1.6+/-0.5 |
| | HBZ-Tg | 37.2+/-3.4 | 45.5+/-4.6 | 46.0+/-4.1 | 35.4+/-4.3 | 6.8+/-1.0 | 4.5+/-1.5 | 9.6+/-1.4 |
| | IL-6 KO | 80.6+/-2.5 | 15.4+/-2.1 | 10.9+/-1.2 | 8.9+/-1.9 | 1.6+/-0.5 | 0.9+/-0.4 | 1.7+/-0.3 |
| | HBZ-Tg/IL-6 KO | 40.4+/-4.2 | 43.4+/-5.0 | 39.2+/-4.8 | 35.5+/-5.8 | 5.9+/-1.3 | 3.0+/-0.9 | 13.1+/-2.8 |
| 28 weeks old | WT | 71.3+/-3.8 | 23.5+/-3.2 | 13.5+/-1.0 | 11.0+/-2.3 | 1.4+/-0.3 | 0.6+/-0.1 | 4.3+/-1.3 |
| | HBZ-Tg | 16.5+/-2.0 | 66.4+/-3.0 | 26.8+/-2.9 | 56.7+/-5.2 | 4.1+/-0.8 | 2.1+/-0.3 | 17.8+/-3.3 |
| | IL-6 KO | 72.9+/-6.8 | 22.8+/-5.6 | 13.5+/-2.4 | 9.7+/-3.6 | 2.1+/-0.6 | 0.7+/-0.3 | 3.2+/-0.9 |
| | HBZ-Tg/IL-6 KO | 13.9+/-4.6 | 62.0+/-6.5 | 36.0+/-2.9 | 42.4+/-5.1 | 4.7+/-0.8 | 2.7+/-0.6 | 17.1+/-4.8 |

**Figure S1. IL-10-producing CD4+ T cells are increased in HBZ-Tg/IL-6 KO mice at 28 weeks old.**

(*A*) Flow cytometric analysis of T-cell subsets. Mouse splenocytes were collected from WT, HBZ-Tg, IL-6 KO and HBZ-Tg/IL-6 KO mice at 28 weeks old. Cells were stained with anti-CD4, anti-CD44, anti-CD62L antibodies for naïve and effector memory T cells, and anti-Foxp3 for Treg cells.

(*B*) Cytokine production by $CD4^+$ T cells. Splenocytes were stimulated with PMA/ionomycin in the presence of protein transport inhibitor for 5 hours, then stained with antibodies to the indicated proteins.

(*C*) The percentage of $CD4^+$ T cells that are positive for each marker or cytokine is shown (n = 4 to 6) (one-way ANOVA with Turkey's multiple comparisons).

Up-regulated gene

| GeneName | logFC | PValue | FDR |
|---|---|---|---|
| Car5b | 6.737845 | 2.50E-121 | 3.14E-117 |
| Zfp503 | 8.699538 | 9.47E-93 | 5.92E-89 |
| Ccr4 | 4.380806 | 2.26E-79 | 9.44E-76 |
| Vax2 | 8.32029 | 6.70E-58 | 1.91E-54 |
| Fam213a | 4.014222 | 7.64E-58 | 1.91E-54 |
| Baiap3 | 5.335091 | 1.01E-57 | 2.11E-54 |
| Calca | 11.91986 | 4.61E-55 | 8.24E-52 |
| Slc1a4 | 4.59841 | 1.11E-50 | 1.73E-47 |
| Trp73 | 8.454542 | 3.70E-50 | 5.15E-47 |
| Neo1 | 5.758186 | 3.42E-49 | 4.29E-46 |
| Dkk3 | 6.38195 | 4.26E-48 | 4.85E-45 |
| Itih2 | 11.91913 | 2.14E-45 | 2.24E-42 |
| Itgae | 3.693676 | 5.30E-45 | 5.11E-42 |
| Clock | 3.196508 | 1.41E-44 | 1.26E-41 |
| Tacstd2 | 5.784546 | 8.91E-44 | 7.43E-41 |
| Ccl5 | 3.648138 | 2.34E-41 | 1.83E-38 |
| Wnt10a | 4.829087 | 2.29E-40 | 1.68E-37 |
| Zan | 5.546501 | 4.51E-40 | 3.14E-37 |
| Rimklb | 6.954414 | 6.59E-39 | 4.34E-36 |
| Tigit | 4.20325 | 1.31E-37 | 8.21E-35 |
| 5330417C22Rik | 6.84193 | 4.39E-32 | 2.62E-29 |
| Cd101 | 3.216451 | 2.85E-30 | 1.62E-27 |
| Ppp2r2c | 7.985721 | 3.19E-30 | 1.74E-27 |
| Calcb | 11.02262 | 3.46E-30 | 1.81E-27 |
| Itih5 | 2.809129 | 5.23E-30 | 2.62E-27 |
| Mmp10 | 5.829464 | 8.52E-30 | 4.10E-27 |
| Vash1 | 7.340381 | 1.19E-28 | 5.34E-26 |
| Tmevpg1 | 4.062615 | 8.27E-28 | 3.57E-25 |
| Epas1 | 3.141413 | 7.00E-27 | 2.92E-24 |
| Pdcd1 | 2.985885 | 2.35E-26 | 9.17E-24 |
| Tbx21 | 2.454325 | 3.65E-26 | 1.38E-23 |
| Cxcr3 | 2.359142 | 5.33E-26 | 1.96E-23 |
| Syne3 | 3.242184 | 3.44E-25 | 1.20E-22 |
| Esm1 | 4.987937 | 1.33E-24 | 4.50E-22 |
| Foxp3 | 2.238101 | 4.89E-24 | 1.61E-21 |
| 1500009L16Rik | 4.012142 | 5.93E-24 | 1.90E-21 |
| Osr2 | 8.333171 | 2.86E-23 | 8.73E-21 |
| Thbs1 | 4.360778 | 3.15E-23 | 9.38E-21 |
| Msl3l2 | 2.506845 | 4.33E-22 | 1.26E-19 |
| Rtkn | 7.015078 | 1.10E-21 | 3.13E-19 |
| Grb7 | 3.455119 | 2.28E-21 | 6.21E-19 |
| Prdm1 | 2.766109 | 3.10E-21 | 8.25E-19 |
| Rcan1 | 2.252883 | 3.29E-21 | 8.59E-19 |
| Xlr3b | 5.839626 | 4.75E-21 | 1.21E-18 |
| Lamc1 | 2.836723 | 4.96E-21 | 1.24E-18 |
| S100a6 | 1.980903 | 6.90E-21 | 1.69E-18 |
| Gzmk | 4.027209 | 1.07E-20 | 2.57E-18 |
| Cxcl3 | 5.660948 | 1.22E-20 | 2.87E-18 |
| Sytl3 | 3.655474 | 1.39E-20 | 3.23E-18 |
| Ednrb | 7.398529 | 1.62E-20 | 3.69E-18 |

Down-regulated gene

| GeneName | logFC | PValue | FDR |
|---|---|---|---|
| Igfbp4 | -2.36933 | 3.53E-29 | 1.63E-26 |
| Dapl1 | -4.57855 | 2.03E-26 | 8.18E-24 |
| Nsg2 | -2.689 | 1.52E-25 | 5.42E-23 |
| Ly6c1 | -2.91314 | 1.63E-23 | 5.09E-21 |
| Gm14085 | -2.83539 | 1.59E-21 | 4.41E-19 |
| Tgfbr3 | -2.25132 | 3.67E-18 | 6.75E-16 |
| Bambi-ps1 | -2.35372 | 3.80E-16 | 6.02E-14 |
| Nrn1 | -3.06585 | 1.15E-15 | 1.71E-13 |
| Satb1 | -1.52801 | 2.12E-13 | 2.39E-11 |
| St8sia1 | -1.67106 | 4.32E-13 | 4.71E-11 |
| Ecm1 | -1.84219 | 1.12E-12 | 1.13E-10 |
| Ugcg | -1.45955 | 5.30E-12 | 4.95E-10 |
| Smad7 | -1.61032 | 6.89E-12 | 6.39E-10 |
| Kif1b | -1.43539 | 1.30E-11 | 1.15E-09 |
| Erdr1 | -2.8781 | 2.01E-11 | 1.68E-09 |
| Treml2 | -1.30601 | 8.06E-11 | 6.15E-09 |
| Sorcs2 | -2.19188 | 9.51E-11 | 7.09E-09 |
| Als2cl | -1.33184 | 1.45E-10 | 1.04E-08 |
| Tmem64 | -1.43601 | 1.89E-10 | 1.31E-08 |
| Gm13546 | -2.77949 | 2.79E-10 | 1.90E-08 |
| Slc16a5 | -2.47443 | 3.25E-10 | 2.20E-08 |
| Dnah7a | -4.01849 | 3.86E-10 | 2.57E-08 |
| Sell | -1.64438 | 4.29E-10 | 2.81E-08 |
| Zbtb16 | -2.81871 | 6.25E-10 | 3.95E-08 |
| Penk | -2.57155 | 6.35E-10 | 4.00E-08 |
| Gramd4 | -1.39909 | 7.40E-10 | 4.61E-08 |
| Pdlim4 | -2.32114 | 8.99E-10 | 5.52E-08 |
| Amigo2 | -1.92952 | 2.10E-09 | 1.21E-07 |
| Sh3gl3 | -3.76089 | 2.28E-09 | 1.29E-07 |
| Lypd6b | -2.53058 | 4.08E-09 | 2.22E-07 |
| She | -3.14345 | 5.31E-09 | 2.84E-07 |
| Arhgap20 | -4.25734 | 7.73E-09 | 3.97E-07 |
| Nrip1 | -1.30339 | 1.02E-08 | 5.11E-07 |
| Vipr1 | -2.16091 | 1.06E-08 | 5.28E-07 |
| Fam101b | -1.35185 | 1.15E-08 | 5.67E-07 |
| Gpm6b | -1.87001 | 1.73E-08 | 8.38E-07 |
| Ramp1 | -1.59596 | 1.74E-08 | 8.40E-07 |
| Atp8b4 | -1.6292 | 2.27E-08 | 1.08E-06 |
| Ifngr2 | -1.2824 | 6.33E-08 | 2.81E-06 |
| Tnfsf8 | -1.72484 | 6.42E-08 | 2.83E-06 |
| Ust | -1.96347 | 8.33E-08 | 3.58E-06 |
| Cd200 | -1.547 | 1.94E-07 | 7.93E-06 |
| Znrf3 | -1.46353 | 1.95E-07 | 7.94E-06 |
| Myb | -1.21434 | 2.13E-07 | 8.55E-06 |
| Cnn3 | -1.64935 | 2.38E-07 | 9.55E-06 |
| Tcf7 | -1.1827 | 3.33E-07 | 1.31E-05 |
| Sema4a | -0.97063 | 4.85E-07 | 1.85E-05 |
| Bach2 | -1.25144 | 5.19E-07 | 1.97E-05 |
| Tubb2a | -1.27521 | 7.59E-07 | 2.80E-05 |
| Grm6 | -2.81241 | 8.02E-07 | 2.94E-05 |

**Figure S2. Top 50 genes which were up-regulated or down-regulated in HBZ-Tg mice compared with WT mice.**

Differentially expressed genes were identified by RNA-seq analysis of $CD4^+$ T cells from WT and HBZ-Tg mice and sorted by FDR.

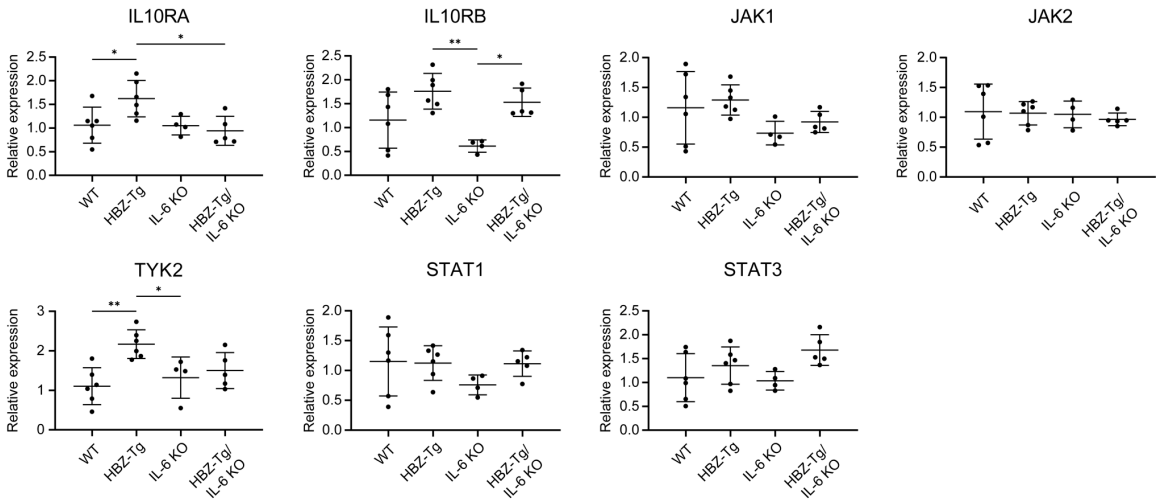



**Figure S3. Expression of genes associated with the IL-10/JAK/STAT signaling pathway in WT, HBZ-Tg, IL-6 KO, and HBZ-Tg/IL-6 KO mice.**

Relative expression values in $CD4^+$ T cells from each strain (n = 3 to 6) were calculated by the ddCt method using a value of WT sample as reference. * $P < 0.05$, ** $P < 0.01$.

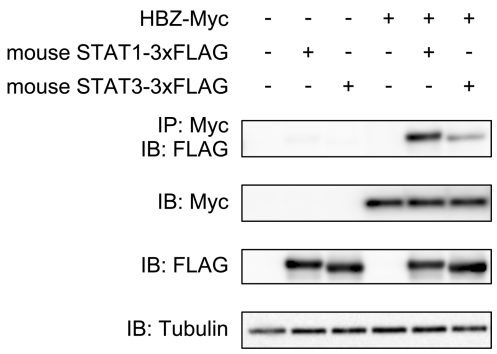



**Figure S4. Co-immunoprecipitation of HBZ and mouse STAT1 or STAT3.**

The indicated expression vectors were co-transfected into HEK293T cells, and protein interactions were analyzed by immunoprecipitation.

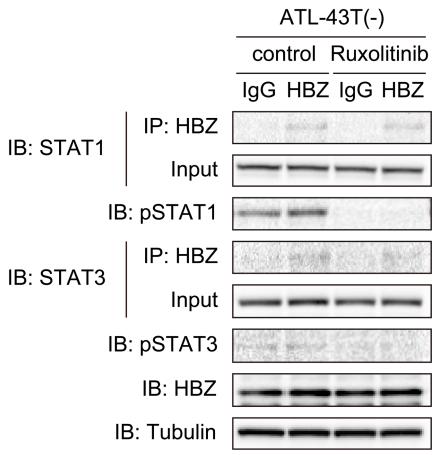



**Figure S5. The interaction between HBZ and STAT proteins in ATL-43T(-) cells**

ATL-43T(-) cells were treated with or without Ruxolitinib for 24 hours. Protein extract was immunoprecipitated with anti-HBZ antibody or control IgG. STAT proteins were detected by anti-STAT1, anti-pSTAT1, anti-STAT3, or anti-pSTAT3 antibody.

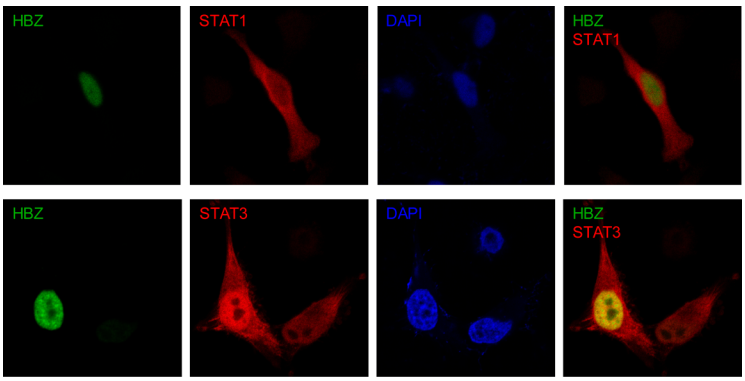



**Figure S6. Co-localization of HBZ and STAT proteins in HeLa cells.**

HBZ-myc and STAT1 or STAT3-3xFLAG were transfected into HeLa cells. Staining was performed using antibodies against myc (green) and FLAG (red). Nuclei were stained with DAPI (blue).

**Table S1. Primers for quantitative RT-PCR**

| gene | | sequence |
|---|---|---|
| *IL10RA* | F | 5'-TCTGTATCACCGAAGCTATGGC-3' |
| | R | 5'-ATGCCGTCCATTGCTTTCAG-3' |
| *IL10RB* | F | 5'-AGATCACTGCAAGCGCACTG-3' |
| | R | 5'-TGTTCATCCGCCAATTCAGC-3' |
| *JAK1* | F | 5'-TCAAGGTTGCCAAACAGCTG-3' |
| | R | 5'-TTGATGAACGGGCCAATGTC-3' |
| *JAK2* | F | 5'-AGCAGCTTGGCAAAGGTAAC-3' |
| | R | 5'-AGTTTCTTCACAGCGACCAC-3' |
| *TYK2* | F | 5'-ATTTCCGCTTGACTGCTGAC-3' |
| | R | 5'-ACAGCTTGGCCTGTACAAAC-3' |
| *STAT1* | F | 5'-AACATCTTGGGTACGCACAC-3' |
| | R | 5'-TGTTCCTTCAGTTGCAGGTG-3' |
| *STAT3* | F | 5'-TGGCACCTTGGATTGAGAGTC-3' |
| | R | 5'-AAACACCAACGTGGCATGTG-3' |
| *RNA18s5* | F | 5'-AACCCGTTGAACCCCATT-3' |
| | R | 5'-CCATCCAATCGGTAGTAGCG-3' |

**SI References**


1. Zhao T*, et al.* (2011) HTLV-1 bZIP factor enhances TGF-beta signaling through p300 coactivator. *Blood* 118(7):1865-1876.
2. Zhao T*, et al.* (2009) Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB. *Blood* 113(12):2755-2764.
3. 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 U S A* 103(3):720-725.