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Figure 1 Effects of IFN-α treatment on HTLV-1 p19 release and viral transcription in various HTLV-1-infected cell lines. A. Expression of HTLV-1 mRNAs (**a**) and proteins (**b, c**) were evaluated by quantitative RT-PCR (**a**), immunoblotting (**b**), and flow cytometry (**c**), respectively, in HTLV-1-infected HUT102, ILT-Hod and ILT-#29 or uninfected Jurkat cell lines. **a.** The mRNA copy numbers measured by using pX or Gag primers were standardized to those for GAPDH and indicated as the means and standard deviations (SD) of duplicate samples. **b.** Cell lysates from indicated cell lines were subjected to an immunoblotting assay with antibodies to Tax (40 kDa) and α-Tubulin (50 kDa). The lysates in lanes 5 and 6 were prepared from ILT-Hod and ILT-#29 cells stimulated with PMA (50 ng/ml) overnight, respectively. **c.** Intracellular Tax proteins in permeabilized cells were stained with Alexa Fluor 488-labeled anti-Tax mAb (open histogram) and mouse IgG3 isotype control antibody (closed histogram). The inserted box indicates Gag expression in ILT-Hod and ILT-#29 cells stimulated with PMA (50 ng/ml) for 17h. **B.** HUT102 (top), ILT-Hod (middle) and ILT-#29 (bottom) cells were cultured for 3 days with or without three doses of IFN-α indicated. HTLV-1 p19 concentrations in the supernatants (left) and Gag mRNA levels were measured by ELISA and quantitative RT-PCR, respectively. Data are presented as the means and SD of duplicate samples. **C.** Frozen stored primary ATL cells were thawed and analyzed for intracellular Tax (top) or Gag (bottom) proteins by flow cytometry immediately (green line) or 24 h after culture with no (black line), 300 IU/ml (red line) or 3000 IU/ml (blue line) of IFN-α in the presence of IL-2 (30 IU/ml). The closed histogram represents samples stained with isotype controls. The mean fluorescence intensity (MFI) of each histogram was indicated in the bar graphs.

In HUT102 cells, IFN-α suppressed HTLV-1 p19-release but not viral transcription, which is in agreement with previous reports [20].

We also examined the effects of IFN-α in primary ATL cells (Figure 1C). In the absence of IFN-α, intracellular expression of HTLV-1 proteins was spontaneously induced in ATL cells within 24 h after the initiation of culture. IFN-α suppressed the induction of Tax expression in these cells at a concentration of 3000 IU/ml more efficiently than 300 IU/ml. IFN-α also suppressed induction of Gag protein expression but equally at two doses.

Because HTLV-1 mRNA expression was suppressed in ILT-Hod and ILT-#29 cells as well as primary ATL cells following IFN-α treatment, we used these ILTs for further study on the effects of IFN-α at a dose of 3000 IU/ml hereafter.

IFN-α reduced Tax protein expression before reduction of pX mRNA

We next examined the time-course of IFN-α effects on Gag and Tax expression at protein and mRNA levels in ILT-Hod and ILT-#29 cells. Expression of intracellular Tax protein decreased within 1 day after addition of IFN-α to both cell lines. Intracellular Tax expression was maintained at lower levels than the control without IFN-α for at least 8 days (Figure 2A, top panels). Intracellular Gag protein expression in IFN-α-treated cells became lower than untreated cells at later time points (3–8 days), although the levels of viral expression fluctuated during culture (Figure 2A, bottom panels). Expression of HTLV-1 mRNAs in both cell lines were comparable to untreated cells or slightly increased in 1 day after IFN-α treatment, despite the reduction in Tax protein. At later time points (3–8 days), HTLV-1 mRNA levels were significantly decreased (Figure 2B). Thus, in IFN-α-treated ILTs, Tax protein was reduced first without apparent reduction in viral transcription, followed by reduction in viral mRNA and other viral protein expression.

We compared the levels of HTLV-1 proteins and mRNAs at 1 day after IFN-α treatment in these ILTs in several experiments, and confirmed that, at this time point, IFN-α reproducibly suppressed Tax protein levels in both cell lines, whereas the effects of IFN-α were inconsistent on Gag protein levels and not suppressive on HTLV-1 mRNA levels measured by using two different primer sets specific for pX and one for Gag regions (Figure 2C).

We further examined the effects of IFN-α on Tax protein and pX mRNA expression in several other ILT lines derived from ATL and HAM/TSP patients (Figure 2D). Although the suppression rates varied among cell lines, IFN-α suppressed intracellular Tax expression in 6 of 7 ILT cell lines tested in 24 h after IFN-α treatment. In ILT-#294 and HUT102 cells, Tax expression was not suppressed by IFN-α. HTLV-1 mRNA levels were not markedly suppressed or even enhanced in some cell lines in 24 h. Transient enhancement of HTLV-1 mRNA levels were sometimes observed also in ILT-Hod or ILT-#29 in 1 day after IFN-α treatment (Figure 2B, C). The effects of IFN-α on cell growth were limited, with mild reductions observed in some ILT lines after 3–4 days of culture (Figure 2E).

PKR was involved in IFN-α-mediated reduction of Tax protein expression

Since the reduction in intracellular Tax protein levels was induced by IFN-α at an earlier stage than for mRNA in ILT cells, we assumed that some post-transcriptional mechanisms such as PKR-induced translational suppression might be involved. We therefore treated ILT-Hod and ILT-#29 cells with IFN-α in the presence of a chemical PKR-inhibitor or its negative-control (Figure 3A). The otherwise decreased levels of Tax protein in both ILTs in the presence of IFN-α were markedly augmented by the PKR-inhibitor. In both ILTs, the negative-control inhibitor did not alter the Tax protein levels. Interestingly, the PKR-inhibitor increased Tax expression in the absence of IFN-α as well especially in ILT-#29 cells (Figure 3A). The enhancement of Tax expression by PKR-

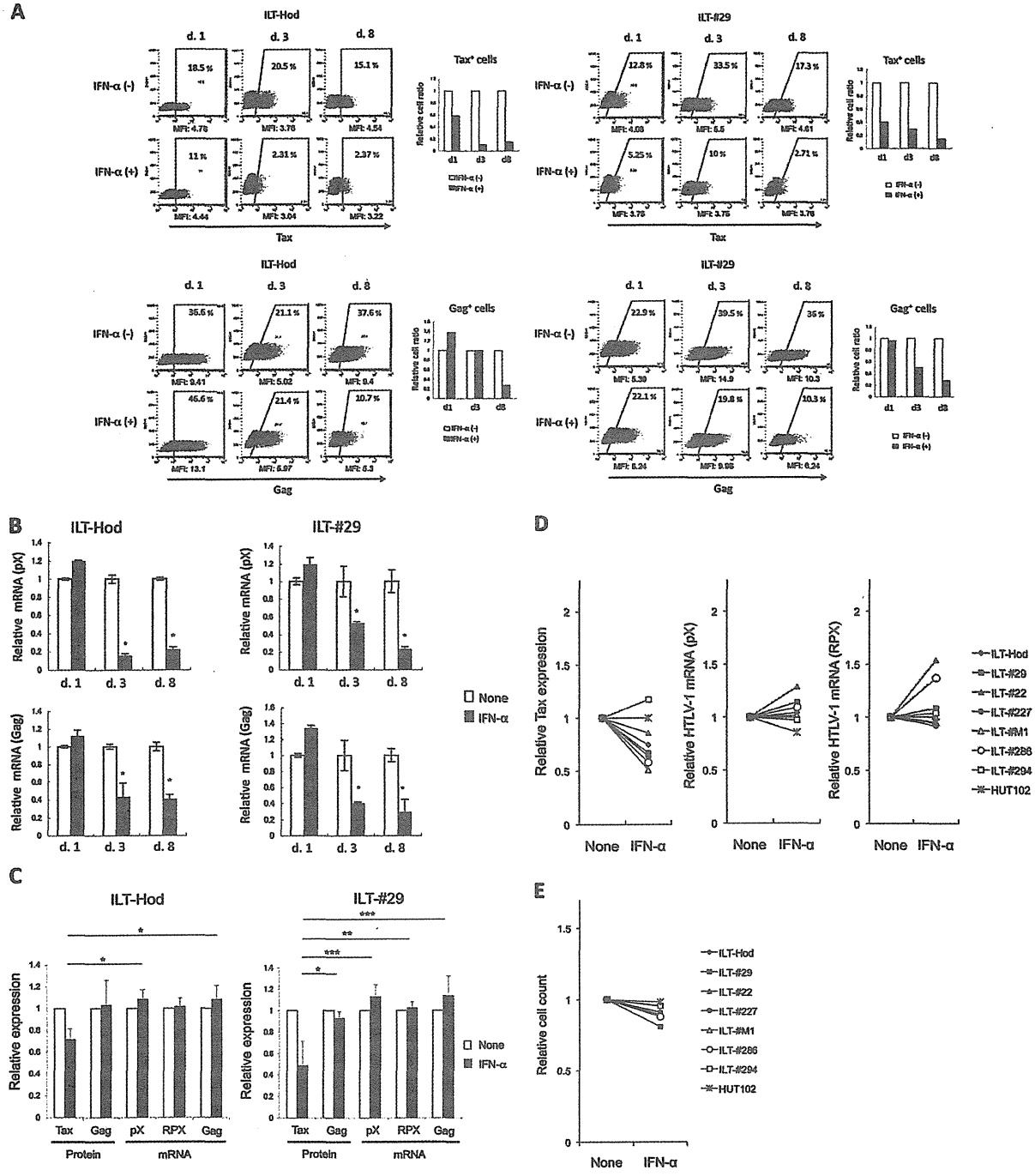

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Figure 2 IFN-α suppressed Tax protein expression before an apparent reduction in HTLV-1 mRNA levels. A. The effects of IFN-α (3000 IU/ml) on intracellular Tax (top) and Gag (bottom) protein expression in ILT-Hod (left) and ILT-#29 (right) cells was evaluated by flow cytometry on days 1, 3, and 8 of culture. Cells stained with isotype antibodies served as negative controls. The values inside the dot plots represent percentages of viral protein-expressing cells, and the relative values in IFN-α-treated (closed bar) against untreated (open bar) samples are shown in the bar graph. The MFI value of the total cell population is indicated below the dot plots. **B.** Expression of HTLV-1 mRNA in the same cell samples prepared in A was evaluated by quantitative RT-PCR using pX (top) and Gag (bottom) primers. Results are standardized and presented as relative values of IFN-α-treated (closed bar) against untreated (open bar) samples. The means and SD of duplicate samples are indicated. *$p < 0.05$. **C.** HTLV-1 proteins (Tax and Gag) and HTLV-1 mRNAs expression in ILT-Hod and ILT-#29 cells were measured 24 h after incubation with (closed bar) or without (open bar) IFN-α, and the relative values were indicated as the means and SD of three independent experiments. Three different primer sets (pX, RPX, and Gag) were used to quantify HTLV-1 mRNAs. **D.** Seven ILT lines from various patients and HUT102 were cultured with or without IFN-α for 24 h, and the proportions of Tax positive cells (left) and the HTLV-1 mRNA quantified using pX (middle) and RPX (right) primers were indicated as relative values against the sample without IFN-α. **E.** Various HTLV-1-infected T cell lines shown in D were cultured with or without IFN-α for 3–4 days, and viable cell numbers analyzed by a colorimetric assay were indicated as relative values.

inhibitor was not a result of transcriptional regulation, as HTLV-1 mRNA levels in the cells treated with PKR-inhibitor were comparable to those with control inhibitor (Figure 3B).

We then assessed PKR mRNA expression in these cell lines (Figure 3C). Both ILT lines expressed higher levels of PKR mRNA than HTLV-1-negative Jurkat and MOLT4 cells (Figure 3C). Moreover, IFN-α treatment further increased PKR mRNA expression in ILTs (Figure 3D). These observations indicated that IFN-α suppressed Tax expression at translational level via PKR in ILTs, and also suggested that similar mechanisms might regulate Tax expression in these cells to some extent without exogenous IFN-α.

Effects of IFN-α and AZT on HTLV-1 expression and cell growth

Combination therapy with IFN-α and AZT has been reported to achieve high response rates especially in patients with smoldering and chronic types of ATL, although patients with acute type ATL frequently relapse after therapy [13]. Despite favorable clinical responses, the combination of IFN-α and AZT reportedly shows minimal effects on the viability of HTLV-1-transformed T cells *in vitro* [16]. As we found that IFN-α affected viral expression in ILT-Hod and ILT-#29 cells in our system, we then examined the effects of IFN-α and AZT using these ILTs.

The effects of these drugs on HTLV-1 expression in ILTs was first evaluated. After three days of incubation, when IFN-α-mediated suppression of intracellular Tax protein expression was clearly observed, similar levels of suppression were produced by treatment with the combination of IFN-α and AZT, but not with AZT alone (Figure 4A).

Next, we assessed the effects of these drugs on cell growth. Treatment of IFN-α alone induced mild suppression of cell propagation in one week of culture, while AZT alone did not. The combination of IFN-α and AZT showed stronger suppression of cell growth than IFN-α alone (Figure 4B). The cell cycle analysis indicated that cells treated with IFN-α alone, but not AZT alone, accumulated in the G0/G1 phase. Combined AZT/IFN-α showed a marked increase in apoptotic cell fractions in both ILT-Hod and ILT-#29 cells (Figure 4C). Expression of Ki-67 was also suppressed in these cells by treatment with IFN-α alone or AZT/IFN-α, but not with AZT alone (Figure 4D).

Therefore IFN-α, but not AZT, induced cell-cycle arrest and suppression of viral expression, while AZT combined with IFN-α induced apoptosis in ILT-Hod and ILT-#29 cells.

Suppression of NF-κB activity by IFN-α treatment

NF-κB pathway is constitutively activated and plays a critical role on cell survival in HTLV-1-infected, through Tax-mediated transactivation and other unknown mechanisms [27-29]. We examined the effects of AZT/IFN-α on NF-κB activity using ILT-Hod and ILT-#29 reporter cells stably expressing the NF-κB-responsive element reporter gene. In both cell lines, NF-κB activity was partly but significantly suppressed by IFN-α alone or in combination with AZT, but not with AZT alone (Figure 5A). The reduction in NF-κB activity by IFN-α was also confirmed by the decreases in the mRNA levels of vascular epithelial growth factor (VEGF), one of the NF-κB-regulated genes, in both ILTs treated with IFN-α (Figure 5B).

Involvement of p53-signalling in IFN-α/AZT-mediated apoptosis in ILTs

We finally assessed the effect of IFN-α and AZT on p53 signaling that is known to be impaired in ATL cells [30]. We measured the phosphorylation of p53 in ILTs by flow cytometry (Figure 6A). The levels of phosphorylated p53 clearly increased in both ILTs following treatment with AZT/IFN-α, while IFN-α alone produced minimal effects.

We also evaluated the activity of the p53 pathway by measuring mRNA levels of p53-responsive genes, BAX and p21 (Figure 6B). Levels of BAX and p21 mRNAs were significantly increased in both cell lines treated

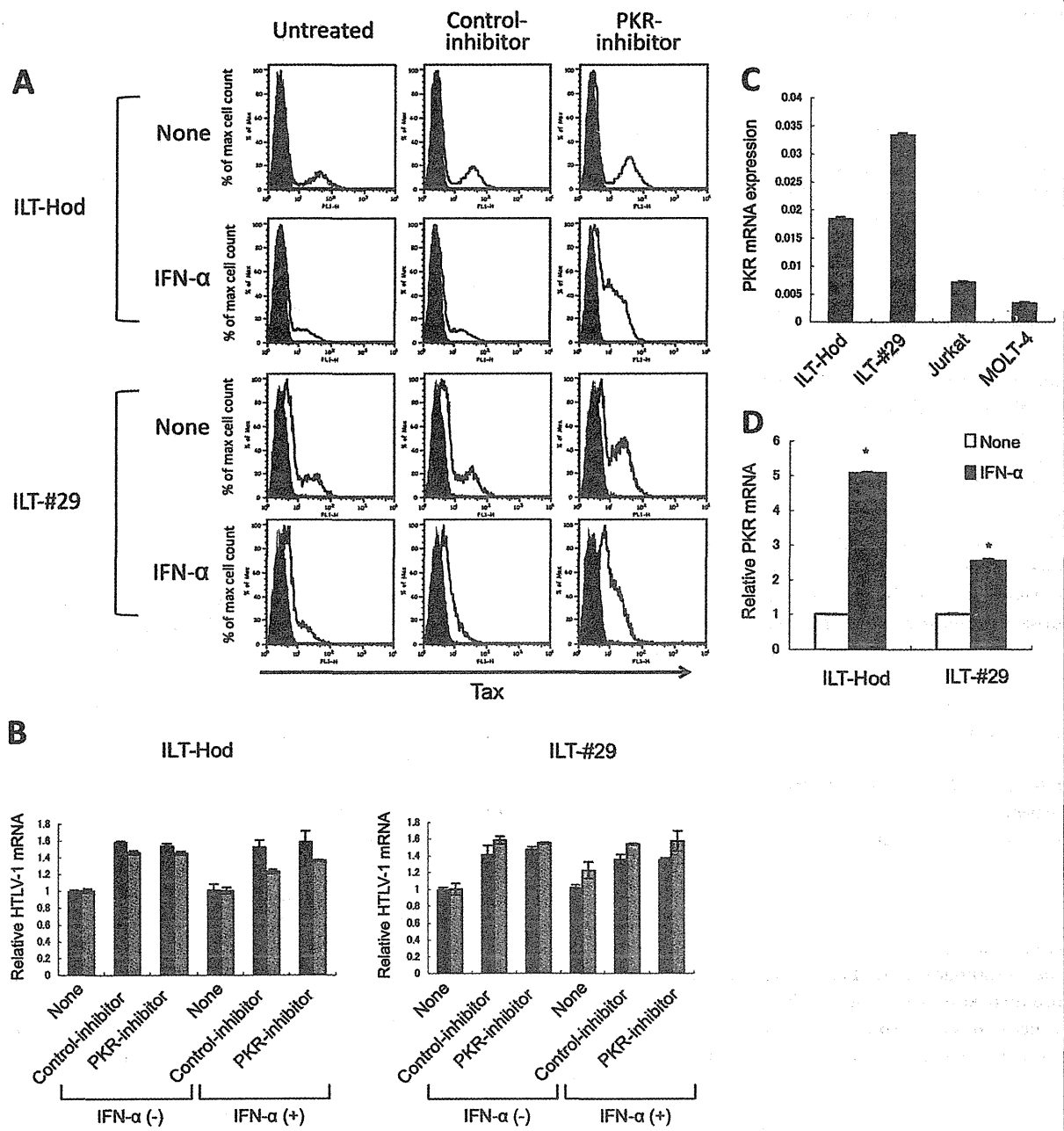

Figure 3 Involvement of PKR in IFN-α-mediated reduction of Tax protein levels in HTLV-1-infected cells. A. ILT-Hod and ILT-#29 cells were incubated with or without the PKR-inhibitor (500 nM) or the negative control-inhibitor (500 nM) for the first 2 h, then further cultured for the next 24 h in the presence or absence of IFN-α (3000 IU/ml) as indicated. Flow cytometry was then performed following stained with anti-Tax (open histogram) and isotype control (closed histogram) antibodies. **B.** HTLV-1 pX mRNAs in the same samples prepared in A were quantified by RT-PCR using two different primer sets (RPX; black bar, and pX; gray bar), standardized to GAPDH mRNAs, and the relative values were indicated as the means and SD of duplicate samples. **C.** PKR mRNAs in ILT-Hod, ILT-#29, and HTLV-1-negative Jurkat and MOLT4 cells were quantified by RT-PCR, standardized to GAPDH mRNA and indicated as the means and SD of duplicate samples. **D.** PKR mRNAs in ILT-Hod and ILT-#29 cells were quantified 24 h after culture in the absence (open bar) or presence (closed bar) of IFN-α (3000 IU/ml), and the relative values are indicated as the means and SD of duplicate samples. *$p < 0.05$.

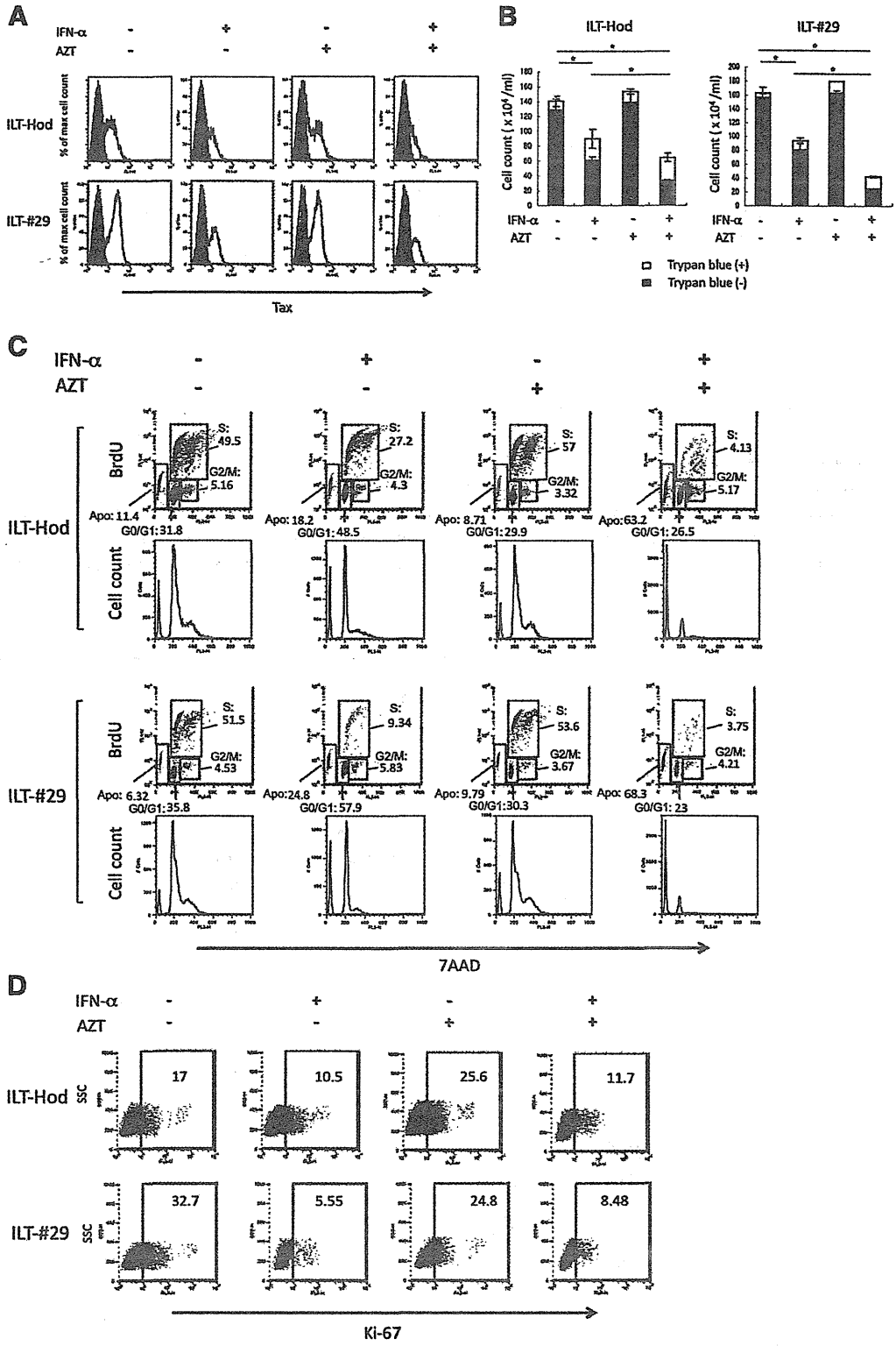

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Figure 4 Effects of IFN-α and AZT on HTLV-1 expression and cell growth of HTLV-1 infected cells. ILT-Hod and ILT-#29 cells (10^6/ml) were cultured in the absence or presence of IFN-α (3000 IU/ml) and/or AZT (10 μM) as indicated, and HTLV-1 expression (**A**), cell growth (**B**), cell cycle (**C**), and Ki-67 expression (**D**) in the cells were evaluated. **A.** Expression of intracellular Tax protein 3 days after the initiation of culture was evaluated by flow cytometry following stained with anti-Tax (open histogram) and isotype control (closed histogram) antibodies. **B.** ILT-Hod and ILT-#29 cells were similarly treated with IFN-α and/or AZT, and maintained with addition of equal volumes of fresh medium without IFN-α or AZT on the day 1 and 3, then viable (closed bar) and non-viable (open bar) cell numbers in cultures were evaluated by trypan blue exclusion on the day 8. *$p < 0.05$. **C.** ILT-Hod and ILT-#29 cells similarly treated with IFN-α and/or AZT were subjected to cell cycle analysis on the day 8. Cultures were treated with BrdU (10 μM) for the last 24 h of culture then permeabilized and incubated with a FITC-labeled mouse anti-BrdU antibody and 7AAD. Cells that are 7AAD-negative can be considered apoptotic (Apo). BrdU-negative and 7AAD-intermediate positive cells are in the G0/G1 phase. BrdU-positive and 7AAD-positive cells are in the S phase. BrdU-negative and 7AAD-highly positive cells are in the G2/M phase. The values in the dot plots indicate the proportion of the cells (%) in each phase. **D.** ILT-Hod and ILT-#29 cells similarly treated with IFN-α and/or AZT were analyzed for intracellular Ki-67 expression by flow cytometry on the day 8. The values in the dot plots indicate the proportion of Ki-67-positive cells (%).

with the combination of AZT and IFN-α. IFN-α alone slightly enhanced BAX and p21 mRNA levels in ILT-#29 cells but not in ILT-Hod cells. Effects of AZT alone were marginal in both cell lines.

The use of a p53-inhibitor partly reduced the apoptotic fraction in AZT/IFN-α-treated ILTs compared with those without inhibitor (Figure 6C). The effects of the p53-inhibitor were limited, however, probably because of a short half-life of the inhibitor.

These observations indicated that the combination of AZT and IFN-α effectively activated p53 pathway that was invoved in cell apoptosis in ILT-Hod and ILT-#29 cells.

Discussion

In the present study, we have demonstrated that IFN-α suppressed HTLV-1 gene expression in infected cells. This is consistent with our previous findings, which indicated that stromal cells suppressed viral expression in HTLV-

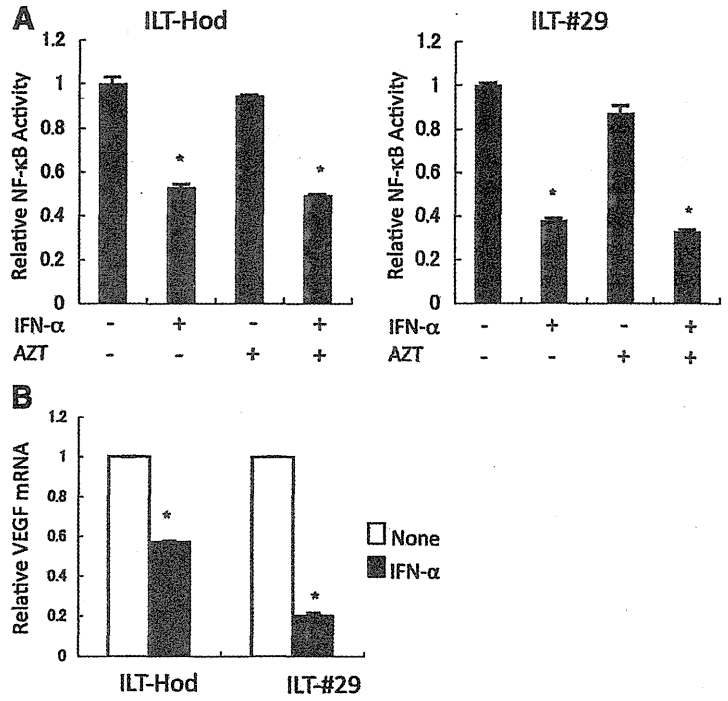

Figure 5 Suppression of NF-κB activity by IFN-α in HTLV-1-infected cells. A. ILT-Hod and ILT-#29 cells that were infected with lentiviral vectors containing reporter gene for the NF-κB responsive element and the TK-promoter several weeks before, were treated with or without IFN-α (3000 IU/ml) and/or AZT (10 μM) for 4 days as indicated. Luciferase activities were measured, and relative NF-κB activities normalized to TK-promoter activities were indicated as means and SD of duplicate samples. *$p < 0.05$. **B.** The levels of mRNA of VEGF, a NF-κB-regulated gene, in ILT-Hod and ILT-#29 cells 3 days after incubation with (closed bar) or without (open bar) IFN-α (3000 IU/ml) were quantified by RT-PCR and standardized to GAPDH mRNA. The relative values are indicated as means and SD of duplicate samples. *$p < 0.05$.

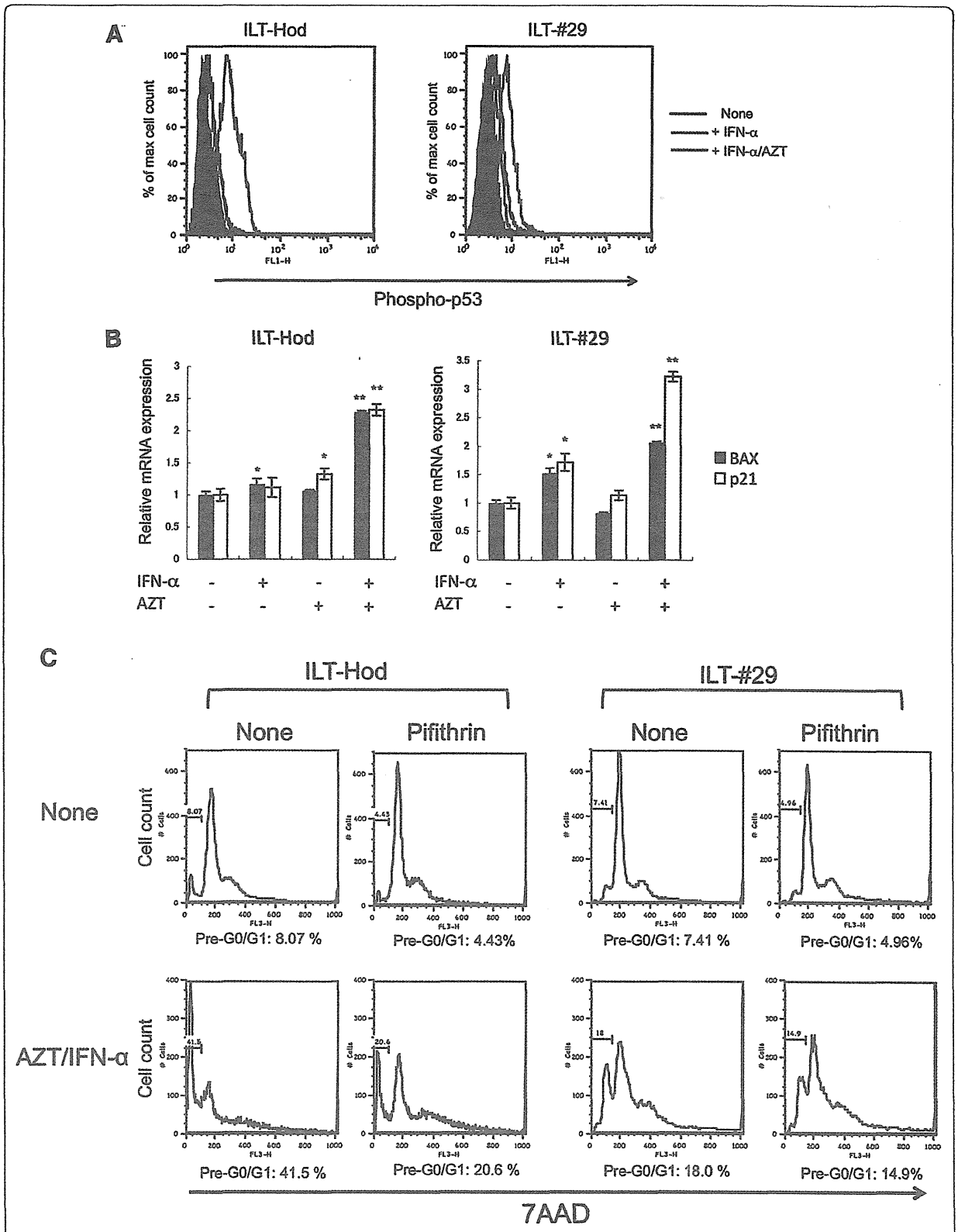

Figure 6 (See legend on next page.)

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Figure 6 Induction of p53-signaling by IFN-α and AZT in HTLV-1-infected cells. A. Intracellular phosphorylated p53 levels in ILT-Hod and ILT-#29 cells were evaluated by flow cytomertry 3 and 4 days after incubation, respectively, in the absence (black line) or presence of IFN-α (3000 IU/ml) alone (blue line), or IFN-α/AZT (10 μM) (red line). The closed histograms indicate cells stained with control antibody. **B.** ILT-Hod and ILT-#29 were treated with IFN-α and/or AZT for 4 days and mRNA expression of BAX (closed bar) and p21 (open bar) was evaluated by quantitative RT-PCR. Results are standardized with the copy number of GAPDH mRNA, and the relative values are indicated as means and SD of duplicate samples. *$p < 0.05$, **$p \leq 0.01$. **C.** ILT-Hod and ILT-#29 cells were cultured with or without IFN-α/AZT in the presence or absence of a p53-inhibitor (Pifithrin-α p-Nitro Cyclic, 1 μM) for 3 days and 5 days, respectively, then the cells were analyzed for the cell cycle by flow cytometry following 7AAD-staining. The proportions of apoptotic cell fractions (pre G0/G1) were indicated below each histogram.

1-infected T-cells via type I IFN when co-cultured [26]. However, these findings conflict with most other reports [16,20-22]. Differences among opposing findings can be attributed to the differences in the HTLV-1-infected cells used. It has been reported that type I IFNs inhibit HTLV-1 p19 release but not viral gene expression in HTLV-1-transformed cells [20]. This was true for HUT102 cells also in the present study, but not for ILT cells (Figure 1B). One of the differences between HUT102 and ILTs is the levels of Tax protein, which is present at much higher levels in HUT102 than ILTs. Because expression of HTLV-1 proteins is barely detectable *in vivo*, we hypothesize that HTLV-1-infected cells in vivo might retain susceptibility to IFNs similarly to ILTs rather than HUT102. Indeed, IFN-α suppressed HTLV-1 gene expression in primary ATL cells that was induced in a short-term culture *in vitro* (Figure 1C).

Reduction in intracellular Tax protein levels preceded transcriptional suppression of viral mRNA in ILTs when treated with IFN-α (Figure 2), indicating involvement of some post-transcriptional mechanisms such as decreased protein translation and/or increased proteolysis [19]. In this study, we found that PKR was involved in IFN-α–mediated Tax suppression (Figure 3). PKR is a ubiquitously expressed serine/threonine kinase, induced by IFNs and activated by double-stranded RNA to phosphorylate its substrates. These substrates include the alpha subunit of translation initiating factor eIF-2, thereby resulting in inhibition of protein synthesis [31-33]. Since the Tax protein positively regulates HTLV-1 transcription through interaction with the HTLV-1 long terminal repeat (LTR) [34,35], it would be reasonable that suppression of HTLV-1 transcription followed the reduction in Tax protein levels. However, the PKR-mediated translational control alone does not explain why Tax protein decreased earlier than Gag protein following IFN-α treatment in ILTs (Figure 2A, C), suggesting the involvement of additional mechanisms to produce preferential reduction of Tax.

It is intriguing that ILTs often show a histogram with two phases in the flow cytometric analysis for HTLV-1 proteins especially for Tax, despite the fact that all the ILT cells are infected with HTLV-1. This suggests that Tax protein levels in ILTs fluctuate between detectable and undetectable levels during culture. For the HUT102 cells, there was always a single peak of Tax-positive cells (Figure 1A). Nevertheless, the HTLV-1 transcription levels are comparable in ILTs and HUT102 (Figure 1A). In addition, the PKR inhibitor abrogated IFN-α-mediated suppression of Tax expression in ILTs without changing mRNA levels (Figure 3A, B). We also found that addition of the PKR inhibitor enhanced Tax expression in the absence of exogenous IFN-α especially for ILT-#29 cells (Figure 3A). Moreover, PKR expression was spontaneously increased in ILTs and further augmented by IFN-α (Figure 3C, D). These findings suggest that Tax protein synthesis might be spontaneously regulated by PKR to some extent in these cells, although it is unclear what activates PKR. If highly structured transcripts from HTLV-1 themselves were the activators of PKR, they might also activate other molecules such as 2', 5'-oligoadenylate synthetase that can also suppress viral expression. HTLV-1 expression might be regulated by such negative feedback systems to maintain equilibrium levels in ILT cells. Further studies will be required to understand the entire system regulating HTLV-1 expression in infected cells.

We noticed some differences with respect to the effects of IFN-α on HTLV-1 gene expression, p19 release, and cell growth in various HTLV-1-infected cell lines, which cannot be fully explained simply by the different levels of Tax expression in these cells, implying the presence of multiple mechanisms resisting against signaling pathways downstream of the IFN-αβ receptor. The mechanisms other than Tax determining IFN susceptibility remain to be clarified.

NF-κB is activated in HTLV-1-infected cells and plays a critical role in survival of these cells [36]. Our results indicate that IFN-α suppressed both viral expression and NF-κB activity; AZT did not affect either of these. Because Tax is a strong activator of NF-κB, IFN-α-mediated reduction of Tax protein levels likely results in IFN-α-mediated suppression of NF-κB (Figure 5A). However, the suppression of NF-κB activity by IFN-α in ILTs was partial. This is presumably attributed to the incompleteness of IFN-α-mediated suppression of Tax expression, and also to the presence of Tax-independent mechanisms for NF-κB activation in these cells.

Although IFN-α inhibited cell growth in ILTs, it was not cytocidal. Cell cycle analysis revealed that IFN-α

induced cell cycle arrest at G0/G1, indicating that IFN-α has only a static effect. Cell apoptosis increased when both AZT and IFN-α were added (Figure 4). It has been reported that type I IFNs induce expression of p53, but do not directly activate it [37]. The p53 transcription factor is activated by various stresses, and mediates cell cycle arrest or apoptosis through induction of many p53-regulated genes [38,39]. HTLV-1-infected cells, including ATL cells, mostly have intact p53 genes, expressing enhanced levels of p53, but its function is impaired [30,40]. Tax can inhibit the functions of p53 through various mechanisms including competition over the co-activator CBP/p300 that is required for trans-activation [27,41,42]. In the present study, we demonstrated that phosphorylation of p53 and expression of the p53-regulated genes (Bax and p21) were markedly enhanced by the presence of both AZT and IFN-α, while IFN-α alone exhibited marginal effects (Figure 6A, B). As exogenous IFN-α reduced Tax protein levels in ILTs (Figures 2A, 4A), it might unlock the Tax-mediated interference on p53 functions and enabled to activate p53-pathway following incorporation of AZT. This is consistent with clinical findings that AZT/IFN therapy is effective on ATL cases without mutations in p53 gene [43].

Conclusions

In conclusion, we have demonstrated that IFN-α can suppress HTLV-1 gene expression in IL-2-dependent HTLV-1-infected cells, and that PKR plays a critical role in the suppression. We further demonstrated that IFN-α and AZT cooperate to activate the p53 pathway and induce apoptosis. Our findings have elucidated previously unknown mechanisms regarding the regulation of HTLV-1 expression in infected cells, and partially explain how the combination of AZT and IFN-α produces therapeutic effects in ATL.

Methods

Cells

The various ILT lines derived from ATL patients (ILT-Hod, ILT-#29, ILT-#22, and ILT-#227) or HAM/TSP patients (ILT-M1, ILT-#286, and ILT-#294) were maintained in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) containing 10% fetal calf serum (FCS; Sigma Aldrich, St. Louis, MO), Antibiotic Antimycotic Solution (Sigma Aldrich) and 30–100 IU/ml of recombinant human IL-2 (Shionogi, Osaka, Japan). The IL-2-independent HTLV-1-infected T-cell line, HUT102, derived from a patient with mycosis fungoides [3], and uninfected T-cell lines, Jurkat [44] and MOLT4 [45] were also used. Mononuclear cells were isolated from the peripheral blood of an acute ATL patient under written informed consent, stored frozen in liquid nitrogen, and used as primary ATL cells for experiments immediately after thawing. This study was approved by the Institutional Review Board of the Tokyo Medical and Dental University.

Antibodies

Alexa Fluor 488-conjugated Lt-4 [46], a mouse monoclonal antibody (mAb) against the HTLV-1 Tax protein, and Alexa Fluor 488 mouse IgG3, κ isotype control (Biolegend, SanDiego, CA) were used for detecting Tax. Mouse ascites containing GIN-7 [47], a mAb against the HTLV-1 p19 Gag protein, and control mouse ascites were used for detecting Gag together with Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM (IgG + IgM) antibodies (KPL, Gaithersburg, MD) as secondary antibody. R-phycoerythrin (R-PE)-conjugated anti-human Ki-67 mouse mAb (BD Pharmingen), Alexa Fluor 488-conjugated rabbit anti-human phosphorylated p53 (Ser15) Ab (Beckman Coulter, CA), and their isotype controls were also used.

Reagents

Natural type human IFN-α (Sumiferon; Dainippon Sumitomo Pharma, Osaka, Japan) was added to cell cultures at various concentrations. Zidovudin (AZT) (Retrovir; GlaxoSmithKline; Research Triangle Park, NC) was used at 10 μM, a concentration inhibiting reverse transcription without cell toxicity [48]. When culturing these cells for longer than 3 days, fresh medium without these reagents was added during culture for maintenance. A chemical PKR-inhibitor ($C_{13}H_8N_4OS$; Calbiochem) and its negative control inhibitor ($C_{15}H_8C_{13}NO$; Calbiochem) were dissolved in DMSO and added at 500 nM in culture 2 h before IFN-α treatment and carried over through the culture. Pifithrin-α p-nitro cyclic, a chemical p53-inhibitor (Calbiochem), was used at 1 μM.

Quantitative RT-PCR and primers

Aliquots (0.5 μg) of total RNA extracted from cells using Isogen (Nippon Gene, Tokyo, Japan) were treated with DNase (Ambion; Austin, TX) and subjected to reverse transcription (RT) with oligo(dT)20 primers followed by PCR using THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan). To quantify HTLV-1 mRNAs, three primer sets were used; Gag primers (forward, 5′-CCT TAC CAC GCC TTC GTA GAA CGC CTC AAC ATA GC-3′; reverse, 5′-TTT GTC TTT GGG GGT CCA GGT CTG ACA AGC CCG CA-3′) located at Gag region, pX primers (forward, 5′-CGG ATA CCC AGT CTA CGT GTT TGG AGA CT-3′; reverse, 5′-GAG CCG ATA ACG CGT CCA TCG ATG GGG TCC-3′) located at pX region, and RPX primers (forward, 5′-ATC CCG TGG AGA CTC CTC AA-3′; reverse, 5′-AAC ACG TAG ACT GGG TAT CC-3′) located at upstream and downstream of the second splice junction site of tax/rex mRNA [6]. The primers specific for PKR (forward, 5′-

CCT GTC CTC TGG TTC TTT TGC T-3'; reverse, 5'-GAT GAT TCA GAA GCG AGT GTG C-3'), VEGF (forward, 5'-GGA GGG CAG AAT CAT CAC G-3'; reverse, 5'-TCG ATT GGA TGG CAG TAG CT-3'), BAX (forward, 5'-GAT GCG TCC ACC AAG AAG CT-3'; reverse, 5'-CGG CCC CAG TTG AAG TTG-3'), p21 (forward, 5'-CCA TGT GGA CCT GTC ACT GT-3'; reverse, 5'-TGG TAG AAA TCT GTC ATG CTG GTC-3'), and GAPDH (forward, 5'-TGA TTT TGG AGG GAT CTC GCT CCT GGA AGA-3'; reverse, 5'-GTG AAG GTC GGA GTC AAC GGA TTT GGT CGT-3') were also used. The thermal cycling protocol involved an initial denaturation at 95°C for 30 s, then 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s, and then detection of fluorescence from SYBR Green. Products were quantified and standardized against GAPDH mRNA copy numbers.

Flow cytometry

For intracellular staining of HTLV-1 antigens, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 100% methanol for 10 min on ice. To detect the Gag protein, cells were serially incubated with GIN-7 or control ascites and FITC-conjugated goat anti-mouse IgG + IgM. To detect the Tax protein, cells were incubated with Alexa Fluor 488-labelled Lt-4 or isotype control antibody. To stain intracellular Ki-67, cells were fixed with 4% paraformaldehyde and permeabilized with a BD Perm/Wash™ Buffer Kit (BD Pharmingen), then incubated with an R-PE-conjugated anti-human Ki-67 mouse mAb or isotype control mouse IgG1, κ antibody. For staining intracellular phosphorylated p53, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 100% methanol for 10 min on ice prior to incubation with antibody. Stained cells were analyzed with a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA) using FlowJo software (Tree Star).

Immunoblotting

HTLV-1-infected or uninfected cells were dissolved in Cell Culture Lysis Reagent (Promega, Madison, WI) containing 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, and 1% Triton X-100, with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and were incubated on ice for 1 hour. The cell lysates were cleared by centrifugation, denatured with SDS sample buffer (Thermo Scientific, Rockford, IL) and 2.5% 2-mercaptethanol (Sigma-Aldrich, St.Louis, MD) at 70°C for 15 min, and 17 μg of proteins were electrophoresed on polyacrylamide gel (Oriental Instruments CO., LTD, Kanagawa, Japan), and then transferred to PVDF membrane (ATTO, Tokyo, Japan). The membranes was blocked with Block Ace (DS Pharma Biomedical Co., Ltd, Osaka, Japan) overnight, and reacted with mouse anti-Tax and anti-α-Tubulin (Cedarlane, Ontario, Canada) antibodies as primary antibodies overnight, followed by exposure to horseradish peroxidase-conjugated sheep anti-mouse IgG whole antibody (GE Healthcare, Pittsburgh, PA) as a second antibody. The reacted bands were visualized by enhanced chemiluminescence using Novel® ECL (Invitrogen, Carlsbad, CA) and analyzed on Image Quant mini LAS 4000 (GE Healthcare).

Cell cycle analysis

Cells were cultured in the presence of 10 μM bromodeoxyuridine (BrdU) for 24 h, fixed and then permeabilized, followed by incubation with a mouse anti-BrdU mAb and 7AAD from BrdU flow Kits (BD Pharmingen), according to the manufacturers' instructions. Stained cells were analyzed with a flow cytometer using CellQuest software (Becton Dickinson). To evaluate cell growth, Trypan blue exclusion test and a colorimetric assay using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) based on formazan color development were used.

Enzyme-linked immunosorbent assays (ELISAs)

The concentration of HTLV-1 p19 in the supernatants from ILT-Hod, ILT-#29 or HUT102 cultures were measured using a RETRO-tek HTLV-1/II p19 antigen ELISA (ZeptoMetrix Corp., Buffalo, NY) according to the manufacturer's instructions.

Reporter assays

Reporter cell lines (ILT-Hod/NF-κB-Luc and ILT-#29/NF-κB-Luc) were established by using a Cignal Lenti-NF-κB reporter Luc Kit (Qiagen, Duesseldorf, Germany) and Cignal Lenti thymidine kinase (TK)-Renilla control (Qiagen). Luciferase assays were conducted with Luciferase or Renilla luciferase assay systems (Promega, Madison, WI) on cell lysates in Renilla luciferase lysis buffer (Promega). Relative NF-κB activity was calculated as the ratio of firefly luciferase to renilla luciferase activities in the same sample.

Statistics

The unpaired *t*-test was performed for statistical significance, and *P* values less than 0.05 were considered significant.

Abbreviations

ATL: Adult T-cell leukemia/lymphoma; AZT: Zidovudin (3'-Azido-3'-deoxythymidine); BrdU: Bromodeoxyuridine; ELISA: Enzyme-linked immunosorbent assay; FCS: Fetal calf serum; FITC: Fluorescein-isothiocyanate; HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis; HSCT: Hematopoietic stem cell transplantation; HTLV-1: Human T-cell leukemia virus type-1; IFN-α: Interferon-α; IgG: Immunoglobulin G; ILT: IL-2-dependent HTLV-1-infected T-cell; mAb: Monoclonal antibody; MFI: Mean fluorescence intensity; PBMC: Peripheral blood mononuclear cell; PKR: RNA-dependent protein kinase; PMA: Phorbol 12-myristate 13-acetate; RT: Reverse transcription; TK: Thymidine kinase; VEGF: Vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions
SK carried out most of the experiments, analyzed data, and wrote the manuscript; MKi and AT carried out certain aspects of the experiments; AH advised on flow cytometry analysis; AS advised on signaling analysis; TM advised on RT-PCR analysis; YT provided HTLV-1-specific monoclonal antibodies; AU provided clinical samples; MKa designed the study, analyzed data, and wrote the manuscript; all authors reviewed and approved the final manuscript.

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References

1. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita KI, Shirakawa S, Miyoshi I: **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* 1981, **78**:6476–6480.
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**: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 USA* 1980, **77**:7415–7419.
4. Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de The G: **Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis.** *Lancet* 1985, **2**:407–410.
5. Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M: **HTLV-I associated myelopathy, a new clinical entity.** *Lancet* 1986, **1**:1031–1032.
6. Kinoshita T, Shimoyama M, Tobinai K, Ito M, Ito S, Ikeda S, Tajima K, Shimotohno K, Sugimura T: **Detection of mRNA for the tax1/rex1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction.** *Proc Natl Acad Sci USA* 1989, **86**:5620–5624.
7. Utsunomiya A, Miyazaki Y, Takatsuka Y, Hanada S, Uozumi K, Yashiki S, Tara M, Kawano F, Saburi Y, Kikuchi H, *et al*: **Improved outcome of adult T cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation.** *Bone Marrow Transplant* 2001, **27**:15–20.
8. Okamura J, Utsunomiya A, Tanosaki R, Uike N, Sonoda S, Kannagi M, Tomonaga M, Harada M, Kimura N, Masuda M, *et al*: **Allogeneic stem-cell transplantation with reduced conditioning intensity as a novel immunotherapy and antiviral therapy for adult T-cell leukemia/lymphoma.** *Blood* 2005, **105**:4143–4145.
9. Yamamoto K, Utsunomiya A, Tobinai K, Tsukasaki K, Uike N, Uozumi K, Yamaguchi K, Yamada Y, Hanada S, Tamura K, *et al*: **Phase I study of KW-0761, a defucosylated humanized anti-CCR4 antibody, in relapsed patients with adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma.** *Journal of clinical oncology: official journal of the American Society of Clinical Oncology* 2010, **28**:1591–1598.
10. Ishida T, Joh T, Uike N, Yamamoto K, Utsunomiya A, Yoshida S, Saburi Y, Miyamoto T, Takemoto S, Suzushima H, *et al*: **Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study.** *Journal of clinical oncology: official journal of the American Society of Clinical Oncology* 2012, **30**:837–842.
11. Hermine O, Bouscary D, Gessain A, Turlure P, Leblond V, Franck N, Buzyn-Veil A, Rio B, Macintyre E, Dreyfus F, *et al*: **Brief report: treatment of adult T-cell leukemia-lymphoma with zidovudine and interferon alfa.** *N Engl J Med* 1995, **332**:1749–1751.
12. Gill PS, Harrington W Jr, Kaplan MH, Ribeiro RC, Bennett JM, Liebman HA, Bernstein-Singer M, Espina BM, Cabral L, Allen S, *et al*: **Treatment of adult T-cell leukemia-lymphoma with a combination of interferon alfa and zidovudine.** *N Engl J Med* 1995, **332**:1744–1748.
13. Bazarbachi A, Plumelle Y, Carlos Ramos J, Tortevoye P, Otrock Z, Taylor G, Gessain A, Harrington W, Panelatti G, Hermine O: **Meta-analysis on the use of zidovudine and interferon-alfa in adult T-cell leukemia/lymphoma showing improved survival in the leukemic subtypes.** *Journal of clinical oncology: official journal of the American Society of Clinical Oncology* 2010, **28**:4177–4183.
14. Harashima N, Kurihara K, Utsunomiya A, Tanosaki R, Hanabuchi S, Masuda M, Ohashi T, Fukui F, Hasegawa A, Masuda T, *et al*: **Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation.** *Cancer Res* 2004, **64**:391–399.
15. Niwa R, Shoji-Hosaka E, Sakurada M, Shinkawa T, Uchida K, Nakamura K, Matsushima K, Ueda R, Hanai N, Shitara K: **Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma.** *Cancer Res* 2004, **64**:2127–2133.
16. Bazarbachi A, Nasr R, El-Sabban ME, Mahe A, Mahieux R, Gessain A, Darwiche N, Dbaibo G, Kersual J, Zermati Y, *et al*: **Evidence against a direct cytotoxic effect of alpha interferon and zidovudine in HTLV-I associated adult T cell leukemia/lymphoma.** *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK* 2000, **14**:716–721.
17. Kchour G, Tarhini M, Kooshyar MM, El Hajj H, Wattel E, Mahmoudi M, Hatoum H, Rahimi H, Maleki M, Rafatpanah H, *et al*: **Phase 2 study of the efficacy and safety of the combination of arsenic trioxide, interferon alpha, and zidovudine in newly diagnosed chronic adult T-cell leukemia/lymphoma (ATL).** *Blood* 2009, **113**:6528–6532.
18. El-Sabban ME, Nasr R, Dbaibo G, Hermine O, Abboushi N, Quignon F, Ameisen JC, Bex F, de The H, Bazarbachi A: **Arsenic-interferon-alpha-triggered apoptosis in HTLV-I transformed cells is associated with tax down-regulation and reversal of NF-kappa B activation.** *Blood* 2000, **96**:2849–2855.
19. El Hajj H, El-Sabban M, Hasegawa H, Zaatari G, Ablain J, Saab ST, Janin A, Mahfouz R, Nasr R, Kfoury Y, *et al*: **Therapy-induced selective loss of leukemia-initiating activity in murine adult T cell leukemia.** *J Exp Med* 2010, **207**:2785–2792.
20. Feng X, Heyden NV, Ratner L: **Alpha interferon inhibits human T-cell leukemia virus type 1 assembly by preventing Gag interaction with rafts.** *J Virol* 2003, **77**:13389–13395.
21. Feng X, Ratner L: **Human T-cell leukemia virus type 1 blunts signaling by interferon alpha.** *Virology* 2008, **374**:210–216.
22. Zhang J, Yamada O, Kawagishi K, Araki H, Yamaoka S, Hattori T, Shimotohno K: **Human T-cell leukemia virus type 1 Tax modulates interferon-alpha signal transduction through competitive usage of the coactivator CBP/p300.** *Virology* 2008, **379**:306–313.
23. Oliere S, Hernandez E, Lezin A, Arguello M, Douville R, Nguyen TL, Olindo S, Panelatti G, Kazanji M, Wilkinson P, *et al*: **HTLV-1 evades type I interferon antiviral signaling by inducing the suppressor of cytokine signaling 1 (SOCS1).** *PLoS Pathog* 2010, **6**:e1001177.
24. Charoenthongtrakul S, Zhou Q, Shembade N, Harhaj NS, Harhaj EW: **Human T cell leukemia virus type 1 Tax inhibits innate antiviral signaling via NF-kappaB-dependent induction of SOCS1.** *J Virol* 2011, **85**:6955–6962.
25. Suzuki S, Zhou Y, Refaat A, Takasaki I, Koizumi K, Yamaoka S, Tabuchi Y, Saiki I, Sakurai H: **Human T cell lymphotropic virus 1 manipulates interferon regulatory signals by controlling the TAK1-IRF3 and IRF4 pathways.** *J Biol Chem* 2010, **285**:4441–4446.
26. Kinpara S, Hasegawa A, Utsunomiya A, Nishitsuji H, Furukawa H, Masuda T, Kannagi M: **Stromal cell-mediated suppression of human T-cell leukemia virus type 1 expression in vitro and in vivo by type I interferon.** *J Virol* 2009, **83**:5101–5108.
27. Yoshida M: **Multiple viral strategies of HTLV-1 for dysregulation of cell growth control.** *Annu Rev Immunol* 2001, **19**:475–496.
28. Jeang KT: **Functional activities of the human T-cell leukemia virus type I Tax oncoprotein: cellular signaling through NF-kappa B.** *Cytokine Growth Factor Rev* 2001, **12**:207–217.
29. Saitoh Y, Yamamoto N, Dewan MZ, Sugimoto H, Martinez Bruyn VJ, Iwasaki Y, Matsubara K, Qi X, Saitoh T, Imoto I, *et al*: **Overexpressed NF-kappaB-inducing kinase contributes to the tumorigenesis of adult T-cell leukemia and Hodgkin Reed-Sternberg cells.** *Blood* 2008, **111**:5118–5129.
30. Takemoto S, Trovato R, Cereseto A, Nicot C, Kislyakova T, Casareto L, Waldmann T, Torelli G, Franchini G: **p53 stabilization and functional**

impairment in the absence of genetic mutation or the alteration of the p14(ARF)-MDM2 loop in ex vivo and cultured adult T-cell leukemia/lymphoma cells. *Blood* 2000, 95:3939–3944.
31. Dey M, Cao C, Dar AC, Tamura T, Ozato K, Sicheri F, Dever TE: Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell* 2005, 122:901–913.
32. Dar AC, Dever TE, Sicheri F: Higher-order substrate recognition of eIF2alpha by the RNA-dependent protein kinase PKR. *Cell* 2005, 122:887–900.
33. Zhang F, Romano PR, Nagamura-Inoue T, Tian B, Dever TE, Mathews MB, Ozato K, Hinnebusch AG: Binding of double-stranded RNA to protein kinase PKR is required for dimerization and promotes critical autophosphorylation events in the activation loop. *J Biol Chem* 2001, 276:24946–24958.
34. Fujisawa J, Seiki M, Kiyokawa T, Yoshida M: Functional activation of the long terminal repeat of human T-cell leukemia virus type I by a trans-acting factor. *Proc Natl Acad Sci USA* 1985, 82:2277–2281.
35. Sodroski JG, Goh WC, Rosen CA, Salahuddin SZ, Aldovini A, Franchini G, Wong-Staal F, Gallo RC, Sugamura K, Hinuma Y, *et al*: trans-Activation of the human T-cell leukemia virus long terminal repeat correlates with expression of the x-lor protein. *J Virol* 1985, 55:831–835.
36. Mori N, Fujii M, Ikeda S, Yamada Y, Tomonaga M, Ballard DW, Yamamoto N: Constitutive activation of NF-kappaB in primary adult T-cell leukemia cells. *Blood* 1999, 93:2360–2368.
37. Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H, Sasaki S, Imai K, Shibue T, Honda K, Taniguchi T: Integration of interferon-alpha /beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature* 2003, 424:516–523.
38. Riley T, Sontag E, Chen P, Levine A: Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* 2008, 9:402–412.
39. Balint EE, Vousden KH: Activation and activities of the p53 tumour suppressor protein. *Br J Cancer* 2001, 85:1813–1823.
40. Newcomb EW: P53 gene mutations in lymphoid diseases and their possible relevance to drug resistance. *Leuk Lymphoma* 1995, 17:211–221.
41. Pise-Masison CA, Mahieux R, Radonovich M, Jiang H, Brady JN: Human T-lymphotropic virus type I Tax protein utilizes distinct pathways for p53 inhibition that are cell type-dependent. *J Biol Chem* 2001, 276:200–205.
42. Ariumi Y, Kaida A, Lin JY, Hirota M, Masui O, Yamaoka S, Taya Y, Shimotohno K: HTLV-1 tax oncoprotein represses the p53-mediated trans-activation function through coactivator CBP sequestration. *Oncogene* 2000, 19:1491–1499.
43. Datta A, Bellon M, Sinha-Datta U, Bazarbachi A, Lepelletier Y, Canioni D, Waldmann TA, Hermine O, Nicot C: Persistent inhibition of telomerase reprograms adult T-cell leukemia to p53-dependent senescence. *Blood* 2006, 108:1021–1029.
44. Schneider U, Schwenk HU, Bornkamm G: Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *International journal of cancer Journal international du cancer* 1977, 19:621–626.
45. Minowada J, Onuma T, Moore GE: Rosette-forming human lymphoid cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes. *J Natl Cancer Inst* 1972, 49:891–895.
46. Lee B, Tanaka Y, Tozawa H: Monoclonal antibody defining tax protein of human T-cell leukemia virus type-I. *Tohoku J Exp Med* 1989, 157:1–11.
47. Tanaka Y, Lee B, Inoi T, Tozawa H, Yamamoto N, Hinuma Y: Antigens related to three core proteins of HTLV-I (p24, p19 and p15) and their intracellular localizations, as defined by monoclonal antibodies. *International journal of cancer Journal international du cancer* 1986, 37:35–42.
48. Mitsuya H, Weinhold KJ, Furman PA, St Clair MH, Lehrman SN, Gallo RC, Bolognesi D, Barry DW, Broder S: 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. *Proc Natl Acad Sci USA* 1985, 82:7096–7100.

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Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/lymphoma

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Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/lymphoma

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Adult T-cell leukemia/lymphoma (ATLL) is an intractable hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1), which infects approximately 20 million people worldwide. Here, we have explored the possible expression of cancer/testis (CT) antigens by ATLL cells, as CT antigens are widely recognized as ideal targets of cancer immunotherapy against solid tumors. A high percentage (87.7%) of ATLL cases (n = 57) expressed CT antigens at the mRNA level: NY-ESO-1 (61.4%), MAGE-A3 (31.6%), and MAGE-A4 (61.4%). CT antigen expression was confirmed by immunohistochemistry. This contrasts with other types of lymphoma or leukemia, which scarcely express these CT antigens. Humoral immune responses, particularly against NY-ESO-1, were detected in 11.6% (5 of 43) and NY-ESO-1–specific CD8$^+$ T-cell responses were observed in 55.6% (5 of 9) of ATLL patients. NY-ESO-1–specific CD8$^+$ T cells recognized autologous ATLL cells and produced effector cytokines. Thus, ATLL cells characteristically express CT antigens and therefore vaccination with CT antigens can be an effective immunotherapy of ATLL. (*Blood.* 2012;119(13): 3097-3104)

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a distinct hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1).[1,2] HTLV-1 is endemic in southwestern Japan, Africa, South America, and the Caribbean Islands, and approximately 20 million people worldwide are infected.[3] A total of 5% of the infected persons develop ATLL after a long latency period.[2] ATLL cells are CD4-positive; and the majority, if not all, of them express the transcription factor FoxP3 (Forkhead Box P3), CD25, CTLA-4, and CCR4 (CC chemokine receptor 4), and are functionally immunosuppressive, thus phenotypically and functionally resembling naturally occurring regulatory T cells (Tregs).[3-9] Because of its immunosuppressive property and resistance to conventional chemotherapy, aggressive ATLL has a poor prognosis with a mean survival time of less than 1 year.[2,8] A recent phase 3 trial of a dose-intensified multidrug chemotherapy for untreated ATLL patients (acute, lymphoma, and unfavorable chronic types) showed a median progression-free and overall survival of only 7.0 and 12.7 months, respectively.[10] This and other reports indicate that chemotherapy alone is of limited success for ATLL and mostly fails to cure the disease.[10,11] Allogeneic hematopoietic stem cell transplantation has been introduced over the past decade as a potential therapy for ATLL with a long-term remission in only a small fraction of patients who are young, well controlled in disease progression, and have an appropriate stem cell source.[12] More effective strategies to treat ATLL are therefore required.

Several HTLV-1 components have been explored as targets for immunotherapy of ATLL. HTLV-1 Tax, which is crucial for ATLL oncogenesis, has generally been considered to be a main target of the host's cellular immune responses. Yet, the frequency of Tax expression in HTLV-1 infected cells reduces in the course of disease progression, and *Tax* transcripts are detected only in approximately 40% of the established ATLL cases,[13] thus limiting Tax-targeted immunotherapy to a subset of patients. HBZ (HTLV-1 bZIP factor), another HTLV-1 component, which contains an N-terminal transcriptional activation domain and a leucine zipper motif at its C-terminal, also plays an important role in the proliferation of ATLL cells and is detectable in almost all ATLL cases.[2] However, CD8$^+$ T cells specific for HBZ could only recognize peptide-pulsed target cells but not ATLL cells themselves.[14] Furthermore, HTLV-1 is transmitted mainly from mothers to infants through breast milk, and such vertical infection in early life may induce tolerance to the virus and result in insufficient HTLV-1–specific T-cell responses.[15,16] For these reasons, targeting the HTLV-1 components alone may be insufficient for successful immunotherapy of ATLL, necessitating identification of novel tumor-associated target antigens for the immunotherapy.

The expression of cancer/testis (CT) antigens, of which more than 100 have been identified so far, is normally limited to human germ line cells in the testis and in various types of human cancers.[17,18] This restricted expression pattern in normal tissues makes them ideal cancer antigens for tumor immunotherapy.

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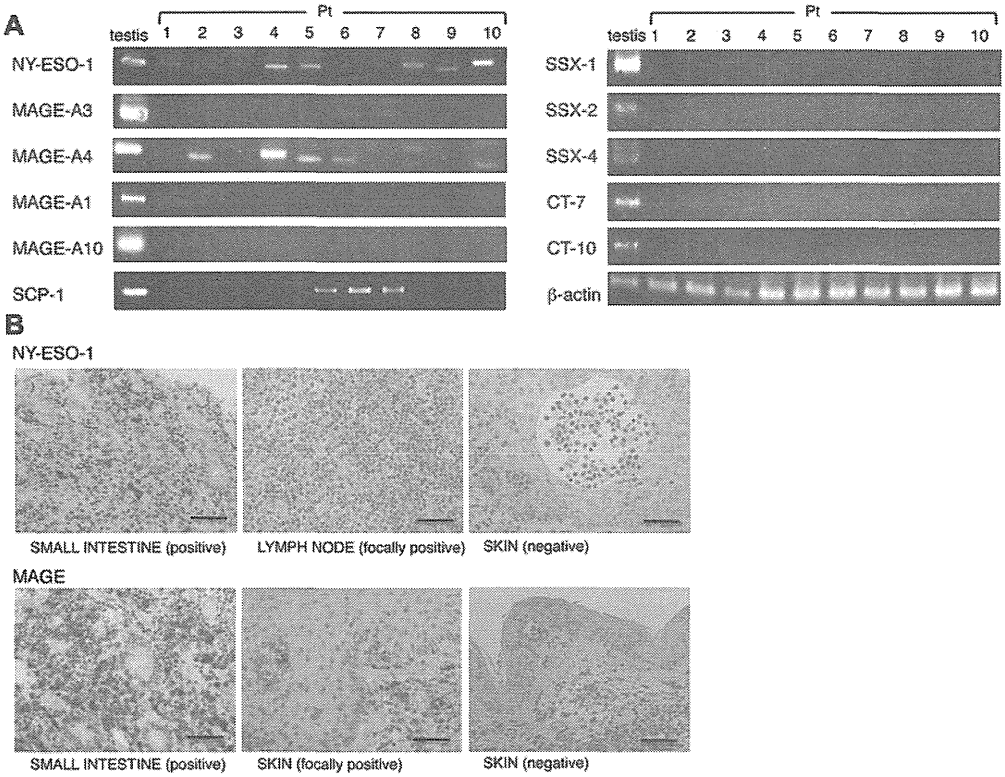

Figure 1. NY-ESO-1, MAGE-A3, and MAGE-A4 are widely expressed by primary ATLL cells. (A) Representative results of RT-PCR analysis for mRNA expression of NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, SSX-4, and SCP-1. (B) ATLL samples were subjected to immunohistochemical staining for NY-ESO-1 (E978) mAb and pan-MAGE (57B) mAb. Bar represents 50 μm. These experiments were performed independently at least twice with similar results.

NY-ESO-1 and MAGE family antigens, a subset of CT antigens, are indeed able to elicit spontaneous humoral and cellular immune responses in cancer patients.[17-19] Clinical trials of CT antigen vaccination are currently under way with several types of vaccine formulation, including peptide, protein, and DNA, and some of the treated patients have experienced clinical benefits from vaccination.[19,20] This promising result of CT antigens as a target of tumor immunotherapy has prompted intensive studies of their expression in a wide range of human cancers. However, detailed analysis of CT antigen expression in hematologic disorders has been limited.[21,22] In the present study, we have investigated possible expression of CT antigens by ATLL cells and possible humoral and cellular immune responses against CT antigens in ATLL patients.

Methods

Primary ATLL cells and peripheral blood

Blood or lymph node samples were obtained from ATLL patients, and mononuclear cells were isolated with Ficoll-Paque. Diagnosis and classification of clinical subtypes of ATLL were according to the criteria proposed by the Japan Lymphoma Study Group.[23] All donors provided informed written consent before sampling according to the Declaration of Helsinki, and the present study was approved by the institutional ethics committees of Nagoya City University Graduate School of Medical Sciences, Osaka University, and Imamura Bun-in Hospital.

ATLL cell lines

ATN-1, ATL102, HUT102, MT-2, and MT-1 were previously described.[24,25] TL-Om1 and TL-Su were kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). MT-4 was purchased from the Health Science Research Resources Bank (Osaka, Japan). MJ was purchased from ATCC. TCL-Kan was kindly provided by Professor M. Kannagi (Tokyo Medical and Dental University, Tokyo, Japan).

Reagents

Phycoerythrin-conjugated anti–IFN-γ (4S.B3) mAb and phycoerythrin-cyanine7–conjugated anti–TNF-α (MAb11) mAb were purchased from eBioscience. Fluorescein isothiocyanate-conjugated anti–IL-2 (MQ1-17H12) mAb and allophycocyanin-cyanine7–conjugated anti-CD8 (SK1) mAb were purchased from BD Biosciences. Anti–NY-ESO-1 mAb (E978, mouse IgG1)[26] and pan-MAGE mAb (57B, mouse IgG1)[27] were purified from hybridoma supernatant by protein G affinity chromatography. Synthetic peptides of NY-ESO-$1_{1\text{-}20}$ (MQAEGRGTGGSTGDADGPGG), NY-ESO-$1_{11\text{-}30}$ (STGDADGPGGPGIPDGPGGN), NY-ESO-$1_{21\text{-}40}$ (PGIPDGPG-GNAGGPGEAGAT), NY-ESO-$1_{31\text{-}50}$ (AGGPGEAGATGGRGPRGAGA), NY-ESO-$1_{41\text{-}60}$ (GGRGPRGAGAARASGPGGGA), NY-ESO-$1_{51\text{-}70}$ (ARASGPGGGAPRGPHGGAAS), NY-ESO-$1_{61\text{-}80}$ (PRGPHGGAASGL-NGCCRCGA), NY-ESO-$1_{71\text{-}90}$ (GLNGCCRCGARGPESRLLEF), NY-ESO-$1_{81\text{-}100}$ (RGPESRLLEFYLAMPFATPM), NY-ESO-$1_{91\text{-}110}$ (YLAMP-FATPMEAELARRSLA), NY-ESO-$1_{101\text{-}120}$ (EAELARRSLAQDAPPLP-VPG), NY-ESO-$1_{111\text{-}130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-$1_{119\text{-}143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-$1_{131\text{-}150}$ (NILTIRLTA-ADHRQLQLSIS), NY-ESO-$1_{139\text{-}160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-$1_{151\text{-}170}$ (SCLQQLSLLMWITQCFLPVF), and NY-ESO-$1_{161\text{-}180}$ (WITQCFLPVFLAQPPSGQRR) were obtained from Invitrogen.

RT-PCR

Total RNA was isolated with RNeasy Mini Kit (QIAGEN). cDNA was synthesized from 0.1 μg of total RNA using SuperScript III reverse

Table 1. Summary of CT antigen expression in ATLL cells from primary ATLL patients

	No. of patients	NY-ESO-1	MAGE A3	MAGE A4
Age (mean, 60.6 y)				
≥ 60 y	29	20	12	20
< 60 y	28	15	6	15
Sex				
Male	30	17	8	17
Female	27	18	10	18
Disease type				
Aggressive	40	25 (62.5%)	14 (35.0%)	26 (65.0%)
Acute	37	22	13	24
Lymphoma	3	3	1	2
Indolent	17	9 (52.9%)	4 (23.5%)	9 (52.9%)
Chronic	11	7	2	8
Smoldering	6	3	2	1

mRNA expression of NY-ESO-1, MAGE-A3, and MAGE-A4 in ATLL cells from primary ATLL patients was analyzed with RT-PCR. CT antigen expression was summarized based on several clinical parameters.

transcriptase kit (Invitrogen) and the Oligo (dT) primer in a total volume of 20 μL. cDNA was amplified in a final volume of 20 μL containing 10μM of each CT antigen primer as reported,[21,28] except NY-ESO-1 (sense, 5′-AGT TCT ACC TCG CCA TGC CT-3′; antisense, 5′- TCC TCC TCC AGC GAC AAA CAA-3′) and 0.2 μL of Ex-Taq polymerase (Takara Bio) according to the instructions provided by the manufacturer.

ELISA

Patients' sera were analyzed by ELISA for seroreactivity to bacterially produced recombinant proteins NY-ESO-1/CTAG1B, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT7/MAGE-C1, CT10/MAGE-C2, SSX1, SSX2, and SSX4.[29] Sera were diluted serially from 1/100 to 1/100 000 and added to low-volume 96-well plates (Corning Life Sciences) coated overnight at 4°C with 1 μg/mL antigen in 25 μL volume and blocked for 2 hours at room temperature with PBS containing 5% nonfat milk. After overnight incubation, plates were extensively washed and rinsed with PBS. Antigen-specific IgG was detected with specific mAb conjugated with alkaline phosphatase (Southern Biotechnology). After addition of ATTOPHOS substrate (Fisher Scientific), absorbance was measured using a fluorescence reader Cytofluor Series 4000 (PerSeptive Biosystems). A reciprocal titer was calculated for each sample as the maximal dilution still significantly reacting to a specific antigen. This value was extrapolated by determining the intersection of a linear trend regression with a cutoff value. The cutoff was defined as 10 times the average of optical density (OD) values from the first 4 dilutions of a negative control pool made of 5 healthy donor sera. In each assay, sera of patients with known presence or absence of specific reactivity were used as controls.

Immunohistochemistry

All tissue specimens were fixed with formalin and embedded in paraffin. Tissue sections of 3 μm thickness on charged glass slides were deparaffinized and rehydrated. Antigen retrieval was performed by autoclave (105°C for 20 minutes) using Tris-EDTA buffer (pH 9.0) as heating solution. Primary mAb for NY-ESO-1 (E978) and pan-MAGE protein (57B) were used at concentrations of 5 μg/mL and 2 μg/mL, respectively. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and 1% sodium azide. Simple stain max-PO (Multi; Nichirei) was used for secondary detection; 3,3′-diamino-benzidine was used as chromogen. Human testicular tissue served as positive control. All histological pictures were captured by NIKON Eclipse microscope and NIKON Image software system (Version 3.22).

In vitro sensitization

NY-ESO-1–specific $CD8^+$ and $CD4^-$ T cells were presensitized as described previously.[30,31] Briefly, $CD8^-$ T cells and $CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) using a CD8 Microbeads and a $CD4^+$ T cell Isolation Kit, respectively (Miltenyi Biotec). The purity of isolated populations was confirmed to be more than 90%. The non-$CD8^+$/$CD4^+$ cell population was pulsed with 10μM of pooled peptides overnight and was used as antigen-presenting cells (APCs). After irradiation, 5 to 10×10^5 APCs were added to round-bottom 96-well plates (Corning Life Sciences) containing 1 to 5×10^5 $CD8^+$ or $CD4^+$ T cells and were fed with IL-2 (10 U/mL; Roche Diagnostics) and IL-7 (20 ng/mL; R&D Systems). Subsequently, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/mL) and IL-7 (40 ng/mL) twice per week.

Intracellular cytokine staining

$CD8^+$ T cells from PBMCs from ATLL patients were presensitized for 10 to 18 days. These presensitized $CD8^+$ T cells were restimulated for 6 hours with peptide-pulsed T-APCs,[31] and GolgiStop reagent (BD Biosciences) was added 1 hour later. Cells were stained for cell surface markers and for intracellular cytokines, such as IFN-γ and TNF-α after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences) and FlowJo Version 7.6.5 software for Macintosh (TreeStar).

Tetramer assay

Tetramer staining was performed as previously described.[31] Briefly, presensitized $CD8^+$ T cells were stained with phycoerythrin-labeled tetramers (prepared by Drs P. Guillaume and I. Luescher at the Ludwig Institute Core Facility, Lausanne, Switzerland) for 15 minutes at 37°C before additional staining with allophycocyanin-cyanine7–conjugated anti-CD8 mAb for 15 minutes at 4°C. After washing, results were analyzed by FACSCanto and FlowJo Version 7.6.5 software.

Statistical analysis

The significance of the difference in each CT antigen expression between 2 groups was assessed by Fisher exact test. P values less than .05 were considered significant.

Results

A subset of CT antigens is highly expressed in ATLL cell lines

To examine possible expression of 11 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, SSX-4, and SCP-1) by ATLL cells, we assessed mRNA expression by RT-PCR in 10 ATLL cell lines (MT-1, MT-2, MT-4, MJ, ATL102, ATN-1, TL-Om-1, TL-Su, TCL-kan, and HUT102). In sharp contrast to a previous report showing that the majority of T-cell lymphomas did not express CT antigens except SCP-1,[21] a high percentage of ATLL cell lines expressed NY-ESO-1, MAGE-A3, and MAGE-A4 (90%, 50% and 70%, respectively) in the present study (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). SCP-1, another CT antigen, was also detected in 40% of ATLL cell lines, as in other T-cell lymphomas (supplemental Table 1).

NY-ESO-1, MAGE-A3, and MAGE-A4 are widely expressed in primary ATLL cells

Given the high percentage of CT antigen mRNA expression in ATLL cell lines, we next examined the expression of 11 CT antigens in primary tumor cells from 57 individual ATLL patients. As shown in Figure 1A, Table 1, and supplemental Table 2, NY-ESO-1, MAGE-A3, and MAGE-A4 mRNA expression was detected in 61.4% (35 of 57), 31.6% (18 of 57), and 61.4% (35 of 57), respectively, of primary ATLL patients. SCP-1 expression was

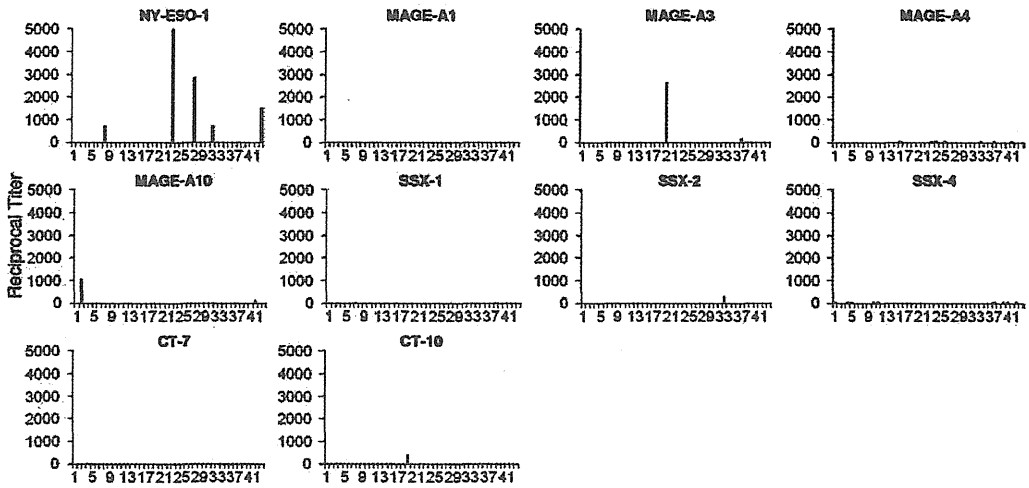

Figure 2. Humoral immune responses against NY-ESO-1 are detected in a subset of patients with ATLL. Sera were collected from 43 primary ATLL patients, and antibody responses against 10 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4) were analyzed by ELISA as described in "ELISA." This experiment was performed at least twice with similar results.

also detected in 23.2% (13 of 56). Among 4 subtypes (acute, lymphoma, chronic, and smoldering) of ATLL, the acute and lymphoma types show aggressive clinical courses (aggressive types), whereas the chronic and smoldering types progress more indolently (indolent types).[2,23] There were no significant differences between the ATLL patients with aggressive and indolent types in the expression of NY-ESO-1, MAGE-A3, and MAGE-A4; yet there was a trend for more frequent expression of CT antigens in aggressive ATLL types (Table 1). Immunohistochemical analysis confirmed the expression of NY-ESO-1 and pan-MAGE at the protein level in samples available for pathologic analysis (Figure 1B). By contrast, expression of other CT antigens was limited: MAGE-A1, 8.8% (3 of 34); MAGE-A10, 0% (0 of 33); CT-7, 0% (0 of 33); CT-10, 0% (0 of 33); SSX-1, 0% (0 of 33); SSX-2, 0% (0 of 33); and SSX-4, 0% (0 of 33) by RT-PCR.

Taken together, CT antigens, such as NY-ESO-1, MAGE-A3 and MAGE-A4, are expressed in a significant fraction of primary ATLL cases, and 87.7% (50 of 57) of ATLL patients expressed at least one of these 3 CT antigens.

Humoral immune responses against NY-ESO-1 are detected in a subset of ATLL patients

We next asked whether ATLL patients spontaneously developed humoral and cellular immune responses specific for CT antigens. Serum samples from 43 primary ATLL patients were assessed by ELISA for the reactivity to 10 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4). Significant antibody titers were detected against NY-ESO-1 in 5 of 43 (11.6%) patients and against MAGE-A1, MAGE-A3, and MAGE-A4 in 0% (0 of 43), 2.3% (1 of 43), and 0% (0 of 43), respectively (Figure 2). Humoral immune responses against CT antigens whose expression was not detected by RT-PCR (MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4) were limited (Figure 2). These data collectively indicate that NY-ESO-1 expressed in primary ATLL cells elicits spontaneous antibody responses in ATLL patients as in patients with NY-ESO-1 expressing solid tumors.[19]

Cellular immune responses against NY-ESO-1 in ATLL patients

We then assessed CT antigen-specific cellular immune responses in ATLL patients. As the majority of ATLL patients are in a severe anemic state as a characteristic of the disease and also because of intensive chemotherapy, we collected PBMCs from patients in partial or complete remission, except patient 8 with an indolent ATLL type. Considering this limitation of available sample sizes and predominant humoral immune responses against NY-ESO-1, we focused on NY-ESO-1 as a parameter of cellular immune responses. With sufficient amounts of PBMCs from 9 ATLL patients (patients 1, 2, 4, 8, 13, 14, 19, 27, and 43), NY-ESO-1 expression by ATLL cells was confirmed by RT-PCR in each patient, except for patient 13 (no detection) and patient 43 (unavailability of sample; supplemental Table 2). $CD8^+$ T cells were presensitized with autologous $CD4^-CD8^-$ PBMCs pulsed with a pool of NY-ESO-1 peptides, and antigen-specific $CD8^+$ T cells were analyzed with NY-ESO-1/HLA tetramers corresponding to the HLA allele of each patient. NY-ESO-1–specific $CD8^+$ T cells were detected in ATLL patients 2, 4, 14, and 43 (Figure 3A). The results indicate that NY-ESO-1–specific $CD8^+$ T-cell responses spontaneously develop in a subset of patients harboring NY-ESO-1 expressing ATLL.

NY-ESO-1–specific $CD8^+$ T cells produce cytokines and recognize an autologous ATLL cell line

With the presence of NY-ESO-1–specific $CD8^+$ T cells in ATLL patients, we further analyzed their cytokine production in response to a pool of NY-ESO-1 peptides or autologous tumor cells. Sufficient amounts of PBMCs for the cytokine analysis were available from 3 ATLL patients (patients 4, 14, and 19), in which NY-ESO-1 expression was confirmed by RT-RCR (supplemental Table 2). NY-ESO-1–specific $CD8^+$ T cells were detected with NY-ESO-1/MHC tetramers in 2 of them (patients 4 and 14), but relevant tetramers were not available for another patient (patient 19). NY-ESO-1–specific $CD8^+$ T cells prepared from these 3 patients by presensitization with NY-ESO-1 peptide-pulsed $CD4^-CD8^-$ PBMCs produced IFN-γ and/or TNF-α by intracellular cytokine staining (Figure 3B). In patient 14, the frequency of NY-ESO-1–specific $CD8^+$ T cells producing IFN-γ was much higher than NY-ESO-1–specific $CD8^+$ T cells detected by NY-ESO-1/HLA-Cw*0304 tetramer, suggesting that this patient may have $CD8^+$ T cells recognizing other epitopes of NY-ESO-1 (Figure 3A-B). Taken together, NY-ESO-1–specific $CD8^+$ T cells were detected in 5 of 9 (55.6%) ATLL patients.

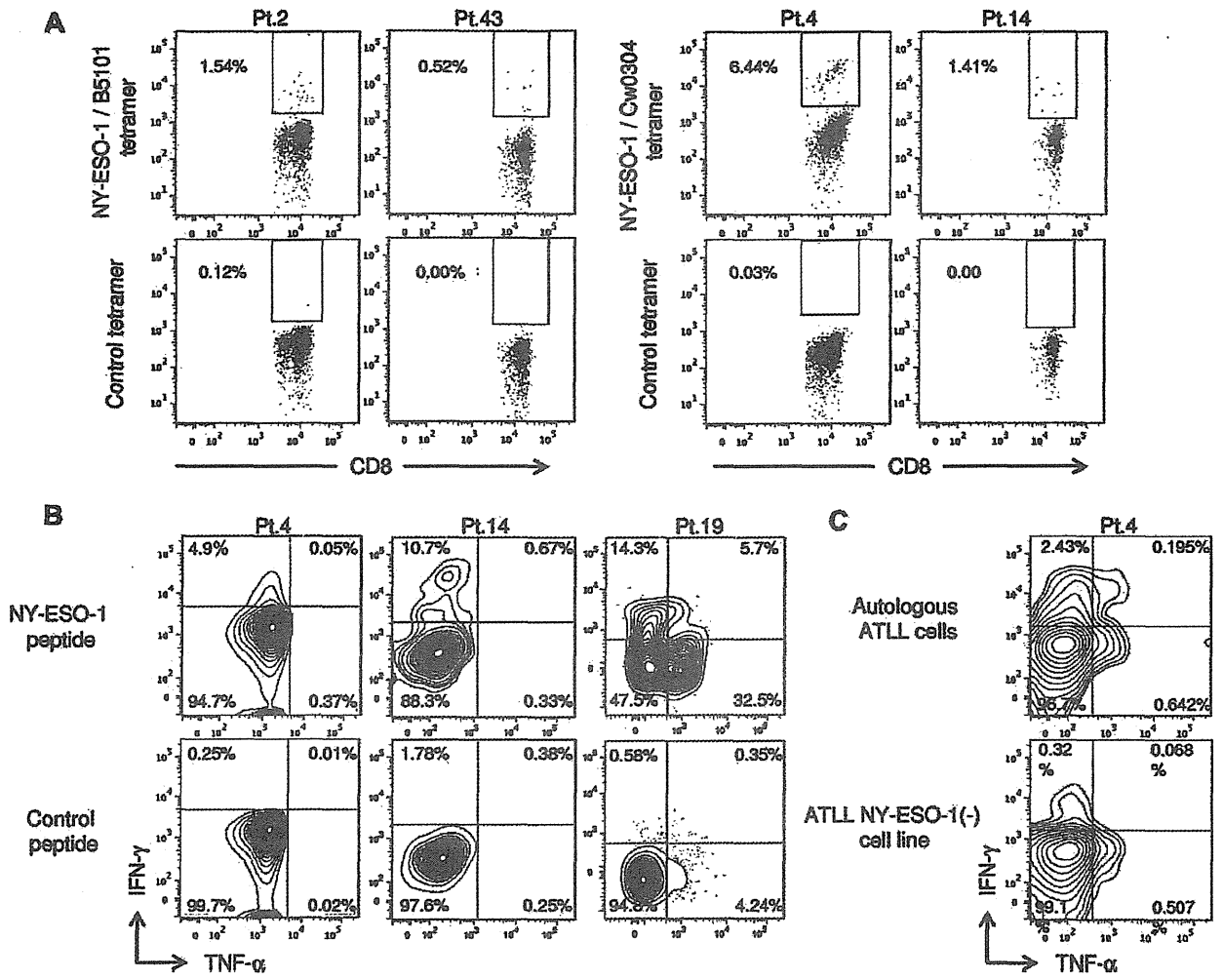

Figure 3. NY-ESO-1–specific CD8⁺ T cells are detected in ATLL patients. CD8⁺ T cells derived from PBMCs of patients 1, 2, 4, 8, 13, 14, 19, 27, and 43 were presensitized by CD4⁻ CD8⁻ PBMCs pulsed with NY-ESO-1 peptides covering the entire sequence of NY-ESO-1 as described in "In vitro sensitization." (A) Induction of specific CD8⁺ T cells was analyzed by staining with NY-ESO-1/HLA tetramers indicated. Cytokine (IFN-γ and TNF-α) secreting capacity of NY-ESO-1–specific CD8⁺ T cells was analyzed by intracellular cytokine staining for recognition of (B) autologous activated T-cell APCs pulsed with NY-ESO-1 peptides or (C) autologous ATLL cells. These experiments were performed independently at least twice with similar results.

We also examined whether NY-ESO-1–specific CD8⁺ T cells from patient 4 recognized autologous ATLL cells. They produced IFN-γ and TNF-α against autologous ATLL cells expressing NY-ESO-1 but not against a control HLA-matched ATLL cell line (ATL-102) without NY-ESO-1 expression (Figure 3C). Collectively, these data indicate that NY-ESO-1–specific CD8⁺ T cells are present in ATLL patients and are able to recognize and kill autologous leukemic cells.

CD4⁺ T-cell responses against NY-ESO-1 in ATLL patients

To determine whether NY-ESO-1–specific CD4⁺ T cells were present in these ATLL patients, CD4⁺ T cells derived from PBMCs obtained from ATLL patients (patients 1, 2, 4, 8, 13, 14, 19, 27, and 43) were presensitized by CD4⁻CD8⁻ PBMCs pulsed with a pool of NY-ESO-1 peptides, and assessed for cytokine production by intracellular cytokine staining. Of 9 patients, NY-ESO-1–specific CD4⁺ T cells were detected only in one patient (patient 19), who was in complete remission after receiving allogeneic hematopoietic stem cell transplantation (Figure 4). Thus, NY-ESO-1–specific CD4⁺ T cells are present in a subset of ATLL patients, but a much lower frequency than CD8⁺ T-cell responses, partly because the presence of ATLL cells, which are CD4⁺, may make the detection difficult and possibly because CD25⁺CD4⁺ Tregs are present in the CD4⁺ T-cell fraction.

Discussion

Since the initial description of ATLL as a unique type of T-cell leukemia/lymphoma,[1] various therapeutic attempts have been made. Yet, the prognosis of ATLL is still poor despite advances in our knowledge regarding the oncogenic process of the disease.[10,23] Here, we have examined CT antigen expression and its immunogenicity in ATLL patients to explore the potential for immunotherapy of ATLL by targeting CT antigens. We found that CT antigens, such as NY-ESO-1, MAGE-A3, and MAGE-A4, were highly expressed in ATLL. In particular, the frequencies of NY-ESO-1 and MAGE-A4 expression (61.4% and 61.4%, respectively) at the mRNA level were higher than or comparable to those in other malignancies. For example, the frequency of NY-ESO-1 and MAGE-A4 expression was 32% to 45% and 28%, respectively, in malignant melanoma, 24% to 33% and 63% to 90.2% in esophageal cancer, 30% to 43%

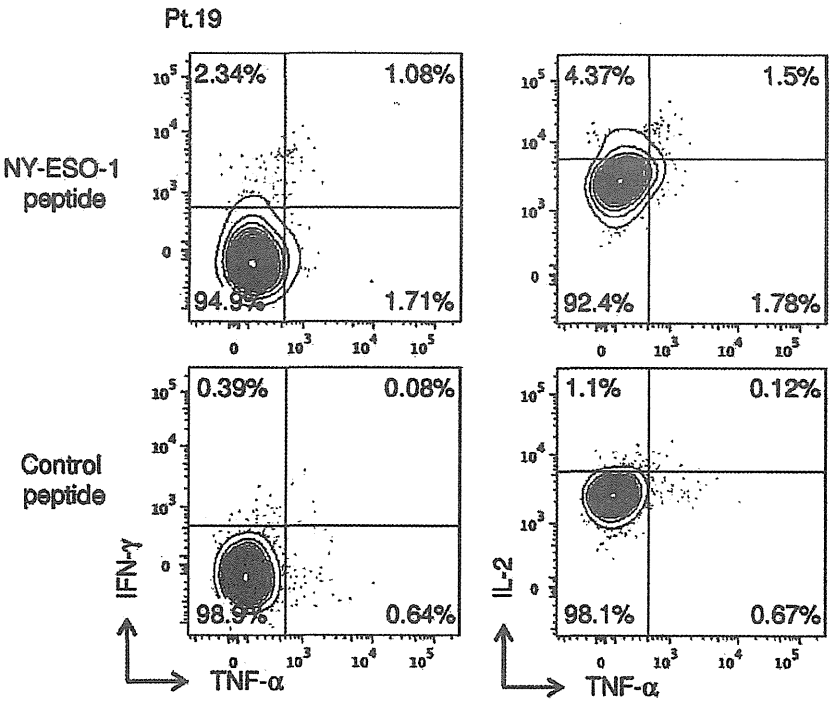

Figure 4. NY-ESO-1–specific CD4+ T cells are present in an ATLL patient (patient 19) receiving an allogeneic hematopoietic stem cell transplantation. $CD4^+$ T cells were presensitized by $CD4^-$ $CD8^-$ PBMCs pulsed with NY-ESO-1 peptide covering the entire sequence of NY-ESO-1 as described in "In vitro sensitization." Induction of NY-ESO-1–specific $CD4^+$ T cells was analyzed by intracellular cytokine staining using autologous activated T-cell APCs pulsed with NY-ESO-1 peptides. These experiments were performed independently twice with similar results.

and 57% in ovarian cancer, and 18% to 35% and 33% in bladder cancer.[19,32,33] In addition, we have revealed that NY-ESO-1 was immunogenic in ATLL patients and elicited specific humoral and cellular immune responses in a subset of patients. These data strongly support CT antigens as novel targets for ATLL immunotherapy.

The frequency of humoral immune responses in ATLL patients was much higher against NY-ESO-1 compared with other CT antigens, such as MAGE-A3 and MAGE-A4. Although NY-ESO-1 expression by ATLL cells is presumably required for antibody induction, the level of NY-ESO-1 mRNA expression did not reflect the induction of humoral immune responses (supplemental Table 2). This lack of correlation appears in part to be the result of immunologic properties of NY-ESO-1 antigen itself.[19,26] It has been shown that polymeric structures of NY-ESO-1 through disulfide bonds and their interaction with calreticulin-TLR4 on immature dendritic cell surface are required to induce phagocytosis of NY-ESO-1 protein and deliver danger signals for making the protein immunogenic.[34] In addition, some non-MAGE-A members of the MAGE family are ubiquitously expressed and therefore possibly in more stable tolerance compared with NY-ESO-1. These properties of NY-ESO-1 and MAGE proteins might make the former more immunogenic than the latter.

Despite high immunogenicity of NY-ESO-1, the frequency (13.6%, 3 of 22) of primary ATLL patients who spontaneously developed NY-ESO-1 antibody responses against NY-ESO-1 expressing leukemic cells was lower compared with the frequencies in patients with malignant melanoma or non-small-cell lung cancers expressing NY-ESO-1 (~ 50%).[19,26] This may reflect the feature of ATLL that tumor cells from the majority of ATLL patients express Foxp3, a key transcription factor for $CD25^+CD4^+$ Tregs.[6,9,35-37] ATLL cells from a subset of patients indeed appear to function as Tregs and contribute to profound immunosuppression that hampers the host's immune responses.[6,36] Alternatively or additionally, the chemokine CCL22 produced by ATLL cells might enhance the migration of CCR4-expressing $CD25^+CD4^+$ Tregs to tumor sites.[38] It remains to be determined whether Tregs in ATLL patients or ATLL cells themselves suppress NY-ESO-1–specific immune responses. Yet, it has been shown that helper T-cell responses against NY-ESO-1 are subject to active suppression by Tregs in patients with solid tumors and healthy persons.[30,37] Further studies are required to understand immunosuppressive property of ATLL to enhance immune responses against CT antigens in ATLL patients.

NY-ESO-1–specific cellular immune responses were detected in a significant number (5 of 9, 55.6%) of ATLL patients in partial or complete remission. This indicates that reducing the number of ATLL cells before CT antigen-targeted immunotherapy may be a crucial component to successfully induce/augment antigen–specific $CD8^+$ T cells. We have recently reported that humanized anti-CCR4 mAb, KW-0761, showed clinically significant antitumor activity as a salvage therapy for patients with relapsed ATLL.[8,39] Patients 2, 4, and 14, from whom we detected NY-ESO-1–specific $CD8^+$ T cells, were in complete or partial remission after anti-CCR4 mAb treatment.[8,39] As CCR4 is expressed on ATLL cells as well as $CD25^+CD4^+FOXP3^+$ Tregs, in addition to T-helper type 2 cells,[8,40,41] anti-CCR4 mAb treatment can reduce not only ATLL cells but also endogenous $CD25^+CD4^+FOXP3^+$ cells,[42] thereby contributing to evoking NY-ESO-1–specific $CD8^+$ T-cell responses. Indeed, the high frequency of NY-ESO-1–specific $CD8^+$ T cells detectable in vitro in ATLL patients could be the result in part of the absence of Tregs. Thus, combining CT antigen-targeted immunotherapy after reduction of endogenous Tregs as well as ATLL cells by anti-CCR4 mAb treatment would be an ideal strategy for ATLL immunotherapy.

NY-ESO-1–specific $CD4^+$ and $CD8^+$ T-cell responses observed in patient 19 who was in a complete remission after allogeneic hematopoietic stem cell transplantation suggest an association of immune responses against CT antigens with a graft-versus-ATLL effect. This indicates that CT antigen-targeted immunotherapy combined with allogeneic stem cell transplantation may augment the efficacy of the current allogeneic hematopoietic stem cell

transplantation for treating ATLL. In addition, as HTLV-1 Tax-specific $CD8^+$ T cells reportedly contribute to graft-versus-ATLL effects,[43] a combination immunotherapy with CT antigen and Tax after stem cell transplantation might also be therapeutically effective. Further, considering the reported high efficacy of NY-ESO-1–targeted adoptive T-cell therapy against malignant melanoma and synovial cell sarcoma,[44,45] similar adoptive T-cell therapy for ATLL could also be effective.

ATLL is, to our knowledge, the first example of high expression of CT antigens in lymphomas or leukemia, as detailed analyses of CT antigen expression in other hematologic disorders have been limited.[21,22] It contrasted with a previous report showing that T-cell lymphoma lacks expression of CT antigens, except SCP-1. As a probable mechanism for transcriptional activation of the CT antigen genes in malignant cells, it has been suggested that the expression of CT antigen is induced by CpG island hypomethylation at the promoter regions.[18] An inflammatory environment created by infection may also trigger NY-ESO-1 expression. It cannot be excluded that viral components may promote this hypomethylation at the promoter regions or elicit inflammatory environment, thereby inducing CT antigen expression. Yet, we failed to observe any correlation between viral gene expression, such as Tax and HBZ and CT antigen expression (supplemental Table 2). In addition, high titers of HTLV-1 Gag/Env antibody responses were not associated with the induction of humoral immune responses against CT antigens (supplemental Table 2). Peripheral T-cell lymphomas, not otherwise specified (PTCL-NOS) are particularly heterogeneous; yet, the CCR4-positive subset of PTCL-NOS might be a distinct disease entity whose clinicopathologic features and genomic profiles were reportedly very similar to the lymphoma type of ATLL.[24,46,47] PTCL-NOS, especially the CCR4-positive subset, have a very poor prognosis like ATLL, and no standard treatment strategies are available.[48] Notably, we have found that CCR4-positive PTCL-NOS also expressed NY-ESO-1 at a high frequency (H.N., T.I., R.U. and S.S., unpublished data, August 2011). These data, when taken together, indicate that the frequent expression of CT antigens in ATLL may not be induced by HTLV-1 infection itself, but rather it may constitute a novel subtype of T-cell leukemia/lymphoma that expresses CCR4 and CT antigens regardless of HTLV-1 infection.

A small population (~ 5%) of HTLV-1–infected persons progresses to ATLL after a long latency period of approximately 50 to 70 years.[2] Although the detailed mechanisms of this leukemogenesis from HTLV-1 infection to ATLL have not yet been well elucidated, host immune responses against HTLV-1–infected cells have been suspected to play an important role.[8,16,49] Interestingly, some of HTLV-1–infected asymptomatic HTLV-1 carriers harbored significant titers of antibody against NY-ESO-1, MAGE-A3, and MAGE-A4, despite that CT antigens, including NY-ESO-1, MAGE-A3, and MAGE-A4, were not detected at the mRNA level in their PBMCs (supplemental Figure 1). This discrepancy raises several possibilities; for example, the number or the frequency of HTLV-1–infected cells expressing CT antigens in circulating PBMCs may be too low to detect the antigen. Alternatively, it is possible that HTLV-1–infected cells expressing CT antigens may be eliminated in HTLV-1–infected asymptomatic carriers by anti-CT antigen-specific immune responses as tumor immunosurveillance,[50] whereas antibody responses may linger on. Future studies with a large cohort of HTLV-1–infected asymptomatic carriers need to address the kinetics of their CT antigen expression during a long latency period and their immune responses against the antigen in the course of ATLL development. The studies will not only provide a rationale for including CT antigens as possible targets of ATLL immunotherapy but also contribute to our understanding of the multistep oncogenesis of ATLL and devising preventive strategies for ATLL by targeting CT antigens.

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This article is dedicated to the memory of Dr Lloyd J. Old.

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Authorship

Contribution: H.N., T.I., S.G., L.J.O., R.U., and S.S. designed the research; H.N., Y.M., T.I., S.G., E.S., F.M., D.S., A.I., and Y.F. performed experiments; T.I., F.M., A.I. A.U., H.I., and R.U. collected samples and obtained clinical data; H.N., Y.M., T.I., S.G., E.S., Y.F., R.U., and S.S. analyzed data; and H.N., Y.M., T.I., S.G., E.S., R.U., and S.S. wrote the paper.

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References

1. 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.
2. 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.
3. Goncalves DU, Proietti FA, Ribas JG, et al. Epidemiology, treatment, and prevention of human T-cell leukemia virus type 1-associated diseases. *Clin Microbiol Rev.* 2010;23(3):577-589.
4. Uchiyama T, Sagawa K, Takatsuki K, Uchino H. Effect of adult T-cell leukemia cells on pokeweed