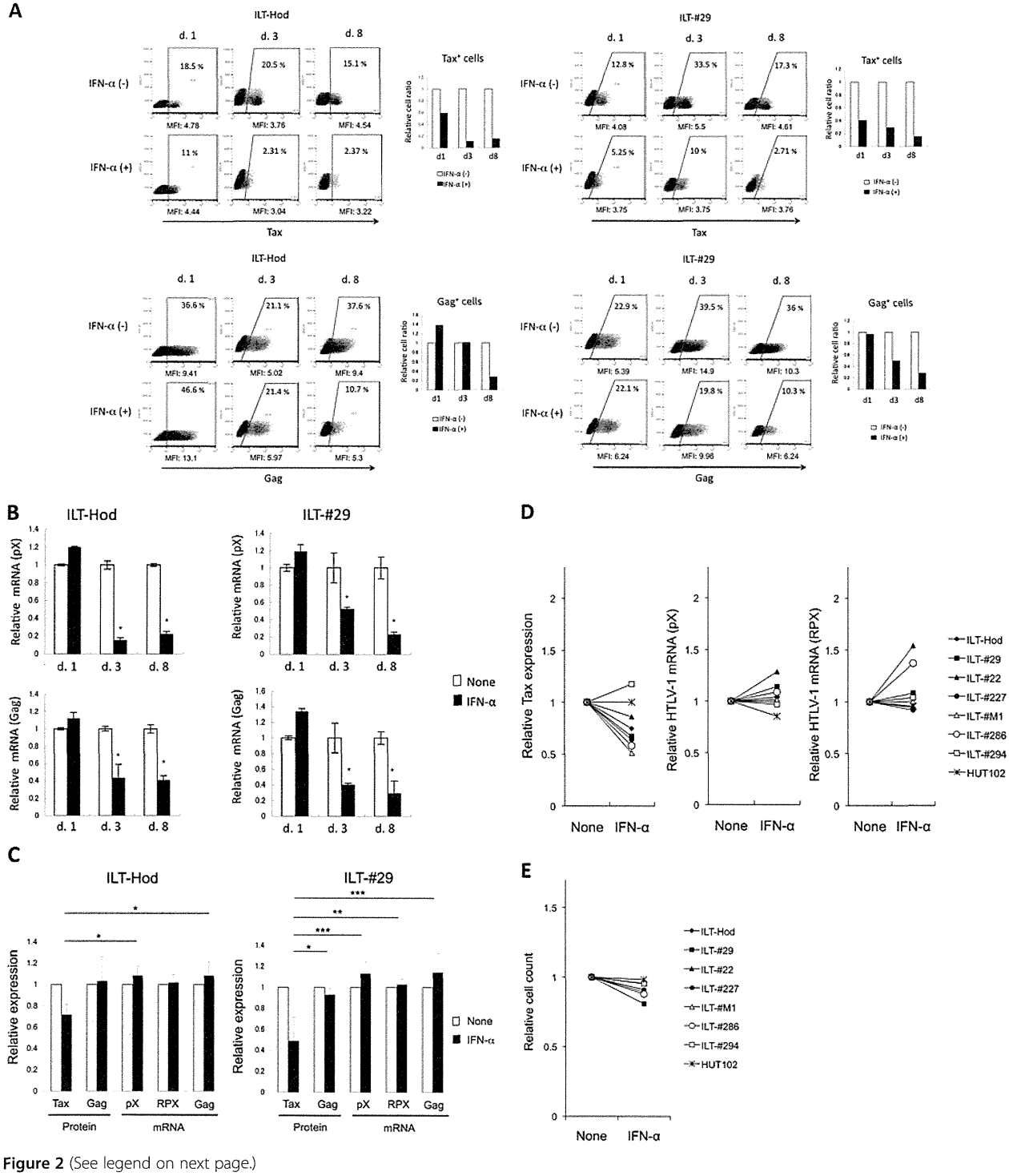

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Figure 2 IFN-α suppressed Tax protein expression before an apparent reduction in HTLV-1 mRNA levels. A. The effects of IFN-α (3000 IU/ml) on intracellular Tax (top) and Gag (bottom) protein expression in ILT-Hod (left) and ILT-#29 (right) cells was 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

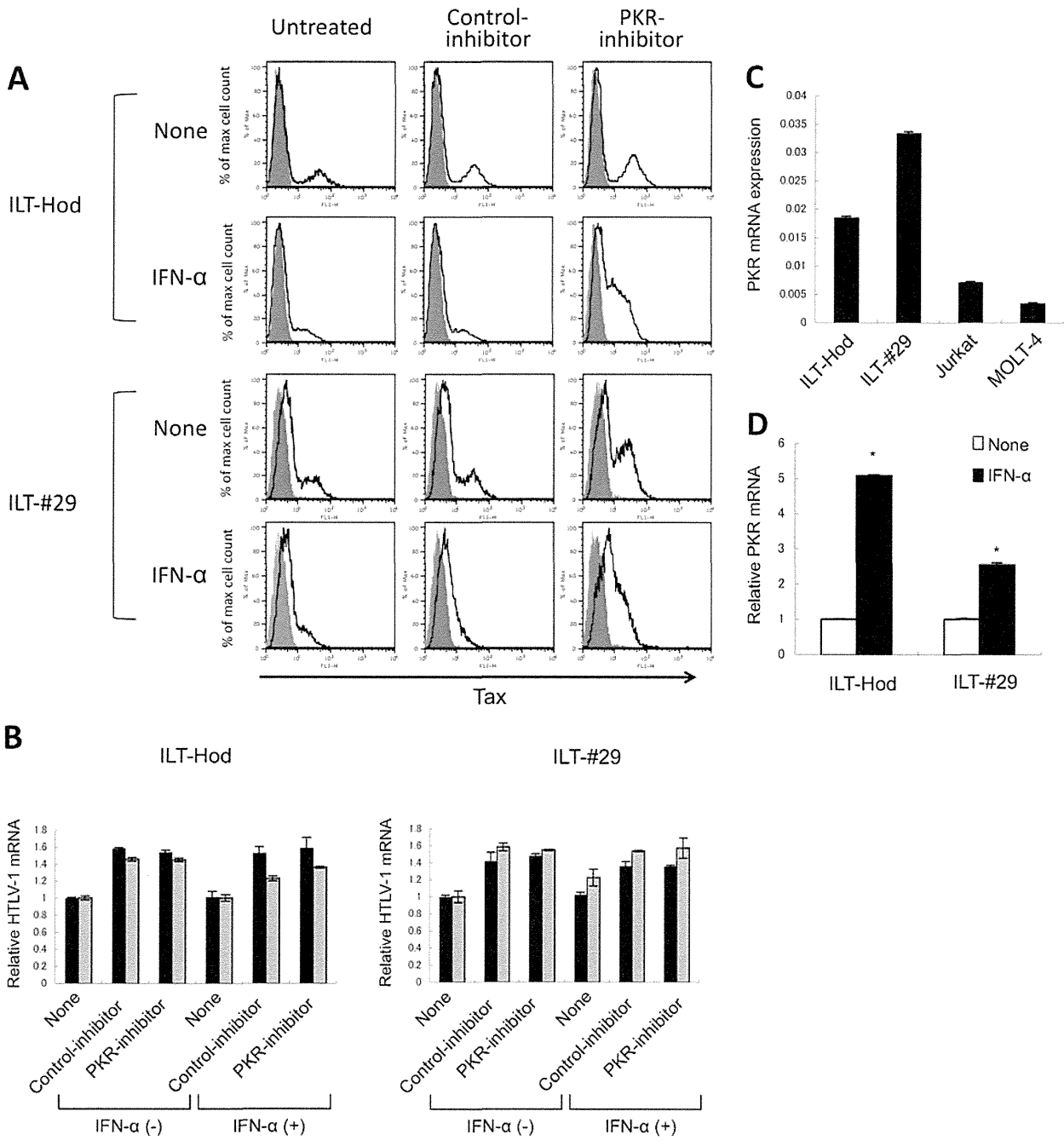

Figure 3 Involvement of PKR in IFN-α-mediated reduction of Tax protein levels in HTLV-1-infected cells. A. ILT-Hod and ILT-#29 cells were incubated with or without the PKR-inhibitor (500 nM) or the negative control-inhibitor (500 nM) for the first 2 h, then further cultured for the next 24 h in the presence 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. *$p < 0.05$.

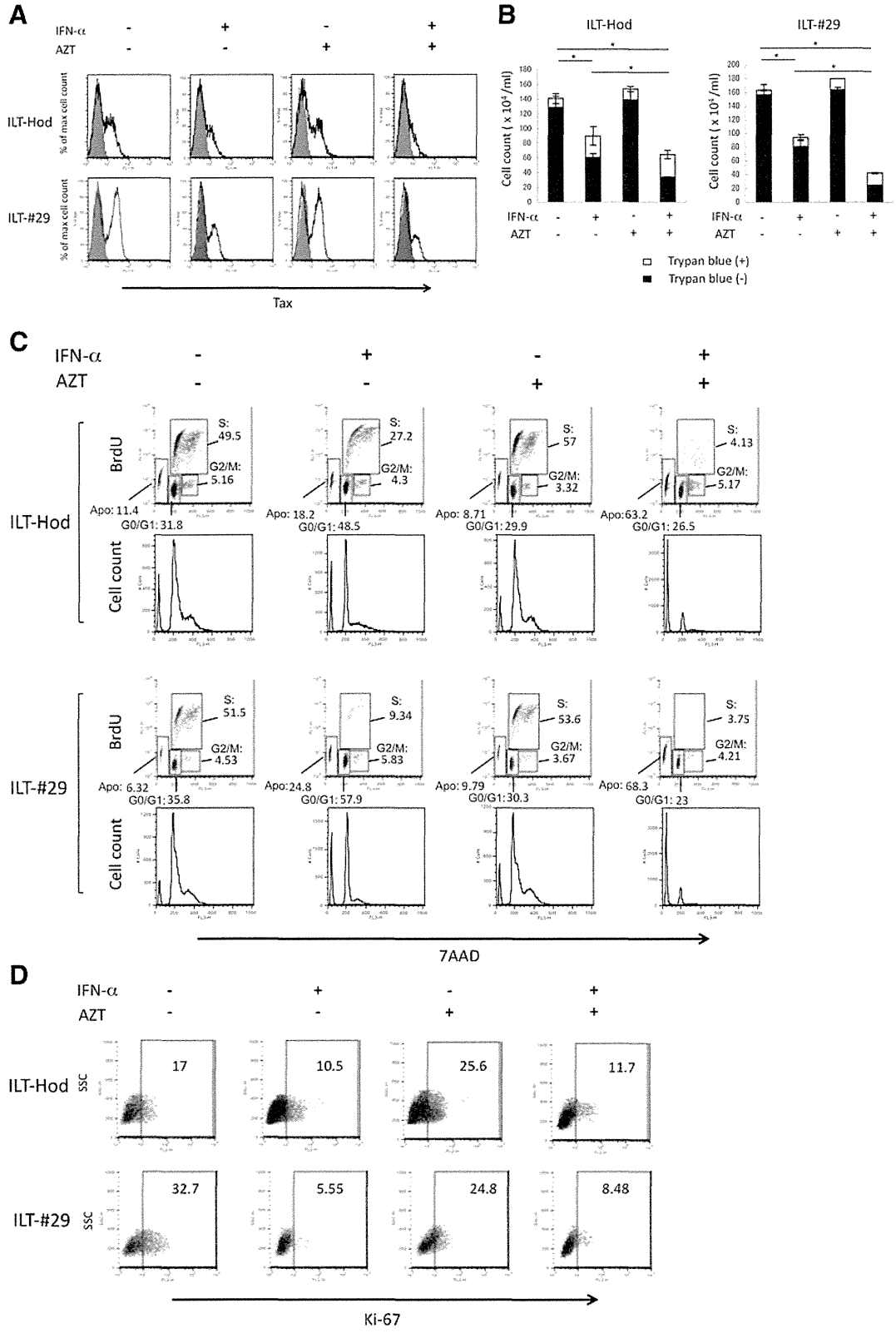

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Figure 4 Effects of IFN-α and AZT on HTLV-1 expression and cell growth of HTLV-1 infected cells. ILT-Hod and ILT-#29 cells (10^6/ml) were cultured in the absence or presence of IFN-α (3000 IU/ml) and/or AZT (10 μM) as indicated, and HTLV-1 expression (**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-

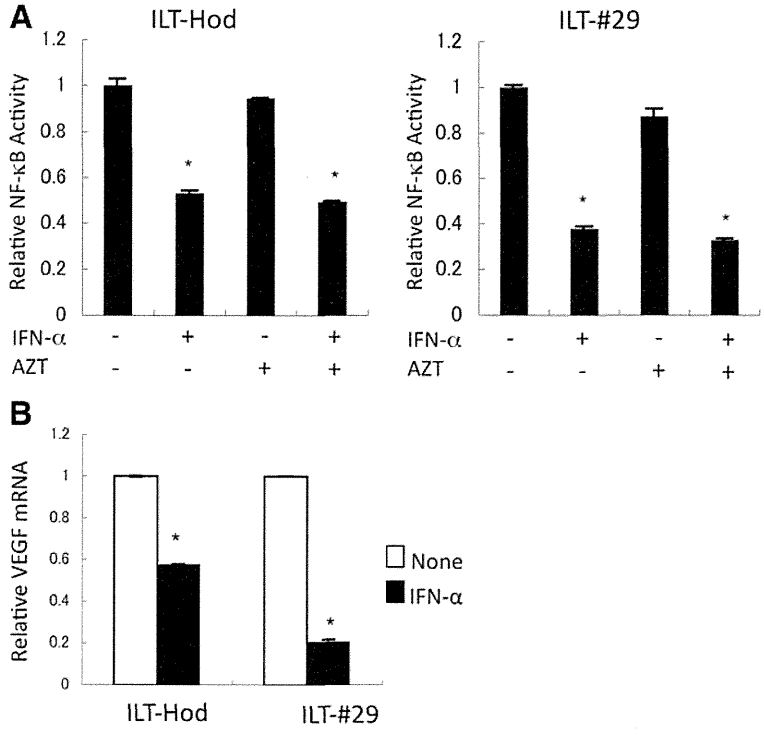

Figure 5 Suppression of NF-κB activity by IFN-α in HTLV-1-infected cells. A. ILT-Hod and ILT-#29 cells that were infected with lentiviral vectors containing reporter gene for the NF-κB responsive element and the TK-promoter several weeks before, were treated with or without IFN-α (3000 IU/ml) and/or 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. *$p < 0.05$.

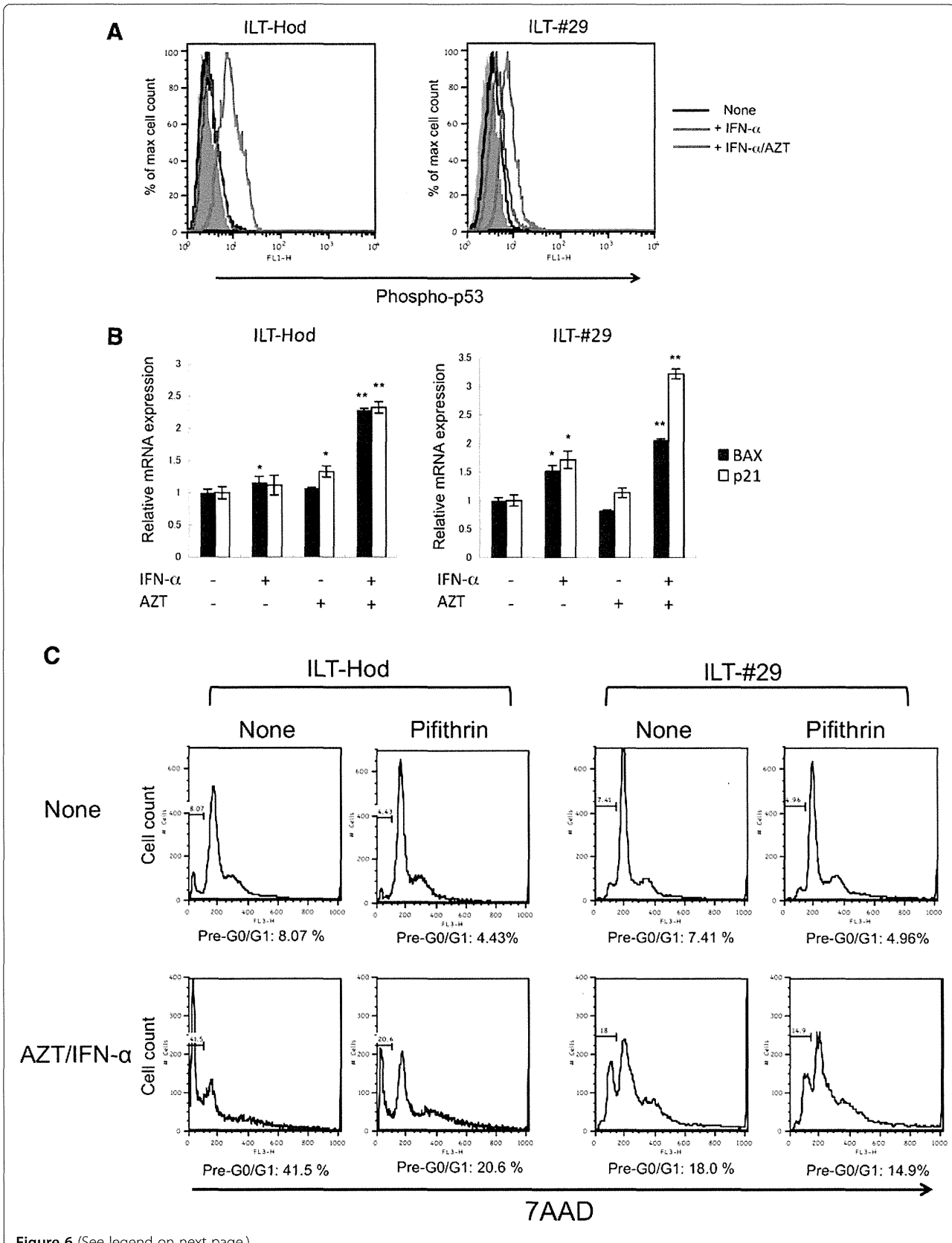

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Figure 6 Induction of p53-signaling by IFN-α and AZT in HTLV-1-infected cells. A. Intracellular phosphorylated p53 levels in ILT-Hod and ILT-#29 cells were evaluated by flow cytomertry 3 and 4 days after incubation, respectively, in the absence (black 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 elF-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 ($C_{15}H_8C_{l3}NO$; 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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Case Report

Stevens–Johnson Syndrome associated with mogamulizumab treatment of adult T-cell leukemia/lymphoma

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We report an adult T-cell leukemia/lymphoma patient suffering from Stevens–Johnson Syndrome (SJS) during mogamulizumab (humanized anti-CCR4 monoclonal antibody) treatment. There was a durable significant reduction of the $CD4^+CD25^{high}FOXP3^+$ regulatory T (Treg) cell subset in the patient's PBMC, and the affected inflamed skin almost completely lacked FOXP3-positive cells. This implies an association between reduction of the Treg subset by mogamulizimab and occurrence of SJS. The present case should contribute not only to our understanding of human pathology resulting from therapeutic depletion of Treg cells, but also alert us to the possibility of immune-related severe adverse events such as SJS when using mogamulizumab. We are currently conducting a clinical trial of mogamulizumab for CCR4-negative solid cancers (UMIN000010050), specifically aiming to deplete Treg cells. (*Cancer Sci* 2013; 104: 647–650)

Adult T-cell leukemia/lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by HTLV-1. The disease is resistant to conventional chemotherapeutic agents, and has a very poor prognosis.[1] Mogamulizumab (KW-0761) is a defucosylated humanized monoclonal antibody targeting CC chemokine receptor 4 (CCR4).[2] A phase I clinical trial for relapsed CCR4-positive peripheral T-cell neoplasms, including ATL, and a phase II study for relapsed ATL have been conducted with mogamulizumab.[3,4] This agent was subsequently approved for the treatment of relapsed or refractory ATL in Japan, the first country in the world to do so, in March 2012. Mogamulizimab went on sale on 29 May 2012. The interim report for the post-marketing surveillance from 29 May to 28 September 2012 revealed skin-related severe adverse events (SAE), as defined by the Medical Dictionary for Regulatory Activities Terminology/Japan, in nine patients. Thus, during only the first 4 months of use, 9 skin-related SAE, including 4 cases of Stevens–Johnson Syndrome (SJS)/toxic epidermal necrolysis (TEN) were reported, with 1 SJS/TEN fatality. These skin-related, potentially fatal SAE are certainly a challenge to the free use of this agent and clearly require investigation. Therefore, here we report an informative ATL patient suffering from SJS on mogamulizumab treatment, focusing on the reduction of the regulatory T (Treg) cell subset ($CD4^+CD25^{high}FOXP3^+$) caused by the antibody.

Case Report

A 71-year old woman was admitted due to elevation of her lymphocyte count. She had been diagnosed as suffering from acute-type ATL nearly 5 months prior to admission. She had received VCAP-AMP-VECP chemotherapy[5] followed by oral sobuzoxane in another hospital, and achieved a transient partial remission. We started mogamulizumab to treat the flare-up of ATL disease (Fig. 1). Grade 1 skin eruptions appeared around her neck after three antibody infusions. Because we were also giving her antibacterial (ciprofloxacin hydrochloride), fungal (itraconazole), pneumocystic (sulfamethoxazole-trimethoprim) and viral (aciclovir) prophylaxes in addition to stomach medicine (lansoprazole), we judged the skin event to be due to drug eruption caused by one of these concomitant drugs. Therefore, we stopped all five, but continued with mogamulizumab. Despite their discontinuation and treatment with topical steroids, the skin rashes continued to worsen. We started the patient on 30 mg oral prednisolone, which improved the skin symptoms. The patient was then able to complete the eight planned infusions, and oral prednisolone was tapered off. She was discharged from hospital 8 days after her eighth infusion (day 65), and thereafter seen as an outpatient. However, she had to be readmitted as an emergency patient at day 75 because of fulminant skin rashes. These included erythemas, scale-like plaques, vesicles, blisters and erosions over many areas of the body. Her lips were swollen and oral mucosa was erosive (Fig. 2a). Skin biopsy revealed marked liquefaction, degeneration and perivascular inflammation with dominant CD8-positive cells but almost complete lack of FOXP3-positive cells (Fig. 2b). We diagnosed her as a SJS, and immediately started steroid pulse therapy (methylprednisolone 500 mg/day ×3 days), followed by oral prednisolone. Her skin and mucosal lesions improved gradually, and became inactive. At the same time, her general condition improved. Thus, we again tapered the steroid dose, and she was discharged at day 144. However, she had to come back yet again as an emergency patient on day 151 for the same reason as before, with fulminant skin rashes. We prescribed her mini-steroid pulse therapy (methylprednisolone 125 mg/day ×1 day), followed by oral prednisolone. Once more, her skin lesions improved gradually. Over this whole period, complete ATL remission was maintained by mogamulizumab. The HTLV-1 provirus load in PBMC pre-treatment, and at days 121 and 162 was 750.1, 0.0 (under the limit of detection) and 0.8 copies/1000 cells, respectively. These post-treatment values are strikingly low, considering that median HTLV-1

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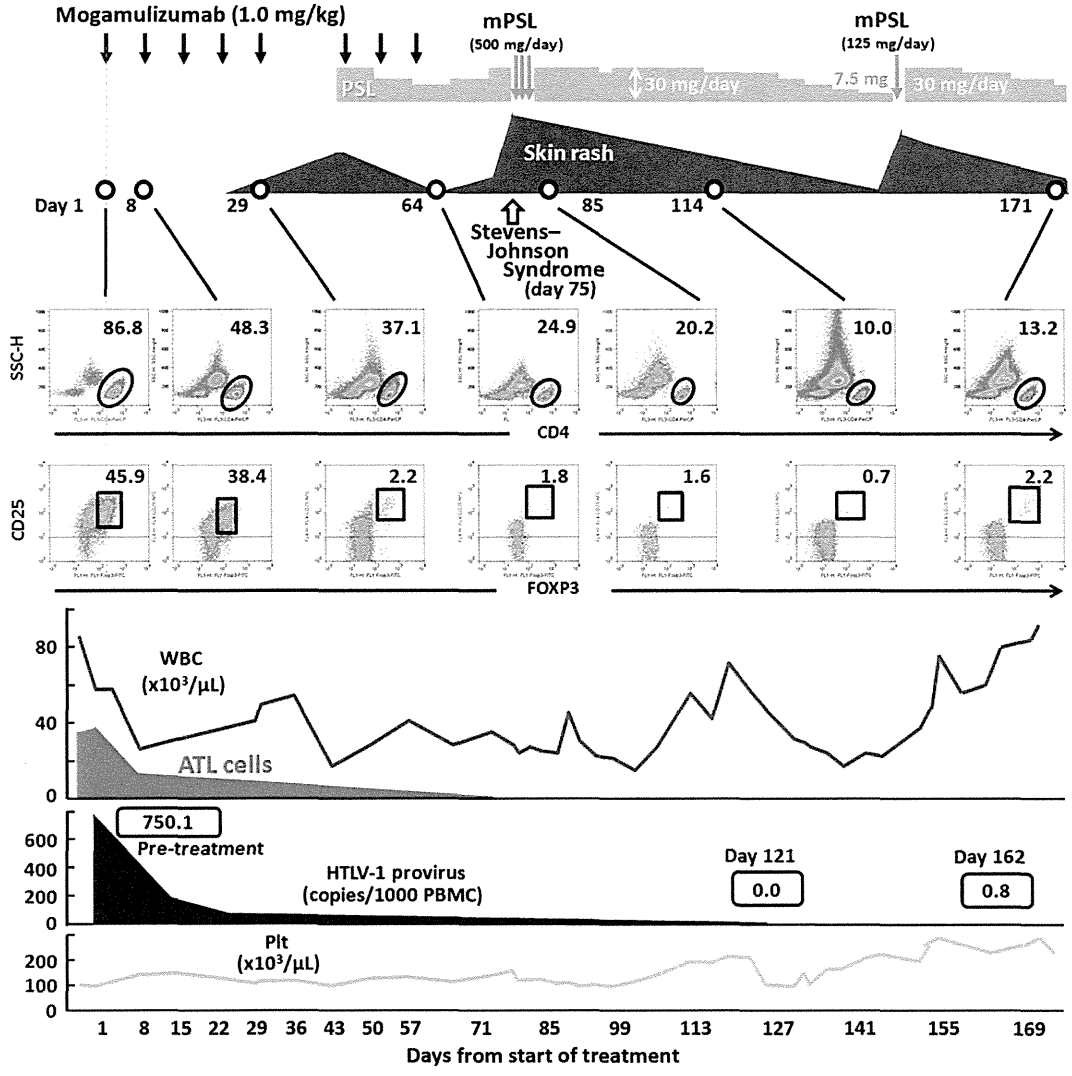

Fig. 1. Clinical course of an ATL patient receiving mogamulizumab monotherapy. ATL; adult T-cell leukemia/lymphoma; mPSL, methylprednisolone; Plt, platelet PSL; prednisolone; WBC, white blood cell.

load in asymptomatic carriers reported by other investigators is 18.0 copies/1000 cells.[6]

We also analyzed CD4, CD25 and FOXP3 expression by PBMC during and after antibody treatment (Fig. 1, middle panels). Before treatment, the majority of the patient's PBMC consisted of CD4-positive and CD25-positive ATL cells. Just before the 5th antibody infusion (day 29), around the time when her skin rash first appeared, the proportion of $CD25^{high}$-$FOXP3^{+}/CD4^{+}$ cells was markedly reduced, to 2.2%. This is low even compared to healthy individuals ($CD25^{high}$-$FOXP3^{+}/CD4^{+}$ cells, mean 3.3%, median 3.3%, range 2.6–4.4%) (Fig. 3). Around the time of SJS onset, the proportion of cells in the Treg subset was further reduced. The proportion of $CD25^{high}FOXP3^{+}/CD4^{+}$ cells at days 64, 85 and 114 was 1.8%, 1.6% and 0.7%, respectively. The striking reduction of the Treg subset persisted until 4 months after the last of the eight antibody infusions (day 171).

Discussion

Drugs often induce adverse cutaneous reactions of varying severity, ranging from simple uncomplicated eruptions to potentially fatal eruptions, such as SJS and TEN, within the spectrum of severe adverse reactions affecting skin and mucosa. Although many factors that might cause variability in the clinical course of such adverse reactions have been suggested, it remains unknown which factors are predominantly involved in these processes. The most prevalent severe drug eruptions are thought to be mediated by drug-reactive T-cells,[7] although we also need to be aware of the alternative view that severe drug eruptions are due to a dysregulated immune system. In this regard, an effect mediated by Treg cells is a likely candidate in severe drug eruptions. Indeed, it is reported that Treg cells can prevent experimentally-induced epidermal injury mimicking TEN in an animal model.[8] Furthermore, Takahashi *et al.* (2009) report that Treg cell function is profoundly impaired in patients with TEN.[9] Consistent with these reports, a marked reduction of the Treg subset was observed in the present case.

Mogamulizumab is the first therapeutic agent targeting CCR4, which is expressed on Treg cells,[10,11] to receive marketing approval anywhere in the world. The reduction of the Treg subset seen here was not specific to the present case, but is commonly observed in ATL patients receiving mogamulizumab. In fact, skin rashes were observed as a frequent non-hematologic adverse event (AE) (63%), mostly occurring

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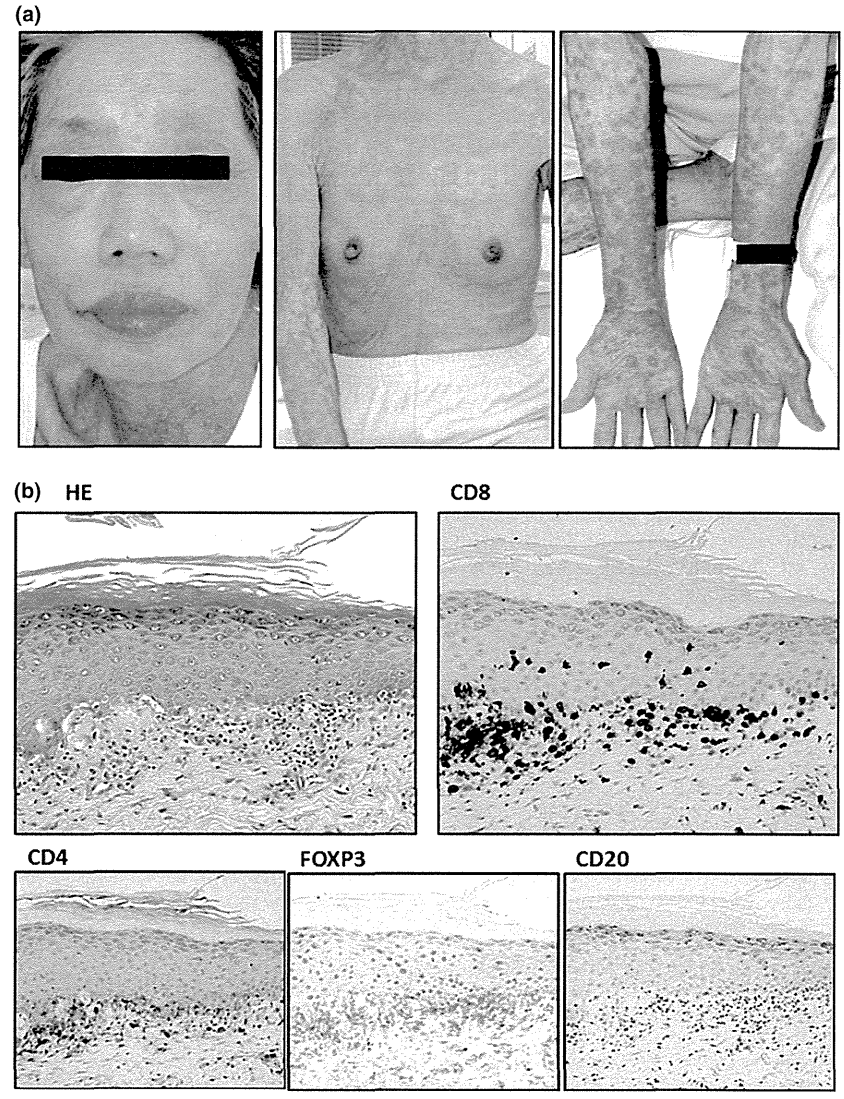
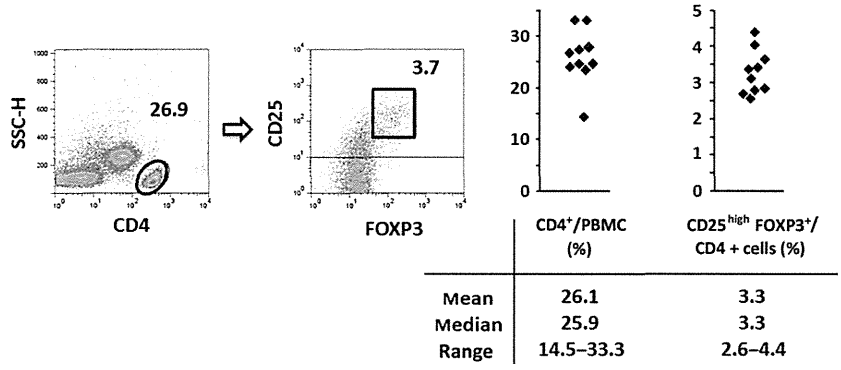


Fig. 2. (a) Macroscopic observations of the patient's skin on the day she was diagnosed with Stevens–Johnson Syndrome. (b) Corresponding skin biopsy showing liquefaction, degeneration and perivascular inflammation with dominant CD8-positive cells but almost no FOXP3-positive cells.



	$CD4^+$/PBMC (%)	$CD25^{high}$ $FOXP3^+$/ CD4 + cells (%)
Mean	26.1	3.3
Median	25.9	3.3
Range	14.5–33.3	2.6–4.4

Fig. 3. $CD4^+CD25^{high}FOXP3^+$ regulatory T cells in PBMC from healthy volunteers (n = 10).

after the fourth or subsequent infusions in the phase II study.[4] The present case was one of these patients. It has been reported that alterations in $CD4^+CD25^+FOXP3^+$ Treg cell frequencies and/or function may contribute to various types of autoimmune diseases.[12] Because the CCR4 molecule aids lymphocyte skin-specific homing,[13] it is not unexpected that skin rashes, which could be an immune-related AE, will be frequently observed in ATL patients receiving mogamulizumab. Because it is an urgent issue to identify which factors determine the severity of immune-related skin disorders associated with mogamulizumab treatment, further investigation on this matter are clearly warranted.

However, reduction of Treg cells is a promising strategy for boosting antitumor immunity in cancer patients, because these cells are increased in the tumor microenvironment and may play an important role in tumor escape from host immunity in several different types of cancer.[14,15] Thus, reduction of Treg cells by mogamulizumab in cancer patients would have both potential benefits leading to enhanced antitumor immunity, but also pose risks of autoimmune disease. The skin-related SAE, including SJS/TEN, are representative of the latter. Currently, several clinical trials of mogamulizumab are being conducted worldwide, not only for ATL, but also other types of lymphoma. In addition, we are currently conducting a clinical trial of mogamulizumab for CCR4-negative solid cancers (UMIN000010050), specifically aiming to deplete Treg cells. Therefore, it is a matter of some urgency to establish the safest and most effective treatment strategies for using mogamulizumab not only in ATL patients but also other types of cancer, to maximize benefit and minimize risk.

In summary, the present case should contribute not only to our understanding of human pathology resulting from therapeutic depletion of Treg cells, but also alert us to the possibility of immune-related SAE, such as SJS/TEN, when using mogamulizumab.

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References

1 Ishida T, Ueda R. Antibody therapy for Adult T-cell leukemia-lymphoma. *Int J Hematol* 2011; **94**: 443–52.
2 Ishii T, Ishida T, Utsunomiya A *et al.* Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. *Clin Cancer Res* 2010; **16**: 1520–31.
3 Yamamoto K, Utsunomiya A, Tobinai 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. *J Clin Oncol* 2010; **28**: 1591–8.
4 Ishida T, Joh T, Uike N *et al.* Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase ii study. *J Clin Oncol* 2012; **30**: 837–42.
5 Tsukasaki K, Utsunomiya A, Fukuda H *et al.* VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. *J Clin Oncol* 2007; **25**: 5458–64.
6 Sonoda J, Koriyama C, Yamamoto S *et al.* HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers is diminished by green tea drinking. *Cancer Sci* 2004; **95**: 596–601.
7 Nassif A, Bensussan A, Boumsell L *et al.* Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. *J Allergy Clin Immunol* 2004; **114**: 1209–15.
8 Azukizawa H, Sano S, Kosaka H, Sumikawa Y, Itami S. Prevention of toxic epidermal necrolysis by regulatory T cells. *Eur J Immunol* 2005; **35**: 1722–30.
9 Takahashi R, Kano Y, Yamazaki Y, Kimishima M, Mizukawa Y, Shiohara T. Defective regulatory T cells in patients with severe drug eruptions: timing of the dysfunction is associated with the pathological phenotype and outcome. *J Immunol* 2009; **182**: 8071–9.
10 Iellem A, Mariani M, Lang R *et al.* Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+) CD25(+) regulatory T cells. *J Exp Med* 2001; **194**: 847–53.
11 Ishida T, Ishii T, Inagaki A *et al.* Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. *Cancer Res* 2006; **66**: 5716–22.
12 Michels-van Amelsfort JM, Walter GJ, Taams LS. $CD4^+$ $CD25^+$ regulatory T cells in systemic sclerosis and other rheumatic diseases. *Expert Rev Clin Immunol* 2011; **7**: 499–514.
13 Campbell JJ, Haraldsen G, Pan J *et al.* The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 1999; **400**: 776–80.
14 Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol* 2012; **13**: e32–42.
15 Ishida T, Ueda R. Immunopathogenesis of lymphoma: focus on CCR4. *Cancer Sci* 2011; **102**: 44–50.

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ORIGINAL REPORT

Multicenter Phase II Study of Mogamulizumab (KW-0761), a Defucosylated Anti-CC Chemokine Receptor 4 Antibody, in Patients With Relapsed Peripheral T-Cell Lymphoma and Cutaneous T-Cell Lymphoma

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ABSTRACT

Purpose
CC chemokine receptor 4 (CCR4) is expressed by peripheral T-cell lymphomas (PTCLs) and is associated with poor outcomes. Mogamulizumab (KW-0761) is a defucosylated humanized anti-CCR4 antibody engineered to exert potent antibody-dependent cellular cytotoxicity. This multicenter phase II study evaluated the efficacy and safety of mogamulizumab in patients with relapsed PTCL and cutaneous T-cell lymphoma (CTCL).

Patients and Methods
Mogamulizumab (1.0 mg/kg) was administered intravenously once per week for 8 weeks to patients with relapsed CCR4-positive PTCL or CTCL. The primary end point was the overall response rate, and the secondary end points included safety, progression-free survival (PFS), and overall survival (OS).

Results
A total of 38 patients were enrolled, and 37 patients received mogamulizumab. Objective responses were noted for 13 of 37 patients (35%; 95% CI, 20% to 53%), including five patients (14%) with complete response. The median PFS was 3.0 months (95% CI, 1.6 to 4.9 months), and the median OS was not calculated. The mean maximum and trough mogamulizumab concentrations (± standard deviation) after the eighth infusion were 45.9 ± 9.3 and 29.0 ± 13.3 μg/mL, respectively. The most common adverse events were hematologic events, pyrexia, and skin disorders, all of which were reversible and manageable.

Conclusion
Mogamulizumab exhibited clinically meaningful antitumor activity in patients with relapsed PTCL and CTCL, with an acceptable toxicity profile. Further investigation of mogamulizumab for treatment of T-cell lymphoma is warranted.

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INTRODUCTION

Mature T/natural killer (NK)-cell neoplasms comprise approximately 20 subclassified heterogeneous groups of non-Hodgkin lymphomas (NHLs) that account for approximately 10% of NHLs in Western countries[1-3] and approximately 25% of NHLs in Japan.[4,5] Mature T/NK-cell neoplasms are largely subdivided into peripheral T-cell lymphoma (PTCL) and cutaneous T-cell lymphoma (CTCL), and different treatment strategies are used for each of these entities.[1,6]

According to the WHO classification, PTCL includes peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), angioimmunoblastic T-cell lymphoma (AITL), and anaplastic large-cell lymphoma (ALCL).[1-3] Cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) and CHOP-like regimens have been widely used as the standard first-line treatment for patients with PTCL.[7,8] With the exception of those patients with anaplastic lymphoma kinase-positive ALCL, the efficacy of these combination therapies is unsatisfactory because those who achieve remission eventually experience relapse and poor outcomes.[3,9] Several agents have been approved by the US Food and Drug Administration for the treatment of relapsed or refractory (Rel/Ref) PTCL: pralatrexate, romidepsin for Rel/Ref PTCL, and brentuximab vedotin for Rel/Ref ALCL. The overall response rates

(ORRs) were reported to be 29% and 25% for PTCL and 86% for ALCL, respectively.[10-12]

CTCL can be classified as mycosis fungoides (MF), Sézary syndrome, or cutaneous ALCL. The majority of cases of CTCL in Japan consist of MF.[13] The therapeutic approaches and outcomes for these conditions are primarily dependent on disease stage.[6,7,14] Patients with advanced stage CTCL who relapse after systemic chemotherapies and those with transformed MF have particularly poor outcomes.[15,16] Recently, the US Food and Drug Administration approved agents for Rel/Ref CTCL treatment, including vorinostat, denileukin diftitox, and romidepsin, with ORRs of 30%, 30%, and 34%, respectively.[17-19] However, there are few treatment options or approved agents for CTCL in Japan, partly because of its low prevalence here.[5,12,13]

CC chemokine receptor 4 (CCR4) is a marker for type 2 helper T cells or regulatory T (Treg) cells and is expressed on tumor cells in approximately 30% to 65% of patients with PTCL.[20,21] CCR4-positive patients (eg, in the PTCL-NOS subgroup) have a shorter survival time when compared with CCR4-negative patients.[21-23] Further, CCR4 expression increases with advancing disease stage in patients with MF/Sézary syndrome.[24]

Mogamulizumab (KW-0761) is a humanized anti-CCR4 monoclonal antibody with a defucosylated Fc region that enhances antibody-dependent cellular cytotoxicity.[25,26] In vitro antibody-dependent cellular cytotoxicity assay and in vivo studies in a humanized mouse model revealed that mogamulizumab exhibited potent antitumor activity against T-cell lymphoma cell lines and against primary CTCL cells from patients.[26-28]

In a phase I study of patients with relapsed adult T-cell leukemia-lymphoma (ATL) and PTCL/CTCL, mogamulizumab was well tolerated up to a dose of 1.0 mg/kg. An ORR of 31% (five of 16) was obtained, including one partial response (PR) among three patients with PTCL/CTCL.[29] Mogamulizumab yielded an ORR of 50% (13 of 26) for relapsed CCR4-positive ATL in a subsequent phase II study.[30] In the United States, a phase I/II study for patients with Rel/Ref CTCL revealed that mogamulizumab was well tolerated with an ORR of 37% (14 of 38, 8% complete response [CR], 29% PR) and a median PFS of 341 days.[31]

The present report describes the results of a multicenter phase II study in Japan that was designed to assess the efficacy and safety of mogamulizumab in patients with relapsed CCR4-positive PTCL or CTCL.

PATIENTS AND METHODS

Study Design and Treatment

This was a multicenter, single-arm phase II study conducted at 15 Japanese centers. At least 35 patients were required to detect a lower limit of the 95% CI that exceeded the 5% threshold, and the expected ORR for mogamulizumab was 25% with a statistical power of 90%.[10,29]

All patients gave written informed consent before enrollment. Patients received intravenous infusions of 1.0 mg/kg mogamulizumab once per week for 8 weeks. Dose modification of mogamulizumab was not allowed. Oral antihistamine and acetaminophen were given before each dose of mogamulizumab as premedication.[29,30] A systemic corticosteroid (hydrocortisone 100 mg intravenously) was also administered before the first dose of mogamulizumab to prevent an infusion reaction. The same dose of hydrocortisone was administered before the second and subsequent administrations at the investigators' discretion. The plasma concentrations of mogamulizumab and antimogamulizumab antibodies in plasma were determined by using enzyme-linked immunosorbent assays.[29,30] Blood samples were collected from all patients who received at least one dose of mogamulizumab at times determined by the protocol for pharmacokinetic analyses. Maximum plasma mogamulizumab concentration and trough concentration parameters were calculated from 0 to 7 days after the eight doses. T-cell subsets and NK cell distribution were also investigated by flow cytometry during and after mogamulizumab treatment. This study was conducted in accordance with the Declaration of Helsinki and in compliance with Good Clinical Practices. The protocol was approved by the institutional review board at each participating institution.

Patients

Patients who were ≥ 20 years of age and who had CCR4-positive PTCL or CTCL with relapse after their last systemic chemotherapy were eligible for participation. Patients who were refractory to their most recent therapy were not eligible for this study. Histopathological subtypes were assessed and reclassified by the Independent Pathology Review Committee according to the 2008 WHO classification.[1] CCR4 expression was determined by immunohistochemistry by using an anti-CCR4 monoclonal antibody (KM2160) and was confirmed by central review, as described previously.[29] In brief, CCR4 expression was classified according to the proportion of stained tumor cells (negative, < 10%; 1+, 10% to <25%; 2+, 25% to < 50%; 3+, ≥ 50%). Staging of nodal/extranodal and/or cutaneous lesions was performed if the lesions met the following requirements: nodal and extranodal lesions were > 1.5 cm in measurable length on cross-sectional computed tomography images, cutaneous lesions were identifiable on visual inspection, and peripheral blood abnormal lymphocyte count was ≥ 1,000/μL and comprised ≥ 5% of total leukocytes. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2. Other notable eligibility criteria regarding laboratory values were as follows: neutrophil count ≥ 1,500/μL, platelet count ≥ 50,000/μL, hemoglobin level ≥ 8.0 g/dL, AST level ≤ 2.5× the upper limit of normal (ULN), ALT level ≤ 2.5× the ULN, total bilirubin level ≤ 1.5× the ULN, and serum creatinine level ≤ 1.5× the ULN. Patients were excluded if they had any severe complications, such as CNS involvement or a bulky lymphoma mass requiring emergent radiotherapy, a history of allogeneic stem-cell transplantation, active concurrent cancers, an active infection, or positivity for hepatitis B virus DNA, hepatitis B surface antigen, hepatitis C virus antibody, or human immunodeficiency virus antibody.

Efficacy and Safety Assessment

The primary objective was to assess the best overall response, and the secondary objectives included assessments of the best response according to disease site, progression-free survival (PFS), and overall survival (OS). Efficacy was evaluated by the Independent Efficacy Assessment Committee according to modified response criteria based on the International Working Group Criteria.[32,33] Cutaneous lesions were evaluated by using the modified Severity Weighted Assessment Tool.[34] In addition, treatment efficacy in patients with CTCL was evaluated by using a Global Response Score.[35] Responses were assessed after the fourth and eighth mogamulizumab infusions and at 2 and 4 months after the end of treatment. Treatment was discontinued if progressive disease (PD) was evident. PD and survival were monitored until at least 4 months after the completion of dosing. For safety evaluations, adverse events (AEs) were graded according to the National Cancer Institute Common Terminology Criteria for AEs, version 4.0.

Statistical Analysis

PFS and OS were analyzed by using the Kaplan-Meier method. PFS was defined as the time from the first dose of mogamulizumab to progression, relapse, or death by any cause (whichever occurred first). OS was measured from the day of the first dose to death by any cause.

RESULTS

Patient Characteristics

Sixty-five patients were screened, and 64 biopsy specimens were histologically confirmed as PTCL or CTCL by the Independent Pathology Review Committee. In total, 50 (78%) of the 64 screened

patients were CCR4-positive. Of these, 38 eligible patients were enrolled in the study and 37 received at least one infusion of mogamulizumab. One patient withdrew because of an infectious complication before dosing. Patient characteristics, histopathology subtypes, and previous systemic therapies are shown in Table 1.

Table 1. Baseline Patient Demographic and Clinical Characteristics

Characteristic*	Patients (N = 37) No.	%	Patients With PTCL (n = 29) No.	%	Patients With CTCL (n = 8) No.	%
Age, years						
Median	64		67		50	
Range	33-80		33-80		36-70	
≥ 65	18	49	17	59	1	13
Sex						
Male	23	62	20	69	3	38
Female	14	38	9	31	5	63
ECOG performance status						
0	24	65	19	66	5	63
1	12	32	10	34	2	25
2	1	3	0	0	1	13
Elevated LDH level†	21	57	18	62	3	38
Bone marrow involvement	7	19	7	24	0	0
No. of previous systemic regimens						
Median	2		2		3	
Range	1-6		1-5		1-6	
1	14	38	13	45	1	13
2	15	41	12	41	3	38
≥ 3	8	22	4	14	4	50
Types of systemic therapy						
Chemotherapy	37	100	29	100	8	100
CHOP/CHOP-like regimen	36	97	29	100	7	88
DeVIC	6	16	4	14	2	25
CHASE	5	14	5	17	0	0
Single-agent therapy	5	14	0	0	5	63
Other	10	27	10	34	0	0
Auto-PBSCT	3	8	3	10	0	0
Radiotherapy	9	24	5	17	4	50
Intensity of CCR4 expression‡						
1+	6	16	4	14	2	25
2+	6	16	4	14	2	25
3+	25	68	21	72	4	50
Histopathology by central review						
PTCL-NOS	16	43	16	55		
AITL	12	32	12	41		
ALCL, ALK negative	1	3	1	4		
MF	7	19			7	88
c-ALCL	1	3			1	13

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; ALK, anaplastic lymphoma kinase; c-ALCL, cutaneous anaplastic large-cell lymphoma; CHASE, cyclophosphamide, cytosine arabinoside, etoposide, and dexamethasone; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; CTCL, cutaneous T-cell lymphoma; DeVIC, dexamethasone, etoposide, ifosfamide, and carboplatin; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; MF, mycosis fungoides; NOS, not otherwise specified; PBSCT, peripheral-blood stem-cell transplantation; PTCL, peripheral T-cell lymphoma.

*Of the 38 patients enrolled, 37 received at least one infusion of mogamulizumab.

†Elevated LDH level: higher LDH level than upper limit of the normal range.

‡The denominator used for the intensity of CC chemokine receptor 4 (CCR4) expression is based on subjects who were positive for CCR4 by immunohistochemistry.

Of the 37 patients who received mogamulizumab, 25 (68%) completed the planned course of eight infusions. Nine patients (24%) discontinued treatment because of PD, and three patients (8%) due to serious AEs.

Efficacy

The ORR for the 37 treated patients was 35% (13 of 37; 95% CI, 20% to 53%), and 14% of patients (five of 37) achieved a CR, of which one was unconfirmed (Table 2). Reponses (CR/PR) were observed in at least one patient with each subtype of disease, but the ORR differed between subtypes. The ORR was 34% (10 of 29; 95% CI, 18% to 54%) in patients with PTCL (three of 16 for PTCL-NOS, six of 12 for AITL, and one of one for ALCL, anaplastic lymphoma kinase-negative) and 38% (three of eight; 95% CI, 9% to 76%) in those with CTCL (two of seven for MF and one of one for cutaneous ALCL). In addition, ORR in patients with CTCL was 50% (four of eight; 95% CI, 16% to 84%) according to the Global Response Score.

Total ORR did not significantly correlate with CCR4 expression level, patient age, or the number of previous chemotherapy regimens. The response rates for lymph node and cutaneous lesions were 33% (11 of 33) and 58% (seven of 12), respectively.

The median PFS was 3.0 months (95% CI, 1.6 to 4.9 months) for the entire population and 2.0 months for patients with PTCL. Although the median OS was not reached for the entire population at the

Table 2. Best Response (N = 37)

Parameter	No. of Patients	No. of Patients With Best Response CR/CRu	PR	SD	PD	Response Rate (%)*
Overall response	37	5	8	13	11	35
Histopathology by central review						
PTCL	29	5†	5	9	10	34
PTCL-NOS	16	1	2	6	7	19
AITL	12	3	3	3	3	50
ALCL, ALK negative	1	1†	0	0	0	100
CTCL	8	0	3	4	1	38
MF	7	0	2	4	1	29
c-ALCL	1	0	1	0	0	100
Age, years						
< 65	19	1†	6	7	5	37
≥ 65	18	4	2	6	6	33
Intensity of CCR4 expression						
1+	6	1	1	3	1	33
2+	6	1	2	2	1	50
3+	25	3†	5	8	9	32
No. of previous systemic regimens						
1	14	3	3	6	2	43
2	15	1	1	6	7	13
≥ 3	8	1†	4	1	2	63

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; ALK, anaplastic lymphoma kinase; c-ALCL, cutaneous anaplastic large-cell lymphoma; CCR4, CC chemokine receptor 4; CR, complete response/complete remission; CRu, uncertain complete response/uncertain complete remission; CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; NOS, not otherwise specified; PD, progressive disease; PR, partial response/partial remission; PTCL, peripheral T-cell lymphoma; SD, stable disease.

*Response rate (%): 100 × number of responders/number of subjects in each category included in the efficacy analysis set.

†Among the patients who showed CR/CRu, one showed CRu.

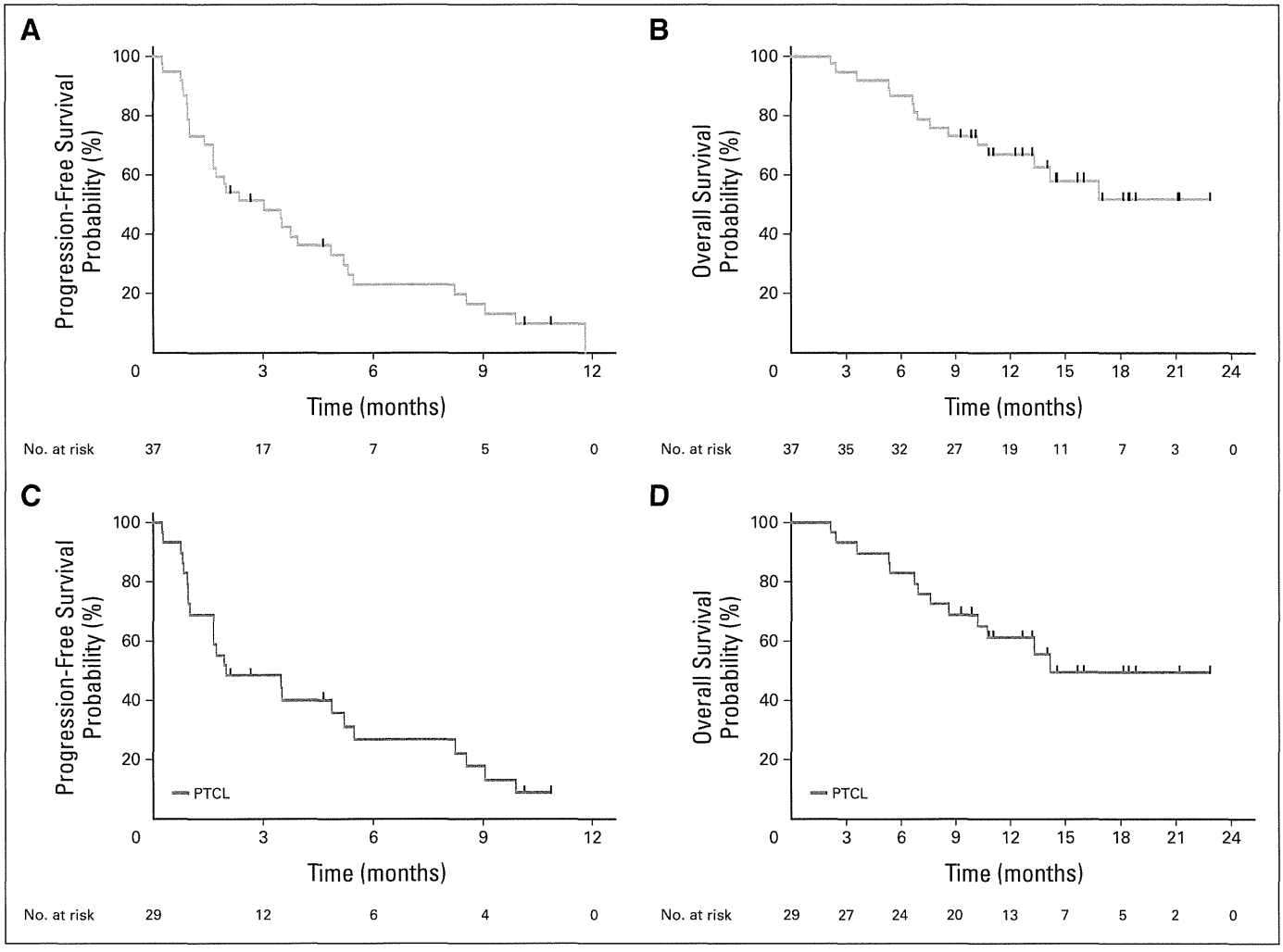

Fig 1. Kaplan-Meier curves of (A) estimated progression-free survival (median, 3.0 months), (B) overall survival (median not reached), (C) progression-free survival in patients with peripheral T-cell lymphoma (PTCL; median, 2.0 months), and (D) overall survival in patients with PTCL (median, 14.2 months).

time of this report, it was 14.2 months for patients with PTCL (Fig 1). Moreover, the median PFS of all 13 responders was 5.5 months, and for PTCL responders (n = 10), it was 8.2 months.

Safety

The most common treatment-related AEs of all grades and treatment-related AEs of grade 3/4 were lymphocytopenia (81%, 73%), neutropenia (38%, 19%), and leukocytopenia (43%, 14%), whereas the most common nonhematologic AE was pyrexia (30%; grade 2 or lower) (Table 3). Lymphocytopenia occurred in 30 patients (81%) and was noted after the first dose in 26 of these patients. For 19 of the patients, lymphocyte counts were $< 800/\mu L$ (grades 2 to 4) before the first dosing. The lymphocyte count ultimately recovered to normal or baseline levels in all patients.

Infusion reaction (24%; grade 2 or lower) occurred primarily at the first infusion, after which it became less frequent, and all patients recovered. No infusion prolongation/interruption was caused by the infusion reaction.

In addition, treatment-related skin disorders were commonly reported (all grades, 51%; grade 3/4, 11%) when grouped according to system organ class. Of the 19 patients who suffered from skin disorder complications, 15 patients experienced improvement, whereas the remaining patients discontinued treatment because of PD or switched to other post treatments. One patient who had a history of psoriasis before the study treatment developed two serious skin disorders (toxicoderma and psoriasis vulgaris) during the study period.

Fifteen serious treatment-related AEs were observed among eight patients (22%); these AEs included grade 3 polymyositis in one patient, grade 2 cytomegalovirus retinitis in two patients, and grade 4 second primary malignancy in one patient with AITL. All patients improved over time, and there were no deaths related to AEs.

Pharmacokinetics and Pharmacodynamics

The mean maximum mogamulizumab concentration and trough mogamulizumab concentration ($\pm$ standard deviation) in plasma after the eighth infusion were 45.9 $\pm$ 9.3 and 29.0 $\pm$ 13.3 μg/mL, respectively. Antimogamulizumab antibodies were not detected after dosing in any patients. These results were consistent with the findings of a previous study of patients with ATL.[30] As an exploratory study, we assessed the effect of mogamulizumab on the number of CD4+/CD25+/Foxp3+ cells (the Treg cell subset) and CD45+/CD16+/CD56+ cells (the NK cell subset). Patients given

Table 3. Treatment-Related Adverse Events (N = 37)

Adverse Event*	All Grades		Grade ≥ 3	
	No.	%	No.	%
Hematologic				
Lymphocytopenia	30	81	27	73
Leukocytopenia	16	43	5	14
Thrombocytopenia	14	38	1	3
Neutropenia	14	38	7	19
Anemia	5	14	2	5
Febrile neutropenia	1	3	1	3
Nonhematologic				
Pyrexia	11	30	0	0
Infusion reaction	9	24	0	0
ALT increased	8	22	1	3
ALP increased	8	22	1	3
Hypophosphatemia	6	16	1	3
Hypokalemia	2	5	1	3
Infection	1	3	1	3
Oral candidiasis	1	3	1	3
Pneumonia	1	3	1	3
Herpes esophagitis	1	3	1	3
Polymyositis	1	3	1	3
Second primary malignancy†	1	3	1	3
Skin and subcutaneous tissue disorders (SOC)	19	51	4	11
Rash papular	6	16	1	3
Rash erythematous	5	14	1	3
Psoriasis	2	5	1	3
Rash maculopapular	2	5	1	3
Toxic skin eruption	2	5	1	3

Abbreviations: ALP, alkaline phosphatase; SOC, System Organ Class (according to the Medical Dictionary for Regulatory Activities).
*Treatment-related adverse events that were reported in at least 15% of patients or that were of grade 3-4 severity.
†Diffuse large B-cell lymphoma was reported in one patient with angioimmunoblastic T-cell lymphoma.

mogamulizumab exhibited a profound depletion of the Treg cell subset during treatment, and cell levels had not returned to baseline 4 months after the last dose (Fig A1). Mogamulizumab also caused a modest decrease in the NK cell subset during treatment (data not shown).

DISCUSSION

This report described results from a single-arm, open-label multicenter phase II study of mogamulizumab in patients with relapsed CCR4-positive PTCL and CTCL.

Mogamulizumab showed promising antitumor activity, with an ORR of 35% (95% CI, 20% to 53%) and a CR/unconfirmed CR of 14%. These data were consistent with those reported with relapsed ATL.[30] It is notable that all three patients who relapsed after autoperipheral blood stem-cell transplantation responded to mogamulizumab. The total ORR is comparable to that of other US Food and Drug Administration-approved drugs, such as pralatrexate and romidepsin.[10,11] However, the present study differed from previous studies in several important respects. Firstly, the patient population was smaller than in the pralatrexate or romidepsin studies. Secondly, since it has been reported that CCR4 expression correlated with advanced disease,[24] it is important to note that although these two studies enrolled relapsed and refractory patients irrespective of their CCR4 expression status, the present study only recruited relapsed patients who were CCR4-positive. However, almost all patients in the present study had good PS compared with those patients in the previous studies. Thirdly, all patients with MF (n = 7) in the present study had relapsed after systemic chemotherapies and were presumed to have advanced stage disease, because all of these patients exhibited clinical skin tumors. Further, four of these seven patients exhibited clinically abnormal lymph node swelling, which does not usually occur at stages lower than IIB.[14,15]

In future study, PFS may also be improved by a longer continuous dosing schedule, such as a phase I/II study for CTCL.[31]

Although the number of patients was relatively small in the present study, the ORR for the AITL group (50%; six of 12) seemed noteworthy, while appearing relatively low in patients with PTCL-NOS (19%; three of 16). However, the three patients with PTCL-NOS who responded to mogamulizumab achieved durable PFS (9.0, 10.1+, and 10.8+ months; +, censored). Further studies are needed to identify which CCR4-positive T-cell lymphoma patients are most likely to benefit from mogamulizumab therapy.

There was no definite correlation between ORR and patient characteristics, such as age, CCR4 expression level, or number of previous systemic regimens. Although our study only included CCR4-positive patients with PTCL and CTCL, a recent US phase I/II study of mogamulizumab included both CCR4-positive and CCR4-negative patients with CTCL.[31] In that study, mogamulizumab exhibited efficacy irrespective of CCR4 expression (positive or negative) or CCR4 expression level, with a continuous dosing schedule.[31] Further studies are needed to define if CCR4 positivity represents a useful predictive biomarker in either PTCL or CTCL.

CCR4-positivity was confirmed in 78% of the 64 screened patients, a higher rate than previously reported.[20,21] However, it is possible that this variation in CCR4 positivity was due to differences in immunohistochemistry assay sensitivity. In our ongoing CTCL phase III study, our protocol permitted recruitment of both CCR4 positive and negative CTCL patients (NCT01728805). This is because the detection limit of CCR4 positivity may not be yet fully established, and mogamulizumab might have antitumor activity against CCR4-negative tumors through the depletion of CCR4-positive regulatory T cells,[36] thus enhancing pre-existing CD8+ cytolytic T-lymphocytes. Based on the latter new concept, an investigator-initiated trial of mogamulizumab against CCR4-negative solid tumors has been initiated (UMIN000010050).

Most of the AEs associated with mogamulizumab were mild and reversible. One patient suffered from polymyositis, an immune-related serious AE, after seven doses of mogamulizumab. The patient improved after steroid pulse therapy, treatment with tacrolimus hydrate, and continuous rehabilitation. Although drug-induced myositis was a possible cause, the relationship between mogamulizumab and myositis was not determined, even after detailed investigation. In our study, skin rash could also represent an immune-related AE, as other immunotherapies, including ipilimumab and zanolimumab, cause similar skin toxicity.[18,36-38] In addition, this may relate to the antitumor mechanism of mogamulizumab, because CCR4 contributes to skin-specific lymphocyte homing.[39] Indeed, a previous study revealed that patients who developed skin disorders ultimately had better therapeutic responses to treatment.[30] In the present study, of the