Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL)


*Department of Biological Science and Technology, Faculty of Industrial Science and Technology, and †Tissue Engineering Research Center, Research Institute of Biological Science, Tokyo University of Science, Noda, Chiba 278-8510, Japan; ‡Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Kagoshima 890-0064, Japan; §Department of Clinical Molecular Biology, Faculty of Medicine, Kyoto University, Kyoto 606-8507, Japan; ‡‡Pharmaceutical Research Laboratory, JT Inc., Takatsuki, Osaka 569-1125, Japan; **Department of Biomolecular Sciences, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan; and ††Department of Surgery, JA Kagoshima Kouseiren Hospital, Kagoshima 890-0062, Japan

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Adult T cell leukemia/lymphoma (ATLL) has been characterized as one of the most aggressive human neoplasias and its incidence is thought to be caused by both genetic and epigenetic alterations to the host cellular genes of T cells infected with human T cell leukemia virus type I (HTLV-I). A multilobulated nuclear appearance is an important diagnostic marker of ATLL, and we have now identified that the molecular mechanisms underlying these formations occur through the multibulb rearrangement via phosphatidylinositol 3-kinase (PI3-kinase) activation by AILIM/ICOS signaling. We also show that PTEN and/or SHIP-1, which are PI3-kinase inhibitors, are thought to be activated by both AILIM/ICOS signaling. Our results suggest that alteration of PI3-kinase signaling cascades, as a result of the down-regulation of inositol phosphatases, induces ATLL-type multilobulated nuclear formation and is also associated with the cellular proliferation of malignant T cell leukemias/lymphomas.

Adult T cell leukemia/lymphoma (ATLL) is one of the most aggressive neoplastic diseases and is caused by human T cell leukemia virus type I infection (HTLV-I; refs. 1–3). Although ~20 million people worldwide are thought to be infected with HTLV-I, ATLL is regarded as a relatively rare occurrence in HTLV-I carriers and the molecular mechanisms underlying the manifestation of nuclear polymorphisms have yet to be fully elucidated.

Malignant T cells in acute-type ATLL patients are activated and induced to proliferate in the lymph nodes (4), which undergo swelling and enter the peripheral blood stream. Therefore, it is plausible that antigentic molecules that are presented in the lymph nodes in ATLL have an important role in the activation of malignant T cells in vivo. During the immune response, T cells are optimally activated in secondary lymphoid tissues, such as the lymph nodes, and a second costimulatory signal, CD28, is necessary for the activation of naïve T cells upon antigen recognition via the T cell receptor/CD3 complex (10, 11). T cell activation also induces the activation-inducible lymphocyte immunomediatory molecule (AILIM)/inducible costimulator (ICOS; refs. 12 and 13). AILIM/ICOS-mediated signals contribute mainly to the regulation of activated T cells and to memory/effecter T cell functions via the phosphatidylinositol 3-kinase (PI3-kinase) cascade (14, 15). Recently, we have demonstrated that AILIM/ICOS signaling in the absence of T cell receptor (TCR)/CD3 stimulation has a distinct biological role in T cell migration and in the polarization of activated T cells through cytoskeletal rearrangement (16).

PI3-kinase is well known to play an essential role in T cell activation and cell motility in not only cells of the immune system but also in other cell types through cytoskeleton rearrangements (17, 18). The activation of PI3-kinase induces the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2), which then binds to the PH domains of intracellular signaling molecules and thus has essential roles in the rearrangement of the cytoskeleton (17–20). In addition, inositol phosphatases, including the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor and Src homology 2 domain containing inositol polyphosphate phosphatase (SHIP), have been implicated in the degradation of PIP3 and shown to antagonize the PI3-kinase/Akt signaling pathway (21–26).

Here, we show that ATLL-type multilobulated nuclei are produced through a distinct rearrangement of microtubules via PI3-kinase cascade that has been activated by both AILIM/ICOS and cell surface molecules. We also demonstrate that the formation of ATLL-type multilobulated nuclei requires the alteration of PI3-kinase/Akt cascade activation via the down-regulation of PTEN and/or SHIP-1. We propose a molecular mechanism for ATLL-type multilobulated nucleus formation and for the development of ATLL.

Materials and Methods

Cell Culture and Treatment. Highly purified T cells (>97%) were isolated from patients diagnosed as having chronic-type ATLL

Abbreviations: ATLL, adult T cell leukemia/lymphoma; HTLV-I, human T cell leukemia virus type I; AILIM, activation-inducible lymphocyte immunomediatory molecule; ICOS, inducible costimulator; PI3-kinase; phosphatidylinositol 3-kinase; PIP3; phosphatidylinositol 3,4,5-trisphosphate.

1R.-I.F., A.H., and A.U. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: t-tsuji@rs.noda.tus.ac.jp.

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and from healthy adult volunteers upon informed consent as described previously (27). Hut102, Jurkat, SupT1, and H9 cells were maintained in RPMI medium 1640 supplemented with 10% FCS. The P13-kinase inhibitor, LY294002 (Psiomega), the microtubule polymerization inhibitor, nocodazol (Sigma), and depolymerization inhibitor, taxol (Paclitaxel; Sigma), were used for analysis of microtubule rearrangement. Treatments were performed on precultures for 30 min at 37°C before stimulation under various conditions.

Fluorescence Microscopy. Cells (3 × 10^4 per well) were stimulated with various mAbs that were precoated at the concentration of 2 μg per well in eight-well chamber slides (Becton Dickinson). Cells were then fixed, permeabilized, and stained with FITC-conjugated anti-α-tubulin (clone DMI, Sigma), rhodamine-conjugated phalloidin (Molecular Probes) and Hoechst dye 33258 (Sigma), as described (16). Cells were analyzed by confocal microscopy using an Axiovert S100 (Carl Zeiss). Image acquisition from the Zeiss inscribe was made with a cooled charged-coupled device camera using QUANTIX (Photometrix, Victoria, Australia), and the images were processed with METAMORPH software (Molecular Devices, Sunnyvale, CA). Cells were also observed by using confocal laser scanning microscopy (LSM-510META, Carl Zeiss). The images acquired by confocal microscopy were then processed with Imaris 4 software (Carl Zeiss). Different numbers and levels of multilobulated nuclear formations were determined in at least 100 cells, in five independent experiments, and the average and the standard error were calculated as the frequency of these formations.

Western Blot Analysis. Cells were lysed as described (27) and the lysates were subjected to electrophoresis on SDS/PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA). The blotted membranes were blocked and subsequently incubated with anti-PTEN polyclonal antibody (anti-PTEN pAb; Cell Signaling Technologies) or anti-SHIP-1 pAb (Upstate) and from healthy adult volunteers upon informed consent as described in Supporting Text. After washing, bound primary Abs were visualized with secondary horseradish peroxidase-conjugated F(ab’)_2 fragment of IgG (ICN/Cappel, Aurora OH) and visualized by using enhanced chemiluminescence (ECL) (Amer sham Pharmacia).

Results

The Involvement of AILIM/ICOS Signaling in the Multilobulated Nucleus Formation. We first investigated whether the induction of costimulatory molecules is involved in typical multilobulated nuclear formations in peripheral T cells isolated from ATLL patients. Chronic-type ATLL-derived T cells, which show relatively low levels of these nuclear polymorphisms, were tested in this experiment via a multilobulated nucleus formation assay because acute-type ATLL-derived T cells have been shown to have already formed these abnormal nuclei in vivo (6–9). The clinical characteristics of the T cell isolates from 11 chronic-type ATLL individuals are summarized in Table 1 and Supporting Text, which are published as supporting information on the PNAS web site.

In T cells isolated from chronic-type ATLL patients, multilobulated nucleus formation was significantly induced by AILIM/ICOS signaling, but not CD28 signaling (data not shown), in the absence of CD3 stimulation (Fig. 1). Fig. 1a shows a typical multilobulated (“flower-like”) nucleus induced by AILIM/ICOS signaling in chronic-type ATLL patients. In such T cells, the nuclear shape is altered such that it forms several deep cavities and results in a multilobulated nucleus with typically three to six small lobules. The frequencies of the appearance of ATLL-type multilobulated nuclei after AILIM/ICOS signaling in our chronic-ATLL patient group are shown in Fig. 1b and were found to be significantly increased when compared to unstimulated T cells isolated from chronic-type ATLL patients (5.90 ± 5.14% to 12.10 ± 6.86%, P = 0.027) but not when compared to healthy donors.

Induction of ATLL-Type Multilobulated Nucleus Formation in AILIM/ICOS-Expressing Jurkat Cells. We transduced a cDNA encoding AILIM/ICOS into the human T cell leukemia cell lines, such as Hut102, H9, SupT1, and Jurkat, in which the expression of AILIM/ICOS could not be detected, and isolated stably expressing clones. Among these AILIM/ICOS-expressing leukemia cells, only AILIM/ICOS-Jurkat cells formed typical ATLL-type multilobulated nuclei after stimulation by exogenous AILIM/ICOS signaling (Fig. 6a, Table 2, and Supporting Text, which are published as supporting information on the PNAS web site). Quantitative evaluation of ATLL-type multilobulated nuclear formations in AILIM/ICOS-Jurkat cells is shown in Fig. 6b and Supporting Text. In AILIM/ICOS-expressing Jurkat cells, ATLL-type multilobulated nucleus formation was dramatically induced with a maximal induction of 63.3 ± 2.2% at 4–8 h after AILIM/ICOS stimulation, followed by a gradual decrease in the frequency of altered nuclei within 48 h (Fig. 2a and 7a and b, which is published as supporting information on the PNAS web site). However, these frequencies were not enhanced with a combination of CD3 stimulation (Fig. 2a). Although the cell adhesion molecules CD29, CD49d, and ICAM-3 are expressed at high levels on the cell surface of AILIM/ICOS-Jurkat cells (Fig. 6c) and the nonstimulated frequencies of multilobulated nuclear formations were significantly higher in these cells than in the controls, these frequencies were at relatively low levels when compared to AILIM/ICOS signaling (Fig. 2a).

We further investigated whether AILIM/ICOS signaling affects cell growth in AILIM/ICOS-Jurkat cells. We also evaluated cell growth in AILIM/ICOS-Jurkat cells after AILIM/ICOS stimulation by using WST-8 reagent. The stimulation
Jurkat cells were stimulated by AILIM CD29, CD49d, or ICAM3 mAbs precoated on slides for 4 h. Values are represented as the mean ± SEM. *, *P < 0.0001 compared with control; **, **P < 0.0005 compared with control. (b) AILIM/ICOS-Jurkat cells were stimulated by anti-AILIM/ICOS, CD29, CD49d, or ICAM3 mAbs precoated at 500 ng per well on 96-well type plates for 2 or 3 days. Cell proliferation was measured with WST-8 reagent by using a Cell Counting kit (Dojindo Labs, Tokyo) according to the manufacturer’s instruction. The cells were cultured for 2 or 3 days, and WST-8 reagent was added for the last 2 h of the culture. Values are represented as the mean ± SEM. *, *P < 0.01 compared with control.

significantly induced the proliferation of AILIM/ICOS-Jurkat cells (Fig. 2b). These results indicate that AILIM/ICOS signaling induces both ATLL-type multilobulated nuclear formation and cellular growth in AILIM/ICOS-Jurkat cells.

The Involvement of Microtubule Rearrangement in ATLL-Type Multilobulated Nucleus Formation. We next performed a time course analysis of nuclear morphological changes and cytoskeletal rearrangements of actin and microtubules during AILIM/ICOS-mediated ATLL-type multilobulated nucleus formation (Fig. 7a and Supporting Text). We also investigated microtubule reorganization during ATLL-type multilobulated nucleus formation by using 3D analyses with confocal microscopy. Our subsequent findings clearly demonstrated that microtubules were bundled and localized within the small cavities between the small nuclear lobules by the 3D analyses with confocal microscopy (Fig. 3a Top). Surprisingly, these bundled microtubules that were present between the lobules formed a loop structure, which then appeared to contract the nuclei and thus induce the formation of small lobules (Fig. 3a Top). The mean values ± SEM, which were calculated from the independent image analysis of five individual nuclei, of the circumference of the microtubule loops at 40, 120, and 240 min after stimulation were found to be 25.2 ± 2.46, 17.8 ± 1.32, and 8.92 ± 1.86 nm, respectively. Ring-like organizations of microtubules were also observed in T cells isolated from one acute-type ATLL patient (Fig. 3b). All of the morphological features of ATLL nuclei and the ring-like microtubule formations were also representative of AILIM/ICOS-Jurkat cells cocultured with CHO cells expressing B7h, the native ligand of AILIM/ICOS (Fig. 7c).

We next examined the effects of the microtubule polymerization and depolymerization inhibitors nocodazol and taxol on these multilobulated nuclear formations. Pretreatment with these agents did not affect filamentous actin formation but inhibited the AILIM/ICOS-mediated nuclear transformations and prevented formation of the bundled microtubule structures (Fig. 3c and Fig. 8, which is published as supporting information on the PNAS web site). These findings indicate that microtubule rearrangements including the contraction of looped microtu-

bules in fact regulate the ATLL-type multilobulated nucleus formation that is induced by AILIM/ICOS stimulation.

The Role of PI3-Kinase in ATLL-Type Multilobulated Nucleus Formation. In the intracellular region of AILIM/ICOS, PI3-kinase binds to the YFMF sequence between residues 180–183 (Fig. 4a and refs. 12 and 13) and the activation of PI3-kinase is essential for T cell activation of AILIM/ICOS-mediated signaling (27). The Y180F mutation resulted in a dramatic decrease in the frequency of nuclear abnormalities when compared to the wild-type AILIM/ICOS-expressing Jurkat cells (Fig. 4b). The PI3-kinase inhibitor, LY294002, also significantly inhibited multilobulated nucleus formation in AILIM/ICOS-Jurkat cells in a dose-dependent manner and this inhibition by LY294002 was also observed in peripheral T cells isolated from chronic-type ATLL patients (Fig. 9, which is published as supporting information on the PNAS web site). Moreover, transduction of a dominant-negative p85 subunit (Δp85), which lacks residues 479–513 of the p85α subunit of PI3-kinase (28), also significantly inhibited ATLL-type multilobulated nucleus formation by AILIM/ICOS stimulation (Fig. 4c). These results indicate that the PI3-kinase cascade induced by AILIM/ICOS signaling is essential for ATLL-type multilobulated nucleus formation.
The nuclear formations in ATLL, a wild-type PTEN-expressing cell line, result from the downstream effectors of Akt signaling pathway not being essential for nuclear formation (Fig. 5a). This finding indicates that the activation of the Akt cascade, effects the nuclear formations induced by AILIM/H20862 with previous reports (25).

ATLL-type multilobulated nucleus formation by AILIM/ICOS-Tyr308 and Ser473 are substituted by alanine, did not inhibit a dominant-negative form of Akt (AktDN), in which both phosphorylated forms of Aktp308 and Aktp473 were not detectable in AILIM/H20862/H9251/H11006. The expression of PTEN by siRNA in AILIM/ICOS expressing SupT1 and Hut 102 cells (Fig. 10, which is published as supporting information on the PNAS web site) and observed that ATLL-type multilobulated nucleus formation in SupT1 and Hut 102 cells was significantly induced to levels of 18.5% and 8.2%, respectively (Fig. 5d). These data indicate that down-regulation of PTEN expression is essential for nuclear transformation in ATLL.

We next analyzed the expression of PTEN and SHIP-1 in peripheral T cells isolated from acute and chronic-type ATLL patients (Table 3, which is published as supporting information on the PNAS web site). The expression of PTEN and SHIP-1, but not p-Akt, was clearly detectable in peripheral T cells isolated from healthy donors (Fig. 5e). In contrast, the expression of PTEN and SHIP-1 in peripheral T cells isolated from acute and chronic-type ATLL patients were significantly decreased and these cells displayed an inverse up-regulation of p-Akt (Fig. 5e and Fig. 11, which is published as supporting information on the PNAS web site).

The Role of the ATLL Multilobulated Nucleus Formation-Associated Genes, PI3-Kinase and PTEN, in Cellular Proliferation. Finally, we examined the effects of the PI3-kinase and PTEN genes, which are associated with ATLL-type multilobulated nucleus formation, upon cellular proliferation in AILIM/ICOS-Jurkat cells. The proliferation of AILIM/ICOS-Jurkat cells was found to be significantly induced by the expression of the constitutively active myristoylated forms of Akt (Myr-Akt) and the PI3-kinase p110 subunit (Myr-p110) in the absence of AILIM/ICOS signaling (Fig. 5f). In contrast, the transduction of wild-type PTEN in these cells strongly reduced their proliferation. These results suggest that the alteration of the PI3-kinase pathway, caused by the disruption of inositol phosphatases, is associated with not only ATLL-type multilobulated nucleus formation but also with the incidence and development of ATLL.

Discussion
Here, we have provided evidence that ATLL-type multilobulated nucleus formation, which is thought to be an important clinical marker of ATLL and to be closely related to the progression and incidence of this cancer, is caused by microtubule constriction via the overactivation of the PI3-kinase cascade. This altered PI3-kinase activity is induced by the down-regulation of the inositol phosphatases, PTEN and/or SHIP-1, after AILIM/ICOS signaling in both peripheral T cells isolated from chronic ATLL patients and in AILIM/ICOS-Jurkat cells. Our results also show that PI3-kinase and PTEN are closely associated with the cellular proliferative capacity.

The PI3-kinase cascade is well known as a regulator of the activation, proliferation, and cytokine production of T cells (17, 18). The major functions of the inositol phosphatases, PTEN and SHIP-1, rely on their phosphatase activity and subsequent antagonism of the PI3-kinase cascade. In addition, the activation levels of the PI3-kinase cascade are also regulated by the expression levels of PTEN and SHIP (21–26). Loss of function of these inositol phosphatases results in the accumulation of PIP3 and the subsequent activation of its downstream effectors, such as the PI3-kinase/Akt cascade (21–26). Jurkat cells, which is the only T cell leukemia cell line that can induce multilobulated nuclear formations after AILIM/ICOS signaling, are deficient in both PTEN and SHIP-1 expression and show a highly inverse up-regulation of PI3-kinase activity and their activation regulates the PI3-kinase cascade via their PIP3 phosphatase PTEN and SHIP-1, rely on their phosphatase activity and associated with the cellular proliferative capacity.
Fig. 5. Overactivation of PI3-kinase is essential for ATLL-type multilobulated nucleus formation and can be observed in T cells isolated from ATLL patients. (a) Western blot analysis showing the expression of PTEN, SHIP-1, total Akt, β-actin, and phosphorylated-Akt (p-Akt) in various human leukemia cell lines. Phosphorylated-Akt was detected with or without AILIM/ICOS signaling. (b) Wild-type AILIM/ICOS-expressing Jurkat cells were transfected with the dominant-negative form of Akt (Akt DN), and then the effects of exogenous gene expression on the frequency of multilobulated nucleus formation were analyzed as described in Fig. 4c. Values are given as the mean ± SEM. * P < 0.0001 compared with mock transfected cells. (c) AILIM/ICOS expressing Jurkat cells were transfected with wild-type PTEN and an EYFP-C1 plasmid control and cultured for 1 day. The cells were then purified and subjected to multilobulated nucleus formation analysis as described in Fig. 4c, and values are given as the means ± SEM. * P < 0.0001 compared with mock transfected cells. (d) A PTEN-specific knockdown was performed by the expression of hairpin siRNA. A PTEN–RNAi plasmid was transfected into AILIM and stable transformants were subjected to ATLL-type multilobulated formation analysis. * P < 0.0001 compared with mock transfected cells. (e) Western blot analysis showing the expression of PTEN, SHIP-1, phosphorylated-Akt (p-Akt), total Akt, and β-actin in T cells isolated from healthy donors and from ATLL patients as indicated. * Not determined. (f) Expression vectors containing myristoylated Akt (Myr-Akt), myristoylated PI3-kinase p110 subunit (Myr-p110), and PTEN cDNAs were transfected into AILIM/ICOS-Jurkat cells, and cell proliferation was measured with WST-8 reagent by using a Cell Counting kit (Dojindo Labs) according to the manufacturer's instruction. The cells were cultured for 3 days, and WST-8 reagent was added for the last 4 h of the culture. Values are represented as the mean ± SEM. * P < 0.0001 compared with control; **, P < 0.0005 compared with control.

p-Akt, as reported (25, 29). Significant decreases in inositol phosphatase levels were also observed in ATLL patient-derived T cells, which resulted in an inverse up-regulation of p-Akt (Figs. 5e and 11). We showed that the alteration in PI3-kinase cascade activity, but not in the activation of Akt, is indeed essential for these multilobulated formations under conditions where inositol phosphatase expression is disrupted.

The PI3-kinase cascade is also thought to play a role in the development of ATLL. We showed in our current study that AILIM/ICOS signaling, and the subsequent alteration of PI3-kinase activity, contributes to the growth rate of AILIM/ICOS-Jurkat cells. Previous studies have demonstrated that the selective suppression of PI3-kinase in T cells by using conditional knockout mice (ptenfl/fl mice), leads to an increase in the levels of mature CD4+ T cells in the periphery (21–23, 30). The subsequent onset of malignant T cell lymphomas, which were classified as CD4+ and not CD8+ T cell lymphomas, could be observed from 10 weeks in these PTEN knockouts, and all of the animals died within 17 weeks (30). Furthermore, transgenic mice expressing an active form of PI3-kinase in their T cells, derived from a thymic lymphoma (p65), developed an infiltrating lymphoproliferative disorder with an increased number of T cells exhibiting a memory CD4+ cell phenotype and a reduced apoptotic response (31). Most ATLL malignant T cells are also classified as CD4+ (5–7). Restoration of SHIP activity in a human leukemia cell line, which has lost expression of endogenous SHIP, down-regulates the constitutively active PI3-kinase/Akt/GSK-3beta signaling pathways and leads to an increased transit time through the G1 phase of the cell cycle (26). Previous reports have also demonstrated that transgenic mice expressing constitutively active myristoylated Akt in T cells had an accumulation of CD4+ T cells and displayed an increased incidence of lymphoma (32). Taken together, these observations suggest that the irregular overactivation of the PI3-kinase cascade, including the disruption of inositol phosphatases such as PTEN and SHIP-1, has an essential role both in the ATLL-specific nuclear phenotype and in the development of ATLL in patients.

Infection of HTLV-I is thought to be essential for the incidence of ATLL (1–3), but in the present study, HTLV-I-negative Jurkat cells also formed ATLL-type multilobulated nuclei. However, it was previously reported that some patients diagnosed with ATLL had typical clinicohematological, morphological, and immunophenotypic characteristics of this cancer but showed no integration of HTLV-I in their leukemic cells and presented with HTLV-I-negative sera (33, 34). Furthermore, previous studies have demonstrated that genetic and epigenetic alterations in host cellular genes are necessary for the development of malignant T cells in ATLL (3, 4). These observations suggested the existence of cellular oncogenesis in HTLV-I-negative ATLL patients and that the phenotype of Jurkat cells is closely related...
both to in vivo leukemogenesis and to the development of ATLL by infection with HTLV-I.

Further studies of the mechanisms underlying both the down-regulation of inositol phosphatases during the development and progression of ATLL, and the downstream effectors of microtubule rearrangement by PIP3, produced by the active PI3-kinase, will provide further important insights into the mechanisms underlying the incidence and progression of ATLL.

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Eleven ATLL patients were included in this study, of which four were men, with an age range of 53-80 years (median age of 66). The clinical characteristics of the T cells isolated from these 11 chronic-type ATLL individuals are summarized in Table 1. The T cell populations in the diseased individuals were found to be predominantly CD4+CD8-CD45RO+ and CD25+, which was a statistically significant finding ($P < 0.001$ compared with healthy donor-derived T cells). In contrast, the prevalence of CD28+ and activation-inducible lymphocyte immunomediatory molecule (AILIM)/inducible costimulator (ICOS)+ T cells did not significantly differ between ATLL patients and healthy donors ($P = 0.440$). Peripheral T cells, isolated from chronic-type ATLL patients, were stimulated with α-AILIM/ICOS mAb for 4 h, and then washed twice with PBS. The remaining cells on chamber slides were fixed, permeabilized, and stained with Hoechst dye 33258. In the T cells, the nuclear transformation, which have small lobules and several cavities, was counted and the frequencies of multilobulated nucleus formation were calculated from the numbers of the nuclear transformed cells among the total observed cells. In the T cells, multilobulated nucleus formation was significantly induced by AILIM/ICOS-signaling, but not CD28-signaling (data not shown), in the absence of CD3-stimulation (Fig. 1). The expression level of AILIM/ICOS in chronic-type ATLL patient-derived T cells was not significantly different from that of healthy donor T cells (Tables 1 and 3). These observations suggest that multilobulated nucleus formation in ATLL patients is induced by the alteration of the intracellular signaling cascade, which incorporates the downstream effectors of AILIM/ICOS-signaling, and not the up-regulation of AILIM/ICOS expression.

**Induction of Nuclear Polymorphisms in Human T Cell Leukemia Cell Lines by AILIM/ICOS-Signaling.** To develop an experimental model for ATLL-type multilobulated nucleus formation, we transduced a cDNA encoding the entire open-reading frame of AILIM/ICOS into human T cell leukemia cell lines Hut102, H9, SupT1, and Jurkat, in which the expression of AILIM/ICOS could not be detected, and isolated stably expressing clones. Morphological changes in the nuclei, induced by AILIM/ICOS-signaling, were classified into four groups; ATLL-type multilobulated, Sezary-like, cerebriform and abnormal (Fig. 6a). These nuclear polymorphisms were found to be dependent on both the cell type and on the frequency of abnormal morphologies that were observed (Table 2). Surprisingly, only AILIM/ICOS-Jurkat cells formed typical ATLL-type multilobulated nuclei following stimulation by exogenous AILIM/ICOS-signaling (Fig. 6a and b and Table 2).

**Quantitative Evaluation of Multilobulated Nucleus Formation in AILIM/ICOS-Expressing Jurkat Cells.** To quantitatively evaluate the abnormal nuclear formations in AILIM/ICOS-expressing Jurkat cells, we designated the levels of the appearance of these structures as class I, II, or III (Fig. 6b). In class I cells, altered nuclei form bends but not cavities. The class II designation describes nuclei in which cavities are partially observed in the outer sides of the nucleus. Class III cells have typical

### Table 1. Clinical and T cell characteristics of both chronic-type adult T cell leukemia/lymphoma (ATLL) patients and healthy donors

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<th>ATLL patients</th>
<th>Healthy donors</th>
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<tbody>
<tr>
<td>Number of individuals</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Age (year), median (range)</td>
<td>66 (53-80)</td>
<td>23.5 (22-55)</td>
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<tr>
<td>Sex (male/female)</td>
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<td>4/4</td>
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<tr>
<td>Clinical subtype</td>
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<td>Normal</td>
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<tr>
<td>WBC (x10^3 ml), median (range)</td>
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<td>Surface-phenotypic markers (% of positive cells)</td>
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<tr>
<td>CD45RO+</td>
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<td>CD4+</td>
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<tr>
<td>CD25+</td>
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</table>

$^a$Patient-derived T cells were stained with antibodies against CD3 (clone HIT3a, BD), CD4 (clone H144B, BD), CD8 (clone 2A3, BD), CD25 (clone 2A3, BD), CD45RA (clone HI100, BD), CD45RO (clone UCHL1, BD), CD152 (clone BNI3, BD) and AILIM/ICOS (clone SA12; Ref 13) and then stained with FITC-conjugated goat anti-mouse IgG F(ab’)$^2$ fragment. After immunostaining, the cells were analyzed on a FACSCalibur flow cytometer using cellquest software package (Becton Dickinson) to determine the mean fluorescence intensities in live cells.

$^b$P<0.001 compared with healthy donor.

$^c$P=0.44 compared with healthy donor.

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**Supporting information**

The Clinical and T Cell Characteristics of both Chronic-Type Adult T Cell Leukemia/Lymphoma (ATLL) Patients and Healthy Donors. Eleven ATLL patients were included in this study, of which four were men, with an age range of 53-80 years (median age of 66). The clinical characteristics of the T cells isolated from these 11 chronic-type ATLL individuals are summarized in Table 1. The T cell populations in the diseased individuals were found to be predominantly CD4+CD8-CD45RO+ and CD25+, which was a statistically significant finding ($P < 0.001$ compared with healthy donor-derived T cells). In contrast, the prevalence of CD28+ and activation-inducible lymphocyte immunomediatory molecule (AILIM)/inducible costimulator (ICOS)+ T cells did not significantly differ between ATLL patients and healthy donors ($P = 0.440$). Peripheral T cells, isolated from chronic-type ATLL patients, were stimulated with α-AILIM/ICOS mAb for 4 h, and then washed twice with PBS. The remaining cells on chamber slides were fixed, permeabilized, and stained with Hoechst dye 33258. In the T cells, the nuclear transformation, which have small lobules and several cavities, was counted and the frequencies of multilobulated nucleus formation were calculated from the numbers of the nuclear transformed cells among the total observed cells. In the T cells, multilobulated nucleus formation was significantly induced by AILIM/ICOS-signaling, but not CD28-signaling (data not shown), in the absence of CD3-stimulation (Fig. 1). The expression level of AILIM/ICOS in chronic-type ATLL patient-derived T cells was not significantly different from that of healthy donor T cells (Tables 1 and 3). These observations suggest that multilobulated nucleus formation in ATLL patients is induced by the alteration of the intracellular signaling cascade, which incorporates the downstream effectors of AILIM/ICOS-signaling, and not the up-regulation of AILIM/ICOS expression.

**Induction of Nuclear Polymorphisms in Human T Cell Leukemia Cell Lines by AILIM/ICOS-Signaling.** To develop an experimental model for ATLL-type multilobulated nucleus formation, we transduced a cDNA encoding the entire open-reading frame of AILIM/ICOS into the human T cell leukemia cell lines Hut102, H9, SupT1, and Jurkat, in which the expression of AILIM/ICOS could not be detected, and isolated stably expressing clones. Morphological changes in the nuclei, induced by AILIM/ICOS-signaling, were classified into four groups; ATLL-type multilobulated, Sezary-like, cerebriform and abnormal (Fig. 6a). These nuclear polymorphisms were found to be dependent on both the cell type and on the frequency of abnormal morphologies that were observed (Table 2). Surprisingly, only AILIM/ICOS-Jurkat cells formed typical ATLL-type multilobulated nuclei following stimulation by exogenous AILIM/ICOS-signaling (Fig. 6a and b and Table 2).

**Quantitative Evaluation of Multilobulated Nucleus Formation in AILIM/ICOS-Expressing Jurkat Cells.** To quantitatively evaluate the abnormal nuclear formations in AILIM/ICOS-expressing Jurkat cells, we designated the levels of the appearance of these structures as class I, II, or III (Fig. 6b). In class I cells, altered nuclei form bends but not cavities. The class II designation describes nuclei in which cavities are partially observed in the outer sides of the nucleus. Class III cells have typical
small nuclear lobules, and are closely similar to the "flower-like" nuclei of aggressive ATL tumors and to AILIM/ICOS-induced multilobulated nuclei in chronic-type ATL derived T cells (Fig. 1a). Hence, we designated the nuclei from class II and III cells as ATL-type multilobulated nuclei and subjected these to further analysis.

The Involvement of Cytoskeletal Rearrangements in ATL-Type Multilobulated Nucleus Formation. The nuclei of AILIM/ICOS-Jurkat cells were observed to gradually form bends during the first 20 min of AILIM/ICOS-stimulation, and a large cavity was generated at the 80-min mark. On the outer side of the folded nucleus, several small cavities were detected at 40 min after stimulation, and small nuclear lobules had completely formed at 4 h. The structures of these multilobulated nuclei were maintained during the 4- to 8-h period after treatment, after which the multilobules were observed to gradually relax (Fig. 7a). It is well known that actin reorganization is closely related to the outside-in signaling of cell-surface molecules such as adhesion proteins. In this regard, filamentous actin could be observed as several spots in the center of the adherent cell surfaces in the early stages following AILIM/ICOS-stimulation.

An AILIM/ICOS-expression vector was transfected into various human T cell leukemia cell lines and stably expressing clones were isolated as described in Materials and Methods. These clones were stimulated by AILIM/ICOS-signaling and nuclear polymorphisms were evaluated as described and classified as indicated in the upper row. The results are expressed as the mean frequency ± SEM, and are representative of three independent experiments. ND: not detected.

Table 3. Adult T cell leukemia/lymphoma patient and healthy donor clinical and T cell characteristics

<table>
<thead>
<tr>
<th></th>
<th>Acute AT</th>
<th>Chronic ATL</th>
<th>Healthy donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>4</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Age (year), median (range)</td>
<td>57 (41-72)</td>
<td>65 (45-79)</td>
<td>29.3 (22-43)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>3/1</td>
<td>3/5</td>
<td>3/0</td>
</tr>
<tr>
<td>Clinical subtype</td>
<td>Acute</td>
<td>Chronic</td>
<td>Normal</td>
</tr>
<tr>
<td>WBC (x10³ ml), median (range)</td>
<td>55.3 (6.7-108.8)</td>
<td>10.7 (7.4-24.1)</td>
<td>6.4 (6.0-11.0)</td>
</tr>
<tr>
<td>Surface-phenotypic mark (%) of positive cells</td>
<td>CD45RO⁺ 83.7</td>
<td>94.2</td>
<td>65.5</td>
</tr>
<tr>
<td></td>
<td>CD4⁺ 95.2</td>
<td>97.4</td>
<td>58.1</td>
</tr>
<tr>
<td></td>
<td>CD8⁺ 4.1</td>
<td>1.1</td>
<td>38.2</td>
</tr>
<tr>
<td></td>
<td>AILIM/ICOS⁺ 3.7</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>CD25⁺ 85.0</td>
<td>71.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Patient-derived T cells were stained as described in Table 1. After immunostaining, the cells were analyzed on a FACSCalibur flow cytometer using cellquest software package (Becton Dickinson) to determine the mean fluorescence intensities in live cells.

Fig. 7. Adult T cell leukemia/lymphoma (ATL)-type multilobulated nuclei of activation-inducible lymphocyte immunomediatory molecule (AILIM)/inducible costimulator (ICOS)-Jurkat cells was induced by AILIM/ICOS signaling. (a) AILIM/ICOS-Jurkat cells were stimulated by AILIM/ICOS for 0-24 h. The cells were stained with rhodamine-phalloidin (red), FITC-conjugated anti-a-tubulin mAb (green) and Hoechst dye 33258 (blue). Cells were analyzed by fluorescent microscopy using an A xiovert 200M and the images were processed with imaris software. (Bar, 10 mm.) (b) AILIM/ICOS-Jurkat cells were stimulated by AILIM/ICOS signaling for the indicated periods. Following stimulation, the frequencies of ATL-type multilobulated formation were measured. Values are represented as the mean ± SEM. (c) AILIM/ICOS-Jurkat cells were seeded onto a confluent layer of human B7h, the native ligand for AILIM/ICOS, expressing CHO cells. After 4 h of coculture, the cells were stained with rhodamine-conjugated phalloidin, FITC-conjugated anti-a-tubulin mAb and Hoechst dye 33258 for filamentous actin (red signal), a-tubulin (green signal), and nuclei (blue signal), respectively. Cells were analyzed by a confocal laser microscopy using an LSM510-META, and the images were processed with imaris 4 software. The images in the lower row represent x-z slices. (Top and Middle) Three-dimensional views at 45° and 90° from x-z projections, respectively. AILIM/ICOS-Jurkat cell was indicated by arrow. (Bar, 10 mm.)
PTEN-specific knock-down by the expression of PTEN-siRNA plasmid. (a) PTEN-specific knockdowns in activation-inducible lymphocyte immunomediatory molecule (AILIM)/ inducible costimulator (ICOS)-SupT1 and Hut102 cells were performed by the expression of hairpin siRNA using a Lipuer.neo+gfp in pSuper RNAi system (OligoEngine, Seattle, WA). To generate the PTEN-RNAi plasmid, a unique 19-bp nucleotide sequence 5'-GGTGAAAGATATCTTCG-3', derived from the mRNA transcript of PTEN, was synthesized and cloned into pSuper.neo+gfp vector according to the manufacturer's instructions. The sequence for PTEN was obtained from GenBank, accession no. NM-000314 (nucleotides 1691-1709). PTEN-RNAi plasmids were transfected into AILIM/ICOS-SupT1 cells, and stable transfectants, which were expressing GFP at high levels, were again isolated by flow cytometry using an EPICS ALTRA (Beckman Coulter) after the G418 selection for 2-3 weeks. Total RNA isolates were prepared from stable transfectants that were highly expressing PTEN-RNAi, using a SV Total RNA Isolation System (Promega). First-strand cDNA, primed with pdN6V random primers, was synthesized by using a First-Strand cDNA Synthesis kit (Amersham Pharmacia). PCR mixtures were prepared using a TaqMan Universal PCR Master Mix (Applied Biosystems) containing 0.9 mM of each primer, and amplification reactions were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 0.25 min and 60°C for 1 min. The sequences of PTEN primer pair are: PTEN (forward: 5'-CCAAATGTTCAAGGGCTGACT-3', reverse: 5'-GAGCTTGCTTCCCGTCTGTTG-3'). The gene expression levels of PTEN were measured by using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR product levels were estimated by the measurement of the intensity of fluorescence of SYBR Green. The expression level of PTEN was normalized to b-actin mRNA. (b) Cell lysates were prepared from the stable transfectants of AILIM/ICOS-SupT1 and Hut102 cells, which were highly expressed of PTEN-siRNA. The expression of PTEN was analyzed by Western blot analysis as described in Materials.

These filamentous actin bodies had assembled at the cell bottom, almost beneath the center of the large nuclear cavity, within the first 2 h and after 4-8 h were observed to have formed a ring-like structure, which gradually expanded (Fig. 7a).

We next investigated whether microtubule filaments are associated with the multilobulated nucleus formation that is induced by AILIM/ICOS-signaling. In AILIM/ICOS-Jurkat cells without stimulation, the microtubule-organizing centre (MTOC) is located to the side of the nuclei and the microtubules are evenly distributed within whole cells (Fig. 7a). After AILIM/ICOS-stimulation in these cells, however, the microtubules were also found to cover the nuclei and bundles of microtubules were also observed in between the small lobular formations after 40 min of stimulation. Furthermore, these bundled microtubules were observed to be integrated into constricted regions within the small nuclear lobules during the 8-h time frame after activation of the AILIM/ICOS cascade, and then gradually diffuse concomitantly with the onset of multilobulated nuclear formations.