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<td>Author(s)</td>
<td>Abe, Masaki; Uchihashi, Kinya; Tsuruda, Kazuto; Osaka, Akemi; Yanagihara, Katsunori; Hasegawa, Hiroo; Yamada, Yasuaki; Kamihira, Shimeru</td>
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<tr>
<td>Citation</td>
<td>European Journal of Haematology, 81(3) pp.209-217; 2008</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2008-09</td>
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<tr>
<td>URL</td>
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<td>(c) 2008 Blackwell Munksgaard; The definitive version is available at <a href="http://www.blackwell-synergy.com">www.blackwell-synergy.com</a></td>
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Note: The table is a transcription from the original document. The text is in English and the table headers are in Japanese.
Foxp3 Expression on normal and leukemic CD4⁺D25⁺T-cells implicated in Human T-cell Leukemia Virus type-1 is inconsistent with Treg cells


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Running title;
Foxp3 expression on normal & leukemic CD25⁺T-cells
Summary

Foxp3 is a master gene of Treg cells, a novel subset of CD4+T-cells primarily expressing CD25. We describe here different features in Foxp3 expression profile between normal and leukemic CD4+CD25+T-cells, using peripheral blood from healthy controls (HCs), HTLV-1-infected asymptomatic carriers (ACs), patients with adult T-cell leukemia (ATL), and various hematopoietic cell lines. The majority of CD4+CD25+T-cells in HCs were positive for Foxp3, but not all CD4+CD25+T-cells in ACs were positive, indicating that Foxp3 expression is not always linked to CD25 expression in normal T-cells. Leukemic (ATL) T-cells constitutively expressing CD25 were characteristic of heterogeneous Foxp3 expression, such as intra- and inter-case heterogeneity in intensity, inconsistency with CD25 expression, and a discrepancy in the mRNA and its protein expression. Surprisingly, the discernible amount of Foxp3 mRNA was detectable even in most cell lines without CD25 expression, a small fraction of which was positive for the Foxp3 proteins. The subcellular localization of Foxp3 in HTLV-1-infected cell lines was mainly cytoplasmic, different from that of primary ATL cells. These findings indicate that Foxp3 has two facets, essential Treg-identity and molecular mimicry secondary to tumorigenesis. Conclusively, Foxp3 in normal T-cells, but not mRNA, is basically potent at discriminating a subset of Treg cells from CD25+T-cell populations, whereas the modulation of Foxp3 expression in leukemic T-cells could be implicated in oncogenesis and have a potentially useful clinical role.

Key Words: Foxp3, Treg, HTLV-1, ATL, CD25
Introduction

A forkhead box protein 3 (Foxp3) is noted as a novel T-cell marker identifying enigmatic Treg cells, and as a master gene regulating development and replication of the cells (1, 2). Recent studies in the scurfy mouse and the human immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) models provide evidence that Foxp3 plays a major role in the differentiation of CD4+CD25+Treg cells (3, 4). Foxp3 in mice has been shown to be exclusively expressed by only CD4+CD25+Treg cells, designated as natural Treg cells that arise in the thymus through a homeostatic process. These findings from the mouse and human models indicate that Foxp3 is a master gene for development of Treg cells, representing a specific marker for identifying a cell-lineage or a subset of Treg cells. In addition to natural Treg cells developing in the thymus, there is another type of Treg cells that acquire the Treg phenotype in mature cells because of antigen stimulation (5). Foxp3 gene transfection can also convert naïve CD4+CD25−T-cells into a regulatory phenotype expressing CD25 and Foxp3, so-called adaptive/inducible Treg cells (6,7). Foxp3 expression is confirmed to be present in a minority of CD4+T-cell clones and CD8+T-cell clones, which is found exclusively in the activation populations (1). This suggests that Foxp3 expression is not always restricted to the natural Treg cell lineage and is inducible after T-cell activation.

Adult T-cell leukemia (ATL), which is a clonal lymphoproliferative neoplasm of post-thymic mature T-cells, presents a CD4+CD25+ phenotype (8). ATL cells have the same markers as those of natural Treg cells, suggesting that ATL originates in natural Treg cells infected with HTLV-1. Recent studies (9,10) have defined the presence of Foxp3 in ATL cells, but the oncological significance of Foxp3 in patients with ATL and asymptomatic Human T-cell leukemia virus-type 1 (HTLV-1) carriers (ACs) is controversial. Interestingly, HTLV-1, a causative retrovirus for ATL, has been reported to infect Treg cells and modulate the expression of Foxp3 (11,12,13). This suggests that Foxp3 is not only relevant as a surrogate molecule of Treg identity, but also likely
involved in the oncogenesis of ATL. Accordingly, we investigated the expression profiles of both Foxp3 mRNA and proteins in normal and leukemic T-cells expressing CD4 and CD25 in samples comprehensively collected from HTLV-1-seropositive people and ATL patients and various hematopoietic cell lines including HTLV-1-related lines. The clinical and oncological implications of alteration in Foxp3 expression were discussed.

Materials and Methods

This study was performed under the approval of the Research Ethics Committee of our institute (No. 079260113). Peripheral blood mononuclear cells (PBMC) were obtained from blood samples of 24 patients with ATL (7 smoldering, 5 chronic, and 12 acute subtypes) and 48 normal control subjects by density gradient centrifugation using Lymphoprep (AXIS-SHIELD, Oslo, Norway). Normal controls were subdivided into healthy controls uninfected with HTLV-1 (HCs) and asymptomatic HTLV-1 carriers (ACs). The subclassification of ATL was based on the criteria of Shimoyama et al. (14).

Cell lines

The cell lines used in this study included six interleukin (IL)-2-dependent ATL cell lines, ST1, KK1, KOB, SO4, LM-Y1 and OMT, two HTLV-1-infected T-cell lines, MT2 and HUT102, and twelve HTLV-1-negative T-cell lines, Jurkat, MOLT4, Raji, CA46, Ramos, U937, K562, THP1, SUDHL4, Cal-1, SKW6-4 and Daudi (15,16). Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. To culture IL-2-dependent cells, the medium was supplemented with 0.5 U/mL of IL-2 (kindly provided by Takeda Chemical Industries, Osaka, Japan).

Flow cytometric analysis of surface markers and intracellular Foxp3

Fluorescein isothiocyanate (FITC)-conjugated anti-CD25, FITC and phycoerythrin (PE)-conjugated mouse IgG1 as a negative control, and Periodimin-chlorophyll-protein complex (PerCP)-conjugated anti-CD4 monoclonal antibodies were purchased from BD Biosciences (San Diego, CA). PE-conjugated anti-FOXP3 mAb (clone 236A/E7) was
purchased from eBioscience (San Diego, CA). PBMC and Cell lines were first stained with anti-CD4 and anti-CD25 or FITC-conjugated mouse IgG1. Intracellular FOXP3 staining was achieved using a PE anti-human Foxp3 Staining set (eBioScience) according to the manufacturer's instructions. Briefly, cells were fixed and permeabilized with Fixation/Permeabilization buffer including paraformaldehyde for 30 min at 4 °C, and then stained with PE-conjugated anti-FOXP3 or PE-conjugated mouse IgG1 for 30 min at 4 °C. Labeled cells were analyzed by flow cytometry with a FACSCalibur (BD Bioscience).

**Polymerase chain reaction (PCR) quantification for FOXP3**

Total RNA was extracted using Isogen (NIPPONGENE, Toyama, Japan) according to the manufacturer's instructions. RNA was further purified by using MessageClean (GenHunter, Nashville, Germany). Total RNA (1 µg) was reverse transcribed using oligo(dT)18 primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and cDNA was synthesized. FOXP3 mRNA levels were quantified by real-time PCR using a commercially available primer set (search. LC. Heidelberg, Germany), LightCycler-Primer Set Human Fox-P3 kit, and SYBR Green I Master kit (Roche, Mannheim, Germany) with the LightCycler 3.1 system (Roche). To assure PCR quality, Porphobilinogen deaminase (PBGD) was measured in the same samples by a LightCycler-Primer Set Human PBGD (search.LC). Cycling conditions were according to the manufacturer's instructions. Since clinical samples consisted of a different mixture of Foxp3 positive and negative cell populations, to compare the expression level of only Foxp3+ cells, we adjusted by dividing the raw load by the percentage of Foxp3+ cells, using the following formula; (Foxp3 copies) / (%Foxp3+ cells) x 100

To examine full-length cDNA of Foxp3, conventional RT-PCR analysis was performed using the primer set of 5'-GCTGATCCTTTTCTGTCAGTCC-3' and 5'-GTGGAAACCTCACTTCTTGGTC-3' for Foxp3. Amplification was established by Phusion™ Flash High-Fidelity DNA Polymerase (FINZYMES, Espoo, Finland). Cycling
conditions were as follows: denaturation at 98°C for 30s, denaturing at 98°C for 10s, annealing and extension at 72°C for 45s for 35 cycles, and extension at 72 °C for 5 min for Foxp3. The polymerase chain reaction (PCR) products were resolved on a 1.5% agarose gel, then visualized by ethidium bromide staining.

**Western blot analysis and antibodies**

Cells were harvested after treatment, washed, and homogenized at 4°C in a lysis buffer (0.1% sodium dodecyl sulfate [SDS], 1% Igepal CA-630, and 0.5% sodium deoxycholate) and a protease inhibitor cocktail (Sigma, St Louis, MO). Cell lysates (30 µg) were resolved by electrophoresis on a 12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking the membrane in 10% FBS and 0.1% Tween 20 in Tris-buffered saline for 1 hour at room temperature, the blots were hybridized overnight at 4°C with primary antibodies. After hybridization with secondary antibodies conjugated with horseradish peroxidase, the immunocomplexes were visualized using an ECL Western blotting detection system (GE Healthcare, Chalfont St. Giles, United Kingdom). The analysis was performed using antibodies to an anti-FOXP3 monoclonal antibody(clone 236A/E7; eBioScience, San Diego, USA) and the monoclonal anti β-actin antibody AC-15 (Sigma).

**Immunocytochemistry for subcellular localization of Foxp3**

Cells smeared on slide glass were fixed by 4% paraformaldehyde at 4°C for 15 minutes and then stained with PE-labeled anti-Foxp3 antibodies for 2h at room temperature after permeabilization by the permeabilized commercial kit (BD Bioscience). The slides were counter-stained with Hoechst 33258 for nuclear stain and fluorescent conjugates of Lectin GS-II for selective stain of the Golgi apparatus (Molecular Probe Inc. OR, USA). Signals were fluoroscopically observed using Leica DM400B Microsystems and the fluorescence images were collected and analyzed with a Leica AF6000 Application Suite (Mannheim, Germany).
Statistical Analysis

Using the Stat View software, the Mann-Whitney U test or Student’s t-test were used to compare data between two groups, and Spearman’s rank correlation was used to examine the two groups.

Results
Flowcytometric detection of Foxp3 proteins

Foxp3 expression was evaluated in normal and leukemic CD4^+T-cells by flow cytometric analysis. Firstly, we examined the frequency of CD25^+Foxp3^+cells in CD4^+T-cells from HCs, ACs, and patients with ATL and in various hematopoietic cell lines, as shown in Fig 1. The mean percentage of CD25^+Foxp3^+cells in CD4^+T-cells was not different between HCs and ACs (3.4 ± 1.7 vs 3.1 ± 1.9), but the number of the cells per mL in ACs was significantly lower (1.5 ± 0.4 x 10^3 vs 1.1 ± 0.3 x 10^3) than that in HCs (p<0.05). There was no correlation between the percentage and aging. In patients with ATL, CD25^+Foxp3^+cells were detectable at the various frequencies from 1.5 to 84% in parallel to the presence of ATL cells defined by morphology, indicating that most ATL cells were positive for Foxp3. On the other hand, in contrast to Foxp3 expression on primary ATL cells, that of ATL-related cell lines was weak fluorescent signal with the subtle shift to the right of relative fluorescence intensity (RFI). To confirm these flow-cytometric findings of the Foxp3 stain, we studied fluoromicroscopically the subcellular localization profiles of Foxp3. As shown in Figure 2, the Foxp3 red fluoro-spots were distributed diffusely in the both cytoplasm and nucleus on primary ATL cells, and locally in the cytoplasm on ATL-related cell lines. To verify whether the localization of Foxp3 was associated to the Golgi apparatus in ATL-related cell lines, a colocalization experiment was performed using a lectin reactive for Golgi apparatus using KOB cell line. As shown in Figure 2 (KOB), the fluorescent red spots were completely overlaid with the Golgi yellow spots, indicating that Foxp3 in KOB cells was
colocalized within Golgi apparatus.

Next, according to the mutual expression profiles of CD25 and Foxp3 expression, CD4+ cells were subdivided into four fractions of CD25*Foxp3* (Treg-type), CD25*Foxp3* (activated type), CD25*Foxp3* (resting type), and CD25*Foxp3* cells, as shown in Figure 3. Most HCs showed the panel A pattern with a majority of CD25* cells co-expressing Foxp3, and most ACs showed the panel-B pattern with a majority of CD25* cells did not expressing Foxp3. On the other hand, the staining profile of primary ATL cells in ATL cases were variable, mainly a Treg-like pattern with weak CD25 and Foxp3 (panel-C), and a mixed pattern of Treg-type cells and activated type CD25* cells (panel-D and E). The intensity of CD25 in ATL cells was prone to decrease more than that of normal Treg cells. Then, to overview the relation of CD25 and Foxp3 expression in inter-cases, a twin plot graph was constructed as shown in Figure 4, displaying the characteristic distribution in each group. Particularly, ATL cases (open triangles) were distributed in three main areas, cluster a; cases with Foxp3* cells=CD25* cells, b; cases with Foxp3* cells<CD25* cells, and c; cases with Foxp3* cell>CD25* cells.

Discrepant expression of Foxp3 in the mRNA and protein

Foxp3 mRNA was quantitatively detected in all 50 samples, regardless of the presence and absence of the proteins, and widely ranged from 700.0 to 1.0 x 10^5. These amplicons amplified by our system were confirmed to be accordant to that of nts 1190 - 1353 bp (NM014009) by sequencing analysis. The quantitative data in each sample are shown in Figure 5, showing that there was no difference in the expression density among HCs, ACs, and patients with ATL, although patients with ATL tended to have a low mRNA dose. Notably, we were able to detect Foxp3 mRNA even in even non-T cell lines, such as K562, Molt4, Ramos et al., despite no or subtle detection of Foxp3 proteins being demonstrable by flow-cytometry. To confirm this discrepancy of the positive mRNA by PCR-quantification and negative protein by the flowcytometry, we
reexamined by Western blot and RT-PCR analyses for full-length cDNA. As shown in Figure 6, similarly to that of the PCR quantification, equal amounts of two isoforms of Foxp3 (one corresponding to the full-length sequence and the other lacking exon 2 (17) were detected in all samples, including non-T-cell lineage cell lines. On the other hand, WB analysis gave clear positive bands in KOB and LM-Y1 (lanes 5 and 6 in Fig.6 B) and faint band in Ramos (lane 8). As described above, Foxp3 in KOB was demonstrated within the Golgi apparatus.

These data on Foxp3 detection at the mRNA and protein levels by the different analyses in various cell lines are summarized in Table 1.

**Discussion**

Firstly, our data showed that the majority of CD4$^+$CD25$^+$T-cells in HCs were positive for Foxp3, but not all CD4$^+$CD25$^+$T-cells in ACs were positive, indicating that Foxp3 expression is not always linked to CD25 expression in normal T-cells. Namely, CD4$^+$CD25$^+$T-cells in ACs were inconsistently composed of the two populations with or without Foxp3, suggesting that the former is Treg cells and the latter is either activated T-cells or aberrant Treg cells down-regulating Foxp3 expression. What does this imbalanced expression of CD25 and Foxp3 in ACs mean? Although we could not define whether the imbalance is a cause or an effect of HTLV-1 infections, Yamano et al. (18) reported the instructive finding that Foxp3 expression in CD4$^+$CD25$^+$T-cells from HAM patients was lower than those from HCs and Tax had a direct inhibitory effect on Foxp3 expression and function of CD4$^+$CD25$^+$cells. This suggests that the imbalance of CD25 and Foxo3 expression in ACs is closely related to HTLV-1 infections. Moreover, this modulation of Foxp3 in carrier stage may be causatively implicated in the frequent complications of auto-immune-like diseases, such as atopic dermatitis, myositis, Sjogren’s syndrome and HTLV-1-associated uveitis (19). All of these clinical observations suggest that cell-mediated immunity is impaired in the HTLV-1 carriers. As
for the underlying mechanism of impaired immunity, Yasunaga et al. reported that the unusual impaired cell-mediated immunity is likely explained by a decrease in naïve T-cells and an increase in activated memory T-cells (20). Although they did not examine Foxp3 expression at that time, the two points of our and their data have something in common. Namely, it is considered that the biological property of HTLV-1 through Tax misleads the immune reaction into immune disturbance with an imbalance in Treg and related cells. In the future, a mutual study of Foxp3 and HTLV-1 carriers could provide a new insight into a clinical role of Foxp3 as well as HTLV-1-associated immune-pathology. Recently, Toulza et al. (21) reported the interesting findings that HTLV-1 infections is associated with abnormal expression of Foxp3 in circulating CD4+ cells and the efficacy of immune control of HTLV-1 infection is mainly determined by the CD4+Foxp3−Tax+ T-cell population.

As for leukemic CD4*CD25*T-cells, our data from ATL cells and ATL-related cell lines showed that Foxp3 expression is heterogeneous and aberrant, including intra- and inter-case heterogeneity in intensity, inconsistency with CD25 expression, and a discrepancy in the mRNA and its protein expression intensity. In particular, the difference in Foxp3 expression profiles between primary ATL cells and ATL-related cell lines is distinct and instructive. That is, the expression of CD25 and Foxp3 has an inverse relation; primary ATL cells are characteristic of weak CD25 and high Foxp3 intensity, while ATL-related cell lines have the distinct features of strong CD25 intensity and either negative or down-regulated Foxp3. The subcellular localization of Foxp3 also is different between them, mainly only cytoplasmic in the cell lines. This heterogeneous expression of Foxp3 in ATL attracts our attention on the origin of ATL cells and the implication of Foxp3 in ATL cell biology. With regard of the cell origin, although ATL originates in mature helper/inducer T-cells, the recent focus has been directed at Treg cells because most ATL cells have the CD25*Foxp3*phenotype. Matsubara et al. (11) speculates on two possible mechanisms. One is a Treg cell origin, in which
HTLV-1-infected Treg cells transform and modulate Foxp3 expression as a result of tumorigenesis, resulting in a Treg phenotype with heterogeneous Foxp3 expression. The other is an HTLV-1-infected CD4⁺CD25⁻T-cell origin, in which leukemic cells adaptively acquire the functional and phenotypical features similar to Treg, namely inducible Treg-like feature. If ATL cell is derived from primary Foxp3-expressing natural Treg cells, Foxp3 expression should be constitutive. However, our data that not all ATL cells are Foxp3⁺cells and Foxp3 is ubiquitously detectable in various cell lines at least at the mRNA level appears to support the latter. Although the present study failed to identify that Tax is implicated in Foxp3 expression as an inducible factor, evidence is accumulating that Treg-like features are easily and frequently induced under pathological conditions in humans (22). However, at present it is lacking in decisive evidence. Accordingly, this does not rule out the other theory, and the two possibilities do not seem to be mutually exclusive in the development of ATL.

Next, as for the oncological implication of Foxp3, our data showed that Foxp3 is not specific for the disease of ATL as well as the linearity, because Foxp3, at least at the mRNA level, was detected in various cell lines derived from B-cells, myeloid cells and dendritic cells. Indeed, some cases of T-chronic lymphocytic leukemia (12), cutaneous T-cell lymphoma and T-cell lymphoma carrying NPM-ALK (23) reportedly were positive for Foxp3. Furthermore, Foxp3 was reported to be positive even in solid tumors, such as pancreatic carcinomas (24). This ectopic expression beyond T-linearity suggests the some implication of Foxp3 in tumorigenesis. From this point of view, a study by Hinz et al (24) is interesting in that Foxp3 ectopically expressed in cancer cells acts as a player of molecular mimicry as Treg function, representing a new mechanism of immune invasion in cancer. Similarly, our previous study showed that primary ATL cells expressing Foxp3 have the ability of anti-proliferative suppression for CD4⁺T-cells in cell-to-cell contact manner (25). This Treg-like function in ATL cells appears to play a role in protecting themselves from immune attack and confers a survival advantage on
Conclusively, Foxp3 protein, but not mRNA, is basically potent at discriminating a subset of Treg cells from the population of normal CD25\(^+\)T-cells, whereas the modulation of Foxp3 in malignant cells assuredly implicates something involved in tumor pathology, indicating the potent capability as a new diagnostic and therapeutic molecule.

**Acknowledgement**

This study was supported financially by Japan Society for the Promotion of Science (no.17390165 and no.18659163).

**References**


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Figure Legends

Figure 1. The frequency (%) of Foxp3-positive cells in CD4\(^+\)T-cells among healthy controls (HCs), HTLV-1-infected asymptomatic carriers (ACs), patients with ATL and cell lines by flowcytometric analysis. That (%) of ATL cases was correlated to the number of leukemic ATL cells detected by morphology.

Figure 2. The subcellular localization of Foxp3 in primary ATL cells, KOB cells and K562 cells by immunocytochemistry.

A; Foxp3 staining (PE), B; Hoechst stain, C; lectin GS-II Golgi stain, D; Merge

Although primary ATL cells and KOB cells shown here were positive for Foxp3 by flowcytometric and Western blot analyses, the immunocytochemistry revealed the different subcellular localization, namely mainly nuclear in primary ATL cells and mostly cytoplasmic in KOB. K562 cells; Negative control.

Figure 3. Representative triple staining patterns for CD4, CD25, and Foxp3 antigens.

%; positive percentage for either Foxp3 or CD25 within CD4\(^+\)T-cells.

(Panel A); a representative case of HCs showing that most CD25\(^+\)T-cells co-expressed Foxp3 (Treg type).

(Panel B); a representative case of ACs showing that most CD25\(^+\)T-cells did not expressed Foxp3, namely they were activated CD25\(^+\)cells.

(Panels C, D, and E); variable ATL patterns showing heterogeneous Foxp3 expression inconsistent with CD25 expression in inter- and intra-case.

Figure 4. A twin dot plot graph of CD25\(^+\)cells (%) and Foxp3\(^+\)cells (%) in each case.
ATL (open triangle) is heterogeneously distributed with the main three clusters (broken line circles), while ATL-related cell lines (solid triangle) are concentrated in one area of CD25$^{\text{high}}$ and Foxp3$^{\text{negative}}$.

HCs and ACs (open and solid circles) are localized at the same area, but ACs are prone to a shift toward the left side, indicating that the number of CD25$^{+}$ cells is large compared to that of Foxp3$^{+}$ cells.

Figure 5. Comparison of the adjusted level of Foxp3 mRNA expression quantified by RT-PCR quantification among HCs, ACs, patients with ATL, HTLV-1-related T-cell lines, and HTLV-1-uninfected hematopoietic cell lines.

No significant difference was observed among HCs, ACs and ATL patients, but the expression levels in the two groups of cell lines were significantly lower than those in the remaining three groups (the Mann-Whitney test).

Figure 6. Conventional RT-PCR analysis for Foxp3 mRNA (A) and Western blot analysis (B).

A: The expected two bands (corresponding to full-length cDNA and splicing form) are observed in all samples tested.

B: Positive bands were observed in lanes 1, 2, 3, 5, and 6.

PC; positive control derived from the manufacturer, HC; healthy control.
Figure 2
Figure 3

(A) HCs

1.6%

5.2%

(Treg type)

1.5%

(Activated type)

(B) ACs

0.6%

2.5%

(C) ATL

23.6%

60.2%

(D) ATL

2.0%

58.2%

(E) ATL

0.4%

15.7%

Figure 4

- 19 -
Figure 5

Figure 6

A: RT-PCR for Foxp3 mRNA

B: WB for Foxp3 protein
Table 1 Summary of Foxp3 and CD25 expression profile in various cell lines.

Although Foxp3 mRNA was detected in all cell lines tested, Foxp3 protein was only detected in only KB & LMY1, all of which were established from ATL cells.

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<td>3.6**</td>
<td>4.4</td>
<td>(+)</td>
</tr>
<tr>
<td>HC</td>
<td></td>
<td>4.4**</td>
<td>4.0</td>
<td>(+)</td>
</tr>
<tr>
<td>Primary ATL cells</td>
<td></td>
<td>82.0**</td>
<td>4.3</td>
<td>(+)</td>
</tr>
</tbody>
</table>

FCM (%) and RFI : flow cytometric positive rate (%) and fluorescence intensity relative to the negative control (RFI).

WB : (+), positive band; (−), negative band; (+/−), faint band.

Tax : PCR quantitative data was categorized into the high, moderate and high degree.