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Citation	Microbes and Infection, 11(12), 964-972 https://doi.org/10.1016/j.micinf.2009.06.007
Issue Date	2009-10
Doc URL	https://hdl.handle.net/2115/39626
Type	journal article
File Information	MI11-12_p964-972.pdf



Functional analysis of Foxp3 and CTLA-4 expressing HTLV-1-infected cells in a rat model

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia (ATL). Some ATL cells express Foxp3, which is known as regulatory T cell (Treg cell) specific transcription factor. It is suggested that Treg cell like suppressive activity of Foxp3 expressing ATL cells is associated to ATL development and related immunodeficiency. To develop an HTLV-1 model system that enables to investigate the association of Treg function in ATL progression, we examined the expression of Foxp3 and CTLA-4, Treg cell-associated factor, in established HTLV-1-infected rat cell lines and their regulatory function. We found the expression of Foxp3 in 9 of 21 and CTLA-4 in 10 of 19 HTLV-1-infected rat cell lines. Moreover, some of the Foxp3 and/or CTLA-4 expressing cell lines suppressed proliferation of naïve T cells that were stimulated with anti-CD3 antibody. Particularly all Foxp3⁺ CTLA-4⁺ cells showed the suppressive activity. Our data suggest the usefulness of our rat model systems for further analysis of the role of Treg cell-associated factors on the development of ATL and related immunodeficiency *in vivo*.

Keywords: HTLV-1; ATL; Foxp3; CTLA-4; animal model

1. Introduction

Natural regulatory T cell (Treg cell) was identified as the $CD4^+ CD25^+ Foxp3^+$ T cell population that was developed in thymus [1]. Treg cells suppress the proliferation of TCR-activated naïve T cells so that this function contributes to maintain immune self-tolerance by suppressing self-reactive T cells. It has been reported that Treg cells use several suppressive mechanisms such as cell contact dependent suppression, regulatory cytokines and Granzyme B and perforin-mediated cytotoxicity depending on the circumstances of their surroundings [1, 2]. Foxp3 has been generally agreed to be a crucial transcription factor that regulates the functions and development of the Treg cells. Exogenous expression of the *Foxp3* gene can convert naïve $CD4^+CD25^-$ T cells into $CD4^+ CD25^+$ regulatory T like cells, and induce Treg cell associated molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) [3]. CTLA-4 is constitutively expressed on Treg cells [4], but its involvement in the suppressive activity of Treg cells has been under debate [5, 6]. Recently CTLA-4 activity for Treg suppressive function *in vivo* and *in vitro* has been demonstrated using Treg specific CTLA-4 conditional knockout (KO) mouse [7].

Adult T cell leukemia (ATL) is neoplastic disease etiologically linked to Human T cell leukemia virus type 1 (HTLV-1). ATL patient causes immunodeficiency associated with

defective cellular immunity [8, 9]. Most of ATL cells exhibit CD4⁺ CD25⁺ T cell phenotype. Several group analyzed the relationship of the ATL cells with Treg cells to explain the reasons for the immunodeficiency in ATL patients. Some ATL cells and HTLV-1-infected human cells express Foxp3 and related molecules, such as CTLA-4 and GITR [10-13]. Moreover, some ATL cells have been reported to suppress T cell proliferation like T reg cells, suggesting the association of Foxp3 expression with the immune escape of ATL cells and immunodeficiency [14].

To analyze the contribution of Foxp3 and Treg associated molecules to development of ATL in more detail, suitable animal model is required. Previously, we established various rat models for HTLV-1 infection including inbred and immunocompromised rats, which allowed the investigation about the ability of T cells to suppress HTLV-1-related malignancy [15, 16]. Moreover, we identified CRM1, a cellular cofactor of Rex that exports viral mRNA from the nucleus to the cytoplasm, to be a major factor, which restricts efficient replication of HTLV-1 in rats [17-19]. Human CRM1 (hCRM1) transgenic (Tg) rat supports replication of HTLV-1 in T cells at the level similar to human T cells *ex vivo* [20].

Since some of the rat HTLV-1-infected cell lines, which we previously established from CRM1 Tg and Wt rats, express CD25, the viral proteins [20], and possess tumorigenic

potency [15], it is interesting to characterize the HTLV-1⁺ rat cells in relation to Foxp3 and Treg cell associated molecules. In this paper, we report Foxp3 and CTLA-4 expression in the HTVL-1-infected rat cell lines for the first time. Moreover some of these cells showed Treg cell like activity that suppresses the proliferation of naïve T cells, which were stimulated through T cell receptor. Our data that indicate the functional similarity between Foxp3 and/or CTLA-4 expressing HTLV-1-infected rat cell lines and human ATL cells suggest the usefulness of our rat model systems for further analysis of Foxp3 and CTLA-4 contribution to development of ATL and related immunodeficiency.

2. Materials and Methods

2.1. Cells

HTLV-1-immortalized cell lines from Wt or Tg rats were established by cocultivating thymocytes or splenocytes with human HTLV-1 producing cell line MT-2, which had been treated with 50 µg/ml of mitomycin C containing medium for 30 min at 37°C. These cells were maintained in the medium supplemented with 10 U/ml of interleukin (IL)-2 (PEPROTECH EC) at the beginning of co-culture. Some cell lines were eventually freed from exogenous IL-2 [15]. We noted “(+)” or “(-)” following cell lines names to distinguish IL-2 dependent or independent stage of each cell line. The HTLV-1-infected rat T cell line FPM1

BP [16] has been described previously.

2.2. Western blotting

Cells were lysed in ice-cold extraction buffer (10 mM Tris-HCl [pH 7.4], 1 mM MgCl₂, 0.5% NP-40) containing protease inhibitor cocktail (Complete mini; Roche Diagnostics). The protein concentration of each sample was determined using a BCA protein assay kit (QB PERBIO). The cell lysates were treated with DNase I (Takara), and then dissolved in sample buffer. The same amounts (approximately 25 µg) of cell lysates were subjected to SDS-PAGE. Following electrophoresis, proteins were transferred to a PVDF membrane and probed with anti- rat Foxp3 (FJK-16s; eBioscience) and anti-β-actin (AC40; Sigma) antibodies followed by secondary antibodies conjugated to horseradish peroxidase. Proteins were visualized by staining with ECL+ (GE healthcare) followed by evaluation with the LAS-1000 plus system (Fuji film) using Image Gauge Version 3.4 software (Fuji film).

2.3. Quantitative RT-PCR of mRNAs

Total RNA was extracted using the Absolutely RNA® Miniprep Kit (Stratagene) and treated with RNase-Free DNase I (Stratagene) to minimize contamination of chromosomal DNA. The RNA concentration was measured by absorbance at 260 nm, and purity was ascertained by the OD₂₆₀/280 ratio.

The *foxp3*, *HBZ* and *g3pdh* mRNA was quantified by real-time PCR using LightCycler PCR instrument (Roche Diagnostics). Quantitative RT-PCR of *foxp3* and *g3pdh* were performed by LightCycler RNA amplification Kit Hybridization Probes (Roche Diagnostics) with the following primer pairs and hybridization probes: *foxp3* forward primer 5'-CAG CAC CTT TCC AGA GTT CTT C -3' and the reverse primer 5'- GCG TGT GAA CCA ATG GTA GAT T-3', the hybridization probes 5'-CCC TTT CAC CTA TGC CAC CCT CAT CC-(FITC)-3' and 5' - (LCRed640)- TGG GCC ATC CTG GAA GCT CCA GAG AGG-3'; *g3pdh* forward primer 5'-AAG GTC ATC CCA GAG CTG AA -3' and the reverse primer 5'-ATG TAG GCC ATG AGG TCC AC-3', the hybridization probes 5'-TCC CAT TCT TCC ACC TTT GAT TGC TGG G- (FITC)-3' and 5'-(LCRed705)-TGG CAT TGC TCT CAA TGA CAA CTT TGT GAA GCT CA-3'. *HBZ* mRNA was quantified using LightCycler RNA amplification Kit SYBR Green 1 (Roche Diagnostics, Mannheim) with the following primer pairs: forward primer 5'-ATG GCG GCC TCA GGG CTG TTT CGA TGC TT-3' and the reverse primer 5'-CTG CCG ATC ACG ATG CGT TT-3'. RNA (200ng) was subjected to RT-PCR reaction according to the instruction manual. The optimum concentration of $MgCl_2$ was 7mM for *foxp3* and *g3pdh*, and 5mM for *HBZ* amplification. 20 μ l of a RT-PCR mixture in a capillary tube containing were subjected to RT-PCR reaction including incubation for

30min at 55°C and 30 sec at 95°C, and then 35 cycles of 5 sec at 95°C and 15 sec at 60°C and 9 sec at 72°C. The copy numbers of cDNA in the samples were estimated based on a standard regression curve using the LightCycler Software version 3 (Roche Diagnostics). The standard curve was obtained by amplifying 1×10^3 to 1×10^7 copies of the Foxp3 and G3PDH cDNA fragments with the corresponding pair of primers. The copy numbers of Foxp3 cDNA were normalized by dividing with those of the G3PDH cDNA in the same samples.

2.4. Analysis of cell surface markers

Expression of cell surface markers was examined by flow cytometry. Briefly, 1×10^6 of cells were stained with various mouse monoclonal antibodies for 30 min on ice, washed three times with 1% BSA in PBS, and then stained with FITC-conjugated goat anti-mouse IgG+IgM antibody. After being washed, the cells were fixed with 1% formalin in PBS, prior to analysis by FACScalibur (Becton Dickinson). Antibodies to rat CD3, CD4, CD25, CD28 and major histocompatibility complex class1 (MHC-1; RT1.A) were purchased from BD Pharmingen Co. Anti-rat CD5 and CTLA-4 (CD154) antibodies were from eBioscience Co.

2.5. Detection of TGF- β 1 in culture supernatant

HTLV-1⁺ cells (10^5 /well) were cultured in 24-well flat-bottom plates for 4 days. The amount of TGF- β 1 in the culture supernatant was quantified using TGF- β 1 enzyme-linked

immunosorbent assay (ELISA) (R&D Systems).

2.6. T cell proliferation analysis

To examine the ability of HTLV-1-infected rat cells to suppress the proliferation of T cells, F344 rat splenocytes (7.5×10^6 cells) were labeled with 10 μ M CFSE solution (Invitrogen) according to the manufacture's manual, and stimulated by plate-coated anti-rat CD3 mAb (G4.18 eBioscience) and 0.5 μ g/ml anti-rat CD28 mAb (JJ319 eBioscience) in 10U/ml of IL-2 containing medium. Then, 5.0×10^6 of mitomycin C (50 μ g/ml)-treated HTLV-1-infected rat cell lines were added in the culture. T cell proliferation was evaluated by calibrating the CFSE dilution with FACScalibur 3 days after commencing the co-culture.

3. Results

3.1. Foxp3 expression in HTLV-1-infected rat cell lines

To assess the expression of Foxp3 in rat HTLV-I-infected cell lines that we established previously, we performed Western blot analysis (Fig. 1) and RT-PCR (Fig. 2A). These analyses revealed that 9 of 21 cell lines expressed foxp3 mRNA and protein (Fig. 1, 2A and Table1). Next, we compared the expression level of foxp3 mRNA among the cell lines. Quantitative RT-PCR analysis revealed that foxp3 mRNA expression level was variable in each cell line (Fig. 2B and Table 1), but parallel to protein level (Fig. 1 and 2B). Interestingly,

we observed the enhanced expression of *foxp3* mRNA in IL-2-dependent cell lines compared with their factor-independent counterparts that were cultured in the absence of IL-2 (Fig. 2B).

To examine the effect of IL-2 on the Foxp3 expression, we cultured the IL-2-independent cell lines in the presence of IL-2 for 3 weeks and compared the Foxp3 expression levels. As shown in Fig. 2C, Foxp3 expression was not restored in the presence of IL-2.

Next, we addressed whether the expression of Foxp3 is associated with HBZ (HTLV-1 bZIP factor) that is a nuclear protein encoded with minus strand RNA of HTLV-1. HBZ is detected in majority of ATL cells whereas Tax expression is often repressed or lacked [21]. As the case of ATL cells, the HBZ mRNA was detected in all of the subjected rat HTLV-1⁺ cell lines albeit with a great variability in the expression level of each cell line (Fig. 2D and Table 1). However, there were no association between HBZ and *foxp3* mRNA expression (Fig. 2E).

3.2. Characterization of Foxp3 expressing cell lines

To characterize the rat Foxp3 positive cell lines in more detail, we examined the production of TGF- β 1 and surface markers of the established cell lines. The TGF- β 1 concentration of culture medium could be detected from 2 of Foxp3 positive and 2 of Foxp3 negative cell lines (Fig. 3A). These data indicate that there are no correlation between TGF- β 1 secretion and Foxp3 expression. As shown in Fig. 3B, all subjected cell lines were CD25 and MHC class 1

positive, whereas CD4 and CD3 expression patterns were variable among the cell lines. Only 2 cell lines exhibited CD4⁺ CD25⁺ phenotypes like regulatory T cell, whereas other 4 cell lines did not express surface CD4. We also examined the expression of CD5 and found that 8 of 10 Foxp3 positive cell lines expressed surface CD5 (Fig. 4A), suggesting significant correlation between surface CD5 and Foxp3 expression (Fig. 4B).

CTLA-4 (CD154) is known as one of the regulatory T cell markers controlled by Foxp3 and suggested its relation to Treg suppressive function [4, 7]. So we addressed the relation of CTLA-4 to Foxp3 expression in HTLV-1-infected rat cell lines. As shown in Fig. 4C, 10 of 18 cell lines exhibited weak CTLA-4 expression. However we did not find the enhanced expression of CTLA-4 in Foxp3 positive cell lines.

3.3 The immunosuppressive activity of Foxp3 positive cell lines

Next, we examined whether Foxp3 and/or CTLA4 expressing HTLV-1-infected rat T cell lines suppress the proliferation of naïve T cells. To assess the suppressive function, rat T cell lines were co-cultured with CFSE-labeled naïve T cells that were stimulated by anti-CD3 monoclonal antibody (mAb). Suppressive effects of HTLV-1-infected cell lines were evaluated by CFSE dilution in the naïve T cells. As shown in Fig. 5, proliferation of naïve T cells was significantly suppressed by 4 of 6 Foxp3 positive cell lines and 2 of 6 cell lines that

were Foxp3 negative but express CTLA-4, Treg associated molecule (Fig. 4C). Notably, all 3 Foxp3⁺ CTLA-4⁺ cell lines showed the suppressive activity (Fig. 4C and 5). These results demonstrate that some HTLV-1-infected rat cell lines have suppressive function as the case of human ATL cells, and suggest that Foxp3 and CTLA-4 may be involved in the suppression.

4. Discussion

Some ATL cells have been reported to express higher level of Foxp3 and exhibited Treg cell like activity [11, 14]. Here we show that some Foxp3 positive HTLV-1-infected rat cell lines, like Treg cells, suppress TCR-stimulated naïve T cell division (Fig. 5). This suppressive function may be dependent on cell-to-cell contact, because the culture medium of suppressive cell lines didn't affect the T cell division (data not shown), consistent with the previous report that described contact dependent Treg like activity of ATL and HTLV-1-infected cells [14]. Therefore, our rat model is useful to examine a role of the suppressive activity of HTLV-1-infected cells in HTLV-1 infection and ATL development.

We found 3 of 4 Foxp3⁺ suppressive cell lines expressed CTLA-4 (Fig. 4C). Moreover, we identified two suppressive cell lines expressing CTLA-4 but not Foxp3 (Fig. 4C and 5), suggesting the association of cellular CTLA-4 expression with suppressive function. Although CTLA-4 contribution to Treg cell function is controversial because Foxp3⁺ Treg cells

differentiated in CTLA-4 KO mice and suppress naïve T cell proliferation [5], it has been recently demonstrated that Treg cells derived from CTLA-4 conditional KO mice show impaired suppressive function [7]. Furthermore, the suppression mechanism has been reported to be mediated by CTLA-4 inducing down-regulation of CD80 and CD86 on antigen presenting cells (APC) [7]. However, in our experiments co-stimulatory signal for naïve T cells was mediated by anti-CD28 mAb but not by APC (Fig. 5). Thus, it is possible that CTLA-4 affected CD80 and CD86 on stimulated T cells directly to suppress their proliferations. This notion is consistent with facts that the expression of CD80 and CD86 is induced by activation of human and mouse T cells and that T cells from CD80/CD86-deficient mice are resistant to suppression by Tregs [6, 22]. Further experiments should reveal the role of CTLA-4 in Treg cell like function of HTLV-1- infected rat cells more clearly.

It has been reported that CTLA-4 expression is normally induced by T cell activation [6] or exogenous Foxp3 gene transduction [3]. In ATL cells, there is positive correlation between foxp3 mRNA expression levels and percentages of CTLA-4 positive leukemic cells [12]. On contrary, we found some CTLA-4⁻ Foxp3⁺ and CTLA-4⁺ Foxp3⁻ cell lines (Fig. 4C and D), suggesting that the Foxp3 expression is not enough to induce CTLA-4 expression and that

Foxp3-independent CTLA-4 induction pathway(s) may be present in some HTLV-1-infected rat cell lines.

Several pathways for induction of Foxp3 has been identified in human and mouse Treg cells [23]. Particularly, IL-2 and TGF- β has been reported as the crucial factors [23]. When we measured the concentration of TGF- β in the culture medium of the HTLV-1-infected rat cell lines, we did not find any correlation between expressions of TGF- β and Foxp3 (Fig. 3A), indicating less contribution of TGF- β to Foxp3 expression in our rat model system. On the other hand, Foxp3 expression was higher in the IL-2 dependent HTLV-1⁺ rat cell lines and then decreased or completely disappeared in the IL-2 independent cells (Fig. 1 and 2B), suggesting a substantial role of IL-2 in maintaining the expression of Foxp3 in the HTLV-1-transformed rat cell lines. It is consistent with the report describing that IL-2 promotes Foxp3 expression in human Treg cells [24]. It has been also demonstrated that the induction of Foxp3 expression is dependent on STAT3/STAT5-associated signal transduction pathway [24]. Moreover, STAT5 has been reported to be activated in the HTLV-1-transformed human cells [25]. Taken together, we propose that IL-2 induces the Foxp3 via STAT dependent way in the HTLV-1-infected cell, especially at the IL-2 dependent stage during the cellular transformation. However, there were some IL-2 dependent HTLV-1-infected cell lines

that do not express Foxp3 (Fig. 1, 2A and B), suggesting the requirement of other factor(s) for the induction of Foxp3.

CD5 is known as a T cell marker and the negative regulator of TCR-mediated signal transduction [26]. Sakaguchi et al. demonstrated that depletion of the CD5^{high} T cell population also depleted majority of CD4⁺ CD25⁺ T cells simultaneously [27]. Recently, the inhibitory role of CD5 has been reported in the murine T reg cell suppressive function [28]. In our data, CD5 expression was significantly correlated with Foxp3 expression in HTLV-1-infected cells (Fig. 4A, B). One of CD5⁻ Foxp3⁺ cell line FWS1-27(-) exhibited suppressive function but not in another cell line FCCT13-1(+). Moreover, a CD5⁺ Foxp3⁻ cell line FWT1(+) could not suppress the naïve T cell proliferations (Fig. 5). Taken together, the results suggest that there is no apparent association between CD5 expression and Treg cell like suppressive function in our system.

Previous reports identified some Foxp3⁺ ATL cells that could not suppress the proliferation of T cells [14]. Similarly, in our rat system there are some Foxp3 positive cell lines, which lack the immunosuppressive function (Fig. 5). These results indicate that Foxp3 may be essential but not sufficient for the T reg like suppressive activity of HTLV-1-infected cell lines. Recent studies have demonstrated transcription factors, NF-AT [29], and AML1/Runx1 [30],

are involved in the induction of suppressive function of Treg cells in concert with Foxp3. These factors may also associate with Treg cell like function of the HTLV-1-infected cell lines. Both types of Foxp3 expressing HTLV-1-infected cell lines that do and do not suppress proliferation of naïve T cells (Fig. 5) may serve for advanced experiments that are focused on NF-AT and/or AML1/Runx1 to reveal the relationships of Foxp3-associated intracellular molecules to Treg cell like functions of HTLV-1-infected cell lines and ATL cells.

There are several reports that suggest relation of Foxp3 expression to ATL progression [13]. Namely, ATL cells or HTLV-1-infected cells that acquire Treg cell like function invoked by Foxp3 may impair the cellular immune reactions to cause exacerbation of HTLV-1-associated diseases. However, there were no useful models to analyze the association of Foxp3 expressing HTLV-1-infected cells with ATL or related immunodeficiency in vivo. Here we identified four HTLV-1 infected cell lines with Foxp3 expression, which have immunosuppressive function. Furthermore, we found three Foxp3⁺ CTLA-4⁺ and two Foxp3⁻ CTLA-4⁺ cell lines that suppress proliferation of naïve T cells. Our data suggest that the cells expressing Treg cell-associated factors such as Foxp3 and CTLA4 are involved in the suppressive functions. Since rat model allows in vivo experiments, the present results may provide the basis for further studies on the relationships of Foxp3 with HTLV-1-associated

pathogenesis.

5. Acknowledgments

We thank A. Hirano, N. Mizuno for excellent technical assistance. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) and the Ministry of Health, Labour and Welfare (Japan) and the Japan Society for the Promotion of Science (Japan).

6. References

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Legends of figures

Fig. 1. Foxp3 expression in established rat HTLV-1 transformed cell lines. Western blot analysis shows the relative levels of Foxp3 in each cell line. Splenic CD4⁺ T cell extract was used as positive control, epithelial cell line FPM-SV/Tax derived from F344 background rat used as negative control. Each protein level was determined on western blots containing 25 µg of total protein per lane.

Fig. 2. Foxp3 and HBZ mRNA expression in established cell lines. (A) Detection of the foxp3 mRNA in cell lines by RT-PCR. RNA extracted from each cell line was subjected to RT-PCR with primers for Foxp3 and with primers for G3PDH as an internal control. (B) The expression level of foxp3 mRNA was measured by quantitative real-time RT-PCR. The copy number of synthesized Foxp3 cDNA was normalized by dividing with the copy number of synthesized G3PDH cDNA in the same sample. Black and grey bar indicate IL-2 dependent and independent cell lines, respectively. (C) Foxp3 protein (upper panel) and mRNA (lower panel) expression of IL-2 independent cell lines that cultured with (+) or without (-) IL-2 (10 U/ml) for 3 weeks. (D) The expression level of HBZ mRNA was measured by real-time RT-PCR. The copy number of synthesized HBZ cDNA was normalized by dividing with the copy number of synthesized G3PDH cDNA in the same sample. Black and white bar indicate

Foxp3 positive and negative cell lines, respectively. The negative control did not show any signals. (E) Correlation between the relative expression of Foxp3 and HBZ mRNAs in the subjected 18 cell lines.

Fig. 3. TGF- β 1 and surface marker analyses of Foxp3 positive or negative cell lines. (A) TGF- β 1 levels in the supernatant of 4-day cultures were quantified by ELISA. (B) The expression of surface antigen CD3, CD4, CD5, CD25, CD28 and MHC1 was analyzed by flow cytometry. Solid histograms show the cells stained with each marker specific monoclonal antibody, and open histograms show the cells stained with control mouse IgG.

Fig. 4. Surface CD5 and CTLA-4 expression of Foxp3 positive or negative cell lines. The expression of surface CD5 (A) and CTLA4 (C) was analyzed by flow cytometry. (A) Cells were stained with anti-rat CD5 (grey) or control (black) antibodies. (C) Cells were stained with the rat CTLA4 (grey) or control (open) mouse IgG. The positive rate of CD5 (B), or CTLA4 (D) expression in Foxp3 positive or negative cell line group. Black area indicates positive rate in each group, and white area indicates CD5 negative rate. The statistical significance of differences was determined by chi square test.

Fig. 5. Suppressive function of Foxp3 and/or CTLA-4 expressing HTLV-1-infected cell lines to stimulated naïve T cells. (A) Suppressive activity of HTLV-1-infected rat cell lines was

estimated based on the percentage of proliferated CFSE-labeled T cells. (*) indicates the $p < 0.05$ compared with CD3 mAb control. (B) Profiles of proliferated CFSE-labeled T cells after co-culture with indicated cell lines (dark histograms) and CD3 mAb control (thin histograms) are presented. M1 region was gated for non-divided T cells estimated with non-stimulated control and M2 region was gated for proliferated cells.

Table 1. Established cell lines and Foxp3 or HBZ expression

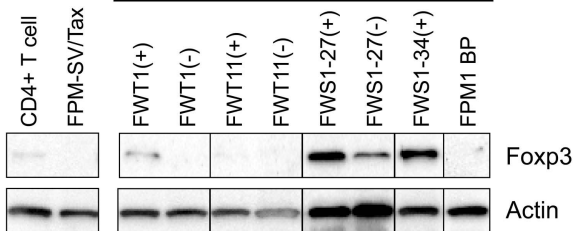
cell line	Dependent or independent ^a :	Presence or absence of ^b :	Detected or not ^c :		Relative mRNA expression values to G3PDH	
			Foxp3 Detection method:			
	IL-2 dependency	hCRM1	Western blot	RT-PCR	Foxp3	HBZ
FWT1(+)	+	–	+	+	0.112	0.060
FWT1(–)	–	–	–	–	0.024	1.394
FWT11(+)	+	–	+	+	0.047	0.045
FWT11(–)	–	–	–	–	0.000	0.068
FWS1(–)	–	–	N.S	–	N.S	N.S
FWS1-27(+)	+	–	+	+	0.056	0.475
FWS1-27(–)	–	–	+	N.S	0.026	1.239
FWS1-34(+)	+	–	+	N.S	0.342	0.382
FPM1 BP	–	–	–	–	0.000	0.112
FCMT1(+)	+	+	+	+	0.115	1.078
FCMT1(–)	–	+	–	N.S	0.000	0.436
FCMS1(–)	–	+	+	+	0.068	1.688
FCMT18(+)	–	+	–	–	0.000	0.429
FCMT18(–)	–	+	N.S	–	0.000	0.113
FCMS18(–)	–	+	–	–	0.000	N.S
FCMT27(–)	–	+	N.S	–	N.S	N.S
FCCT13-1(+)	+	+	+	+	0.111	2.056
FCCT13-1(–)	–	+	–	–	0.000	8.260
FCCT13-2(+)	+	+	+	N.S	0.040	2.076
FCCS13-1(+)	+	+	–	–	0.000	1.630
FCCS13-1(–)	–	+	–	–	0.000	2.943
FCCS13-2(–)	–	+	N.S	–	N.S	N.S

a +, IL-2 dependent stage; -, IL-2 independent stage

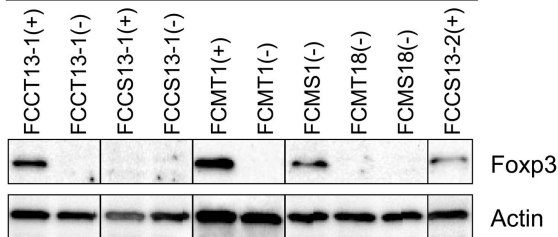
b +, Positive; -, Negative

c +, Detected; -, Not detected; N.S, Not subjected

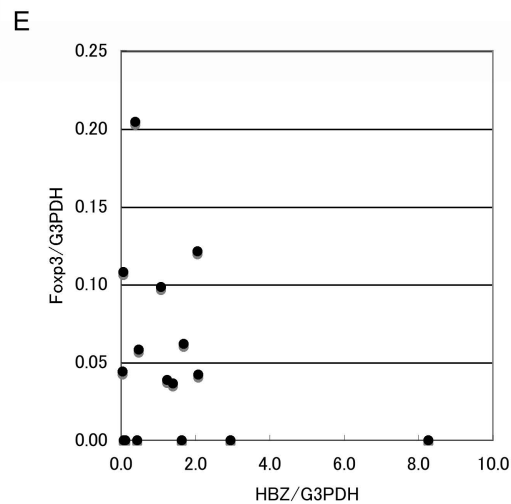
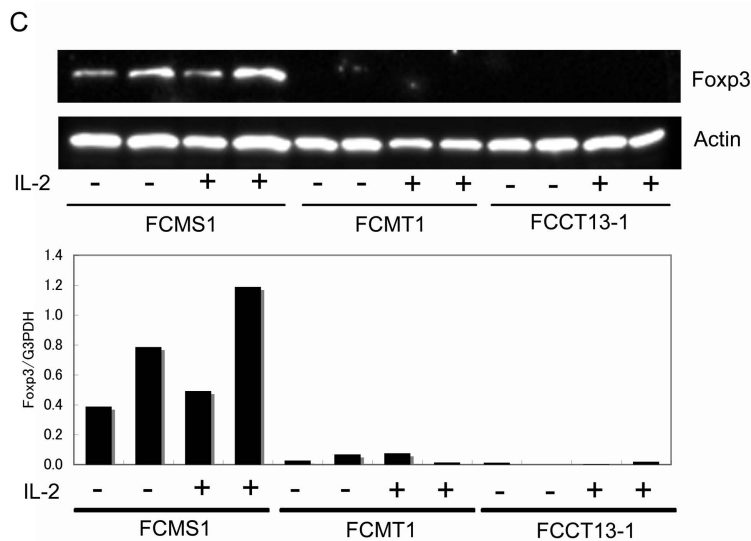
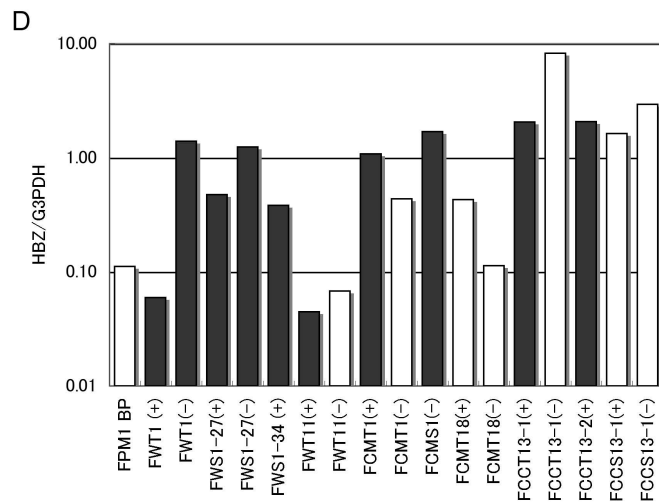
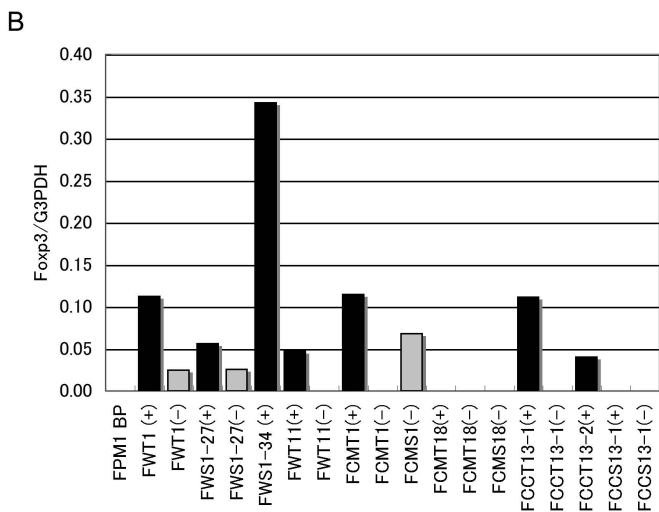
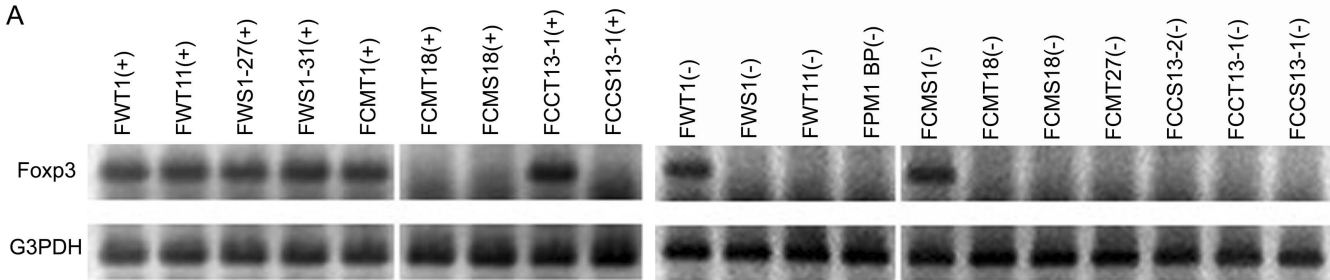
Wt Cell lines



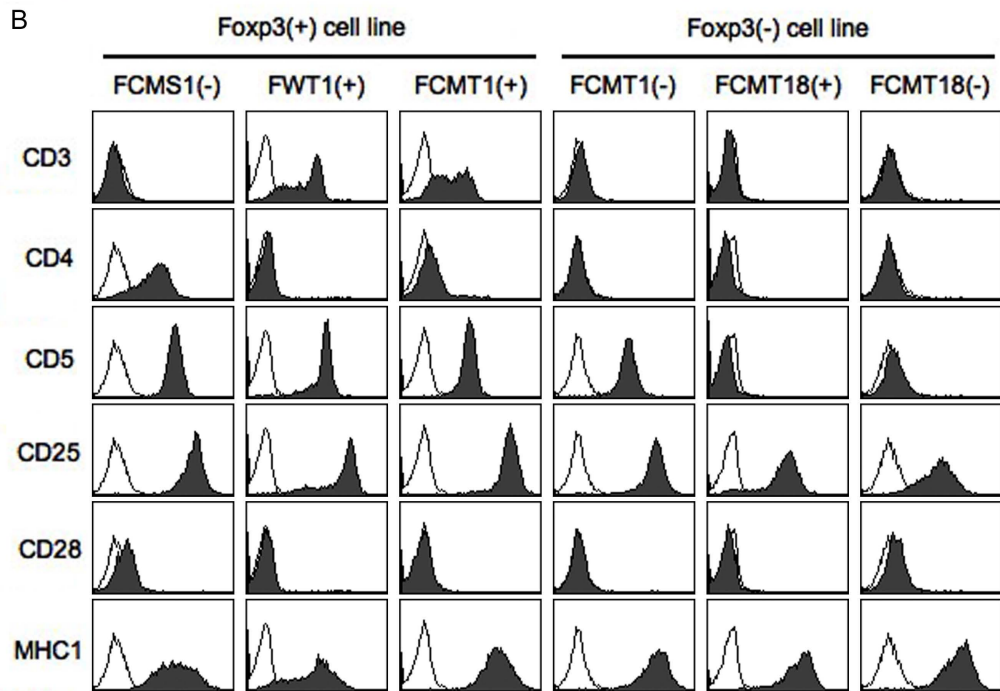
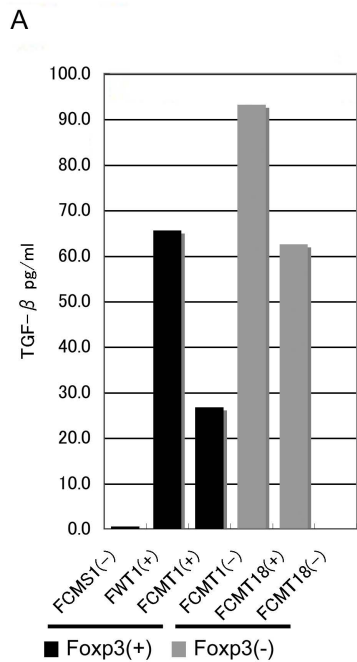
hCRM1 Tg Cell lines



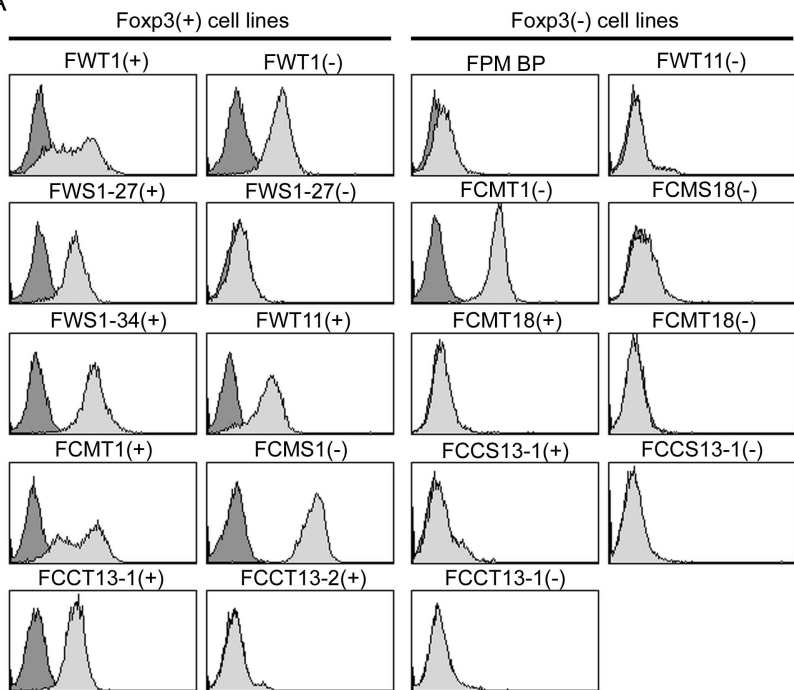
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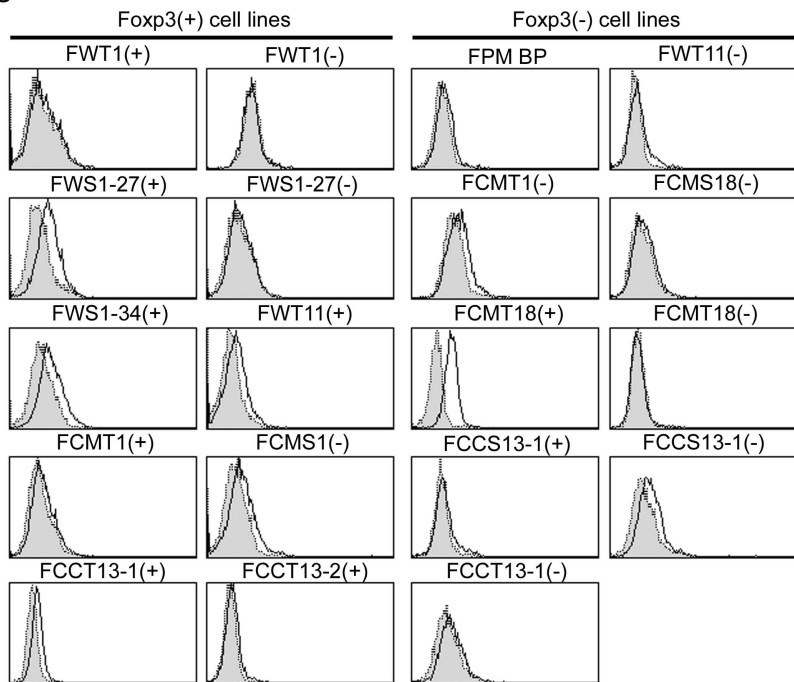
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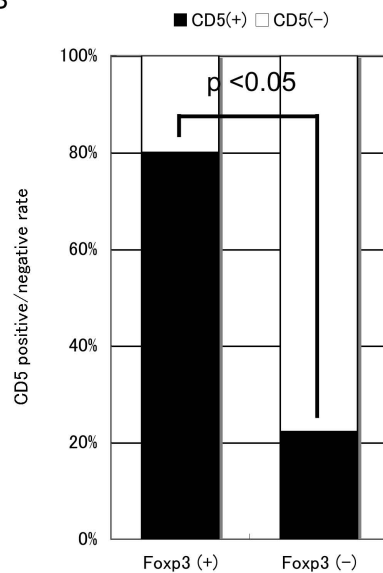
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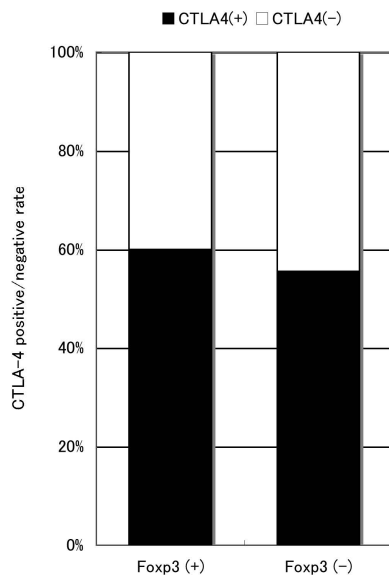
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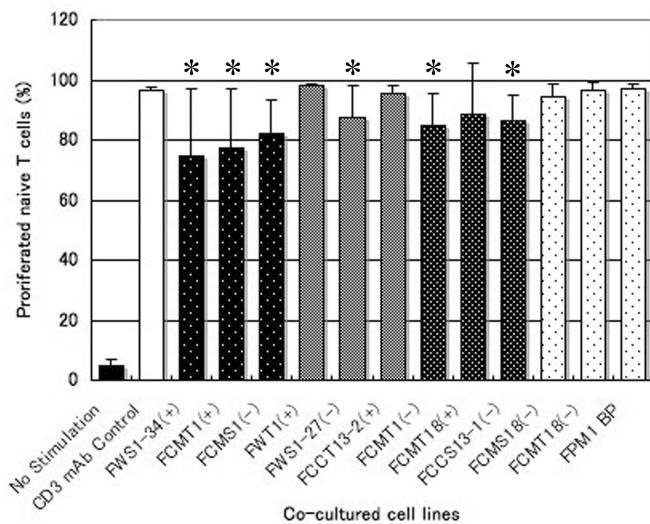
B



D

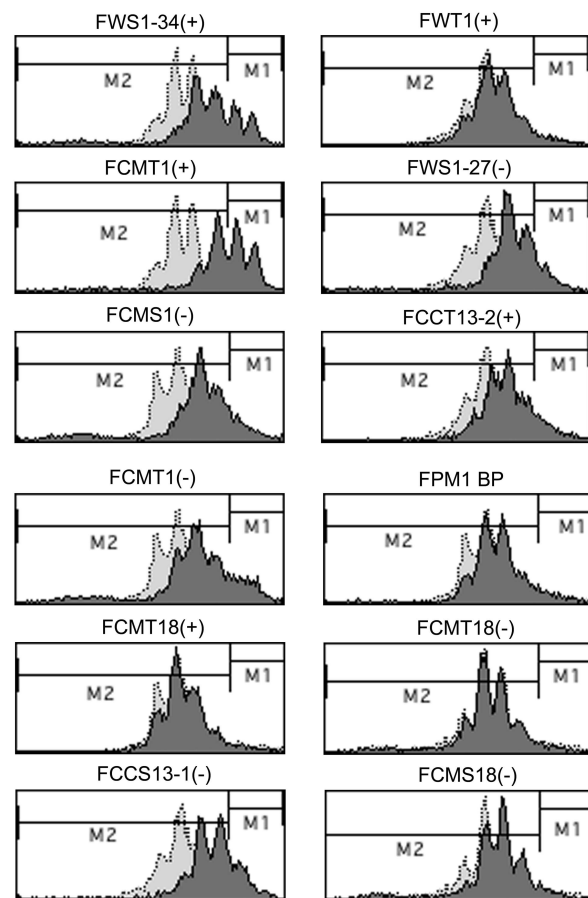


A



- Foxp3(+) CTLA4(+) cell lines
- ▒ Foxp3(+) CTLA4(-) cell lines
- ▤ Foxp3(-) CTLA4(+) cell lines
- ▥ Foxp3(-) CTLA4(-) cell lines

B



1cm