Table 2. The incidence of inflammatory cell infiltration in systemic organs in male FW-pX rats

Organs	Incidence (%)
Heart	26/29 (90)
Liver	24/29 (83)
Kidney	22/29 (76)
Salivary gland	16/23 (70)
Skin	18/29 (62)
Pancreas	18/29 (62)
Skeletal muscle	16/29 (55)
Lung	5/29 (17)

syngeneic or autologous transplantation [19], and production of autoantibodies is sometimes accompanied with the disease [17], autoimmune mechanisms seem to be involved in the pathogenesis. It is considered that putative disruption of the thymus by conditioning regimens and/or acute GVHD may induce abortive negative selection of autoreactive T cells [17, 18, 20, 21]; however, details remain unclear. To better understand

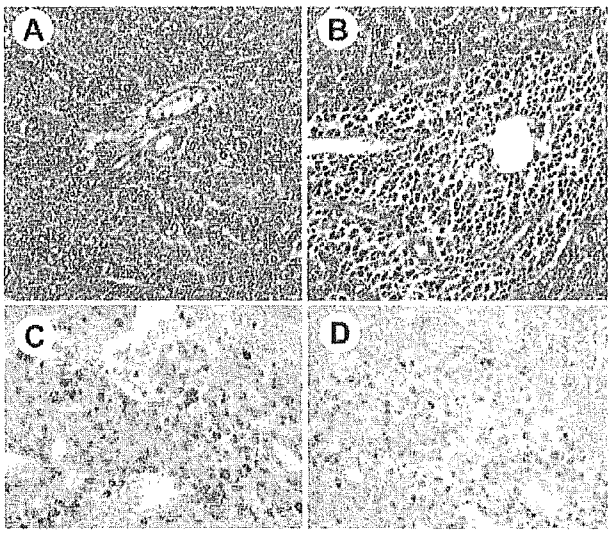

Fig. 7. Adoptive transfer experiments. Splenocytes isolated from 4-week-old FW-wt (A) and FW-pX rats (B–D), respectively, were stimulated by PMA (20 ng/ml) and ionomycin (2 μg/ml) for 24 h, and then injected intravenously into FW-wt rats (1×10^8/rat) which had been given sublethal total-body irradiation (9 Gy) (B–D). Five days later, recipient rats were killed for pathological examinations. Splenocytes from one donor were injected into one recipient. Experiments were repeated independently, and five rats were examined in each experimental group. Tissue sections of the liver from recipients were stained with H&E (A, B) and with anti-CD3 (CL020AP) (C) and CD68 (ED-1) (D) Ab [original magnification: ×200 (A, B), ×400 (C, D)]. Photographs show representative findings of reproduced results.

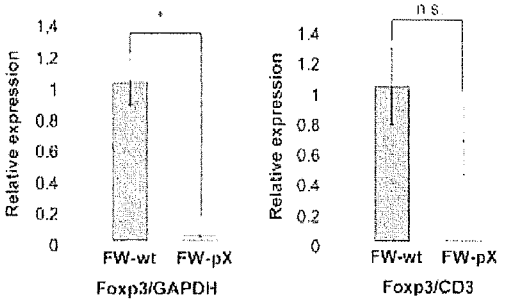

Fig. 8. Comparative analyses of mRNA expression of the Foxp3 gene in FW-pX and FW-wt rats. PBMC were separated from FW-pX and FW-wt rats (6 weeks old, n=3), respectively. Total RNA extracted from the cells was reverse-transcribed, and then the random-primed cDNA served as a template. Quantitative real-time PCR monitored by the SYBR Green I dye was carried out using the ABI PRISM 7700 Sequence Detector System. Amounts of the specific mRNA were quantified at the point where the system detected the uptake in exponential phase of PCR accumulation, and were standardized by the GAPDH gene or the gene for CD3 δ chain. The expression level in PBMC from FW-wt rats was set as 1.0, and the relative expression was calculated. Data are represented as mean ± SD in experiments done in triplicate; $^*p<0.05$, n.s.: not significant.

the pathophysiology of chronic GVHD, development of highly satisfactory animal models is needed.

In the present study, we found that FW-pX rats (F1 generation of F344 lck-pX rats and nontransgenic Wistar rats) showed atrophy of the thymus, lymphocytopenia, structural alteration of lymphoid tissues, and inflammatory cell infiltration into multiple organs. These disorders resembled chronic GVHD in patients given HSCT. Adoptive transfer of FW-pX splenocytes could induce lymphocytic infiltration into sublethally irradiated wild-type syngeneic recipients, clearly indicating that autoimmune mechanisms are involved in the pathogenesis of the disorders in FW-pX rats. These findings suggested that FW-pX rats spontaneously developed chronic GVHD-like autoimmune diseases. Among the strain combinations of mating, incidence of the disease was highest in male FW-pX rats (90%), though 0–20% of other F1 strains of lck-pX rats showed a similar phenotype (data not shown). The significance of strain combination or sex dependency of the disease will be considered in our ongoing works.

We previously reported that transgenic rats established in WKAH strains which expressed the HTLV-1 *pX* gene without tissue specificity developed systemic autoimmune diseases [9]. Although atrophy of the thymus was sometimes observed, there was no significant association with the development of most diseases in these rats (env-pX rats). Moreover, lymphocytopenia, which is a characteristic of GVHD, is never seen in env-pX rats. Therefore, regardless of the common transgene, it is considered that FW-pX and

env-pX rats develop autoimmune diseases via diverse pathogenesis.

In the FW-pX thymus, atrophy occurred by increased apoptosis of thymic epithelial cells and CD4 CD8 DP thymocytes from early days after birth. Thymic epithelial cells are essential for positive and negative selections of T precursors, and CD4 CD8 DP thymocytes are strongly influenced by cortical epithelial cells in the differentiation process [22]. According to this dogma, it is considered that severe depletion of CD4 CD8 DP thymocytes may be mediated by early diminution of thymic cortical epithelial cells in FW-pX rats. Tax (protein product of the HTLV-1 *pX* gene) plays paradoxical roles in proliferation and apoptosis depending on host cell types and conditions [6, 23]. Putative genetic factors in (F344 x Wistar) F1 rats may be associated with apoptosis of thymic cortical epithelial cells expressing the *pX* gene. Interestingly, CD8 SP cells were noted at a higher ratio than CD4 SP cells in FW-pX thymocytes, suggesting that abortive differentiation of T cells occurred in neonatal days. Nuclear factor (NF)-κB plays an important role in the differentiation into CD8 SP cells from CD4 CD8 DP thymocytes [24]. Since Tax activates NF-κB [25], the relative dominancy of CD8 SP cells compared to CD4 SP cells may be mediated, at least in part, by the *pX* transgene in thymocytes.

However, framework of the thymus appeared to be essential for impairment of T cell differentiation in FW-pX rats, because lethally irradiated nontransgenic FW-wt rats reconstituted with FW-pX BM cells never showed a similar alteration of profile of thymocytes or developed GVHD-like diseases (unpublished results). The altered profile of thymocytes corresponded with numerical dominancy of CD8 T cells compared to CD4 T cells in the peripheral blood and also in the sites of inflammation in FW-pX rats. Analyses for phenotype of peripheral CD4 and CD8 T cells showed that these cells were activated in FW-pX rats. Further investigations are needed to determine the causal association of the *pX* transgene with vanishing of thymic epithelial cells especially in the cortex, depletion of CD4 CD8 DP thymocytes, and generation and activation of autoreactive T cells.

Neonatal thymectomy, leading to lack of peripheral T-reg cells, generally results in organ-specific but not systemic autoimmune diseases in mouse models [26, 27]. Teshima et al. [28] reported that BM chimeras defective in expression of MHC class II on thymic APC led to impaired differentiation of T cells and resulted in development of systemic autoimmune diseases. Thymectomy of recipients prior to BM transplantation prevented the diseases in their model, suggesting that framework of the thymus was essential for generation of autoreactive T cells. The collective evidence suggests that systemic autoimmune diseases may occur when abortive differentiation of T cells in the thymus induces both active generation of autoreactive T cells and lack of peripheral T-reg cells. Our FW-pX rats, in which both impaired T cell differentiation associated with atrophy of the thymus and inflammatory cell infiltration into multiple organs related to autoimmune response are evident, may be useful for understanding the mechanisms of generation of autoreactive T cells and loss of peripheral T-reg cells.

Although several models of chronic GVHD have been reported [29–31] in which animals were given irradiation and lymphoid cell transplantation, influences of diverse factors including irradiation and acute GVHD complicated the pathogenesis in these models. FW-pX rats spontaneously developed chronic GVHD-like systemic autoimmune diseases, following abortive differentiation of T cells in the thymus at early ages in the newborn. This rat model may shed light on the pathogenesis of chronic GVHD and also other systemic autoimmune diseases whose etiology is unknown.

Materials and methods

Rats

Inbred F344 and closed colony Wistar rats were purchased from SLC (Shizuoka, Japan) and Charles River (Kanagawa, Japan), respectively. F344 lck-pX rats (line 38) [10] were maintained at the Institute of Animal Experimentation of Hokkaido University Graduate School of Medicine. All experiments using rats were done according to the Guide for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine (http://www.hoku-dai.ac.jp/animal/houki/ hokudaisisin.html).

Mating

FW-pX rats were obtained by mating of male lck-pX rats with female Wistar rats. Since epithelial thymoma occurred more frequently in male than in female lck-pX rats (reason currently unknown), we used male lck-pX rats for mating in the present study. All FW-pX rats were screened for the *pX* gene by genomic PCR as described [10]. FW-wt rats were obtained by mating of nontransgenic male F344 rats with female Wistar rats, and served as controls. Eight-week-old rats were used for mating.

Monoclonal antibodies

Murine mAb used were anti-rat CD3 (CL020AP for immunohistochemistry; Cedarlane, Fornby, Canada; and G4.18 for FCM; PharMingen, San Diego, CA), CD4 (OX-35; PharMingen), CD8 (OX-8; PharMingen), CD11b/c (OX-42; PharMingen), CD25 (OX-39, PharMingen), CD44 (OX-49, PharMingen), CD62L (HRL1; PharMingen), CD68 (ED-1; Serotec, Oxford, UK), MHC class II (MRC OX-6, Serotec), αβ TCR (R73; PharMingen), and anti-human cytokeratin (AE1+AE3 and MNF116; DAKO, Glostrup, Denmark). We have previously

shown that AE1+AE3 and MNF116 were cross-reactive with rat epithelial cells [32]. Mouse IgG1 and IgG2 (CBL600P and CBL601P, respectively; Chemicon International, Temecula, CA) served as isotype controls.

Histopathology and immunohistochemistry

Tissue samples were fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin blocks. Each 4-μm section was stained with hematoxylin and eosin (H&E). For immunohistochemistry, an avidin-biotin immunoperoxidase kit (DAKO) was used. After immunostaining, tissue sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Immunofluorescence staining

To determine the co-localization of staining for cytokeratin and ssDNA, tissue sections were incubated with MNF116 (mouse monoclonal IgG1) and rabbit anti-ssDNA antisera (A4506; DAKO) followed by incubation with Alexa Fluor 488-goat anti-mouse IgG (Molecular Probes, Leiden, Netherlands) and Alexa Fluor 594-goat anti-rabbit IgG (Molecular Probes). After being washed with PBS, the sections were mounted in Fluorescent Mounting Medium (DAKO). Immunofluorescence was detected using a confocal microscope (1×70; OLYMPUS, Tokyo, Japan). TUNEL was done using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI).

Flow cytometry

Expression of cell surface molecules was analyzed by FCM. To detect apoptotic cells, thymocytes were stained using Annexin-V-FLUOS Staining Kit (Roche, Mannheim, Germany). FACScan (Becton Dickinson, Franklin Lakes, NJ) was used for these purposes.

Quantitative real-time RT-PCR

Total RNA was isolated from various organs of FW-pX rats, using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Complementary DNA was synthesized from total RNA, using a random primer set and murine Moloney leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time RT-PCR was done using the ABI PRISM 7000 Sequence Detection System (Applied Biosystem, Foster City, CA) with SYBR Green I as a double-stranded DNA-specific binding dye, and the reaction was continuously monitored based on the fluorescence levels, according to Wittwer et al. [33]. Each cDNA was amplified, using QuantiTect SYBR Green Master Mix (QIAGEN) containing 0.3 μM of the specific primers for the *pX* gene (sense: 5′-GTCTTCTTTTCGGATACCCAGTCTA-3′; antisense: 5′-AAG-GAGGGGAGTCGAGGGATAAGGA-3′) in a total volume of 10 μl, according to the following PCR conditions: 50°C for 2 min, 95°C for 15 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. The relative expression of the *pX* mRNA was analyzed by the ΔΔCT method [34]. GAPDH was used as a control for the amount of RNA (sense: 5′-ATGGGAGTTGCTGTTGAAGT-CA-3′; antisense: 5′-CCGAGGGCCCACTAAAGG-3′). Each reaction was done in triplicate.

Adoptive transfer

Mononuclear cells were isolated from the BM and spleen of 4-week-old FW-pX rats using Histopaque-1083 (Sigma-Aldrich, St. Louis, MO). For BM cell transfer experiments, isolated cells from the BM (1×10^7) were injected intravenously into FW-pX rats which had been lethally irradiated (12 Gy). Two months after transplantation, recipients were killed for examinations. For spleen cell transfer experiments, isolated splenocytes were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. For activation of lymphocytes *in vitro*, 20 ng/ml PMA and 2 μg/ml ionomycin were added to the culture medium, as described [35]. Twenty-four hours later, cells were harvested, washed twice, and injected intravenously into FW-wt rats (1×10^8/rat) with sublethal irradiation (9 Gy). Five days later, recipient rats were killed for pathological examinations. As negative controls, similarly irradiated FW-wt rats given splenic mononuclear cells from FW-wt rats with same treatments were used.

Comparison of the expression levels of the Foxp3 gene

Total RNA was extracted from PBMC of FW-pX and FW-wt rats (6 weeks old), respectively. In both groups, three rats were used for the experiments. Complementary DNA was synthesized, and then quantitative real-time RT-PCR was performed, using the specific primers for the Foxp3 gene (sense: 5′-GAGCCAGCTCTACTCTGCAC-3′, antisense: 5′-CCTCGAA-GACCTTCTCACAA-3′), the gene for CD3 δ chain (sense: 5′-cgaatgtgccagaactgtgt-3′, antisense: 5′-agtgtcaacagccccagaaa-3′), and the GAPDH gene. Each reaction was done in triplicate.

Statistical analysis

Data are represented as mean ± standard deviation (SD). Statistical significance between any two groups was determined by two-tailed Student's *t*-test. p values less than 0.05 were considered to be significant.

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Aberrant gene expression by $CD25^+CD4^+$ immunoregulatory T cells in autoimmune-prone rats carrying the human T cell leukemia virus type-I gene

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Keywords: animal model, Foxp3, JAK/STAT, SOCS, T-reg

Abstract

Transgenic rats expressing the *env-pX* gene of human T cell leukemia virus type-I under the control of the viral long terminal repeat promoter (env-pX rats) developed systemic autoimmune diseases. Prior to disease manifestation, the immunosuppressive function of $CD25^+CD4^+$ T (T-reg) cells was impaired in these rats. Since T cell differentiation appeared to be disordered in env-pX rats, we assumed that the impairment of T-reg cells might be caused by an abortive differentiation in the thymus. However, reciprocal bone marrow transfers between env-pX and wild-type rats revealed that direct effects of the transgene unrelated to the thymus framework induced the abnormality of T-reg cells. To identify molecular changes, comparative analyses were done between env-pX and wild-type T-reg cells. Expression of the *Foxp3* gene and cell-surface markers supported a naive phenotype for env-pX T-reg cells. Array analyses of gene expression showed some interesting profiles, e.g. up-regulation of genes associated with the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways in env-pX T-reg cells. Additionally, expression of the suppressor of cytokine signaling (SOCS) family genes, which inhibit the JAK/STAT signals, was extremely low in env-pX T-reg cells. These findings suggest that the transgene may mediate the down-regulation of the SOCS family genes and that subsequent excess signals through the JAK/STAT pathways may result in the loss of function of env-pX T-reg cells. We suggest that investigation of the pathology of T-reg cells in our autoimmune-prone rat model may aid in understanding the roles of T-reg cells in human autoimmune diseases.

Introduction

Human T cell leukemia virus type-I (HTLV-I) is pathogenically associated with not only adult T cell leukemia (1, 2) but also a number of inflammatory diseases such as myelopathy (3, 4), uveitis (5) and probably arthropathy (6), Sjögren's syndrome (7), T cell alveolitis (7, 8) and infective dermatitis (9). We reported earlier that transgenic rats expressing the *env-pX* gene of HTLV-I under the control of the viral long terminal repeat promoter developed a wide spectrum of collagen vascular diseases, including destructive polyarthritis resembling rheumatoid arthritis, necrotizing arteritis mimicking polyarteritis nodosa, sialoadenitis similar to Sjögren's syndrome, myocarditis, myositis and dermatitis (10). The transgene was expressed constitutively in all the organs of these rats (env-pX rats) without tissue or cell specificity. Since rheumatoid factors and anti-nuclear and anti-DNA autoantibodies were present in sera, env-pX rats seemed to be a prototype model for autoimmune diseases. Prior to development of diseases, progenitors for B cells and osteoclasts were shown to increase in the bone marrow (BM) (11). Peripheral T cells were pre-activated to express CD54 (ICAM-1) and CD80/86 before these rats developed diseases and showed a high response against several mitogenic stimuli *in vitro* (12). Thymus framework carrying the transgene was responsible for the development of autoreactive T cell-mediated necrotizing

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arteritis, thus suggesting that T cell differentiation in the thymus might be disordered in these rats (13). Recently, we found that immunoregulatory functions of peripheral $CD25^+CD4^+$ T (T-reg) cells were impaired in young env-pX rats without disease manifestation, though the number of the cells was equivalent to that in age-matched wild-type WKAH rats (14). On the other hand, it is known that T-reg cells are generated in the normal thymus (15, 16). Therefore, in the present study, we aimed to determine if functional alterations of T-reg cells in env-pX rats would be caused by an abortive differentiation in the thymus or by direct effects of the transgene on these cells. In addition, to examine aberrant molecular expression in env-pX T-reg cells, comparative analyses were done between env-pX and control WKAH rats, using flow cytometry, cDNA arrays and real time quantitative reverse transcriptase (RT)–PCR.

Methods

Rats

Six-week-old male env-pX rats [HTLV-I *env-pX* transgenic rats established in WKAH strains (10)] and non-transgenic WKAH rats were used. These rats were maintained at the Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine. Experiments using animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

Antibodies

Anti-rat CD3, CD4, CD25 (IL-2Rα chain), CD28, CD45RC, CD54 (ICAM-1), CD122 (IL-2Rβ chain),and TCR mAbs were purchased from Pharmingen (San Diego, CA, USA).

BM transfer

Mononuclear cells were prepared from the BM of env-pX and WKAH rats using Lympholyte Rat (Cedarlane, Ontario, Canada) and were then used as BM cells. Microscopic examinations revealed that all env-pX rats used as BM donors were disease-free. The BM cells were injected via the tail vein of recipient rats that had been lethally irradiated using 12 Gy from a ^{60}Co source. BM cells from one donor (1×10^7 per rat) were transplanted into one recipient. In each group of donor/recipient combination, at least three pairs of transplantation were done. Two months after the transplantation, all rats were killed and $CD25^+CD4^+$ T cells were isolated, as described below.

Cell sorting

Mononuclear cells were prepared from the spleen of each rat, using Lympholyte Rat, then stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 mAbs. $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were isolated using FACSVantage (Becton Dickinson, Franklin Lakes, NJ, USA). Purity of the sorted cells exceeded 95%.

Cell proliferation assay

Mononuclear cells were prepared from the cervical lymph nodes of WKAH rats, using Lympholyte Rat. After 1 h of incubation in plastic dishes, adherent and non-adherent cells were collected. The adherent cells were treated with mitomycin C (25 μg ml^{-1}) for 1 h and served as antigen-presenting cells (APCs). $CD25^-CD4^+$ T cells were separated from the non-adherent cells, using a magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany), and served as responders. The responder cells (1×10^5) and mitomycin C-treated APCs (2×10^4) were mixed in tissue culture wells (96-well round-bottom plates) coated with anti-CD3 antibody, as described (12). Splenic $CD25^+CD4^+$ T cells (2×10^4) isolated from rats with BM transplantation using FACSVantage were added to the wells, and these cells were incubated for 96 h. [^{3}H]Thymidine ([^{3}H]TdR) (18.5 kBq) was pulsed 16 h prior to harvest of the cells. Proliferation of cells was quantified by [^{3}H]TdR uptake.

Extraction of total RNA

Total RNA was extracted from FACS-sorted $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells using RNAeasy columns (Qiagen, Valencia, CA, USA).

The cDNA array analysis

For cDNA array analysis, we prepared original filters equipped with 271 rat genes. Details on the filter preparation are described elsewhere (17). Total RNA of each sample was treated with DNase I (TAKARA Shuzo, Kyoto, Japan), and poly $(A)^+$ mRNA was purified using the mRNA purification kit (MagExtractor, TOYOBO, Osaka, Japan). Biotin-labeled cDNA probes were generated using Gene Navigator cDNA Amplification System ver.2 (TOYOBO), and then hybridized to the cDNA array filters using PerfectHyb Hybridization Solution (TOYOBO), according to the manufacturer's instructions. Hybridized signals were developed using Phototope Star kits (New England Biolab, Beverly, MA, USA), detected using FluorS and Quantity One v4.2.1 software (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using ImaGene 4.0 software (BioDiscovery, Segundo, CA, USA). Intensities less than the mean value of signals on spots of the negative control DNA fragment were excluded as false signals. Mean value of the intensity at spots of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for standardization of each filter.

Real time quantitative RT–PCR

The purified total RNA was reverse transcribed using M-MLV RT (Invitrogen, Carlsbad, CA, USA), and the random-primed cDNA served as a template. Real time quantitative PCR was done, using SYBR Green I dye (Applied Biosystems, Foster City, CA, USA). Primer sets for the *Foxp3*, *JAK1*, *STAT1*, suppressor of cytokine signaling (SOCS) family and *GAPDH* genes are listed in Table 1. Amplification was carried out at 45 cycles of two-step PCR (95°C for 30 s, 60°C for 30 s) after the initial denaturalization (95°C, 15 min), using an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). The amount of specific mRNA was quantified at the point where the system detected the uptake in exponential phase of PCR accumulation, and the ratio to that of the *GAPDH* gene was calculated for each sample.

Table 1. Primers used for the real time quantitative RT–PCR

Name of Genes	Direction	Sequence
Rat *Foxp3*	sense	GAGCCAGCTCTACTCTGCAC
	anti-sense	CCTCGAAGACCTTCTCACAA
Rat *JAK1*	sense	CATCCCAGTCTCTGTGCTGA
	anti-sense	AGCAGCCACACTCAGGTTCT
Rat *STAT1*	sense	TCACCATTGTTGCAGAGAGC
	anti-sense	CGATCGGATAACACCTGCTT
Rat *SOCS1*	sense	CCTCCTCGTCCTCGTCTTC
	anti-sense	AAGGTGCGGAAGTGAGTGTC
Rat *SOCS2*	sense	CAGATGTGCAAGGACAAACG
	anti-sense	AATGCTGAGTCGGCAGAAGT
Rat *SOCS3*	sense	CCTTTGAGGTTCAGGAGCAG
	anti-sense	GTAGCCACGTTGGAGGAGAG
Rat *CIS*	sense	TGTGCATAGCCAAGACGTTC
	Anti-sense	GGGTGCTGTCTCGAACTAGG
Rat *GAPDH*	Sense	ATGGGAGTTGCTGTTGAAGTCA
	Anti-sense	CCGAGGGCCCACTAAAGG

Statistics

For cell proliferation assay and real time quantitative RT–PCR, the Mann–Whitney *U* test was applied for statistical analysis. A *P*-value of <0.05 was regarded as significant.

Results

The transgene in BM cells but not in the thymus framework was responsible for functional alterations of T-reg cells in env-pX rats

To determine which was mainly implicated in the impairment of immunoregulatory function of env-pX T-reg cells, the transgene in the thymus framework or in the T-reg cells, reciprocal BM transfers were done between disease-free env-pX rats and wild-type WKAH rats. Splenic $CD25^+CD4^+$ T cells were isolated 2 months post-transplantation, after which the immunosuppressive function of the cells was assayed (Fig. 1). When $CD25^+CD4^+$ T cells from lethally irradiated env-pX rats reconstituted by WKAH BM cells were added to the mixed culture of WKAH $CD25^-CD4^+$ T cells and mitomycin C-treated APCs, anti-CD3 antibody-induced cell proliferation was significantly suppressed as in the control experiments using $CD25^+CD4^+$ T cells from WKAH to WKAH BM transfers. By contrast, the immunosuppressive function of $CD25^+CD4^+$ T cells from lethally irradiated WKAH rats reconstituted by env-pX BM cells was completely absent as in the control experiments with BM transfers from env-pX to env-pX rats. These findings clearly indicated that the transgene in BM cells rather than in the thymus framework was critically involved in the pathology of T-reg cells in env-pX rats.

Expression level of the Foxp3 gene was equivalent in env-pX and WKAH T-reg cells

The *Foxp3* is a master gene and the best marker for T-reg cells (18, 19). The real time quantitative RT–PCR revealed that the *Foxp3* gene was expressed at a significantly higher level in env-pX $CD25^+CD4^+$ T cells than in $CD25^-CD4^+$ T cells (Fig. 2). The relative expression (when the expression level in $CD25^-CD4^+$ T cells was set as 1) reached 26.2 ± 3.9 in env-pX rats, which was equivalent to the value (31.0 ± 14.0) in wild-type WKAH rats.

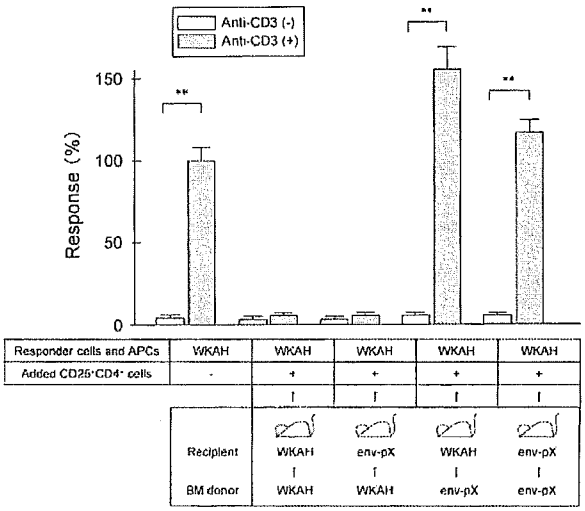

Fig. 1. Analysis of the immunosuppressive function of $CD25^+CD4^+$ T cells from rats that had undergone BM replacement. Six-week-old disease-free env-pX rats and wild-type WKAH rats were lethally irradiated and then reconstituted by reciprocal transplantation of BM cells. Two months later, splenic $CD25^+CD4^+$ T cells were isolated from recipients, using FACSVantage. These cells (2×10^4) were added to mixed cultures of WKAH CD25 $CD4^+$ responder T cells (1×10^5) and mitomycin C-treated APCs (2×10^4) in tissue culture wells coated with anti-CD3 antibody (hatched columns) or uncoated (open columns). After 96 h of incubation, cell proliferation was measured based on $[^3H]$TdR uptake. The uptake when responder cells were stimulated by anti-CD3 antibody in the absence of T-reg cells was set as 100. Data are represented as mean ± SD of percentage in experiments done in triplicate. (**$P < 0.01$.)

Difference in surface molecules on T-reg cells was nil between env-pX and WKAH rats

We previously reported that there was no significant difference in the surface expression of CD25 (IL-2Rα chain), CD80, CD86 and membrane-bound transforming growth factor (TGF)-β1 on T-reg cells of env-pX and WKAH rats (14). In the present study, we found that surface expression of TCR, CD28, CD45RC and CD122 (IL-2Rβ chain) on env-pX T-reg cells was equivalent to that on WKAH T-reg cells (Fig. 3). The expression of CD54 (ICAM-1) on env-pX T-reg cells was also similar to that on WKAH T-reg cells (data not shown). The combined evidence suggests that env-pX T-reg cells may not exhibit an activation phenotype because cell-surface expression of molecules including CD25, CD45RC, CD54, CD80, CD86 and CD122 are normally altered when T cells are activated (12, 20–23).

Comparison of gene expression profiles in T-reg cells of env-pX and WKAH rats

For comparative analyses of gene expression profiles in T-reg cells of env-pX and WKAH rats, cDNA array analysis was done using original filters equipped with 271 probes for rat genes associated with apoptosis, signal transduction, cell cycle regulation and so on (17). About one-third of the genes tested

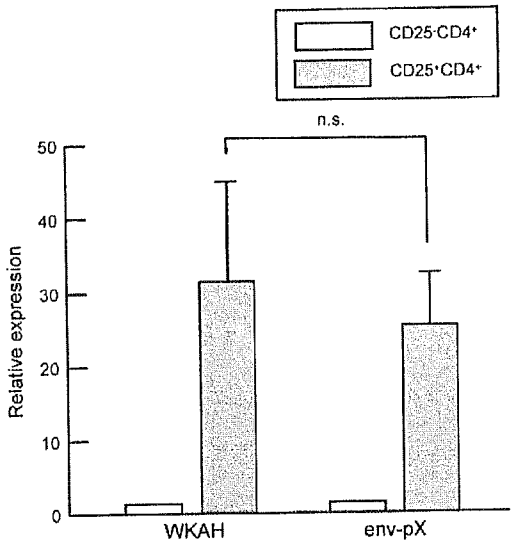

Fig. 2. Comparative analyses of mRNA expression of the *Foxp3* genes in $CD25^+CD4^+$ (hatched columns) and CD25 $CD4^+$ T cells (open columns) of env-pX and WKAH rats, using real time quantitative RT-PCR. Splenic $CD25^+CD4^+$ and CD25 $CD4^+$ T cells were isolated from env-pX and WKAH rats, respectively (6-week-old, disease-free), using FACSVantage. Total RNA extracted from the cells was reverse transcribed, and then the random-primed cDNA served as a template. The real time quantitative PCR monitored by the SYBR Green I dye was carried out, using the ABI PRISM 7700 Sequence Detector System. Amounts of the specific mRNA were quantified at the point where the system detected the uptake in exponential phase of PCR accumulation, and the ratio to the housekeeping *GAPDH* gene was calculated for each sample. The expression in CD25 $CD4^+$ T cells from each group of rats was set as 1 and data are represented as mean ± SD of relative expression in experiments done in triplicate. (n.s.: not significant)

could be evaluated. Expression levels of many genes were higher in env-pX T-reg cells than those in WKAH T-reg cells (Table 2), while a smaller number of genes had decreased in env-pX T-reg cells (Table 3). The expression levels of *CD25* (IL-2Rα chain) and *CD122* (IL-2Rβ chain) genes were increased in env-pX T-reg cells (3.7- and 4.8-fold, respectively). However, in our experiments, no significant difference in the surface expression of CD25 (14) or CD122 (Fig. 3) on T-reg cells was evident between env-pX and WKAH rats, suggesting that the transgene in env-pX T-reg cells did not induce cell-surface protein expression, but increased mRNA expression of *CD25* and *CD122* in the cells. A similar observation was noted for CD54 (ICAM-1, data not shown). Further investigations for the putative post-transcriptional events in env-pX T-reg cells are needed to understand the discrepancy.

Some characteristic gene expression profiles were recognized in env-pX T-reg cells, e.g. lack of regulation of cell cycle [expression of both activators, including cyclins and cyclin-dependent kinases (CDKs), and CDK inhibitors were altered], dysregulation of apoptosis (expression of apoptosis-inducible genes such as caspases was increased, while other apoptosis inducers *FADD* and *TRADD*, and anti-apoptotic molecules such as *Bcl-2*, were decreased) and up-regulation of genes associated with the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (expression of *JAK1, JAK2, STAT2* and *STAT6* was increased).

High expression of the JAK1 and STAT1 genes and low expression of the SOCS family genes in env-pX T-reg cells

Using real time quantitative RT-PCR, we examined the expression of the *JAK1* and *STAT1* genes. The expression level of *JAK1* gene was significantly higher in env-pX T-reg cells than in WKAH T-reg cells (Fig. 4A), corresponding to the cDNA array results. A similar tendency was observed in the *STAT1* gene that could not be evaluated by the cDNA array for unknown reasons (data not shown). The SOCS family molecules have been shown to inhibit the JAK/STAT pathways activated by several cytokines (24, 25). When we examined the family, *SOCS1, SOCS2, SOCS3* and cytokine-inducible Src homology 2 protein (*CIS*) genes, all were at extremely low expression levels in env-pX T-reg cells compared with findings in WKAH T-reg cells (Fig. 4B).

Discusssion

Peripheral $CD25^+CD4^+$ T (T-reg) cells are engaged in inhibiting proliferation of autoreactive T cells and in the maintenance of immunologic self-tolerance (26). Athymic nude mice which had been given peripheral lymphocytes-depleted T-reg cells from histocompatible BALB/c mice developed T cell-mediated autoimmune diseases, including gastritis, thyroiditis and insulin-dependent diabetes mellitus, and adoptive transfer of BALB/c T-reg cells to these mice suppressed development of the diseases (27–29). Recent studies revealed that the transcription factor Foxp3 is a critical mediator of the development of T-reg cells (18, 19). Scurfy mice, in which the *Foxp3* gene is deficient so that T-reg cells are not generated, develop fatal lymphoproliferative and autoimmune disorders (30). In addition, it has been shown that the number of T-reg cells is reduced in autoimmune-prone strains such as non-obese diabetic, New Zealand Black (NZB), New Zealand White (NZW) and (NZB × NZW) F1 mice (31). The combined evidence suggests that lack of T-reg cells may be pathogenically associated with the development of autoimmune diseases in mice.

On the other hand, we have reported that functional but not quantitative alterations of T-reg cells are evident in HTLV-I *env-pX* transgenic rats before they develop autoimmune diseases (14). In these rats, autoreactive T cells against the vasculature may not be eliminated in the thymus and T cell-mediated necrotizing arteritis occurs (13). Since the commitment to T-reg cells by Foxp3 has been suggested to occur in the normal thymus (15, 16, 32), we asked if the functional alterations of *env-pX* transgenic T-reg cells are caused by an abortive differentiation of T cells in the env-pX thymus. Contrary to our expectation, reciprocal BM transfers between disease-free env-pX rats and wild-type WKAH rats suggest that the abnormality of T-reg cells in env-pX rats is caused by direct effects of the transgene rather than by an abortive differentiation in the thymus.

Expression level of the *Foxp3* gene in $CD25^+CD4^+$ T cells was equivalent in env-pX and wild-type WKAH rats. Although peripheral T cells from env-pX rats are ready to be activated

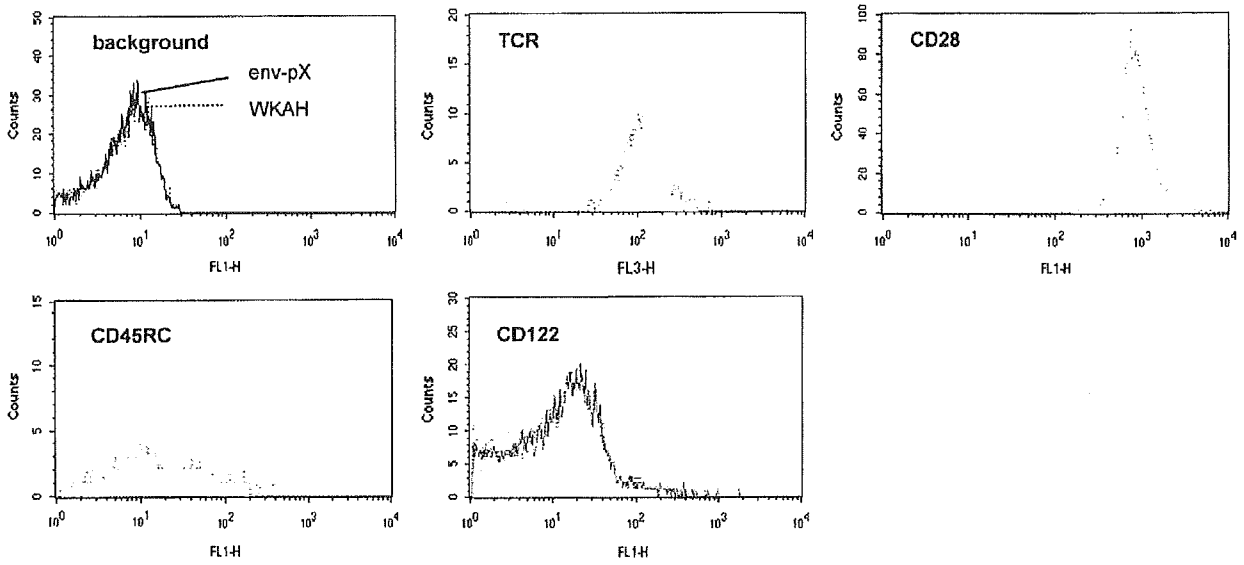

Fig. 3. Comparative analyses of cell-surface molecules on T-reg cells of env-pX and WKAH rats. $CD4^+$ T cells were separated from the spleen of each rat (6-week-old, disease-free), using the magnetic cell sorting system, and then stained with PE-conjugated anti-CD25 and FITC-conjugated antibodies for TCR, CD28, CD45RC or CD122 (IL-2Rβ chain). Flow cytometry was done using FACSCalibur (Becton Dickinson), and then $CD25^+$ cells were gated to obtain histograms using Cell Quest software (Becton Dickinson). Lines and dots represent env-pX and WKAH T-reg cells, respectively. Experiments were conducted at least twice, and representative results are shown.

in vitro (12) and activated T cells express CD25 on the cell surface (22), our results of the real time quantitative RT-PCR for the *Foxp3* gene suggest that $CD25^+CD4^+$ T cells isolated from env-pX rats before they developed diseases might not contain activated $CD4^+$ T cells expressing CD25. In addition, a significant difference in expression of cell-surface molecules including TCR, CD25 (IL-2Rα chain), CD28, CD45RC, CD54 (ICAM-1), CD80, CD86, CD122 (IL-2Rβ chain) and membrane-bound TGF-β1 on T-reg cells was not evident in env-pX and WKAH rats. It is shown that expression of CD122 was increased in human leukemia cells transformed by HTLV-I (22, 23). The lower expression level of p40Tax (a product of the *env-pX* gene) in our transgenic model than in HTLV-I-transformed T cells (10) may be associated with the difference between human and rat cells with regard to the effect of the HTLV-I gene on expression of CD122. These findings suggest that env-pX T-reg cells may be phenotypically naive.

However, the cDNA array analysis showed some characteristic features of gene expression of env-pX T-reg cells, suggesting lack of regulation of the cell cycle, dysregulation of apoptosis and increased signal transduction through the JAK/STAT pathways. The aberrant expressions of cell cycle-related genes may correspond to our finding that env-pX T-reg cells show autologous and anti-CD3 antibody-induced proliferation (14). As alterations in gene expression of apoptosis-related molecules in env-pX T-reg cells were contradictory, it remains to be clarified whether env-pX T-reg cells would be prone to apoptosis. Expression of genes that belong to the mitogen-activated protein (MAP) kinase cascade such as MAP kinase p42 and MAP kinase kinase-3, those in the downstream of JAKs (33), was also increased in env-pX T-reg cells (see Table 2). Moreover, the expression level of genes targeted by the JAK/STAT signals including cyclins and p21Waf1 (34) in T-reg cells was higher in env-pX rats than in wild-type rats. The collective evidence suggests that the JAK/STAT pathways are activated in env-pX T-reg cells. This may be also related to the loss of anergic features of T-reg cells in env-pX rats (14).

Many cytokines, if not all, that are related to immune responses utilize the JAK/STAT pathways (35, 36). The SOCS family molecules were shown to complete a negative feedback loop to attenuate signal transduction through the JAK/STAT pathways (24, 25). Recent studies have shown that the SOCS family genes are expressed at high levels in T-reg cells, suggesting that these molecules contribute to the anergic and immunosuppressive phenotypes of these cells (37). Real time quantitative RT-PCR showed increased expression levels of the *JAK1* and *STAT1* genes in env-pX T-reg cells. Although it remains to be clarified if phosphorylation of the JAK/STAT kinases is actually augmented in env-pX T-reg cells, our data do correspond to the report that the JAK/STAT pathways were activated in T cells transformed by HTLV-I (38, 39). Interestingly, we noted a marked and significant reduction of the SOCS family, *SOCS1*, *SOCS2*, *SOCS3* and *CIS* genes in env-pX T-reg cells. Regulation of the expression of SOCS family molecules is poorly understood; however, IL-4, IL-12 and IFN-γ have been shown to increase expression of these genes in T cells (40). In our cDNA array analysis, the expression of the *IL-12 p35* gene in env-pX T-reg cells was one-tenth of that in wild-type T-reg cells (see Table 2). This may relate to the low-level expression of the SOCS family genes in env-pX T-reg cells. Since p40Tax encoded by the transgene impacts on the expression of several host genes and

Table 2. Genes expressed at a higher level in env-pX T-reg cells than in WKAH T-reg cells[a]

Functional category	Accession number	Name of genes	Ratio (env-pX/WKAH)
Secreted protein	AF022952	VEGF-β	7.2
	NM133519	IL-11	2.2
	L00981	TNF-β1	2.2
Surface receptor	NM019178	Toll-like receptor 4	>41.6[b]
	NM031048	LIFR	>27.5[b]
	NM012673	CD90 (Thy-1)	>22.2[b]
	U90610	*CXCR-4*	>20.3[b]
	NM000395	IL-3/IL-5/GM-CS Rβ	>9.7[b]
	AJ554216	RT-1Da (MHC class II)	>9.5[b]
	NM012967	CD54 (ICAM-1)	26.9
	NM012752	CD24	8.2
	NM012830	CD2	6.5
	AB015747	IL-4R	6.2
	NM013195	CD122 (IL-2Rβ)	4.8
	X74917	TCRβ	4.8
	NM013163	CD25 (IL-2Rα)	3.7
Cell cycle regulator	XM342638	Cdk6	>39.5[b]
	XM235633	cyclin T1	>38.9[b]
	NM171993	p55	>38.9[b]
	NM130860	Cdk9	>16.1[b]
	XM340763	cyclin F	>13.5[b]
	D14015	cyclin E	>12.3[b]
	XM214007	cyclin I	>12.1[b]
	NM031550	p16Ink4a	>9.6[b]
	U24174	p21Waf1	>9.3[b]
	D16308	cyclin D2	>8.4[b]
	NM131902	p18	4.9
	NM031762	p27Kip1	3.7
Signal transduction	M64300	MAP kinase p42	>18.2[b]
	X93150	MKK-3	>17.4[b]
	XM225262	RIP	>9.1[b]
	U13396	JAK2	>7.9[b]
	NM011948	MEK kinase-4	10.0
	NM030857	Lyn	8.5
	AJ000556	JAK1	3.4
Transcription factor	AF055292	STAT6	>20.8[b]
	NM019963	STAT2	>13.2[b]
	NM030867	IκB-α	16.7
Apoptosis-related	AF244366	FLIP	>17.7[b]
	NM016787	Nip2	>17.1[b]
	XM344434	Rb 1	>13.6[b]
	XM235060	RAID	>12.7[b]
	NM012922	Caspase-3	>11.5[b]
	NM022612	Bim	>9.5[b]
	NM181628	tsg101	>8.7[b]
	NM012762	Caspase-1	>7.3[b]
	XM213712	PMS2	15.1
	NM053905	Lamin B1	11.7
	NM021846	Mcl-1	11.2
	NM133381	CBP	8.0
	NM022522	Caspase-2	3.4

[a]Gene expression profiles in T-reg cells were compared between env-pX and WKAH rats, using the cDNA array technique. In both groups, T-reg cells from six rats were collected. The mRNA was extracted from the respective pooled T-reg cells. The expression of each gene was standardized by expression of the housekeeping *GAPDH* gene, and ratio (env-pX/WKAH) was calculated.
[b]Since expression of the gene was below the detection threshold in WKAH T-reg cells, the ratio (env-pX/WKAH) is represented as the value of env-pX T-reg cells.

Table 3. Genes expressed at a lower level in env-pX T-reg cells than in WKAH T-reg cells[a]

Functional category	Accession number	Name of genes	Ratio (env-pX/WKAH)
Secreted protein	NM053390	IL-12 p35	0.1
	NM024388	NGF β polypeptide	0.0
	NM019165	IL-18	0.0
	NM022177	FGF-2	0.0
	NM012589	IL-6	0.0
Surface receptor	NM017183	CXCR-2	0.2
	NM031132	TGFβ-RII	0.0
Cell cycle regulator	NM007628	cyclin A1	0.3
	XM342812	cyclin C	0.3
	NM031020	p38	0.0
Signal transduction	NM012855	JAK3	0.5
	NM012758	Syk	0.4
	NM012755	Fyn	0.0
	NM011237	Rad9 homolog	0.0
Transcription factor	NM012912	ATF-3	0.3
	L26267	IκB-ε	0.0
Apoptosis-related	NM152937	FADD	0.4
	XM341671	TRADD	0.3
	NM016993	Bcl-2	0.2
	XM225039	ING1	0.1
	NM021755	Lamin A	0.0
	NM031606	PTEN	0.0
	NM031535	Bcl-XL	0.0
	NM053420	Nip3	0.0

[a]Gene expression profiles in T-reg cells were compared between env-pX and WKAH rats, using the cDNA array technique. In both groups, T-reg cells from six rats were collected. The mRNA was extracted from the respective pooled T-reg cells. The expression of each gene was standardized by expression of the housekeeping *GAPDH* gene, and the ratio (env-pX/WKAH) was calculated.

modulates molecular functions (41), it is possible that unidentified pathways associated with the transgene are implicated in the down-regulation of the SOCS family genes in env-pX T-reg cells.

HTLV-I p40Tax associates with nuclear factor (NF)-κB and activates the JAK/STAT kinases (39). In env-pX T-reg cells, excess signals through the JAK/STAT pathways may be transduced by the activation of NF-κB and removal of the negative regulation mediated by the SOCS family molecules. Immunoregulatory functions of T-reg cells are attenuated when exposed to a high dose of IL-2 (26). Since IL-2 transduces the JAK/STAT signals (35, 36), it is considered that the excess JAK/STAT signals mediated by p40Tax and NF-κB, mimicking signals by IL-2, may result in the loss of immunoregulatory function of env-pX T-reg cells. Studies are ongoing to clarify the relationship between HTLV-I p40Tax and the down-regulation of the SOCS family molecules.

Mutations in the *Foxp3* gene cause immune dysregulation, polyendocrinopathy, enteropathy and X-linked (IPEX) syndrome in humans (42). However, it remains unclear whether lack and/or dysfunction of Foxp3 play pathogenic roles in patients with other common autoimmune diseases. Analyses using not only mouse models with quantitative alterations of T-reg cells but also our rat model exhibiting functional alterations of the cells may aid in understanding the pathogenic roles of T-reg cells in patients with autoimmune diseases.

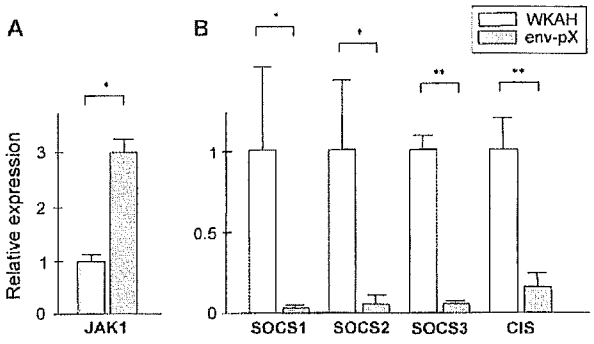

Fig. 4. Comparative analyses of mRNA expression of the *JAK1* and SOCS family genes in T-reg cells of env-pX (hatched columns) and WKAH rats (open columns), using real time quantitative RT–PCR. Splenic $CD25^+CD4^+$ T cells were isolated from env-pX and WKAH rats (6-week-old, disease-free), using FACSVantage. Total RNA extracted from the cells was reverse transcribed, and then the random-primed cDNA served as a template. The real time quantitative PCR monitored by the SYBR Green I dye was carried out, using the ABI PRISM 7700 Sequence Detector System. Amounts of the specific mRNA were quantified at the point where the system detected the uptake in exponential phase of PCR accumulation, and the ratio to the housekeeping *GAPDH* gene was calculated for each sample. The expression of each gene in WKAH T-reg cells was set as 1, and data are represented as mean ± SD of relative expression in experiments done in triplicate. ($*P < 0.05$, $**P < 0.01$.)

Acknowledgements

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Abbreviations

APC	antigen-presenting cell
BM	bone marrow
CDK	cyclin-dependent kinase
CIS	cytokine-inducible Src homology 2 protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
$[^3H]$TdR	$[^3H]$thymidine
HTLV-I	human T cell leukemia virus type-I
JAK	Janus kinase
MAP	mitogen-activated protein
NF	nuclear factor
NZB	New Zealand Black
NZW	New Zealand White
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
RT	reverse transcriptase

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Virus-induced dysfunction of CD4+CD25+ T cells in patients with HTLV-I–associated neuroimmunological disease

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CD4+CD25+ Tregs are important in the maintenance of immunological self tolerance and in the prevention of autoimmune diseases. As the CD4+CD25+ T cell population in patients with human T cell lymphotropic virus type I–associated (HTLV-I–associated) myelopathy/tropical spastic paraparesis (HAM/TSP) has been shown to be a major reservoir for this virus, it was of interest to determine whether the frequency and function of CD4+CD25+ Tregs in HAM/TSP patients might be affected. In these cells, both mRNA and protein expression of the forkhead transcription factor Foxp3, a specific marker of Tregs, were lower than those in CD4+CD25+ T cells from healthy individuals. The virus-encoded transactivating HTLV-I *tax* gene was demonstrated to have a direct inhibitory effect on Foxp3 expression and function of CD4+CD25+ T cells. This is the first report to our knowledge demonstrating the role of a specific viral gene product (HTLV-I Tax) on the expression of genes associated with Tregs (in particular, *foxp3*) resulting in inhibition of Treg function. These results suggest that direct human retroviral infection of CD4+CD25+ T cells may be associated with the pathogenesis of HTLV-I–associated neurologic disease.

Introduction

The human T cell lymphotropic virus type I (HTLV-I) is an exogenous human retrovirus that is associated with chronic, persistent infection of human T cells. While the majority of infected individuals remain healthy, lifelong asymptomatic carriers, approximately 2–3% develop an aggressive mature T cell malignancy termed adult T cell leukemia, and another 0.25–3% develop an inflammatory disease of the CNS termed HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP) (1–3). Furthermore, in some HAM/TSP patients, other autoimmune diseases characterized by multiorgan lymphocytic infiltrates, including uveitis, arthritis, polymyositis, Sjögren syndrome, atopic dermatitis, and alveolitis, have been reported (4, 5). Patients with HAM/TSP have high frequencies of HTLV-I–infected T cells and heightened virus-specific immune responses, including increased proinflammatory cytokine production (6–8). One of the most striking features of the cellular immune response in HAM/TSP patients is the increased numbers of HTLV-I–specific CTLs, which are lower or absent in asymptomatic carriers (9). In some HLA-A*201 HAM/TSP patients, the frequency of Tax11–19–specific CTLs can be as high as 30% of total CD8+ T cells in peripheral blood (10) and even higher in cerebrospinal fluid (6). Neuropathological findings have demonstrated focal infiltrates of T cells and macrophages in the CNS (11). These observations have suggested that inflammatory T cells (particularly virus-specific CD8+ CTLs) may play an immunopathologic role in this disorder.

Recently, a large body of information has demonstrated that CD4+ Tregs constitute an important component of the normal, healthy immune response. These cells are engaged in the maintenance of immunologic self tolerance by actively suppressing the activation and expansion of self-reactive lymphocytes that may cause autoimmune disease (12, 13). The majority of these Tregs constitutively express CD25 (the IL-2 receptor α chain). The normal CD4+CD25+ Treg population constitutes 5–10% of peripheral CD4+ T cells in mice and 1–2% in humans (only the CD4+CD25^{high} T cells exhibit similar regulatory function in humans) (14). Removal or functional alteration of this population from normal rodents leads to the spontaneous development of various autoimmune diseases (12, 13). CD4+CD25+ Tregs have unique immunological characteristics. For example, they do not proliferate in response to antigenic stimulation in vitro and can potently suppress the proliferation of other CD4+ or CD8+ T cells induced either by polyclonal or antigen-specific stimuli (12, 13). Costimulation with anti-CD28 or provision of exogenous IL-2 inhibits the suppressive ability of these CD4+CD25+ Tregs (15, 16). They constitutively express gene products of glucocorticoid-induced TNF receptor family–related (GITR) receptors and cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) (17–21). Furthermore, it has been reported that *forkhead transcription factor* (*foxp3*) gene is specifically expressed in Tregs and is required for their development and function (22–24). Interestingly, mice of the *foxp3* mutant strain, or scurfy mice, succumb to a CD4+ T cell–mediated, lymphoproliferative, and autoimmune disease characterized by multiorgan lymphocytic infiltrates and overproduction of proinflammatory cytokines (25–27). Furthermore, similar immunological abnormalities are observed in CTLA-4–defi-

Nonstandard abbreviations used: AC, HTLV-I–infected asymptomatic carrier; GITR, glucocorticoid-induced TNF receptor family–related; HAM/TSP, HTLV-I–associated myelopathy/tropical spastic paraparesis; HD, healthy donor; HTLV-I, human T cell lymphotropic virus type I.

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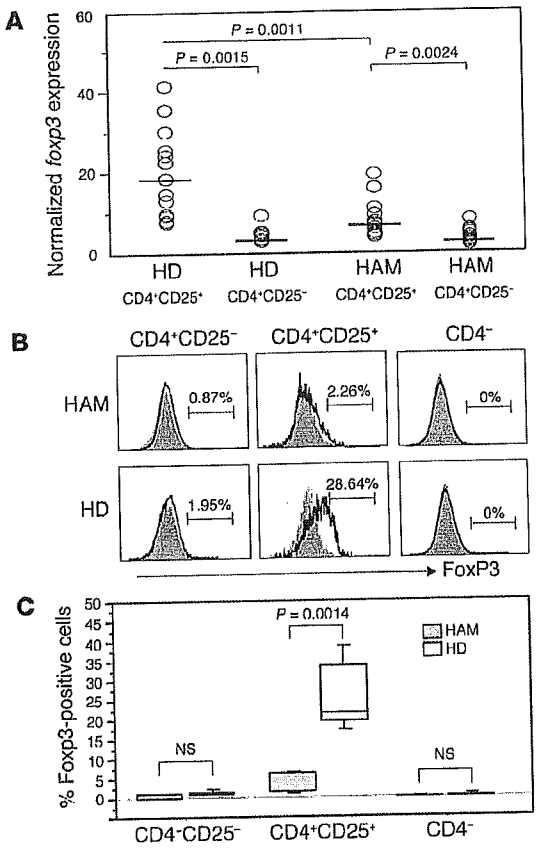

Figure 1
Decreased Foxp3 expression in CD4+CD25+ T cells from HAM/TSP patients. (**A**) Quantitative expression of *foxp3* mRNA was determined by real-time RT-PCR. The level of *foxp3* mRNA expression was calculated as the relative quantity of *foxp3* mRNA expression divided by the relative quantity of endogenous control *HPRT* mRNA expression, as described in Methods. The data represent isolated cell subsets (CD4+CD25+ or CD4+CD25-) from 13 uninfected HDs and 13 HAM/TSP patients (HAM). *Foxp3* mRNA expression was significantly reduced in the CD4+CD25+ T cell subset from HDs compared with that from HAM/TSP patients. (**B**) A representative histogram of intracellular expression of Foxp3 protein showing results from flow cytometric analysis of PBMC samples from HAM/TSP patients and HDs. Foxp3 protein expression was detected in the CD4+CD25+ T cell subset from HDs but not in CD4+CD25- or in total CD4- T cell subsets. In contrast, the number of Foxp3-positive cells in CD4+CD25+ T cells from HAM/TSP patients was clearly reduced. (**C**) Data represent averaged percentage of Foxp3-positive cells in each T cell subset. The percentage (mean ± SD) of Foxp3-positive cells in CD4+CD25+ T cells of 8 HAM/TSP patients (3.09% ± 1.04%) was significantly lower than that of 8 HDs (25.9% ± 8.23%; $P = 0.0014$). No difference in the protein expression levels of Foxp3 was observed in CD4+CD25- or CD4- cells between HAM/TSP patients and HDs.

cient mice (28, 29). HAM/TSP patients share many immunological characteristics with the scurfy *foxp3* mutants and CTLA-4–deficient mice, including the in vitro spontaneous lymphoproliferation of predominantly CD4+ T cells and clinical manifestations associated with autoimmune disease characterized by multiorgan lymphocytic infiltrates and overproduction of proinflammatory cytokines. It was therefore of interest to determine the frequency and function of CD4+ Tregs in patients with HAM/TSP.

We have recently demonstrated that in HAM/TSP patients, the CD4+CD25+ T cell population is the main reservoir for HTLV-I: more than 90% of these cells contain HTLV-I proviral DNA, and they express HTLV-I *tax* mRNA at significantly higher levels than in CD4+CD25- cells (30). Moreover, these HTLV-I–infected CD4+CD25+ T cells were not functionally suppressive but rather were shown to be stimulatory for the HTLV-I Tax-specific proliferation of CD8+ T cells (30). Therefore, we have hypothesized that HTLV-I infection of CD4+CD25+ T cells may alter the regulatory function of this population of CD4+ cells or that the proportion of Tregs may be decreased in HAM/TSP patients. To answer these questions, we developed a quantitative TaqMan PCR assay for the detection of human *foxp3* mRNA and a FACS assay for the detection of Foxp3 protein. We have shown that *foxp3* mRNA expression in CD4+CD25+ T cells of HAM/TSP patients is lower than that of HDs. In addition, CD4+CD25+ T cells of HAM/TSP patients have lower levels of expression of Foxp3 protein as well as other Treg markers such as CTLA-4 and GITR but were overproducing proinflammatory cytokines such as IL-2 that are known to inhibit CD4+CD25+ regulatory activity. Importantly, we have also demonstrated defects in the regulatory function of HTLV-I *tax* gene–transfected CD4+CD25+ T cells. In an attempt to define which HTLV-I virus gene(s) may be associated with the dysregulation of Foxp3, we have transfected the HTLV-I–transactivating *tax* gene into CD4+CD25+ T cells from HDs and have demonstrated a Tax-specific inhibition of *foxp3* expression that can suppress CD4+CD25+ Treg function. Collectively, these results demonstrate that a consequence of HTLV-I infection of CD4+CD25+ T cells in HAM/TSP patients (30) is the suppression in both the frequency and function of CD4+ Tregs, which may be associated with a break in immunological self tolerance resulting in the HTLV-I–associated disorders with multiorgan lymphocytic infiltrates.

Results

Decreased foxp3 expression in CD4+CD25+ T cells from HAM/TSP patients. To assess whether CD4+CD25+ cells in HAM/TSP patients have altered expression of Foxp3, we isolated CD4+CD25+ and CD4+CD25- T cells from PBMCs of HAM/TSP patients, HTLV-I–infected asymptomatic carriers (ACs), and uninfected healthy donors (HDs) and quantified the expression levels of *foxp3* by real-time RT-PCR. The percentages (mean ± SD) of $CD4^+CD25^{high}$ T cells in PBMCs of HAM/TSP patients, ACs, and HDs were 19.52% ± 9.00%, 5.30% ± 1.62%, and 2.19% ± 1.07%, respectively (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI200523913DS1). As expected, *foxp3* mRNA expression levels were significantly higher ($P = 0.0015$) in CD4+CD25+ cells compared with CD4+CD25- cells from 13 HDs (Figure 1A). Similarly, *foxp3* expression levels were also higher in CD4+CD25+ cells compared with CD4+CD25- T cells from 13 HAM/TSP patients ($P = 0.0024$). However, the expression of *foxp3* in the HAM/TSP CD4+CD25+ population (6.81 ± 4.77; see Methods) was significantly lower (approximately 2.5-fold; $P = 0.0011$) than that observed in HD CD4+CD25+ cells (16.01 ± 10.76; see Methods) (Figure 1A). *foxp3* expression levels in CD4+CD25+ cells from 2 ACs were comparable to levels observed in cells from HDs (Table 1). No difference in the expression levels of *foxp3* mRNA was observed among HAM/TSP, AC, and HD

Table 1
foxp3 mRNA expression in CD4+CD25+ T cells and CD4+CD25- T cells from HAM/TSP patients, ACs, and HDs

	HDs[A]		ACs[B]		HAM[A]	
	Mean	SD	Mean	SD	Mean	SD
CD4+CD25+	16.01	10.76	13.62	0.17	6.81	4.77
CD4+CD25-	2.61	1.62	3.73	0.30	2.48	1.74

Data represent normalized *foxp3* mRNA expression. HAM, HAM/TSP patients. [A]$n = 13$. [B]$n = 2$.

$CD4^+CD25^-$ cells. These results are in agreement with previous studies of both mouse and human (22, 31) Tregs demonstrating that the transcription factor Foxp3 is preferentially expressed in $CD4^+CD25^+$ T cells. However, the *foxp3* expression was reduced in $CD4^+CD25^+$ T cells from patients with HAM/TSP.

Loss of foxp3 protein expression on $CD4^+CD25^+$ T cells from HAM/TSP patients. As we had shown that the level of *foxp3* mRNA was significantly decreased in $CD4^+CD25^+$ T cells from HAM/TSP patients compared with HDs, we wished to determine whether comparable reductions in Foxp3 protein expression could also be demonstrated. Therefore, we investigated the intracellular expression of Foxp3 protein in PBMCs from HAM/TSP patients and HDs using flow cytometry with a commercially available anti-human Foxp3 antibody. Analysis of Foxp3 protein expression in subpopulations of lymphocytes from 8 HDs revealed significant staining, as expected, in the $CD4^+CD25^+$ T cell subset but not the $CD4^+CD25^-$ or $CD4^-$ T cell subsets (Figure 1, B and C). A representative histogram is shown in Figure 1B. The percentage (mean ± SD) of Foxp3-positive cells in $CD4^+CD25^+$ T cells from 8 HDs was 25.9% ± 8.23% (Figure 1C). This is consistent with the hypothesis that only a subset of the $CD4^+CD25^+$ T cell population may be $CD4^+$ Tregs (12, 13). In contrast, the percentage (mean ± SD) of Foxp3-positive cells in $CD4^+CD25^+$ T cells from 8 HAM/TSP patients was significantly reduced to 3.09% ± 1.04% ($P = 0.0014$) (Figure 1C). A representative histogram is shown in Figure 1B. No difference in the protein expression levels of Foxp3 was observed in $CD4^+CD25^-$ or $CD4^-$ cells between HAM/TSP patients and HDs (Figure 1B). These results support the finding that *foxp3* mRNA is reduced in $CD4^+CD25^+$ cells from HAM/TSP patients compared with HDs (Figure 1A) and continue to suggest that dysregulation of Tregs may be contribute to the pathogenesis of this disorder.

Reduced expression of regulatory cell surface marker and increased proinflammatory cytokine production in $CD4^+CD25^+$ T cells from HAM/TSP patients. Tregs have been characterized by their constitutive expression not only of Foxp3 but also of cell surface proteins such as CD25, CD38, CD62L, CD69, CTLA-4, and GITR (17–21, 23, 32, 33). To determine the levels of these cell surface molecules, we investigated their expression in $CD4^+CD25^+$ T cells from both HAM/TSP patients and HDs. As shown in Table 2, $CD4^+CD25^+$ T cells from HAM/TSP patients showed lower expression of CD38 ($P = 0.0003$), CD62L ($P = 0.0374$), CD69 ($P = 0.0101$), CTLA-4 ($P = 0.0104$), and GITR ($P = 0.0010$) molecules than those from HDs, while the expression of HLA-DR was not significantly different. We confirmed a decrease in CD45RA expression ($P = 0.0112$) and an increase in CD45RO expression ($P < 0.0001$) in $CD4^+CD25^+$ T cells from HAM/TSP patients (Table 2), as had been previously reported (34, 35). We also investigated intracellular cytokine expression in $CD4^+CD25^+$ T cells. The expression of proinflammatory cytokine such as IL-2 ($P = 0.0011$) and IFN-γ ($P = 0.0034$) was significantly increased in HAM/TSP patients compared with HDs, whereas there were no significant differences in expression of Th2 cytokines such as IL-4 and IL-10 (Table 2). Collectively, these results demonstrate a reduction in cell surface molecules, particularly GITR and CTLA-4, which have been associated with $CD4^+$ Tregs, on HAM/TSP $CD4^+CD25^+$ cells (17–21). These findings are consistent with our previous observations on reduced Foxp3 expression (Figure 1).

Lack of regulatory function in $CD4^+CD25^+$ T cells from HAM/TSP patients. While we have shown a decrease in *foxp3* mRNA and protein expression in HAM/TSP $CD4^+CD25^+$ cells as well as other cell surface markers that characterize $CD4^+$ Tregs, it remains to be determined whether this corresponds to a reduction in Treg function. To determine the effect of HAM/TSP $CD4^+CD25^+$ cells on T cell regulatory function, we performed functional CFSE proliferation assays. As shown Figure 2, HD $CD4^+CD25^-$ T cells specifically proliferated upon stimulation with anti-CD3 antibody. As expected, addition of irradiated, sorted allogeneic HD $CD4^+CD25^+$ (which did not proliferate; data not shown) to these HD $CD4^+CD25^-$-responding cells resulted in an inhibition of proliferation consistent with a Treg function of HD $CD4^+CD25^+$ cells (14, 36, 37). In contrast, coculturing irradiated HAM/TSP $CD4^+CD25^+$ cells with HD $CD4^+CD25^-$ cells did not suppress the proliferative capacity of these anti-CD3–stimulated, responding $CD4^+CD25^-$ cells (Figure 2). These results suggest that Treg function in $CD4^+CD25^+$ cells from HAM/TSP patients is dysregulated.

HTLV-I Tax suppresses foxp3 expression. Since Foxp3 message and protein expression were significantly reduced in HAM/TSP $CD4^+CD25^+$ cells relative to those from HDs, we hypothesized that the virus-encoded transactivating *tax* gene (38, 39) might be associated with this reduction. To investigate this possibility, we transfected an HTLV-I *tax* DNA vector known to express high levels of HTLV-I Tax protein (40) into purified $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells from 7 HDs using a highly efficient electroporation transfection system (greater than 70% of transfected

Table 2
Cell surface marker expression and proinflammatory cytokine production in CD4+CD25+ T cells from HAM/TSP patients and HDs

	HAM ($n = 6$)		HDs ($n = 6$)		
	Mean	SD	Mean	SD	*P* value[A]
CD45RA	5.34	4.95	28.9	19.8	$P = 0.011$
CD45RO	95.1	3.17	71.6	9.89	$P < 0.0001$
CD27	45.9	22.8	64.1	8.97	NS
CD28	88.4	12.5	53.8	31.8	$P = 0.0472$
CD38	8.6	1.51	21.3	6.23	$P = 0.0003$
HLA-DR	36.2	22.9	15.5	16.4	NS
CD69	1.87	1.11	24.5	19.5	$P = 0.0101$
CTLA-4	0.13	0.14	3.83	5.63	$P = 0.0104$
GITR	2.58	3.1	13.2	5.5	$P = 0.0010$
IL-4	2.82	0.89	2.82	3.26	NS
IL-10	0.2	0.12	0.68	0.53	NS
IL-2	38.7	21.5	2.9	1.79	$P = 0.001$
INF-γ	17.5	10.3	2.62	2.61	$P = 0.0034$

[A]According to Student's *t* test. Data represent percentages in $CD4^+CD25^+$ cells from HAM/TSP patients and HDs.

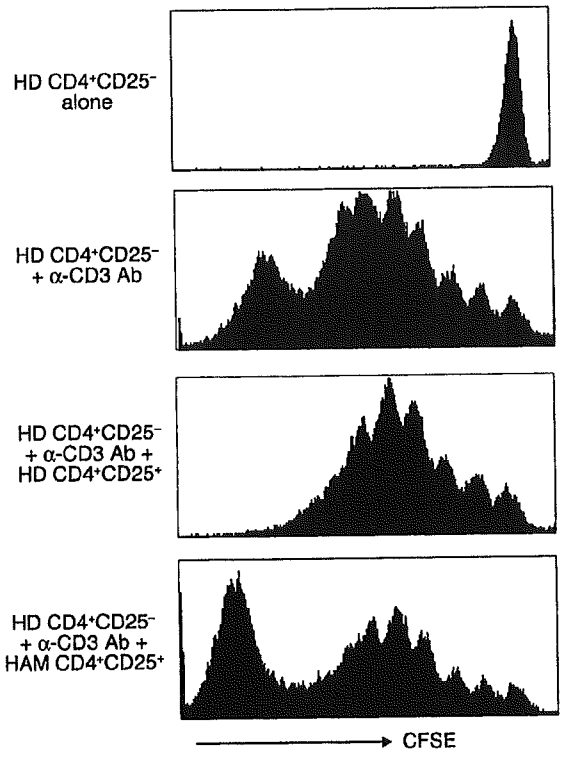

Figure 2
Lack of regulatory function in CD4+CD25+ T cells from HAM/TSP patients. A total of 1 × 10^5 CD4+CD25- T cells/well from HDs were labeled with CFSE. They were cultured for 6 days in the culture medium in the absence or presence of 2.5 μg/ml anti-CD3 antibody (top 2 panels). They were also cultured for 6 days in 2.5 μg/ml anti-CD3 antibody added to culture medium with 1 × 10^5 irradiated allogeneic CD4+CD25+ T cells from HDs or with 1 × 10^5 irradiated CD4+CD25+ T cells from HAM/TSP patients (bottom 2 panels). The data indicate that regulatory function in CD4+CD25+ T cells from HAM/TSP patients is reduced in comparison with that in CD4+CD25+ T cells from HDs. Failure of CD4+CD25+ T cells to suppress lymphoproliferation of activated HD cells was observed in separate experiments with cells from 4 HAM/TSP patients, while suppression of activated HD cell proliferation by allogeneic HD CD4+CD25+ T cells from 2 HDs was demonstrated.

cells expressed the transgene). We measured *foxp3* mRNA expression using real-time RT-PCR before and after transfection. As shown in Figure 3, in all donors, the *foxp3* mRNA expression level in CD4+CD25+ T cells was significantly decreased by transfection with HTLV-I *tax* DNA ($P = 0.018$). By contrast, there was no significant difference in the level of *foxp3* message in CD4+CD25- T cells before and after HTLV-I *tax* DNA transfection (Figure 3, A and B). When CD4+CD25+ T cells from HDs were transfected with another HTLV-I gene expression vector, HTLV-I *env*, no change in *foxp3* mRNA expression level was observed (Figure 3B). These results support the hypothesis that the transactivating HTLV-I *tax* gene is associated with the reduction in *foxp3* message and protein expression observed in HAM/TSP CD4+CD25+ T cells.

Loss of regulatory function in HTLV-I tax–transfected HD CD4+CD25+ T cells. As we had demonstrated that HTLV-I tax significantly reduced *foxp3* messenger RNA levels in HTLV-I *tax*–transfected HD CD4+CD25+ cells, it was of interest to determine whether this also corresponded to a reduction in T cell regulatory function in this population of cells. As shown in Figure 4 (a representative experiment using cells from 3 different HDs), HD CD4+CD25- T cells alone proliferated upon stimulation with anti-CD3 antibody, while the capacity of HD CD4+CD25+ regulatory cells to proliferate upon this stimulus was significantly diminished. As expected, addition of HD CD4+CD25+ to autologous HD CD4+CD25--responding cells demonstrated an inhibition of proliferation. In contrast, coculturing of HTLV-I Tax–transfected HD CD4+CD25+ cells (which induced a reduction in *foxp3* message; Figure 3) with HD CD4+CD25- failed to suppress the proliferation of these anti-CD3–stimulated, responding CD4+CD25- cells (Figure 4). These results support the hypothesis that the reduction of levels in Foxp3 in HAM/TSP CD4+CD25+ cells is mediated through infection with HTLV-I and may result in dysregulation in Treg function of HTLV-I–infected CD4+CD25+ Tregs.

Discussion

Naturally arising CD4+CD25+ Tregs are engaged in dominant control of self-reactive T cells, contributing to the maintenance of immunological self tolerance. It has been known that *foxp3* is specifically expressed in CD4+CD25+ Tregs and is a key gene for the development and function of Tregs (22–24). Therefore, to test the hypothesis that HTLV-I–infected CD4+CD25+ T cells may lack regulatory potential in HAM/TSP patients, we measured *foxp3* gene expression quantitatively and demonstrated that Foxp3 expression in CD4+CD25+ T cells of HAM/TSP patients was lower than that in cells of HDs (Figure 1). This result suggested 3 possibilities: HTLV-I has a direct inhibitory effect on Foxp3 expression; the frequency of Tregs is decreased in the CD4+CD25+ T cell population of HAM/TSP patients; or HAM/TSP patients have genetically determined low expression of *foxp3* gene. Although these possibilities are not mutually exclusive, to address whether HTLV-I has direct inhibitory effect on the Foxp3 expression, we tested the effect of HTLV-I *tax* gene transfection on *foxp3* expression in CD4+CD25+ T cells from HDs. As shown in Figures 3 and 4, it was demonstrated that HTLV-I Tax had a direct inhibitory effect on Foxp3 expression and inhibited the regulatory function of CD4+CD25+ T cells from HDs. These results suggest that HTLV-I has the potential to induce the diminution of CD4+CD25+ Treg function through the suppression of Foxp3 expression. Moreover, this is the first report to our knowledge demonstrating the role of a specific viral gene product (HTLV-I Tax) on the expression of Foxp3 that results in inhibition of Treg function. Potentially other viruses tropic for CD4+ cells may have similar effects on this important function of Tregs, as has been recently reported for HIV (41, 42).

The analysis of cell surface markers and cytokine production of CD4+CD25+ T cells from HAM/TSP further supports the observations of reduced Foxp3 levels. CD4+CD25+ T cells from HAM/TSP patients expressed lower levels of CTLA-4 and GITR molecules. CTLA-4 and GITR have also been reported to be constitutively expressed on Tregs and play a key role in normal CD4+CD25+ Treg function (17–21). Therefore, reduced expression of CTLA-4 and GITR on CD4+CD25+ T cells of HAM/TSP patients may suggest decreased frequency of Tregs in HAM/TSP patients. However, as the CTLA-4 expression on CD4+CD25+ T cells is not decreased in scurfy *foxp3* mutant mice (23), this low expression of CTLA-4 on CD4+CD25+ T cells of HAM/TSP patients is not caused by low

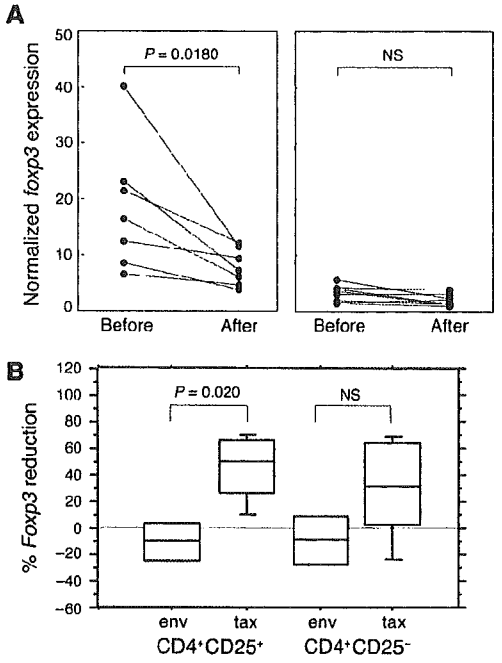

Figure 3
HTLV-I Tax suppresses Foxp3 expression. Purified CD4+CD25+ T cells and CD4+CD25− T cells from HDs were transfected with the HTLV-I *tax* gene (n = 7) or HTLV-I *env* gene (n = 4). The *foxp3* mRNA expression in these T cell populations before and after transfection was measured by real-time RT-PCR. (**A**) The *foxp3* mRNA expression level in CD4+CD25+ T cells was significantly decreased by transfection with HTLV-I *tax* gene (P = 0.018). By contrast, there was no significant decrease in *foxp3* mRNA expression in CD4+CD25− T cells. (**B**) *foxp3* mRNA expression was significantly decreased in HTLV-I *tax*–transfected CD4+CD25+ T cells compared with HTLV-I *env*–transfected CD4+CD25+ T cells (P = 0.020). There was no significant difference between the *foxp3* mRNA expression in HTLV-I *tax* transfected CD4+CD25− T cells and that in HTLV-I *env*–transfected CD4+CD25− T cells. env, HTLV-I *env* gene; tax, HTLV-I *tax* gene.

Foxp3 expression. HTLV-I may have direct suppressive effect on CTLA-4 expression. Furthermore, CD4+CD25+ T cells from HAM/TSP patients overproduced proinflammatory cytokines such as IL-2 and IFN-γ (Table 2) and may contribute to the spontaneous lymphoproliferation that has been observed in such patients (43, 44). It has been reported that normal CD4+CD25+ Tregs do not produce IL-2 by themselves and lose regulatory function in the presence of exogenous IL-2 (15, 16). Therefore, increased production of IL-2 may further support the hypothesis that CD4+CD25+ T cells from HAM/TSP patients have a defect in Treg function.

Activated T cells are increased in HAM/TSP patients (Table 2), and this raises the possibility that there may be a dilution of Tregs rather than a functional decrease in this population. To minimize this concern, we selected the CD25+ population from HAM/TSP patients based on gates set on $CD25^{high}$ in HDs during FACS sorting. A number of studies have shown that predominantly $CD25^{high}$ T cells possess regulatory functions, while $CD25^{low}$ represent activated T cells (14, 37, 45). Importantly, we have direct evidence that the introduction of HTLV-I *tax* downregulated *foxp3* expression in HD CD4+CD25+ T cells, while HTLV-I *env* did not (Figure 3B). This downregulation of *foxp3* was associated with a decrease in Treg function (Figure 4).

To demonstrate functional dysregulation, we also compared the ability of CD4+CD25+ Tregs isolated from HDs and from HAM/TSP patients to suppress plate-bound CD3-activated CD4+CD25− T cells from HDs. As shown in Figures 2 and 4, proliferation of plate-bound CD3-activated CD4+CD25− cells was diminished by 30% (Figure 4) with HD CD4+CD25+ T cells, while HAM/TSP CD4+CD25+ T cells (Figure 2) or HTLV-I tax-transfected T cells (Figure 4) did not suppress T cell proliferation. Collectively, these data suggest defects in the function of HAM/TSP CD4+CD25+ Tregs. The suppression of activated CD4+CD25− T cells by Tregs we observed is consistent with previous reports (12, 14, 16, 46), although Baecher-Allan et al. have demonstrated inhibition of CD4+CD25+ Treg function when responding CD4+CD25− cells were stimulated with high concentrations of plate-bound CD3 (46). Difference in these 2 studies could be explained by the different ratios of responding suppressor T cells used. In the present study, we demonstrated the suppressive function using a 1:1 ratio of CD4+CD25+ Tregs to responder cells.

It has been reported that naturally present Tregs may act to hamper effective immune responses to invading pathogenic microbes (33, 47, 48). For example, in mice infected with Friend retrovirus, it was demonstrated that CD4+ Tregs were increased in number and showed immunosuppressive activity. These CD4+ Tregs had increased expression of CD38+ and CD69+ (33). In contrast, the expression of CD38+ and CD69+ on CD4+CD25+ T cells was decreased in HAM/TSP patients and did not show immuno-

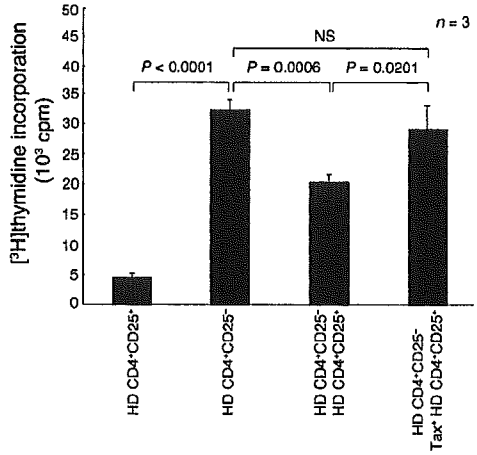

Figure 4
Loss of regulatory function in HTLV-I tax–transfected HD CD4+CD25+ T cells. CD4+CD25+ or CD4+CD25− T cells from uninfected HDs were stimulated with 2.5 μg/ml anti-CD3 antibody and irradiated PBMCs and cultured for 4 days (HD CD25+ and HD CD25−). Furthermore, to compare the suppressive activity of HD CD4+CD25+ T cells before and after HTLV-I *tax* gene transfection, CD4+CD25− T cells from HDs were stimulated with 2.5 μg/ml anti-CD3 antibody and irradiated PBMCs and cultured for 4 days in the presence of equal numbers of HD CD4+CD25+ T cells or HTLV-I *tax*–transfected HD CD4+CD25+ T cells (Tax+ HD CD25+). After culture, [³H]thymidine was added for additional 16 hours. The suppressive activity of CD4+CD25+ T cells from HDs was inhibited by transfection with the HTLV-I *tax* gene. Data represent the mean of experiments with cells from 3 HDs.

suppressive activity. These results suggest that CD38$^+$ and CD69$^+$ are also important cell surface markers that may distinguish human Tregs from effector T cells, as reported previously in studies on rodents (32, 33).

It has been reported that microbial infection can dysregulate Tregs to suppress pathologic antimicrobial immune responses that cause tissue damage (i.e., immunopathologic response) (49, 50). For example, in SCID mice chronically infected with *Pneumocystis carinii*, transfer of T cells depleted of CD4$^+$CD25$^+$ Tregs elicited severe pneumonitis, whereas transfer of T cells not depleted of Tregs did not (49). Thus, in controlling microbial immunity, the frequency of CD4$^+$CD25$^+$ Tregs may play an important role. However, it is not known how these T cells contribute to the regulation of antimicrobial immune responses. The increased expression of CD28 molecules and decreased expression of CTLA-4 on CD4$^+$CD25$^+$ T cells in HAM/TSP patients (shown in this study) may therefore serve to regulate this population of cells (51). CD28 and CTLA-4 share the same ligands (CD80 and CD86) on APCs, and CD28 has much lower affinity for CD80 and CD86 than CTLA-4 (52). CTLA-4 has been reported to be required for the suppressive function of Tregs. In contrast, stimulation through CD28, with concurrent TCR stimulation, abrogates suppressive function (15, 16). CD4$^+$CD25$^+$ T cells have been reported to be a major reservoir of HTLV-I and to present HLA-virus peptide complexes (30). This increased expression of HTLV-I peptide/HLA complexes on CD4$^+$CD25$^+$ cells may increase activation of these cells by signaling through CD28, resulting in the loss of T cell regulatory/suppressive activity. Further comparative analysis of the expression of these molecules on CD4$^+$CD25$^+$ T cells between healthy individuals infected with HTLV-I and patients with HAM/TSP will be necessary to confirm these hypothesis.

In summary, it was demonstrated that in CD4$^+$CD25$^+$ T cells from HAM/TSP patients that were preferentially infected with HTLV-I, Foxp3 expression was lower than that in cells from HDs. HTLV-I Tax had a direct inhibitory effect on Foxp3 expression and inhibited the regulatory function of CD4$^+$CD25$^+$ T cells from HDs. Furthermore, compared to CD4$^+$CD25$^+$ T cells from HD, CD4$^+$CD25$^+$ T cells from HAM/TSP patients showed lower expression of constitutive molecules of Tregs such as CD38, CD62L, CD69, CTLA-4, and GITR and overproduced proinflammatory cytokines such as IL-2 and IFN-γ. In addition, loss of function of CD4$^+$CD25$^+$ T cells has also been reported in other autoimmune disorders such as type 1 diabetes, rheumatoid arthritis, and multiple sclerosis, a neurodegenerative disorder of unknown etiology (37, 45, 53). The finding that autoreactive T cells in patients with autoimmune diseases are more easily activated (54, 55) than those in healthy individuals suggest that CD4$^+$CD25$^+$ Tregs may play a role in controlling the development of autoimmunity. A dysfunction in Tregs in HAM/TSP is consistent with the hypothesis that an autoimmune component may also contribute to the pathogenesis of HAM/TSP (reviewed in ref. 56). Although it has been well demonstrated that the removal or functional alteration of CD4$^+$CD25$^+$ Tregs from normal rodents leads to the spontaneous development of autoimmune diseases, how these cells lose their suppressive function in human disease is unknown. This study suggests the hypothesis that the direct human retrovirus infection of CD4$^+$CD25$^+$ T cells may contribute to a dysregulation of CD4$^+$CD25$^+$ Tregs in a human retrovirus-associated neurologic disease.

Methods

Subjects and cell preparation. The PBMCs were prepared by centrifugation over Ficoll-Hypaque gradients (BioWhittaker) from 13 HAM/TSP patients, 13 HTLV-I–seronegative HDs, and 2 ACs, and the cells were viably cryopreserved in liquid nitrogen until tested. HAM/TSP was diagnosed according to WHO guidelines (57). HTLV-I seropositivity was determined by ELISA (Abbott Laboratories), with confirmation by Western blot analysis (Genelabs Technologies Inc.). Blood samples were obtained after informed consent as part of a clinical protocol reviewed and approved by the NIH institutional review panel. CD4$^+$ T cells were negatively selected from the PBMCs with magnetic beads (MACS CD4$^+$ T cell isolation kit; Miltenyi Biotec) according to the manufacturer's instructions. These selected CD4$^+$ T cells were stained with anti-CD25 FITC (Caltag Laboratories) and sorted into CD4$^+$CD25$^+$ (sorted CD25$^+$ cells were gated on high levels of expression of CD25 in HDs during FACS sorting; Supplemental Figure 1) and CD4$^+$CD25$^-$ T cells using FACSVantage (BD).

Foxp3 expression analysis by real-time RT-PCR. Total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and cDNA was synthesized from extracted RNA using TaqMan Gold RT-PCR Kit using Random Hexamer primer (Applied Biosystems). *foxp3* mRNA expression was quantified by real-time PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems). Real-time RT-PCR was performed using the protocol described in our previous report (10), with some modification. Sample cDNA from 100 ng RNA was applied per well and analyzed. Samples were run in duplicate, and the mean values were used for calculation. The primer set for *foxp3* was 5′-GGCCCTTCTCCAGGACAGA-3′ and 5′-GCTGATCATGGCTGGGTTGT-3′. The probe for *foxp3* was 5′-FAM-ACTTCATGCATCAGCTCTCCACTGTGGATTAMRA-3′. Amplification was carried out at 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles at 95°C for 15 seconds and 60°C for 1 minute in a total volume of 50 μl. We used the human housekeeping gene *hypoxanthine ribosyl transferase* (*HPRT*) primers and probe set (Applied Biosystems) to calculate for normalized values of *foxp3* mRNA expression. The normalized values in each sample were calculated as the relative quantity of *foxp3* mRNA expression divided by the relative quantity of *HPRT* mRNA expression. The values were calculated by the following formula: normalized *foxp3* expression = $2^{\text{Ct value of } HPRT - \text{Ct value of } foxp3}$.

Flow cytometric analysis. PBMCs were immunostained with various combinations of the following fluorescence-conjugated antibodies: CD25 (CALTAG Laboratories), CD4, CD45RA, CD45RO, CD27, CD28, CD38, CD62L, HLA-DR, CD69, CTLA-4 (BD Biosciences – Pharmingen), and GITR (R&D Systems). These cells were also intracellularly stained with the following antibodies: IL-2, IFN-γ, IL-4, IL-10 (BD Biosciences – Pharmingen), and Foxp3 (Abcam Inc). Flow cytometric analysis was performed on a FACSCalibur cytometer (BD Biosciences). Data processing was accomplished with CELLQuest software (BD).

Transfection. The sorted cells from HDs were harvested in a seeding condition of 1×10^6 cell/ml and were incubated for 2 hours at 37°C in RPMI 1640 supplemented with 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM glutamine (culture medium). The cells washed once in PBS and resuspended in the specified electroporation buffer (Nucleofector solution; Amaxa) to a final concentration of 1×10^6 cells/100 μl. Then 2 μg of HTLV-I *env* plasmid DNA or HTLV-I *tax* plasmid DNA (kindly provided by D. Derse, National Cancer Institute-Frederick, Frederick, Maryland, USA) were added to the cell suspension and they were transfected using T cell Nucleofector kit (Amaxa) according to the manufacturer's instructions. After electroporation, the cells were immediately suspended in 2 ml of culture medium and cultured overnight at 37°C in a 5% CO_2 incubator.

Proliferation assay by CFSE. A total of 1×10^5 CD4$^+$CD25$^-$ T cells/well from HD were labeled with CFSE using Vybrant CFDA SE Cell Tracer Kit

(Invitrogen Corp.) according to the manufacturer's instructions. Cells were incubated for 6 days in the culture medium with or without 2.5 μg/ml anti-CD3 antibody in round-bottomed 96-well plates. In some cultures, 1×10^5 irradiated allogeneic $CD4^+CD25^+$ T cells from HDs or 1×10^5 irradiated allogenic $CD4^+CD25^+$ T cells from HAM/TSP patients were added. Cells were subjected to flow cytometric analysis.

Proliferation assay by liquid scintillation counter. For the proliferation assay of T cells from HDs, 1×10^4 $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells/well from HDs were cultured in 200 μl culture medium (RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 5% human AB serum) in round-bottomed 96-well plates. These cell populations were stimulated with 2.5 μg/ml anti-CD3 antibody (OKT-3; BD) in the presence of 5×10^4 irradiated PBMCs. After 4 days culture, 1 μCi tritium thymidine ([^{3}H]TdR)/well was added for additional 16 hours. A liquid scintillation counter was used to measure proliferation. Furthermore, to compare the suppressive effect on the cell proliferation between $CD4^+CD25^+$ T cells and HTLV-I *tax* gene transfected $CD4^+CD25^+$ T cells, 1×10^3 $CD4^+CD25^-$ T cells/well from HD were stimulated with 2.5 μg/ml anti-CD3 antibody (OKT-3) in the presence of 5×10^4 irradiated PBMCs, then cocultured with 1×10^4 $CD4^+CD25^+$ T cells/well or with 1×10^4 HTLV-I *tax*-transfected $CD4^+CD25^+$ T cells/well. After 4 days culture, 1 μCi [^{3}H]TdR/well was added for additional 16 hours. A liquid scintillation counter was used to measure proliferation.

Statistical analysis. Student's *t* tests were used for the significance of data comparison.

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