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ORIGINAL ARTICLE

FOXP3$^+$CD25$^-$ Tumor Cells with Regulatory Function in Sézary Syndrome

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Cutaneous T-cell lymphoma (CTCL) has been suggested by *in vitro* experiments to represent a malignant CD4$^+$ T-cell proliferation with a regulatory T-cell (Treg) phenotype (CD4$^+$CD25$^+$FOXP3$^+$). We investigated percentages of FOXP3$^+$ and CD25$^+$ cells in the blood of 15 Sézary, 14 mycosis fungoides (MF), and 10 psoriasis (Pso) patients and 20 normal healthy donors (NHDs). We found similar numbers of FOXP3$^+$ cells in MF (10.4% of blood CD4$^+$ cells) and Pso (11.1%) patients and NHDs (9.8%). In 8 of 15 (53%) Sézary patients, significantly reduced percentages of FOXP3$^+$ cells were seen in blood (2.9%) and skin (10.4%). Interestingly, 6 of 15 (40%) Sézary patients showed significantly increased percentages of FOXP3$^+$ cells (39.7% (blood), 20.3% (skin)); however, these cells did not express CD25. In these latter patients, clone-specific TCR-Vβ-chain antibodies were used to demonstrate that these FOXP3$^+$CD25$^-$ cells were monoclonal CTCL tumor cells. FOXP3$^+$CD25$^-$ CTCL tumor cells showed a highly demethylated status of the foxp3 gene locus similar to Treg cells, and they were functionally able to suppress IL-2 mRNA induction in TCR-stimulated conventional T cells. Thus, FOXP3$^+$CD25$^-$ CTCL tumor cells with functional features of Treg cells define a subgroup of Sézary patients who might carry a different prognosis and might require differential treatment.

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INTRODUCTION

Primary cutaneous T-cell lymphomas (CTCLs) represent a heterogeneous group of rare lymphoproliferative disorders characterized by skin-homing monoclonal proliferations of CD4$^+$ T lymphocytes (Willemze *et al.*, 2005). The major variant is mycosis fungoides (MF), which is confined to the skin and has a slowly progressing course over years to decades. The less common Sézary syndrome (SS) carries a worse prognosis (Klemke *et al.*, 2005) and is characterized by erythroderma, generalized lymphadenopathy, and leukemic blood involvement with >1000 Sézary cells per μl (Vonderheid *et al.*, 2002). Both variants of CTCL are clinically associated with a marked immunosuppression that is often associated with a fatal outcome, especially in patients with SS.

In 1995, Sakaguchi *et al.* identified CD4$^+$CD25$^+$ T cells that control immune responses to non-self antigens by suppressing conventional T-cell (Tcon) activity (Sakaguchi *et al.*, 1995). These suppressive cells represent a small subset of T cells (approximately 10% of peripheral CD4$^+$ T cells) and were termed regulatory T (Treg) cells (Sakaguchi *et al.*, 2007; Sakaguchi and Powrie 2007). Treg cells are important because depletion of CD25$^+$ cells leads to a variety of autoimmune inflammatory diseases, whereas reconstitution with CD4$^+$CD25$^+$ T cells can inhibit disease development (Setoguchi *et al.*, 2005). In 2001, research on scurfy mice, which spontaneously develop severe autoimmunity, showed that the disease was due to a single mutation of the foxp3 gene on the X chromosome (Brunkow *et al.*, 2001). Mutations of the human foxp3 gene were found to be the cause of a similar human disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Bennett *et al.*, 2001). FOXP3 is abundantly expressed in natural Treg cells, and is so far the most reliable molecular marker for these cells (Roncador *et al.*, 2005). However, naive T cells in humans readily express FOXP3 upon TCR stimulation, although the expression is generally much lower and only transient compared with that of natural Treg cells (Gavin *et al.*, 2006; Pillai *et al.*, 2007; Walker *et al.*, 2003; Yagi *et al.*, 2004). Still, quantifying the degree of DNA demethylation in the foxp3 locus enables differentiation

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Abbreviations: CTCL, cutaneous T-cell lymphoma; FACS, fluorescence-activated cell sorter; MF, mycosis fungoides; NHD, normal healthy donor; Pso, psoriasis; SS, Sézary syndrome; Tcon, conventional T cell; Treg, regulatory T cell

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between Treg cells and activated FOXP3$^+$ Tcon cells (Baron *et al.*, 2007; Janson *et al.*, 2008).

Despite their importance in controlling autoimmunity, Treg cells can also suppress antitumor immune responses in various cancers (Wang and Wang 2007). Therapeutically, antitumor immune responses can be enhanced after depletion of Treg cells during vaccination, for example, in glioma patients (Grauer *et al.*, 2008). Not only solid tumors but also non-Hodgkin lymphomas can induce functional Treg cells and thus worsen prognosis (Mittal *et al.*, 2008).

The fact that CTCL is a disease of malignant CD4$^+$ T cells associated with clinically evident immunosuppression made it an interesting model for the investigation of various immune functions. On the basis of *in vitro* cell culture studies, Berger *et al.*, 2005 suggested that CTCL represents a malignant proliferation of Treg cells, which could explain its immunosuppressive nature. This could not be immediately confirmed *in vivo*, as multiple laboratories, including our own, failed to demonstrate the FOXP3 expression of skin-infiltrating CTCL tumor cells and, rather, showed that FOXP3$^+$ cells in MF skin lesions were bona fide Treg cells (Banham *et al.*, 2008; Gjerdrum *et al.*, 2007; Klemke *et al.*, 2006). High numbers of FOXP3$^+$ cells in skin specimens from MF lesions correlated with early stages of MF and a better prognosis than did MF skin infiltrates with fewer FOXP3$^+$ cells (Gjerdrum *et al.*, 2007). However, a recent study identified FOXP3$^+$ CTCL tumor cells in the skin of 4 of 11 patients and the expression of low-molecular splice forms of FOXP3 in the peripheral blood of 4 of 6 patients with SS (Krejsgaard *et al.*, 2008).

Here, we characterized Treg cells in the peripheral blood of 29 CTCL patients by analyzing the expression of FOXP3, CD25, and skin-homing markers. In a subgroup comprising 40% of our Sézary patients, we found FOXP3 mRNA and protein expressions of CTCL tumor cells, which were identified by monoclonal TCR-Vβ-chain expression. FOXP3$^+$ CTCL tumor cells showed a highly demethylated status of the foxp3 gene locus, similar to Treg cells. Finally, FOXP3$^+$ CTCL tumor cells isolated from Sézary patients also showed regulatory functions like those of Treg cells, as demonstrated by the suppression of IL-2 mRNA induction in TCR-stimulated Tcon cells.

RESULTS

CD4$^+$CD25$^+$FOXP3$^+$ cells are not significantly reduced in the peripheral blood of patients with SS compared with MF, psoriasis, and healthy controls

To identify Treg cells in the peripheral blood of CTCL patients and controls (normal healthy donors (NHDs) and psoriasis (Pso)), we measured CD4, CD25, and FOXP3 expression by flow cytometry, according to the strategy presented in Supplementary Figure S1. In our previous study, we found a lack of Treg cells in Sézary patients, as demonstrated by the CD4$^+$CD25^{++} expression in the blood and the FOXP3 expression in the skin (Klemke *et al.*, 2006). To better characterize Treg cells in CTCL patients, we analyzed FOXP3 expression in the blood from Sézary and MF patients and, as

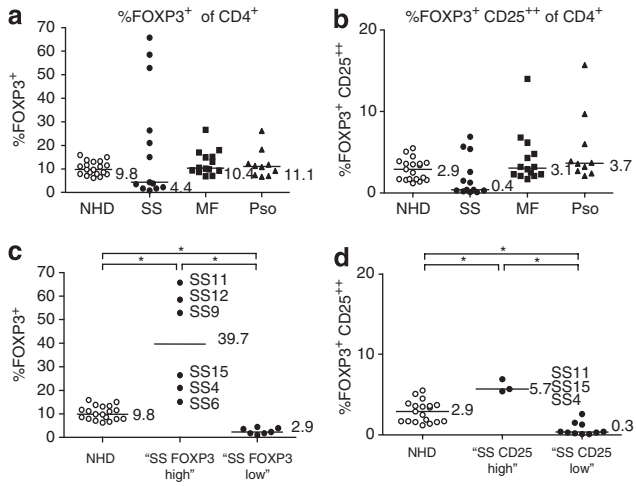

Figure 1. Quantification of FOXP3$^+$ and FOXP3$^+$CD25^{++} Treg cells in normal healthy donors (NHDs) and patients with SS, MF, and Pso. The percentages of FOXP3$^+$ Treg cells and FOXP3$^+$CD25^{++} Treg cells among CD4$^+$ cells in different patient groups were measured using flow cytometry according to the strategy presented in Supplementary Figure S1. Every symbol represents a single individual (NHD, $n=18$; SS, $n=13$; MF, $n=14$; Pso, $n=10$). The median for each group is shown by the black line. Identification numbers of individual patients are shown in (**c**) and (**d**). No significant differences could be detected between the groups as evaluated by the Wilcoxon signed-rank test (**a** and **b**). (**c**) Further analysis of the SS patients compared with NHDs revealed one group of patients with significantly reduced numbers of FOXP3$^+$ cells (''SS FOXP3 low'') and a second group with significantly increased numbers of FOXP3$^+$ cells (''SS FOXP3 high''), $*=P<0.05$. (**d**) The analysis of FOXP3$^+$CD25^{++} cells also identified a ''SS FOXP3/CD25 low'' and a ''SS FOXP3/CD25 high'' group compared with NHDs, $*=P<0.05$ (**d**).

controls, from Pso patients and NHDs. We found similar percentages of FOXP3$^+$ cells in NHDs (9.8 ± 2.9% (SD) of CD4$^+$ cells, $n=20$), MF (10.4 ± 5.4%, $n=14$) and Pso (11.1 ± 6%, $n=10$) patients. Percentages of FOXP3$^+$CD25^{++} cells in NHDs (2.9 ± 1.3%), MF (3.1 ± 3.2%) and Pso (3.7 ± 4.3%) patients were also comparable (Figures 1a and b, Table 1). In line with our previous findings (Klemke *et al.*, 2006), the percentages of FOXP3$^+$ (4.4 ± 23,9%, $n=15$) and FOXP3$^+$CD25^{++} cells (0.4 ± 2.4%) were reduced in Sézary patients (Figures 1a and b). However, these differences were not significant because of the wide spread of values in the Sézary group.

Sézary patients show heterogeneous expression levels of FOXP3 in peripheral blood and skin

A more detailed analysis of the Sézary patient group revealed two different subgroups. In all, 53% (8 of 15) of Sézary patients showed significantly reduced percentages of FOXP3$^+$ (2.9 ± 1.3% of CD4$^+$ cells) and FOXP3$^+$CD25^{++} cells (0.3 ± 0.8%) (''SS FOXP3 low'') when compared with those from NHDs (both $P<0.05$) and Pso and MF patients. Interestingly, 40% (6 of 15) of Sézary patients showed significantly increased numbers of FOXP3$^+$ cells (39.7 ± 21.6%, $n=6$) (''SS FOXP3 high''), and a subset of these also

Table 1. Laboratory data of patients with Sézary syndrome established at the time of this study

Patient	WBC (4200–10200 per µl)	CD4 (400–1800 per µl)	CD4/CD8 (0.9–3.5)	Sézary cell count (%)[1]	$CD4^+$ $CD7^-$(per µl)	TCR-R	TCR-Vβ	$FOXP3^+$ cells per µl Blood	$FOXP3^+$ of $CD4^+$ (%) Blood	$FOXP3^+$ of $CD4^+$ (%) Skin
$SS1^{(-)}$	14900	7570	98	90	7483	mc	Vβ2	295	3.9	n.d.[3]
$SS2^{(-)}$	8110	2097	5.6	46	1930	mc	Vβ18	82	3.9	6.9
$SS3^{(-)}$	6540	1223	5.4	40	1024	mc	Vβ1	12	1	9
$SS4^{(+)}$	4440	1347	6.4	60	1007	mc	Vβ7.1	283	21	25.6
SS5	51000	7678	97	70	6528	mc	Vβ17	n.d.[3]	n.d.[3]	n.d.[3]
$SS6^{(+)}$	9850	3554	48,5	n.d.[2]	2955	mc	Vβ22	537	15.1	n.d.[3]
$SS7^{(-)}$	11800	1399	5.2	10	25	mc	Vβ2	83	5.9	6.9
$SS8^{(-)}$	5140	1453	19	23	1277	mc	Vβ1	70	4.8	7.5
$SS9^{(+)}$	6180	1065	9.9	40	626	mc	Vβ18	969	91	15.6
$SS10^{(-)}$	4900	1532	15.3	90	1435	mc	–	35	2.3	n.d.[3]
$SS11^{(+)}$	9240	1106	11.2	50	966	mc	–	727	65.7	18.2
$SS12^{(+)}$	4620	198	1.3	n.d.[2]	121	mc	Vβ2	116	58.5	31.4
$SS13^{(-)}$	10400	4129	32	80	3890	mc	–	70	1.7	n.d.[3]
$SS14^{(-)}$	13500	2297	90	n.d.[2]	2070	mc	Vβ4	39	1.7	18.2
$SS15^{(+)}$	10700	1269	7.2	6	192	mc	–	335	26.4	10.8

mc, monoclonal; SS, Sézary syndrome: $^{(+)}$"SS FOXP3 high"; $^{(-)}$"SS FOXP3 low"; TCR-R, T-cell receptor rearrangement of peripheral blood mononuclear cells; WBC, white cell blood count.
[1]Determined by peripheral blood smear.
[2]Not detectable by morphology.
[3]Not determined.
Ranges of normal values and used units are indicated in the first row.

had increased percentages of $FOXP3^+CD25^{++}$ cells (5.2 ± 0.8%, $n=3$) in comparison with those from NHDs (both $P<0.05$) (Figures 1c and d). Figures 2a and b show original flow cytometry data in order to illustrate the varying levels of $FOXP3^+$ cells in $CD4^+$ cells in different patients. In patient SS1, we hardly detected any $FOXP3^+$ cells, and the few that were found were $CD25^-$(Figure 2a). In patient SS9, nearly all $CD4^+$ cells expressed FOXP3 at levels almost as high as those in regular Treg cells (as can be noticed by the overlay of $CD4^+$ cells from an NHD, Figure 2b). Finally, in some patients, for example, SS12, half of the $CD4^+$ cells were $FOXP3^+$. Interestingly, almost all $FOXP3^+$ cells of the patients were $CD25^-$, and the few $FOXP3^+CD25^+$ cells detected probably reflect bona fide Treg cells. Only in patients SS4, SS11, and SS15 did we find substantial levels of CD25 on $FOXP3^+$ cells. In addition, the percentage of $FOXP3^+$ cells also differs in the skin between the two Sézary patient groups, with a trend toward higher percentages of skin-infiltrating $FOXP3^+$ cells for the "SS FOXP3 high" group (Table 1). "SS FOXP3 high" patients have a mean of 20.3 ± 8.2% (range 11–31%) $FOXP3^+$ cells of $CD4^+$ cells in the skin infiltrate in contrast to a mean of 10.4 ± 5.3% (range 7–18%, $P=0.063$) $FOXP3^+$ cells of $CD4^+$ cells in patients of the "SS FOXP3 low" group. This finding suggests that in the "SS FOXP3 high" patients, CTCL tumor cells may express FOXP3 *in vivo*, similar to what has been shown by Berger *et al.*, 2005 for CTCL cells after *in vitro* culture with dendritic cells.

A subgroup of patients with SS has high numbers of $FOXP3^+$ CTCL tumor cells in the peripheral blood

The high number of $FOXP3^+$ cells in the blood of almost half of the Sézary patients could be due to FOXP3 expression in monoclonal CTCL tumor cells or to the expansion of polyclonal natural Treg cells. To distinguish between these possibilities, each Sézary patient was screened with a panel of TCR-Vβ-chain antibodies as described elsewhere (Morice *et al.*, 2004). We were able to detect a predominant T-cell population by an identical TCR-Vβ-chain expression in 11 of 15 (73%) Sézary patients (Table 1) as described previously, because the panel of available TCR-Vβ-chain antibodies covers only about 70% of the TCR-Vβ-chain repertoire (Klemke *et al.*, 2008; Morice *et al.*, 2004; Morice *et al.*, 2006). In some Sézary patients, the CTCL tumor cells as identified by the identical "clonal" TCR expression also express FOXP3 (Figure 3), but, interestingly, express very low levels of CD25 (data not shown). In an NHD, as well as in patient SS3, $FOXP3^+$ cells were equally distributed within the monoclonal and polyclonal T-cell populations, both showing only a few $FOXP3^+$ cells (Figures 3a and b). Patient SS9 demonstrated an intermediate FOXP3 expression within the "clonal" T-cell population (Figure 3c). Finally, a high

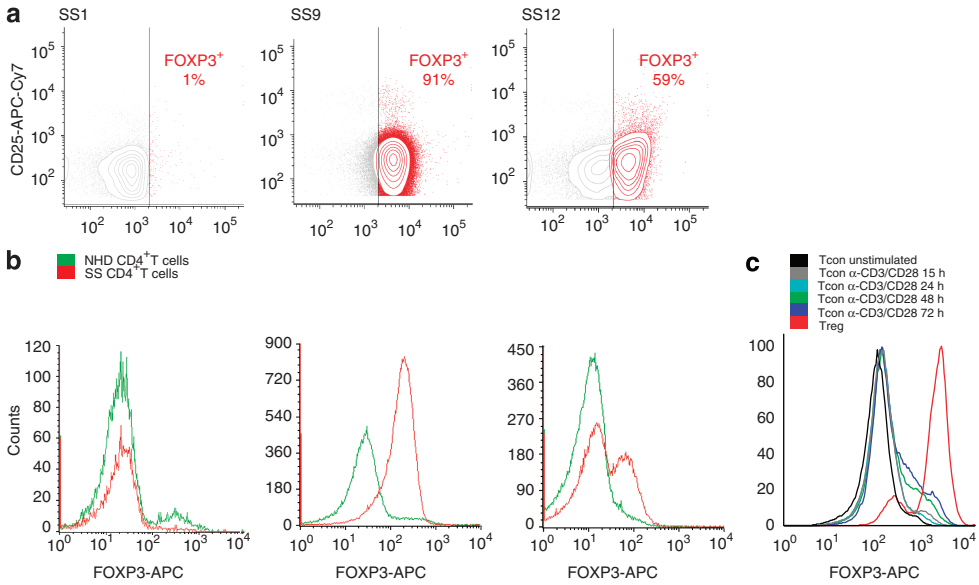

Figure 2. FOXP3 and CD25 expression levels in patients with Sézary syndrome (SS). FOXP3 expression was compared with CD25 expression of $CD4^+$ cells (''dot blots in A''). (**a**) FOXP3 expression identifies two groups of SS patients: an ''SS FOXP3 low'' group (shown as SS1) and an ''SS FOXP3 high'' group (shown as SS12 and SS9). (**b**) To better illustrate the different levels of FOXP3 expression, the SS patients were compared with a normal healthy donor (NHD) in a FOXP3 histogram overlay. (**c**) As a control, Tcon cells (Tcon) from an NHD were stimulated with α-CD3/α-CD28 for 15–72 hours (one representative donor shown) showing induction of FOXP3 in contrast to unstimulated Tcon cells, but to a lesser extent than was found in Treg cells (Treg).

FOXP3 expression of all CTCL tumor cells was seen in patient SS12 (Figure 3d). This finding was verified by staining with two different FOXP3 antibodies (PCH101 and 236A/E/), confirming its specificity (Tran *et al.*, 2007) (Figure 3). Furthermore, we excluded nonspecific Ab binding using isotype control antibodies (data not shown). Finally, we were able to identify increased Foxp3 mRNA expression levels in TCR-Vβ-chain CTCL tumor cells of ''SS FOXP3 high'' patients that were sorted by fluorescence-activated cell sorting (FACS), which were similar to those in expanded Treg cells from NHD, although lower than in freshly isolated Treg cells from NHD (Figure 4). Furthermore, the Foxp3 mRNA expression levels of ''SS FOXP3 high'' patients were similar to those of Tcon cells stimulated with α-CD3/α-CD28 for 15–23 hours. However, the FOXP3 mRNA expression in activated Tcon cells was only transient and decreased below that of unstimulated Tcon cells after 14 days (Figure 4). FOXP3 protein expression was also seen in stimulated Tcon cells, but to a lesser extent than in Treg cells (Figure 2c). In addition, FOXP3 expression is stable in CTCL tumor cells, as reanalyses of FOXP3 protein expression in several $FOXP3^+$ patients after a few months still yielded an identically high FOXP3 expression in tumor cells (data not shown). In contrast to this, the FOXP3 expression in Tcon cells was shown to be only transient after *in vitro* activation (Figure 4 and Allan *et al.*, 2007; Pillai *et al.*, 2007 and Walker *et al.*, 2003). Patients belonging to the ''SS FOXP3 low'' group were found to express low levels of FOXP3 mRNA similar to shortly stimulated (3 hours) or unstimulated Tcon cells from NHDs (Figure 4). In summary, this clearly shows that in a subgroup of 40% of Sézary patients, CTCL tumor cells express FOXP3 mRNA and protein.

$FOXP3^+$ CTCL tumor cells exhibit functions of naturally occurring Treg cells

The fact that the ''clonal'' $FOXP3^+$ cells of the ''SS FOXP3 high'' subgroup of Sézary patients are $CD25^-$ argues for the idea that these are CTCL tumor cells rather than expanded Treg cells. However, Treg cells might lose CD25 expression on prolonged proliferation. To test whether $FOXP3^+$ CTCL tumor cells ($FOXP3^+$ ''clonal'' cells) might have originated from Treg cells or whether they gained the function of Treg cells because of the expression of FOXP3, we investigated whether they demonstrate the features and functions of naturally occurring Treg cells. It was recently shown that it is possible to distinguish Treg cells from activated $FOXP3^+$ Tcon cells by quantifying the degree of DNA methylation in the human foxp3 locus (Baron *et al.*, 2007; Janson *et al.*, 2008). We have used this technique to investigate the methylation status of the foxp3 locus of TCR-Vβ-chain FACS-sorted CTCL tumor cells. Patients of the ''SS FOXP3 high'' group demonstrated a highly demethylated foxp3 gene locus similar to that of Treg cells from NHDs or an intermediate status of the foxp3 gene locus with higher demethylation than in Tcon cells but less than in Treg cells (Figure 5a). In contrast, ''SS FOXP3 low'' patients showed a similar methylation status of the foxp3 gene locus, as did Tcon cells from NHDs (Figure 5a). Demethylation in $FOXP3^+$ tumor cells can be observed although the analyzed patients are all female, even though one of the X-chromosomal foxp3 loci is usually silenced and thus methylated. An aberrant methylation pattern does not seem to be a general feature of Sézary tumor cells, as it is observed only in $FOXP3^+$ and not in $FOXP3^-$ tumor samples.

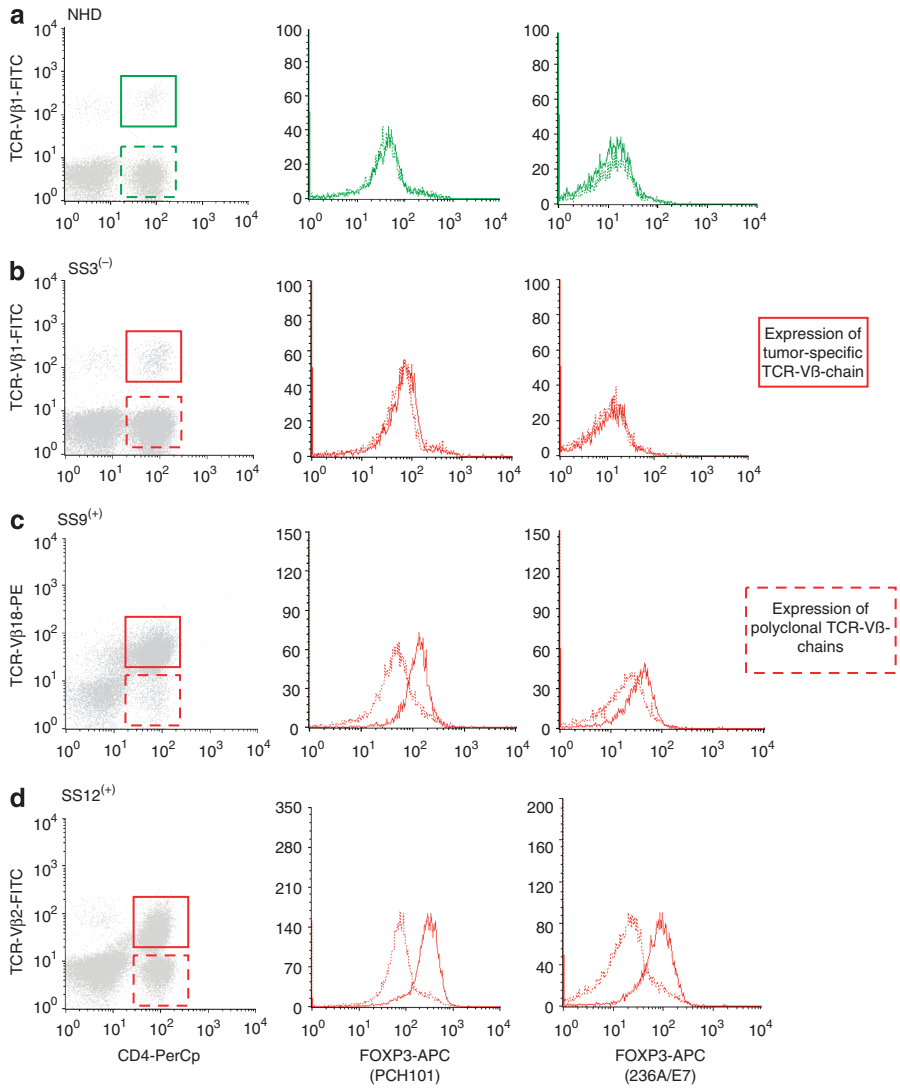

Figure 3. In a subgroup of Sézary (SS) patients, the cutaneous T-cell lymphoma (CTCL) tumor cells express FOXP3. Using a large panel of α-TCR-Vβ-chain mAbs, we first determined the appropriate mAb with which to detect clonal CTCL tumor cells. The mAb was then used in multicolor staining in combination with FOXP3. The identical surface expression of a given TCR-Vβ-chain within the lymphocyte population identifies CTCL tumor cells ($CD4^+TCR\text{-}V\beta^+$; solid square) and distinguishes them from the remaining polyclonal lymphocytes ($CD4^+TCR\text{-}V\beta^-$, dotted square). Both cell populations were analyzed for their FOXP3 expression with two antibodies (PCH101 and 236A/E7). The results of a normal healthy donor (NHD) (**a**, in green) and of three SS patients (**b**–**d**, in red) are shown. In the NHD, the solid gate identifies all TCR-Vβ1$^+$ cells that are of polyclonal nature and thus similar to the rest of the cells in the dotted gate, and both populations therefore show identical FOXP3 expression patterns (**a**).

Recently our group demonstrated that Treg cells suppress the induction of Th1 cytokine mRNA (for example, IL-2 and IFN-γ) of Tcon cells as early as 1 h after stimulation (Oberle *et al.*, 2007). Here, we tested the capacity of CTCL tumor cells from both patient groups to suppress IL-2 mRNA induction of Tcon cells after TCR stimulation. TCR-Vβ-chain FACS-sorted $FOXP3^+$ CTCL tumor cells suppressed TCR-induced IL-2 mRNA expression in Tcon cells to a similar extent, as did naturally occurring Treg cells from NHDs (Figure 5b). In contrast, $FOXP3^-$ CTCL tumor cells from patients of the ''SS FOXP3 low'' group were not able to suppress TCR-induced IL-2 mRNA induction in Tcon cells (Figure 5b). Thus, $FOXP3^+$ CTCL tumor cells have suppressive function and a demethylated foxp3 gene locus, and are therefore similar to naturally occurring Treg cells.

DISCUSSION

In this study, we provide evidence for $FOXP3^+$ CTCL tumor cells *in vivo*. In accordance with our previous results showing a lack of $CD4^+CD25^+$ cells in Sézary patients (Klemke *et al.*, 2006), we identified a subgroup of Sézary patients with significantly reduced percentages of $FOXP3^+$ and $FOXP3^+CD25^+$ cells in peripheral blood in contrast to MF and Pso patients and NHDs. Interestingly, 40% of Sézary patients presented significantly increased numbers of $FOXP3^+$ and $FOXP3^+CD25^+$ cells in the blood, and also—

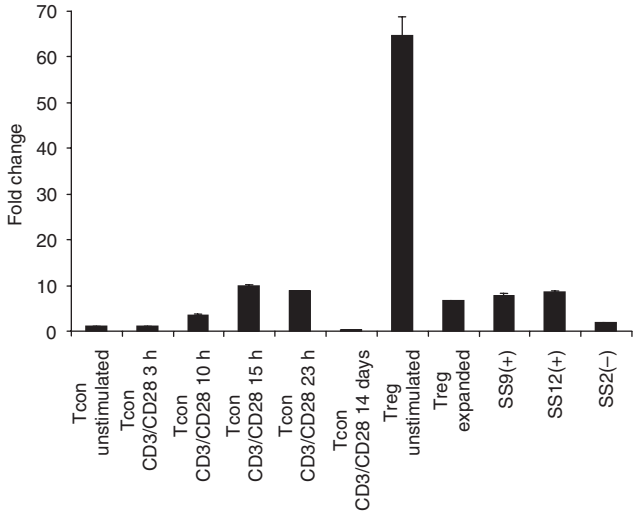

Figure 4. FOXP3$^+$ cutaneous T-cell lymphoma (CTCL) tumor cells express FOXP3 mRNA at a level similar to that of expanded Treg cells. FOXP3 mRNA levels were determined by quantitative reverse transcriptase–PCR in TCR-Vβ-chain CTCL tumor cells that were sorted by fluorescence-activated cell sorting from FOXP3$^+$ and FOXP$^-$ Sézary (SS) patients. As controls, normal healthy donors (NHDs) were analyzed with regard to FOXP3 mRNA expression in both unstimulated and α-CD3/α-CD28-stimulated Tcon cells (Tcon), unstimulated Treg cells (Treg), and expanded Treg cells (3–23 hours and 14 days, expanded for 20 days with α-CD3, α-CD28, and IL-2). One representative NHD is shown. Fold inductions relative to unstimulated Tcon cells (set to 1) are denoted. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase, with error bars representing the standard deviation of the mean. Subgroups of SS patients are labeled (+) for "SS FOXP3 high" or (−) for "SS FOXP3 low."

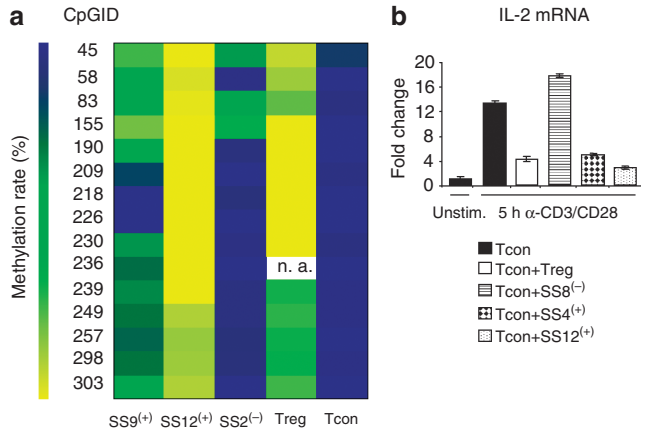

Figure 5. FOXP3$^+$ CTCL tumor cells show DNA demethylation of the foxp3 gene locus and suppress IL-2 mRNA induction in TCR-stimulated Tcon cells comparable to Treg cells from normal healthy donors. (**a**) Cell populations were sorted by fluorescence-activated cell sorting according to their CD4 and CD25 or TCR-Vβ-chain expression. Total DNA from sorted cells was extracted and subjected to bisulfite sequencing to assess CpG methylation of the foxp3 gene locus. The methylation rate of characteristic CpG sequences in the TSDR (Treg-specific demethylated region) of the Foxp3 gene, which consists of amplicon 5, is depicted by the color scale. The DNA methylation rate of three Sézary patients (SS), as well as Treg and Tcon cells from a representative NHD. (**b**) For suppression assays, responder Tcon cells from an NHD were co-cultured with Treg cells (NHD) or Sézary tumor cells in a 1:1 ratio and stimulated with α-CD3 and α-CD28. Responder Tcon cells alone, either unstimulated (unstim.) or stimulated, were used as control. To exclude cell density effects, double amounts of Tcon cells alone were also used as control (not shown). Treg cells were isolated by flow sorting for expression of CD4 and high expression of CD25, whereas Tcon cells were CD25 depleted and CD4$^+$ purified by MACS technology. After the co-culture assays, responder Tcon cells were separated from Treg cells by staining for CD4 and CD25 and from tumor cells by CD4 and TCR-Vβ staining and subsequent flow sorting. After stimulation with α-CD3 and α-CD28 for 5 hours, only Treg cells express CD25, whereas Tcon cells are not yet positive for the activation marker, CD25, on the surface. RNA was extracted from the responder Tcon cells and subjected to quantitative reverse transcriptase–PCR. IL-2 mRNA expression, normalized to glyceraldehyde-3-phosphate dehydrogenase, is shown relative to unstimulated Tcon cells (set to 1). Error bars represent standard deviations of the mean. Subgroups of SS patients are labeled with (+) for "SS FOXP3 high" or (−) for "SS FOXP3 low." n. a., not amplified.

to a lesser degree—in the skin. This could be attributable to the fact that the majority of skin biopsies were taken at the time of diagnosis, in contrast to most of the blood samples, which were taken during the course of disease in order to obtain fresh cells for flow cytometry and functional assays. Our finding of FOXP3$^+$ CTCL tumor cells supports and extends a study that identified increased FOXP3 protein and mRNA expression in the peripheral blood of 6 of 31 (20%) Sézary patients (Capriotti *et al.*, 2008). This retrospective study did not correlate FOXP3 expression with tumor cell markers and did not analyze skin specimens. Krejsgaard *et al.* (2008) used immunohistochemistry to detect the FOXP3 expression of skin-infiltrating CTCL tumor cells—identified by morphology—and low-molecular splice forms of FOXP in the peripheral blood of Sézary patients. In our study, we identified FOXP3 protein and mRNA expression in clonally expanded CTCL tumor cells by using clone-specific TCR-Vβ-chain antibodies for co-stainings.

Similar to our results in Sézary patients, it was shown in several studies that adult T-cell leukemia/lymphoma cells express FOXP3 (Chen *et al.*, 2006; Karube *et al.*, 2004; Karube *et al.*, 2008; Kohno *et al.*, 2005; Matsubara *et al.*, 2005; Shimauchi *et al.*, 2008; Abe *et al.*, 2008). Interestingly, some patients with adult T-cell leukemia/lymphoma show clinical features similar to those of patients with MF (Willemze *et al.*, 2005). The FOXP3 expression of adult T-cell leukemia/lymphoma tumor cells was shown to correlate with infection by human T-lymphotropic virus-1 (Walsh *et al.*, 2006). Infection with Epstein–Barr virus might also lead to FOXP3$^+$ tumor cells, as observed in patients with post-transplant lymphoproliferative disorder (Ebert *et al.*, 2008). However, infections with human T-lymphotropic virus-1 and Epstein–Barr virus are usually not seen in Sézary patients. FOXP3 expression of tumor cells was recently also shown for a number of solid tumors, including malignant melanoma, prostate cancer, colon cancer, and, less frequently, in breast cancer cell lines (Ebert *et al.*, 2008). Thus, FOXP3 expression is not restricted to Treg cells but is also seen in various tumor cells.

CTCL tumor cells represent monoclonal proliferations of CD4$^+$ lymphocytes. Treg cells were shown to be a subpopulation of CD4$^+$ lymphocytes, characterized by

FOXP3 and CD25 expression. Therefore, the question was whether CTCL tumor cells in Sézary patients also express CD25. We failed to detect high expression levels of CD25 in the majority of patients, as almost all $FOXP3^+$ cells of the patients were $CD25^-$. Only in three patients did we find substantial levels of CD25. Although FOXP3 binds to the CD25 promoter and is therefore capable of inducing CD25 expression, $FOXP3^+CD25^-$ cells with suppressive function were also found in mice (Masteller *et al.*, 2005; Wan & Flavell 2005; Fontenot *et al.*, 2003). It could be speculated that control of the CD25 promoter by FOXP3 is lost in CTCL tumor cells. Furthermore, Capriotti *et al.* (2008) presented similar results in Sézary patients with a high tumor burden. In their study, 8 of 31 Sézary patients showed CD25 expression that did not always correlate with FOXP3 expression. The expression of CD25 by circulating neoplastic cells of leukemic CTCL seems to be low and quite variable (Jones *et al.*, 2004; Talpur *et al.*, 2006; Waldmann *et al.*, 1984).

The quantification of Foxp3 mRNA underscored our protein data in that only ''SS FOXP3 high'' cells expressed substantial amounts of Foxp3 mRNA. Foxp3 mRNA expression of those tumor cells was not as high as that in freshly isolated Treg cells from NHD, but it was comparable to that in Treg cells from NHDs, which were expanded *in vitro* for 2–3 weeks, and to Foxp3 mRNA expression levels detected in 15–23-hour TCR-stimulated Tcon cells. These data might be indicative of a high expansion rate of CTCL tumor cells *in vivo*.

CTCL tumor cells might have acquired FOXP3 expression by activation. It was recently shown and confirmed by us (Figures 2c and 4) that activated human Tcon cells transiently express FOXP3 (Allan *et al.*, 2007) and can gain suppressive abilities (Pillai *et al.*, 2007 and Walker *et al.*, 2003). Other investigators have shown that FOXP3 expression does not correlate with suppressive function (Tran *et al.*, 2007 and Zheng *et al.*, 2008). Baron *et al.* (2007) found that DNA demethylation of the foxp3 locus discriminates Treg cells from activated $FOXP3^+$ Tcon cells in that several regions of the foxp3 locus are demethylated in Treg, but methylated in Tcon cells. In particular, the TSDR (Treg-specific demethylated region) of the foxp3 gene, which consists of amplicon 5, was shown to be highly demethylated, specifically in Treg cells. Representative patients expressing FOXP3 exhibit a stronger demethylation of the foxp3 locus than do Tcon and $FOXP3^-$ CTCL tumor cells, the latter carrying a methylation pattern resembling the one of Tcon cells. This indicates that the FOXP3 expression observed in CTCL tumor cells in the ''SS FOXP3 high'' group of Sézary patients is due to the regulation of foxp3 gene transcription and not (only) to altered stability of FOXP3 mRNA or protein. Another group demonstrated that the features of Treg cells (FOXP3 expression and IL-10 production) were induced in CTCL tumor cells by common γ-chain-signaling cytokines, such as IL-2 and IL-15, and do not represent a fully predetermined, constitutive phenotype independent of the local environmental stimuli to which these malignant $CD4^+$ T cells become exposed, for example, in the skin (Kasprzycka *et al.*, 2008).

To determine whether $FOXP3^+$ CTCL tumor cells represent a malignant proliferation of Treg cells, functional studies of their suppressive capacity are crucial. Because CTCL tumor cells are difficult to cultivate *in vitro,* long-term assays to assess suppressive activity, such as proliferation assays, are difficult to perform. Therefore, we tested suppressive capacity by analyzing IL-2 mRNA suppression, which was shown to be suppressed in Tcon cells by Treg cells within a few hours (Oberle *et al.*, 2007). $FOXP3^+$ CTCL tumor cells from the ''SS FOXP3 high'' subgroup of patients were able to suppress IL-2 mRNA induction in TCR-stimulated Tcon cells in contrast to $FOXP3^-$ CTCL tumor cells, which failed to do so. This clearly shows that FOXP3 expression of CTCL tumor cells is associated with Treg-like functions. The functional relevance of FOXP3 expression for the suppression of Tcon cells could be demonstrated in some patients with adult T-cell leukemia/lymphoma (Chen *et al.*, 2006; Matsubara *et al.*, 2005), but not in others (Shimauchi *et al.*, 2008). It remains an open question whether $FOXP3^+$ CTCL tumor cells are capable of suppressing an antitumor immune response. However, normal Treg cells might control tumor cell growth in CTCL patients, as reflected by the association of high numbers of Treg cells with a better prognosis in patients with MF and $CD30^+$ CTCL (Gjerdrum *et al.*, 2007; Gjerdrum *et al.*, 2008), in contrast to the more aggressive course observed in Sézary patients who lack normal Treg cells (Klemke *et al.*, 2006) or exhibit Treg cells without suppressive activity (Tiemessen *et al.*, 2006). Extracorporeal photopheresis has been used to treat SS for more than 20 years. It was shown to induce antigen-specific Treg cells in mice (Maeda *et al.*, 2005), further supporting a beneficial effect of Treg cells in the prognosis, course, and treatment of CTCL. It can be speculated that TCR-specific Treg cells might be able to specifically suppress the growth of CTCL tumor cells and thereby control tumor-cell expansion in the skin. This would be supported by the observation of a better prognosis for CTCL patients with increased numbers of Treg cells. In nonlymphoid cancer, Treg cells will not have a direct influence on tumor cells, but they will inhibit the T cells that should fight the cancer. In CTCL, it is possible that a direct effect of Treg cells on tumor cells outweighs their suppressive effect on the immune system in general.

The $FOXP3^+$ CTCL tumor cells behave in a manner functionally similar to Treg cells despite the fact that they exhibit phenotypical differences (for example, in CD25 expression). It remains an open question whether $FOXP3^+$ CTCL tumor cells acquired FOXP3 expression either during the natural course of the disease or because of the choice of treatment. In our study population, we identified two untreated Sézary patients with $FOXP3^+$ CTCL tumor cells (Table 2). The majority of patients in both groups received extracorporeal photopheresis and IFN-α, which makes it difficult to identify a particular therapy leading to FOXP3 expression in tumor cells. Another point of interest is the clinical relevance of our observation regarding prognosis and response to treatment. We compared different clinical parameters of ''SS FOXP3 low'' with ''SS FOXP3 high'' patients. We could not find significant differences between

Table 2. CTCL patients' characteristics regarding clinical data, therapy, and outcome

Patient	Age[1]	Gender	Stage[2]	Duration of disease[3]	CTCL-SI[4,5]	Therapy[5]	Outcome
SS1$^{(-)}$	63	M	IVA	9	65	ECP	Died
SS2$^{(-)}$	62	F	III	56	52	ECP, IFN-α	PR
SS3$^{(-)}$	56	M	IVA	98	62	ECP, IFN-α	Died
SS4$^{(+)}$	66	M	IVA	34	65	ECP, IFN-α, PUVA	PR
SS5	70	F	IVA	46	65	ECP, IFN-α, PUVA	Died
SS6$^{(+)}$	51	M	III	30	55	Untreated	SD
SS7$^{(-)}$	82	M	III	16	49	Untreated	Died
SS8$^{(-)}$	59	F	IVA	76	65	ECP, IFN-α	PR
SS9$^{(+)}$	74	F	IVA	55	73	ECP, IFN-α, PUVA	PR
SS10$^{(-)}$	69	F	IVB	34	84	Alemtuzumab (α-CD52)	Died
SS11$^{(+)}$	79	M	III	86	55	ECP, IFN-α	Died
SS12$^{(+)}$	86	F	III	25	54	Topical steroids	SD
SS13$^{(-)}$	65	F	IVA	14	65	ECP, IFN-α, PUVA	PR
SS14$^{(-)}$	71	M	III	97	54	Topical steroids	SD
SS15$^{(+)}$	75	M	III	19	50	Untreated	CR
MF1	70	F	IB	16	20	Untreated	PR
MF2	38	M	IB	41	17	UVB 311, topical steroids	PR
MF3	37	F	IB	48	14	Topical steroids	PR
MF4	82	F	IB	39	9	UVB 311, topical steroids	PR
MF5	53	F	IB	37	14	Topical steroids	SD
MF6	67	F	IB	168	6	Topical steroids	PR
MF7	50	M	IB	17	9	IFN-α, PUVA	CR
MF8	76	M	IB	301	6	Topical steroids, PUVA	PR
MF9	71	M	IB	181	3	Untreated	CR
MF10	52	M	IA	197	3	Topical steroids	CR
MF11	71	M	IB	169	11	Topical steroids	PR
MF12	58	F	IVA	243	0	Untreated	CR
MF13	40	M	IB	29	9	Topical steroids	CR
MF14	80	F	IB	37	6	UVB 311	PR

CR, complete remission; ECP, extracorporeal photopheresis; f, female; IFN, interferon; m, male; MF, mycosis fungoides; PR, partial remission; PUVA, psoralen+UVA; SD, stable disease; SS, sézary syndrome: $^{(+)}$, ''SS FOXP3 high''; $^{(-)}$, ''SS FOXP3 low''; TNM, tumor node metastasis.
[1]In years.
[2]According to the TNM classification (Bunn and Lamberg, 1979).
[3]In months.
[4]According to Klemke *et al.* (2005).
[5]At the time of this study.
Cut-off date: 31 March 2008.

the two Sézary patient groups in CTCL severity index, tumor burden (CD4/CD8 ratio, number of circulating Sézary cells), and duration of disease. However, there seems to be a trend toward a better prognosis for the ''SS FOXP3 high'' group. In this population, one of six patients died, whereas four of eight patients in the ''SS FOXP3 low'' group died (Table 2). Unfortunately, the two subgroups were too small for a statistical comparison. In patients with MF and CD30$^+$ CTCL, high numbers of skin-infiltrating FOXP$^+$ cells were shown to be associated with a favorable prognosis (Gjerdrum *et al.*, 2007; Gjerdrum *et al.*, 2008). Another study found a worse prognosis for Sézary patients with FOXP3 expression (Capriotti *et al.*, 2008). The clinical relevance of our findings needs to be confirmed in a larger cohort of well-characterized Sézary patients. The EORTC (European Organization for Research and Treatment of Cancer) cutaneous lymphoma task force is planning a multicenter study to evaluate diagnostic markers, including FOXP3, in SS, which could possibly answer the above-mentioned questions.

In conclusion, in a subgroup of Sézary patients, CTCL tumor cells show FOXP3 expression and Treg-like functions, for example, foxp3 gene locus demethylation and suppression of IL-2 mRNA in TCR-stimulated Tcon cells. FOXP3$^+$ CTCL tumor cells differ from Treg cells in an inconsistent CD25 expression and a lower Foxp3 mRNA expression. The modulation of FOXP3 expression in leukemic T cells could be implicated in oncogenesis and potentially has a useful clinical role. It has to be elucidated whether the FOXP3 expression of CTCL tumor cells is of prognostic relevance, and whether FOXP3 expression is acquired or whether CTCL represents a malignant expansion of Treg cells. Thereafter, it should be possible to develop new treatment options that target CTCL tumor cells more specifically.

PATIENTS AND METHODS

Patients

A total of 29 CTCL patients diagnosed according to the WHO (World Health Organization)–EORTC classification of cutaneous lymphomas (Willemze *et al.*, 2005), the tumor-node-metastasis staging classification (Bunn and Lamberg, 1979), and the criteria of the International Society of Cutaneous Lymphomas (Vonderheid *et al.*, 2002) were included in the study, representing MF stages IA ($n=1$), IB ($n=12$), and IVA ($n=1$) and SS stages III ($n=7$), IVA ($n=7$), and IVB ($n=1$) (Table 2). For controls, we investigated blood samples from Pso patients ($n=10$) and NHDs ($n=20$). Informed consent was obtained from all participants before inclusion. The study was conducted according to the ethical guidelines at our institution and the Helsinki Declaration, and approved by the Ethics Committee II of the Ruprecht-Karls-University of Heidelberg, Germany.

Cell isolation, antibodies, flow cytometry, and histology

Peripheral blood lymphocytes were separated by Ficoll gradient centrifugation and monocyte depletion. The following antibodies were used for staining of the lymphocytes as previously described: α-CD4-PerCp, α-CD25-APC-Cy7 (BD Pharmingen, Heidelberg, Germany), α-CLA-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany), and α-FOXP3-APC (clones PCH101 and 236/E7, eBioscience, Heidelberg, Germany) (Fritzsching *et al.*, 2005; Oberle *et al.*, 2007). Flow cytometry was carried out on a FACSCanto II (Becton Dickinson, Heidelberg, Germany), and data were analyzed using FACSDiva and CellQuest software (Becton Dickinson). For RNA and DNA analyses, cells were sorted using CD4, CD25, and TCR-Vβ-chain (Immunotech/Beckman Coulter, Fullerton, CA) expression with a FACS DIVA sorter (Becton Dickinson). For suppression assays, Treg and Tcon cells were isolated using MACS technology by CD4 and CD25 expression (Miltenyi Biotec) (Fritzsching *et al.*, 2005; Oberle *et al.*, 2007).

Skin biopsies were taken at the first presentation of the patients before onset of treatment and were fixed in formalin. Hematoxylin and eosin, CD4 (clone 1F6, Novocastra, Newcastle Upon Tyne, UK), and FOXP3 stainings were carried out as described elsewhere (Poenitz *et al.*, 2003; Klemke *et al.*, 2006; Roncador *et al.*, 2005). All specimens were blinded and analyzed as previously described (Djemadji-Oudjiel *et al.*, 1996; Klemke *et al.*, 2006).

Cell culture

Freshly isolated T cells were cultured in X-Vivo-15 medium (Cambrex, Verviers, Belgium) supplemented with 1% Glutamax (Invitrogen, Karlsruhe, Germany). Treg cells were either used immediately or stimulated overnight with 1 μg ml^{-1} α-CD3 and 50 U ml^{-1} IL-2, or expanded for up to 3 weeks with 0.1 μg ml^{-1} α-CD3, 300 U ml^{-1} IL-2, and 1 μg ml^{-1} α-CD28, and re-stimulated every week.

DNA preparation and foxp3 promoter methylation analysis

Genomic DNA from freshly prepared, FACS-sorted cells was isolated using the DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Analysis of the DNA methylation status of the foxp3 locus was carried out using bisulfite sequencing by Epiontis, Berlin, Germany as previously explained (Baron *et al.*, 2007).

Suppression assay, RNA preparation, and quantitative FOXP3 and IL-2 reverse transcriptase–PCR

The assays were carried out as previously described (Oberle *et al.*, 2007). Briefly, freshly isolated HLA-A2$^-$ CD4$^+$CD25$^-$ (Tcon) and HLA-A2$^+$ CD4$^+$CD25^{high} (Treg) or CTCL tumor cells were cultured alone or together in a 1:1 ratio and stimulated with soluble α-CD3 (0.2 μg ml^{-1}) and α-CD28 (0.5 μg ml^{-1}) mAbs. After stimulation for 3–5 hours, the cells were labeled with antibodies against CD4, CD25, HLA-A2, and the respective TCR-Vβ-chain for CTCL tumor cells, separated by cell sorting on a FACS DIVA, and immediately lysed in RNA extraction lysis buffer. RNA isolation, cDNA preparation, and quantitative PCR were performed as described elsewhere (Fritzsching *et al.*, 2005; Oberle *et al.*, 2007).

Statistical analysis

Data are presented as individual values and as mean or median. The Wilcoxon signed-rank test was used to compare the results of the various patient groups, including multiple comparisons. The test was considered significant with $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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