

CD164 and FCRL3 Are Highly Expressed on CD4 + CD26 – T Cells in Sézary Syndrome Patients

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Sézary syndrome (SS) cells express cell surface molecules also found on normal activated CD4 T cells. In an effort to find a more specific surface marker for malignant SS cells, a microarray analysis of gene expression was performed. Results showed significantly increased levels of mRNA for CD164, a sialomucin found on human CD34 + hematopoietic stem cells, and FCRL3, a molecule present on a subset of human natural T regulatory cells. Both markers were increased in CD4 T cells from SS patients compared with healthy donors (HD). Flow cytometry studies confirmed the increased expression of CD164 and FCRL3 primarily on CD4 + CD26 – T cells of SS patients. Importantly, a statistically significant correlation was found between an elevated percentage of CD4 + CD164 + T cells and an elevated percentage of CD4 + CD26 – T cells in all tested SS patients but not in patients with mycosis fungoides and atopic dermatitis or HD. FCRL3 expression was significantly increased only in patients with high tumor burden. CD4 + CD164 + cells displayed cerebriform morphology and their loss correlated with clinical improvement in treated patients. Our results suggest that CD164 can serve as a marker for diagnosis and for monitoring progression of cutaneous T-cell lymphoma (CTCL)/SS and that FCRL3 expression correlates with a high circulating tumor burden.

Journal of Investigative Dermatology (2014) **134**, 229–236; doi:10.1038/jid.2013.279; published online 18 July 2013

INTRODUCTION

Sézary syndrome (SS), the leukemic variant of cutaneous T-cell lymphoma (CTCL), is a malignancy of skin-trafficking CD4 T cells. The diagnosis is based predominantly on tissue biopsy showing atypical, epidermotropic CD4 T cells in the epidermis, and by microscopic examination of peripheral blood buffy coats for presence of lymphocytes with atypical cerebriform appearing nuclei, known as Sézary cells. In addition to examination of tissue biopsies and blood, flow cytometry is now a widely accepted diagnostic tool. However, as SS cells express molecules also present on normal activated CD4 T cells, diagnosis based on the phenotype of circulating malignant cells can be difficult (Kim *et al.*, 2005).

The malignant SS cells have been phenotyped as central memory cells expressing CD4 + CD26 – CD45RO +. The ability of the malignant cells to localize to the skin is facilitated by skin addressins CLA (cutaneous lymphocyte antigen) and

CCR4 (chemokine (C-C motif) receptor 4), whereas the presence of CCR7 facilitates entry into lymph nodes (Ferenczi *et al.*, 2002; Sokolowska-Wojdylo *et al.*, 2005a, b; Campbell *et al.*, 2010). Advancing disease in SS patients correlates with the gradual decline in the TCR repertoire, eventually resulting in the presence of malignant CD4 T cells expressing a single TCR V β (Yawalkar *et al.*, 2003). Furthermore, molecules such as NKp46 and CD158k/KIR3DL2, receptors originally identified on natural killer cells, ganglioside GD3 (CD60), and syndecan 4 (SD-4), present on activated normal T cells, were also found to be expressed at elevated levels mainly in SS patients with high tumor burden (Poszepczynska-Guigne *et al.*, 2004; Campbell *et al.*, 2010; Scala *et al.*, 2010; Bensussan *et al.*, 2011; Chung *et al.*, 2011). Interestingly, T-plastin, an intracellular protein, has been found exclusively in the malignant circulating CD4 T cells in SS patients, but its intracellular expression and lack of specific antibodies applicable for flow cytometry diminish its usefulness as a diagnostic marker (Kari *et al.*, 2003; Su *et al.*, 2003).

The identification of a clonal malignant TCR V β population of CD4 T cells in patients facilitates diagnosis and monitoring of the Sézary cells. However, SS patients without an identifiable circulating clone can pose a diagnostic and therapeutic monitoring challenge, particularly as loss of CD26 is indicative of the malignant cells, but it does not distinguish them from normal populations of CD4 + CD26 – cells present in the circulation (Bernengo *et al.*, 2001; Jones *et al.*, 2001; Sokolowska-Wojdylo *et al.*, 2005b).

In our attempt to find a specific surface marker for malignant cells, CD4 T cells isolated from SS patients and

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Abbreviations: AD, atopic dermatitis; CTCL, cutaneous T-cell lymphoma; FDR, false discovery rate; FCRL3, FC-receptor-like 3; HD, healthy donors; MF, mycosis fungoides; PBMC, peripheral blood mononuclear cell; SD, syndecan 4; SS, Sézary syndrome

Received 21 December 2012; revised 3 May 2013; accepted 20 May 2013; accepted article preview online 21 June 2013; published online 18 July 2013

healthy donors (HD) were subjected to microarray analysis of global gene expression. These studies revealed that CD164 and FC-receptor-like 3 (FCRL3) were expressed at significantly higher levels in CD4 T cells of patients as compared with HD. CD164, a sialomucin adhesion receptor demonstrated on a population of CD34+ hematopoietic progenitor cells, has been reported to be expressed in <3% of peripheral CD3 T cells in healthy volunteers (Watt *et al.*, 1998; Zannettino *et al.*, 1998). FCRL3 is a member of the FCRL gene family encoding proteins FCRL 1–6 that are homologous to the classical Fc receptors. FCRL3 expression is found in 40% of naturally occurring human CD4+CD25+Foxp3+ T regulatory cells, and functional studies showed that CD4+CD25+FCRL3+ cells are nonresponsive to anti-CD3/CD28, IL-2, phytohemagglutinin, or concanavalin A stimulation (Nagata *et al.*, 2009). Subsequent studies published by Swainson *et al.* (2010) demonstrated that FCRL3+ cells exhibit a CD4+ memory T-cell phenotype and that expression of FCRL3 correlates with high levels of programmed cell death-1 receptor.

In this study we provide evidence that CD164 may serve as an early detection marker for SS in patients with low-to-high tumor burden, and that FCRL3 expression correlates with disease progression.

RESULTS

Increased mRNA expression of CD164 and FCRL3 genes in CD4 T cells from SS patients as assessed by microarray analysis and QRT-PCR

We performed a microarray analysis of global gene expression on CD4 T cells isolated from SS patients to identify surface markers specific for the malignant cells. We compared gene expression in CD4 T cells from patients with high tumor burden ($n=2$, $\geq 50\%$ of malignant cells in circulation), medium tumor burden ($n=2$, 50–20% of malignant cells), and low tumor burden ($n=2$, $\leq 20\%$ of malignant cells) and in HD ($n=3$).

A total of 1,219 genes were identified as significantly differentially expressed ($P<0.05$, false discovery rate (FDR) $<10\%$) and differed by at least 80% of the levels between patients with high, medium, and low tumor burden and HD. We chose two genes coding for cell surface molecules not previously associated with CTCL. CD164 exhibited a significant difference between all 6 SS patient samples and 3 HD ($P=0.004$, FDR=7%). FCRL3 was the most differentially expressed gene between patients with high and medium tumor burden versus normal samples ($P=0.006$, FDR=10%), but was less informative for patients with low tumor burden (Figure 1).

To validate the expression of CD164 and FCRL3 on the SS malignant cells, we assessed mRNA levels for these genes in CD4 T cells of 11 patients with high tumor burden, 16 with medium-to-low tumor burden (6 medium tumor burden and 10 low tumor burden), and 9 HD by quantitative real-time reverse-transcriptase-PCR (QRT-PCR). In the same cohort of patients, we also assessed expression of T-plastin. As shown in Figure 2, CD164 was significantly expressed in the cells of patients with high, medium, and, importantly, low tumor burden compared with healthy controls. FCRL3, as well as T-plastin, were both expressed by CD4 T cells from patients

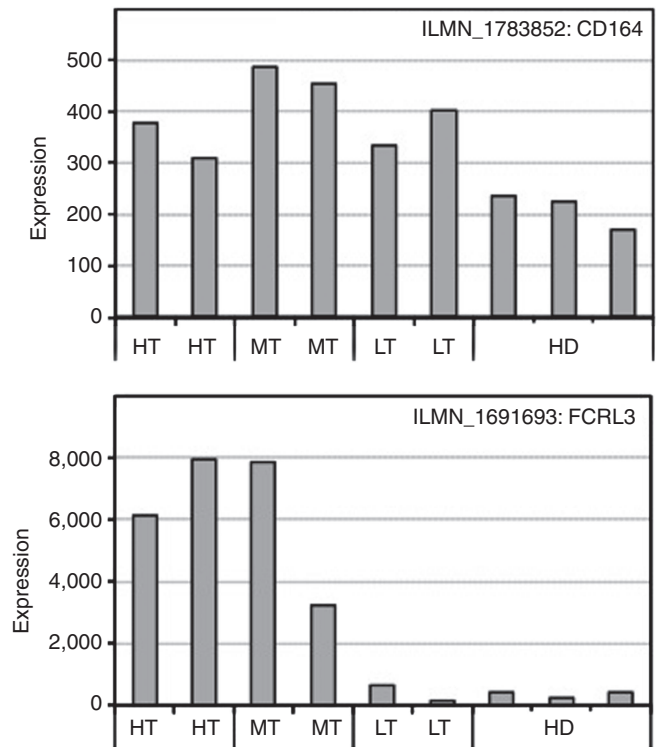


Figure 1. Increased mRNA expression of CD164 and FCRL3 genes in CD4 T cells from Sézary syndrome patients as assessed by microarray analysis. The expression of CD164 and FCRL3 in CD4 T cells isolated from peripheral blood mononuclear cells (PBMCs) of Sézary patients with high tumor (HT), medium tumor (MT), and low tumor (LT) burden and healthy donors (HD). Normalized signals with the indicated genes are shown.

with high-to-medium but not low tumor burdens. These data strongly suggest that malignant cells, particularly in patients with advanced disease, express all three molecules.

Of note, in addition to CD164, FCRL3, and T-plastin, we also assessed mRNA expression of SD-4 and NKp46 (Bensussan *et al.*, 2011; Chung *et al.*, 2011). SD-4 mRNA expression was significantly increased in patients compared with HD ($P=0.01$), whereas NKp46 mRNA was inconsistently expressed and not significantly increased among SS patients (data not shown).

The acquisition of CD164 correlates with the loss of CD26 expression in SS patients; FCRL3 is present mainly in CD4 T cells in patients with high tumor burden

Peripheral blood mononuclear cells (PBMCs) from SS patients (high/medium/low tumor burden), mycosis fungoides (MF) patients without known blood involvement, HD, and atopic dermatitis (AD) patients were analyzed for the presence of CD164 and CD26 on their CD4 T cells by flow cytometry. The percentages of CD4+CD164+ as well as CD4+CD26– T cells were significantly increased in SS patients when compared with MF and AD patients or HD (P -values for both markers were <0.0001). Importantly, we found a statistically significant correlation between an elevated percentage of CD4+CD164+ T cells and an elevated percentage of CD4+CD26– T cells in all 59 SS patients (Pearson's correlations: $\rho=0.674$, $P<0.0001$) but not in MF patients

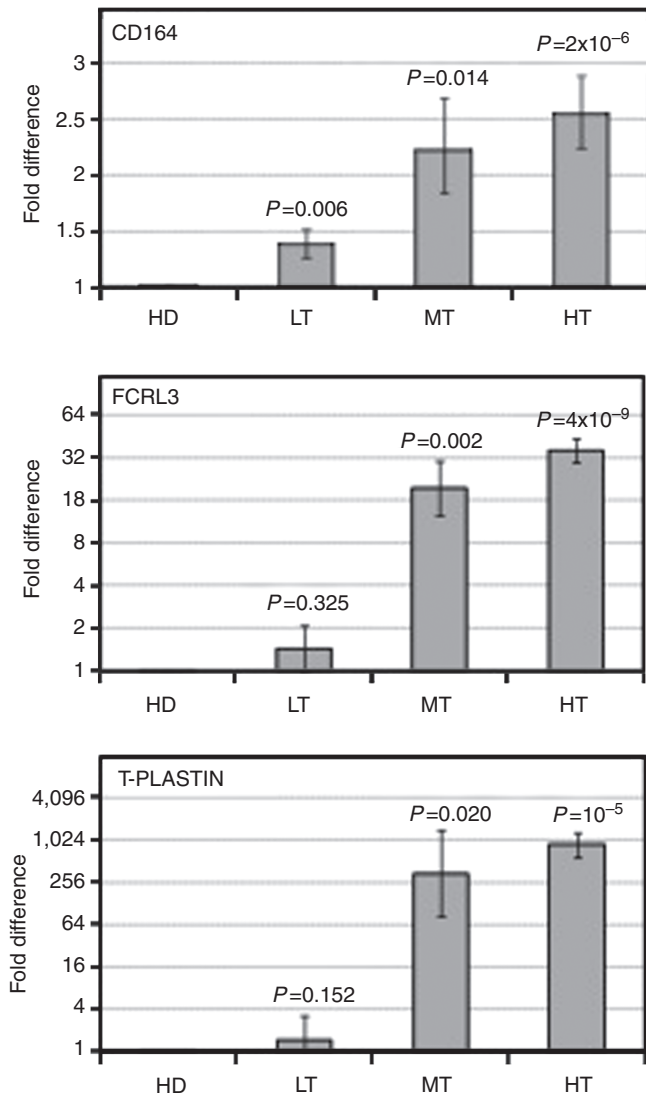


Figure 2. CD164, FCRL3, and T-plastin mRNA expression in CD4 T cells from Sézary syndrome patients as assessed by quantitative real-time reverse-transcriptase-PCR (QRT-PCR). CD4 T cells were isolated from peripheral blood mononuclear cells (PBMCs) of 11 patients with high tumor burden (HT; $\geq 50\%$ malignant cells), 6 with medium tumor burden (MT; 50–20% malignant cells), and 10 with low tumor burden (LT; $\leq 20\%$ malignant cells), and 9 healthy donors (HD). Total RNA was extracted from CD4 T cells followed by QRT-PCR to assess mRNA levels. Fold difference for CD164, FCRL3, and T-plastin is calculated versus expression levels in cells from healthy volunteers. Error bars represent SEM.

($n = 10$, $\rho = -0.372$, $P = 0.33$) or HD ($n = 14$, $\rho = -0.39$, $P = 0.21$). Figure 3a shows percentages of CD4 + CD26 $^-$ and CD4 + CD164 $^+$ T cells detected in SS patients (mean CD26 $^-$: 59.2%, SEM: 3.7, mean CD164 $^+$: 34.7%, SEM: 3.7), MF patients (mean CD26 $^-$: 36.6%, SEM: 4.6, mean CD164 $^+$: 3.0%, SEM: 0.7), and HD (mean CD26 $^-$: 19.6%, SEM: 2.6, mean CD164 $^+$: 1.2%, SEM: 0.2). Compared with MF and HD, the percentage of CD4 + CD26 $^-$ T cells in SS patients was also significantly increased ($P < 0.02$). Consistent with the data shown in Figure 3a, CD164 protein was detected on the surface of patients with high ($n = 37$) medium ($n = 14$), and, importantly, low ($n = 8$) tumor burden

and was significantly increased in all three groups compared with HD or MF patients without peripheral blood involvement and in AD patients (Figure 3b). Interestingly, CD164 expression was not detected in CD4 T cells from six AD patients (mean CD164 $^+$: 1.2%, SEM: 0.50). It should be mentioned that only 4 out of 59 SS patients (6.8%) were low expressers of CD164, with $< 5\%$ of CD4 T cells expressing CD164 (mean: 1.8%, SEM: 0.84).

A statistically significant increase in FCRL3 protein expression was found only in patients with a high tumor burden in the circulation (Figure 3c). On average, 46.6% of CD4 T cells from patients with high tumor burden expressed FCRL3 (SEM: 6.35) on their surface compared with 5.1% of CD4 + FCRL3 $^+$ cells in patients with medium tumor burden (SEM: 1.58) and 2.7% of CD4 + FCRL3 $^+$ cells in patients with low tumor burden (SEM: 0.87). The mean expression of FCRL3 on CD4 T cells from MF patients and HD was 13% (SEM: 3.5) and 5.6% (SEM: 2.0), respectively (Figure 3c).

The detailed flow cytometric analysis confirmed the results suggested by the data shown in Figure 3a–c that CD164 and FCRL3 are mainly expressed on CD4 + CD26 $^-$ T cells (Figure 3d and Supplementary Table S1 online).

Among patients with high tumor burden, whose CD4 T cells were mainly CD26 negative and expressed a single TCRV β as defined by antibodies, CD164 and FCRL3 were expressed predominately on CD4 + CD26 $^-$ V β $^+$ T cells (Figure 3d, patient 1, upper panel). Similarly, in patients without an identifiable TCRV β , including patients with low tumor burden, CD164 was predominantly expressed on CD4 + CD26 $^-$ T cells (Figure 3d, patient 2, lower panel). FCRL3 expression was rarely evident in CD4 T cells in patients with medium-to-low tumor burden.

CD4 + CD164 $^+$ cells display cerebriform morphology

CD4 + CD164 $^+$ cells and CD4 + CD164 $^-$ cells were isolated from PBMCs of highly leukemic patients and processed to create 1- μ m-thick section slides used for the assessment of Sézary cells. Representative photographs are shown in Figure 4a and b. CD4 + CD164 $^+$, but not CD4 + CD164 $^-$ T cells, manifested a high degree of cerebriform morphology. In addition, we sorted CD4 T cells into CD4 + CD164 $^+$ and CD4 + CD164 $^-$ cells, placed them on glass slides and analyzed them for potential differences in cell size between the two groups. As shown in Figure 4c and d, CD4 + CD164 $^+$ T cells were much larger in size compared with the CD4 + CD164 $^-$ T cells. Although the nuclear morphology cannot be fully assessed using this method, the differences in size between CD4 + CD164 $^+$ and CD4 + CD164 $^-$ T cells further suggest that CD4 CD164 $^+$ T cells may represent the malignant population, a finding consistent with Clark *et al.* (2011), who showed that malignant CTCL cells have a high-scatter profile.

Clinical improvement in disease status of SS patients correlates with a decreasing percentage of CD4 + CD26 $^-$ CD164 $^+$ FCRL3 $^+$ circulating T cells

We next attempted to determine whether clinical improvement in disease status can be monitored by assessing changes

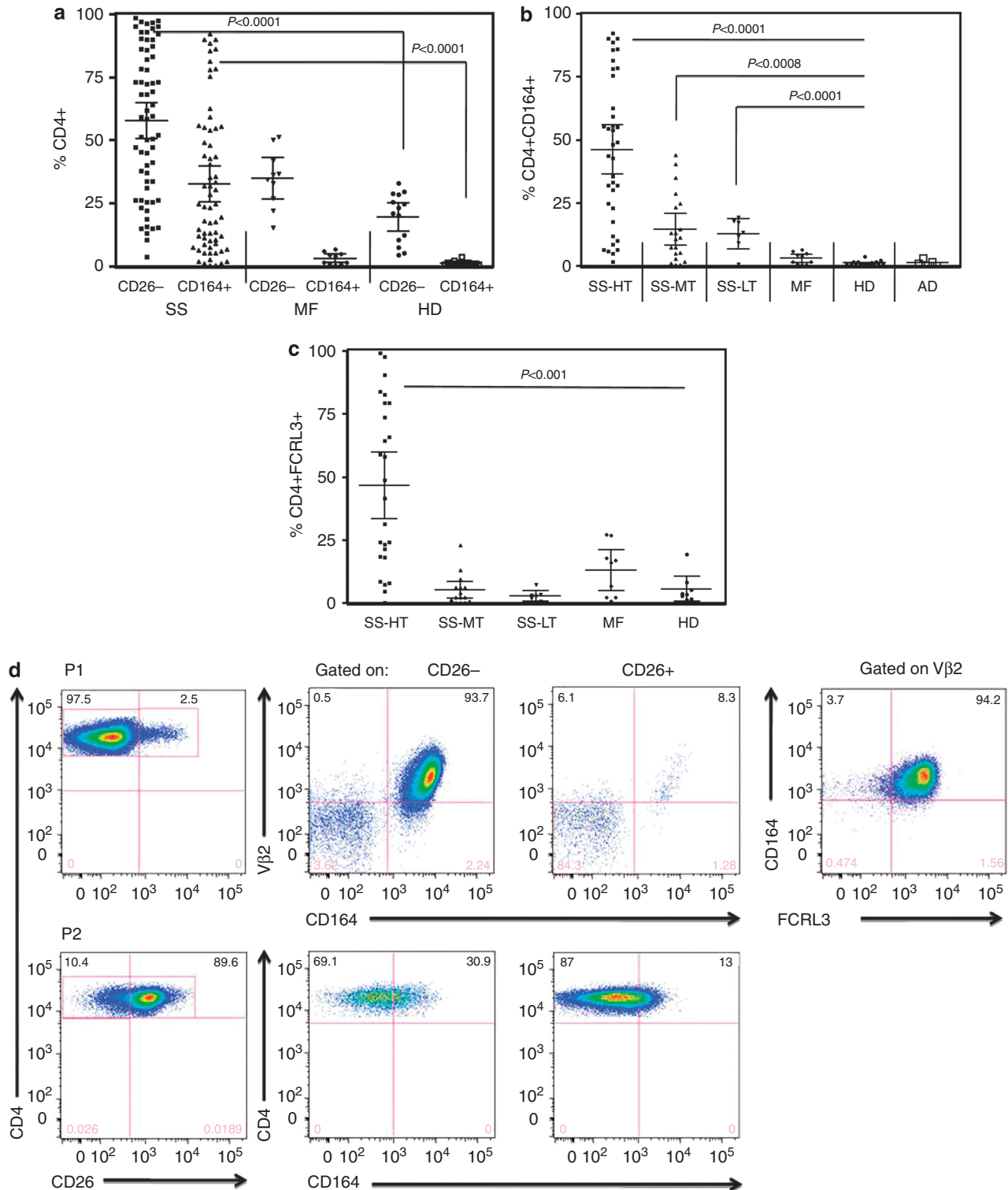


Figure 3. The acquisition of CD164 correlates with the loss of CD26 on CD4 T cells of patients; FCRL3 is predominantly expressed in CD4 T cells of patients with high tumor burden. The cell surface expression of CD26, CD164, and FCRL3 was assessed by flow cytometry. (a) Sézary syndrome (SS) patients ($n = 59$) demonstrate significantly higher percentages of CD4+CD164+ and CD4+CD26- T cells compared with mycosis fungoides (MF) patients ($n = 10$), or healthy donors (HD; $n = 14$). (b) Percentage of CD4+CD164+ T cells is significantly higher in patients with high tumor (HT; $n = 37$), medium tumor (MT; $n = 14$), and low tumor (LT; $n = 8$) burden compared with MF, HD, or atopic dermatitis (AD) patients ($n = 6$). (c) Percentage of CD4+FCRL3+ T cells is significantly higher only in CD4 T cells of HT patients ($n = 26$) but not in MT ($n = 15$) or LT ($n = 7$) patients as compared with HD. Results are expressed as mean with 95% confidence interval. (d) CD164 and FCRL3 are predominately expressed on CD4+CD26- and CD4+CD26- β T cells. Shown results are from one representative patient (out of 7) with a TCRV β defined by antibody (upper panel) and from one representative patient (out of 14) whose TCRV β was not defined by antibody (lower panel). The peripheral blood mononuclear cells (PBMCs) of patients were collected before initiation of systemic therapy at the University of Pennsylvania.

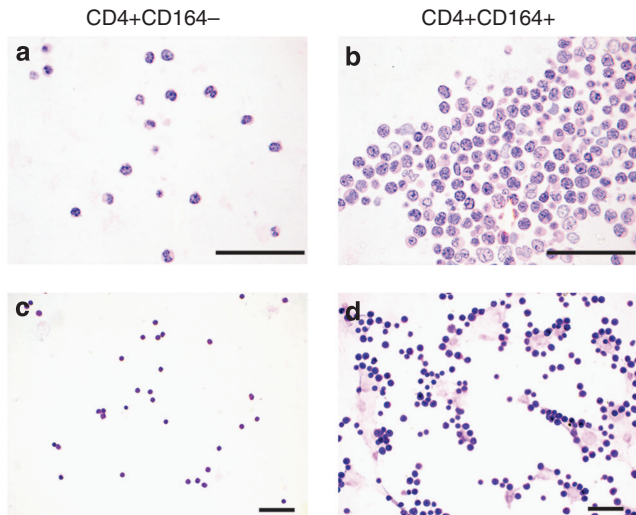


Figure 4. CD4 + CD164 + cells demonstrate the phenotype of malignant Sézary cells. For assessment of cellular morphology, (a, b) CD4 + CD164 + cells (5×10^6 , 85% purity) and CD4 + CD164 – cells (2.9×10^6 , purity 69%) from a patient with high tumor burden were recovered using an anti-PE column. Cells were processed to obtain a 1- μ m-thick section, stained with hematoxylin and eosin (H&E), and assessed for the presence of malignant cells. Cellular morphology was examined using an Olympus BX51 microscope. Images were captured with a Leica DFC 420 camera. Data shown are from one representative patient out of five. For size assessment, (c, d) CD4 T cells from a patient with high tumor burden were sorted into CD4 + CD164 + and CD4 + CD164 – (purity of both populations 98%), placed on glass slides, air dried, stained with H&E, and analyzed using a Zeiss Axiophot microscope. Images were captured using a Leica DFC 450 camera. Shown images are from one representative patient out of six. Bars = 100 μ m.

in the expression of CD164 and FCRL3 in the CD4 T cells of patients. We focused on three originally highly leukemic patients who had achieved a complete clinical remission with resolution of all skin lesions and lymphadenopathy while being treated with multimodality immune therapy. As shown in Figure 5, on completion of treatment, two patients with an identifiable V β clone experienced a marked decrease in the percentage of V β + CD4 T cells (patients 1 and 2), and all three patients had decreases in the percentages of CD26 –, CD164 +, and FCRL3 + CD4 T cells. These results suggest that the expression of CD164 as well as FCRL3 in the CD4 T cells of SS patients can serve as a surrogate marker of disease progression and circulating tumor burden, and that these molecules may be used to monitor therapeutic efficacy in CTCLs.

DISCUSSION

Our data demonstrate previously unreported high expression of CD164 and FCRL3 on CD4 T cells from SS patients. Importantly, our results show a statistically significant correlation between CD164 acquisition and loss of CD26 expression; high CD164 expression correlates with increased percentages of CD26 – T cells.

The potential for CD164 to serve as a marker for malignant cells is underscored by: (1) the presence of CD164 on CD4 T cells of SS patients with a wide range of tumor burdens;

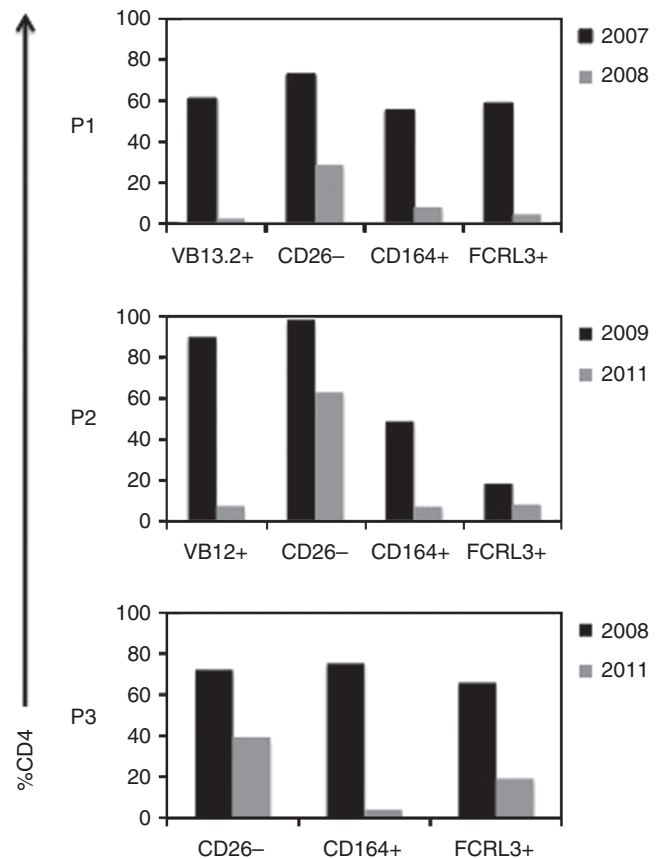


Figure 5. Clinical remission of disease correlates with the disappearance of CD4 + CD26 – T cells expressing V β , CD164, and FCRL3. Peripheral blood mononuclear cells (PBMCs) from three patients with high tumor burden were analyzed by multicolor flow cytometry to assess the expression of the molecules on CD3 + /CD4 + T cells. Samples of patient's PBMCs were collected before the onset of systemic treatment and during clinical remission. Patient 1 (P1) received extracorporeal photopheresis (ECP), IFN- α , and psoralen plus UVA (PUVA), whereas patients 2 and 3 (P2 and P3) received ECP, IFN- α , and total skin electron beam therapy.

(2) the absence of CD164 on CD4 T cells from HD and patients with AD; (3) morphological examination of purified CD4 + CD164 + T cells demonstrating the morphology of malignant Sézary cells; and (4) the disappearance of CD4 + CD164 + T cells in three patients who experienced clinical remission as a result of treatment.

Recently, CD164 expression was shown in human prostate cancer cells and in cell lines derived from human breast carcinoma. It has been also identified as a new marker for acute lymphoblastic leukemia (Havens *et al.*, 2006; Coustan-Smith *et al.*, 2011; Leccia *et al.*, 2012). There is evidence suggesting an important role for CD164 in prostate cancer cell metastasis and in the development of colorectal cancer (Matsui *et al.*, 2000; Havens *et al.*, 2006).

It is not yet clear what drives the expression of CD164 on SS CD4 T cells. Based on our preliminary results, TCR stimulation with anti-CD3/28 or polyclonal stimulation with phytohemagglutinin does not play a key role in upregulating CD164 expression in the CD4 T cells of patients. In response to either stimulus, the percent of CD4 + CD164 + T cells rose from

0.1% (unstimulated group) to 5.5%, whereas CD25 expression increased from 2% (unstimulated) to nearly 40% (data not shown). It is also highly unlikely that systemic treatment such as IFN or extracorporeal photopheresis increases CD164 or FCRL3 expression, as we observed longitudinal decreases, rather than increases, in the percentages of CD4 + CD164 + / FCRL3 + T cells among SS patients over their treatment course with these agents.

Interestingly, CXCL12 (chemokine (C-X-C motif) ligand 12) has been shown to stimulate the expression of both CD164 mRNA and protein in human prostate cancer cells, but it remains to be established whether it has a similar role in CTCLs. CXCL12 was abundantly expressed in the skin of SS patients as demonstrated by Narducci *et al.* (2006). Moreover, inhibition of enzymatic activity of CD26 enhanced the CXCL12-induced migration of the CTCL cell line Hut78 (Narducci *et al.*, 2006). Our preliminary data showed increased CXCL12 mRNA expression in the skin of three patients with high tumor burden and three with medium tumor burden whose CD4 T cells were used in these studies as compared with HD (M. Wysocka, unpublished data). Thus, it is conceivable that increased levels of CXCL12 in SS patients may contribute to the elevated CD164 expression, although it may not be an exclusive factor. The presence of another factor, presently unknown, contributing to the tumor transformation, loss of CD26, and acquisition of CD164 expression in the CD4 T cells of patients cannot be ruled out, given that patients with a low tumor burden in the circulation have percentages of CD4 + CD26 – cells comparable to HD, yet still express CD164 in their CD4 T cells.

Considering that absence of CD26 and presence of CD164 on the cell surface enhances CXCL12-mediated cell migration, our findings strongly suggest that CD164 defines a population of malignant CD4 T cells with the ability to invade skin and possibly lymph nodes and bone marrow.

The phenotype of CD4 + FCRL3 + T cells in SS patients differs from the described phenotype of naturally occurring T regulatory cells in that the freshly isolated cells from SS patients typically lack Foxp3 expression. We found no correlation between FCRL3 and CD25 expression in patients with high tumor burden, as CD4 T cells were highly positive for FCRL3 (>82%) but only marginally so for CD25 (<10%). However, the presence of FCRL3 protein on CD4 T cells from this group of patients and the presence of FCRL3 mRNA in patients with medium tumor burden may indicate a gradual loss of responsiveness of patients' CD4 T cells to cytokines and TCR stimulation, as has been previously suggested (Fargnoli *et al.*, 1997; Wysocka *et al.*, 2004). We previously showed that some SS patients have an increased expression of programmed death-1 receptor on CD4 T cells, a molecule that has been associated with decreased immune responsiveness (Shimauchi *et al.*, 2007; Samimi *et al.*, 2010). Recently, Chung *et al.* (2011) demonstrated that association of SD-4 present on SS patients' CD4 T cells, with dendritic cell-associated heparan sulfate proteoglycan-integrin ligand (DC-Hil) leads to attenuation of TCR-induced proliferation of CD4 T cells. We have found significantly elevated levels of mRNA for SD-4 in our cohort of SS patients (data not shown),

supporting the notion that SD-4 may also contribute to this unresponsive phenotype.

Currently, we do not have a full understanding of the biological significance of FCRL3 expression in the CD4 T cells of SS patients. Similarly, the functional significance of CD164 expression on patients' cells awaits a better understanding. However, the statistically significant expression of CD164 on CD4 T cells from patients but not from HD, and the significant correlation between acquisition of CD164 and loss of CD26 expression, all indicate that CD164 may be a potentially interesting marker for malignant SS cells. Furthermore, our preliminary data showing a lack of CD164 expression on CD4 T cells from six patients with severe-to-moderate AD suggests that CD164 may be restricted to SS, but certainly further studies are needed to fully assess this marker specificity. Our future studies will focus on understanding the biological significance of CD164 and FCRL3, particularly with regard to their association with malignant transformation in CTCLs and their expression in other inflammatory skin diseases.

In summary, the presence of a positive marker such as CD164 that distinguishes between normal, immunocompetent CD4 T cells and malignant CD4 T cells in SS will facilitate earlier diagnosis as well as therapeutic monitoring of disease status. It will also further our understanding of the underlying mechanisms responsible for the transition from activated normal CD4 T cells to malignant CD4 T cells and may eventually lead to a more targeted SS therapy.

MATERIALS AND METHODS

Patients

SS patients were diagnosed on the basis of clinical, histopathologic, and immunohistologic criteria (Murphy, 1988). To assess the numbers of circulating malignant T cells, PBMCs of patients were analyzed by flow cytometry for the presence of CD4⁺/CD26[–]/CD7[–] cells and by examination of 1 μ m sections of formalin-fixed peripheral blood buffy coats for lymphocytes with atypical cerebriform appearing nuclei (Introcaso *et al.*, 2005). Patients with erythroderma and circulating malignant T cells were defined to have SS (Olsen *et al.*, 2007). Donation of peripheral blood samples by patients and healthy volunteers was according to protocols approved by the University of Pennsylvania Institutional Review Board.

The studies were conducted in accordance with the Declaration of Helsinki Principles, and all participants provided written informed consent.

Isolation of CD4 T cells and CD4 + CD164 + cells

CD4 T cells from patients or healthy volunteers were isolated from freshly collected PBMCs as previously described, using Dynal CD4 Positive Isolation Kit (Invitrogen Dynal, Oslo, Norway) (Rook *et al.*, 1995). Pure CD4 T cells were used in microarray and QRT-PCR studies. To assess cellular morphology, CD4 T cells were stained with anti-CD164-PE antibody and recovered using anti-PE microbead columns from Miltenyi Biotec (Auburn, CA) or sorted using BD (San Jose, CA) FACS Aria II SORP into CD4 + CD164 + and CD4 + CD164 – cells. CD4 T cells were isolated from patients currently undergoing treatment in the CTCL clinic. Patients were routinely treated with extracorporeal photopheresis combined with Bexarotene, IFN- α , or IFN- γ .

Preparation of cells for morphological assessment

For assessment of cellular morphology, CD4+CD164+ T cells or CD4+CD164- T cells, previously separated using anti-PE columns, were processed in JB4 to obtain 1- μ m-thick sections according to the standardized procedure for analysis of the presence of S  zary cells in peripheral blood buffy coats adapted by the Division of Dermatopathology, Department of Dermatology, University of Pennsylvania (Philadelphia, PA). For size assessment, CD4+CD164+ T cells or CD4+CD164- T cells, previously sorted, were placed on glass slides, air-dried, hematoxylin and eosin stained, and microscopically analyzed.

Flow cytometry analysis

To analyze the phenotype of CD4 T cells from patients and HD, PBMCs were stained with antibodies anti-CD3, anti-CD4, anti-CD26, anti-CD164, and anti-CD25 purchased from BD Biosciences (San Jose, CA) and anti-FCRL3. Production and characterization of anti-FCRL3 antibody, H5, has been described previously (Nagata *et al.*, 2009). To assess the viability of analyzed cells, the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit from Invitrogen (Grand Island, NY) was used.

Cells were analyzed with the LSRII flow cytometer (Becton Dickinson, San Jose, CA) at the Flow Cytometry and Cell Sorting Core, Abramson Cancer Center, University of Pennsylvania. A total of 100,000 events were collected to analyze CD4 T cells. The data were further analyzed by FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Pearson's correlation coefficients were estimated to assess the correlation between CD164+ and CD26- expression on CD4 T cells. Student's *t*-test was used to assess differences between groups in levels of expression. Tests were considered statistically significant using a two-sided *P*-value of 0.05. The statistical package R (www.r-project.org) was used for analysis.

Microarray studies: preparation of CD4 T-cell RNA

Gene expression was analyzed in CD4 T cells from six SS patients and three HD. RNA was processed as previously described and hybridized on Illumina (Valencia, CA) Human WG 8v2 microarray chips (Showe *et al.*, 2009). All arrays were processed in the Wistar Institute Genomics Facility (Philadelphia, PA).

Data preprocessing

Array data were processed by Illumina's BeadStudio software and expression levels exported for analysis. The gene-wise, median correlation of each array compared with all other arrays was computed to ensure that no outliers existed within the data. The expression levels were then quantile normalized and noninformative probes (those with detection *P*-value >0.05 in all samples) were removed to reduce experimental noise. Ultimately, 23,050 probes that targeted known genes were used in our analysis.

Gene expression analysis

A one-way analysis of variance test was conducted on the data to identify genes differentially expressed between any tumor samples versus HD or high/medium tumor burden samples versus HD separately. FDR was estimated using the procedure of Storey and Tibshirani (2003). The data analysis was conducted using functions of MATLAB

7.2 (Natick, MA) and significance was defined at $P \leq 0.05$ and FDR $\leq 10\%$.

Quantitative real-time RT-PCR

Total RNA was extracted from CD4 cells using TRIzol Reagent (Ambion, Grand Island, NY). Complementary DNA was synthesized from 1 μ g of RNA using High Capacity RNA to cDNA Kit (Applied Biosystems, Grand Island, NY). QRT-PCR was performed using Taqman gene expression assays: 18s rRNA, CD164, FCRL3, T-plastin, SD-4, and NKp46 according to the manufacturer's protocols (Applied Biosystems). Comparison of expression levels of a gene between pairs of groups was done using the Δ Ct method.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Xuming Mao for his suggestions and valuable discussions. This work was supported by NCI grant R01CA122569 and the Translation Research Grant from the Leukemia and Lymphoma Society (to AHR); and NCI grants R01 CA 132098 (to LCS). The Wistar Institute Genomic and Bioinformatics facilities were supported by Cancer Center Support grant P30 CA010815. SN and TI were recipients of a NIH COBRE grant (1P20RR024219-01A2).

SUPPLEMENTARY MATERIAL

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

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