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Blood Flow Cytometry in Sézary Syndrome: New Insights on Prognostic Relevance and Immunophenotypic Changes During Follow-up.

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The fluorochrome-conjugated monoclonal antibodies used against T-cell antigens included anti-CD2 FITC, PE, or PE-Cy7 (clone S5.2); anti-CD3 FITC, PE, or PerCP-Cy5.5 (clone SK7); anti-CD4 FITC, PE, or V450 (clone SK3); anti-CD5 FITC or APC (clone L17F12); anti-CD7 FITC or PE (clone 4H9) or anti-CD7 PerCP-Cy5.5 (clone M-T701); anti-CD8 PE or APC-Cy7 (clone SK1); anti-CD26 PE (clone L272); anti-CD27 FITC or APC (clone M-T271); anti-CD28 PE (clone CD28.2); anti-CD38 PE or PE-Cy7 (clone HB-7); anti CD45RA FITC (clone 4KB5); anti-CD45RO PE (clone UCHL1); anti-CD45 PerCP or V500 (clone HI30); and CD62L APC (clone DREG-56), all from BD Biosciences, as well as anti-CD26 APC (clone CLB-22C3) from Caltag Laboratories (Burlingame, CA). The negative staining threshold was established by the addition of an isotype-matched control tube. The analysis of the TCR-V β repertoire was done using a commercially available kit (IOtest Beta Mark TCR-V β Repertoire Kit; Beckman Coulter, Miami, FL) designed to quantitate 24 different TCR-V β specificities covering approximately 70% of the normal human TCR-V β repertoire.

Over the past 2 years, the panel was enhanced by the addition of anti-KIR3DL2/CD158k PE (clone AZ158k), kindly provided by Innate-Pharma (Marseille, France), as well as anti-PD1/CD279 PerCP-Cy5.5 (clone EH12.1) and anti-NKP46/CD335 PE (clone 9E2/Nkp46), both from BD Biosciences.

Neoplastic T-cell populations were identified on the basis of patterns of aberrant antigen expression with respect to the normal internal phenotypic populations according to definitions by Vaughan et al⁷ and Jamal et al.³¹ A change in immunophenotype over time was defined as a gain or loss of an antigen on atypical cells stable in at least two consecutive samples. A modification of antigen intensity was accepted only when there was a log difference of at least 0.5. To identify any shifts in relative fluorescence over time caused by changes in reagents or instruments, we compared levels of antigen expression with normal internal cell populations.

Molecular Analysis

TCR- γ Gene Rearrangement Analysis

PCR was routinely used to detect the presence of a clonal TCR rearrangement. PCR with primers directed against the TCR- γ gene was performed as previously described.²⁸ TCR γ -GR was studied on serial blood determinations by GeneScan capillary electrophoresis analysis, as previously described, in 24 of 107 patients.³⁰

DNA Methylation PCR Array for the CD26/Dipeptidyl Peptidase IV Gene

The Methyl-Profiler qPCR Primer Assay for Human DPP4 (CpG Island 03780): MePH03780-2 (Qiagen, Valencia, CA) uses the MethylScreen technology (Orion Genomics, St Louis, MO). This method is based on the detection of the leftover input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme. These enzymes will digest unmethylated and methylated DNA, respectively. The remaining DNA is quantified after digestion by real-time PCR in each individual enzyme reaction, using primers that flank a promoter (gene) region of interest. The relative fractions of hypermethylated, intermediately methylated, and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a no-enzyme added reaction.

Three digestion reactions were prepared, three with each of the enzymes and one not digested reaction (Mo, Ms, Md, and Msd), according to the Qiagen instructions. The SYBR Green quantitative PCR array was performed as follows: 95°C for 10 minutes, three cycles at 99°C for 30 seconds and 72°C for 1 minute, and 40 cycles at 97°C for 15 seconds and 72°C for 1 minute. SYBR Green fluorescence from each well during the annealing step of each cycle was detected and recorded. EpiTect Methyl PCR Array Ct analysis was performed on an Excel-based data template, available at www.sabiosciences.com/dna_methylation_data_analysis.php.

The minimum level of hypermethylation considered positive can be set at 10% to 20%. However, this is dependent on the ratio of target vs nontarget cells present in the sample (ie, normal cells mixed with cancerous cells). To overcome this threshold problem, we checked methylation on 98%/99.5% CD4+ purified cells.

Statistical Analyses

Statistical analyses were performed using the STATA 12.0 statistical software (StataCorp LP, College Station, TX). Data that were significant according to the Kolmogorov-Smirnov tests were considered to have a non-Gaussian distribution and were described by median and range. The cell percentages were compared

between groups using a one-way nonparametric analysis of variance (Kruskal-Wallis) test, followed by Dunn's multiple-comparison post hoc test for multiple groups. Data were considered significant when $P < .05$. Overall survival (OS) was calculated from the date of SS diagnosis to the date of death or last checkup for all patients. In the univariate analyses, product limit estimates were derived using the Kaplan-Meier method, and statistical comparisons were carried out by the log-rank Mantel-Cox test. Univariate/multivariate analyses were carried out to evaluate the influence that different variables had on OS. In the univariate/multivariate analysis, sex, B1/B2 blood stage, the presence of dim marker expression on atypical cells, loss of T-cell markers, and CD7 and CD26 expression (positive/mixed vs negative) were dichotomized; age at diagnosis, number of atypical cells, percentage of atypical cells, and CD4/CD8 ratio were continuous.

Results

Patient Characteristics

A total of 107 patients with SS (58 males and 49 females, median age 69.5 years at diagnosis) were studied with a median follow-up of 24.9 months (range, 1.2–128.9 months). At diagnosis, all patients had pruriginous erythroderma (two of 107 also displayed skin nodules), diffuse superficial adenopathies, atypical circulating SCs ($>1,000$ cells/ μL), and circulating T-cell clones detected by TCR-g chain analysis with the use of PCR. An identical clone was evidenced in all patients tested ($n = 50$), both in blood and skin, by GeneScan analysis. Twelve patients had a history of MF and 20 a long-standing erythroderma that had been misdiagnosed as reactive dermatitis.

The median WBC count at diagnosis was 11,220/ μL (range, 3,270–61,670/ μL). The median percentage and absolute lymphocyte count were 39% (range, 10%–95.1%) and 4,314/ μL (range 1,002–53,036/ μL), respectively.

Immunophenotypic Findings: Heterogeneity at Diagnosis

The most commonly observed lymphocyte immunophenotypic pattern in patients with SS was CD3+, CD5+CD4+, CD8-, CD27+, CD28+, and CD45RO+, with a consequent increase in the CD4/CD8 ratio (median, 11.19; range, 0.95–990). The most common aberrancy at diagnosis was a lack of CD26 expression Figure 1A, Figure 1B, and Figure 1C (central dot plots) and was observed in 96 (89.7%) of 107 cases. While 11 patients (10.3%) showed a "mixed" phenotype with a variable percentage of atypical CD26+ SCs Figure 1D, Figure 1E, and Figure 1F (central dot plots), this was predominant in only three cases (Figure 1E). In 84 of 107 cases, clonality was investigated by means of anti-TCR antibodies: in 50 (40%) cases, a clonal population was positively identified. The CD4+CD26- subset in the 96 CD26- patients with SS (median, 66; range, 16–97.50) matched with the atypical population detected by the analysis of the TCR-V β chain variable region and/or by T-marker dim expression (linear regression analysis: $R^2 = 0.77$). A high variability in CD7 expression was documented: 51 (47.7%) patients were CD7- (Figure 1A,D, dot plots on the right), 26 (24.3%) were CD7+ (Figure 1C,F, dot plots on the right), and 30 (28%) displayed the simultaneous presence of CD7+ and CD7- SCs (Figure 1B,E, dot plots on the right). A total of 28 (26.2%) of 107 cases had a loss of T-cell markers other than CD26 and CD7. The most frequent was a CD2 loss in 23 of 28 cases, with four complete Figure 2B (central dot plot) and 19 partial Figure 2A (central dot plot), followed by a CD4 loss in five of 107 cases (complete in two and partial in three). One case showed a coexistent CD2 and CD4 loss, and another had a CD45RO- CD45RA- phenotype. None of the 107 patients with SS showed either a CD3 or CD5 loss.

Either a "dim" or, more rarely, a "bright" population was found in 103 (96.3%) of 107 cases. The most frequently expressed "dim" markers were CD3 (82/107 [76.6%]), CD4 (52/107 [48.6%]), CD2 (47/107 [43.9%]), and CD7 (21/107 cases, with 20% considering only the positive and mixed cases). Several patients had more than one "dim" population (Figure 2A, central dot plot); the most common was CD2dimCD3dim (31 cases [29.0%]; 21/31 were CD2dimCD3dimCD4dim).

CD38 expression was significantly lower (median, 17.5%; range, 1%–94%) than in healthy participants (median, 65%; range, 20%–88%) in all but six patients. The CD4+CD38- population (median, 70.0%; range, 5%–97.90%) matched with the CD4+CD26- one (linear correlation = 0.86 in CD26- patients with SS) (Figures 2A and 2B, dot plot on the right).

Beginning in 2010, CD279/PD1, KIR3DL2/CD158k, and NKP46/CD335 were added to our panel and investigated in 17 of 107 patients. CD279/PD1 was constantly detected on SCs Figure 3A and Figure 3B (second dot plot from right), whereas CD158k was expressed in only 40% of patients (Figure 3B, second dot plot from left), and NKP46 was negative (Figures 3A and 3B, first dot plot from right) in all but one of the patients.

Both subsets within the “CD26-mixed” cases belonged to the same TCR-V β clone, as demonstrated by flow cytometry (Figures 1D, 1E, and 1F, central dot plots) and GeneScan analysis Figure 4, which clearly identified the presence of a single neoplastic clone. It was possible to repeat the GeneScan analysis during follow-up in four of 11 patients, confirming the stability of the clone.

No relevant phenotypical differences were found in the 12 patients with a prior MF diagnosis with respect to SS right from the beginning: two of 12 showed immunophenotypic heterogeneity at diagnosis with a CD26+ subset, one was CD7+, two were CD7 mixed, and nine were CD7-.

Immunophenotypic Stability During Follow-up

A stable phenotype over time was documented in 73 (76.8%) of 95 patients with SS with serial determinations (minimum of three samples), in contrast to an unstable one observed in 22 (23.2%) of 95. Phenotypic changes concerned a single antigen in 17 cases and two simultaneous antigens in five cases. In detail, 11 (12.1%) patients acquired a CD4+CD26+ subset variable from 2% to 86%, and two lost the small CD4+CD26+ fraction that had been detected at diagnosis. Figure 5 shows the variations over time of the CD4+CD26 \pm subpopulations according to treatment in a patient; interestingly, Gene Scan analyses performed during time showed no molecular changes despite the phenotypical switch observed. Positive or negative changes in CD7 expression were observed in nine (8.5%) patients (four cases from negative to mixed or positive and five cases from mixed to negative or positive). Five cases had changes in CD2 expression (four from negative to positive and one from positive to negative). No variations in fluorescence intensity were observed during follow-up, and the “dim” or “bright” CD3 or CD4 populations detected at diagnosis remained stable over time. In 11 of 22 cases, the onset of a new population was preceded by a treatment-induced, almost complete disappearance of circulating SCs (alemtuzumab in seven patients and fludarabine in four patients).

CD26/Dipeptidyl Peptidase IV Gene Hypermethylation

The DNA methylation profile of the CpG islands for the promoter region of CD26/dipeptidyl peptidase IV (DPP-IV) on PBLs was examined in 10 patients with SS and five healthy donors. Since the results were ambiguous, we decided to repeat the experiment on CD4+ purified lymphocytes (five patients with SS and two healthy donors) and report the data as follows: all SS cases with a CD26- phenotype (CD4+CD26- population >85%) were hypermethylated, whereas a lower level of hypermethylation was observed (82% hypermethylated and 18% unmethylated) in an SS case with a consistent CD4+CD26+ percentage (24%). All normal samples were unmethylated Table 1.

Univariate/Multivariate Analyses of Prognostic Factors

Five- and 10-year OS was 31.6% and 15.4%, respectively, and 68 patients died during follow-up. The following parameters detected at diagnosis were considered for univariate survival analysis: sex, age, percentage and absolute number of circulating SCs, CD4/CD8 ratio, B1/B2 blood stage, previous MF diagnosis, marker loss (CD7, CD26, and others), and the presence of “dim” populations Table 2. The parameters related to tumor burden (ie, percentage and absolute SC number) were both identified as having a statistically significant association with poor survival, whereas sex and age, previous MF diagnosis, and phenotypic aberrancies related to CD7 Figure 6A and CD2 (data not shown) expression at diagnosis had no impact on survival. Interestingly, patients characterized by the presence of a variable proportion of CD26+ atypical cells at diagnosis showed a statistically significant higher OS (P = .050) Figure 6B. CD26 expression maintained a significant role (hazard ratio [HR], 2.94; P = .045) in the Cox multivariate model when corrected for main EORTC prognostic factors: B1/B2 blood stage (HR, 2.14; P = .013) and CD4/CD8 ratio (HR, 1.00; P = .031) (Table 2).

Discussion

CTCLs are a clinically heterogeneous group derived from skin-homing T lymphocytes with different biological profiles and clinical course.^{32,33} Since a large series of patients with SS with a median follow-up of 24.9 months was available to us, we were able to carry out a retrospective immunophenotypic study to evaluate the efficacy that flow cytometry has in the quantification of atypical circulating SCs. This method was applied to define the most frequent aberrancies that had a diagnostic impact and to investigate the phenotype stability over time.

The most frequent aberrancies observed in more than 90% of patients were a defective CD26 expression or a modification of antigen expression intensity (dim or bright), whereas CD7 expression always varied greatly. Since 1987, our group^{15,34} has focused on the CD26 molecule, underlying its importance in SS diagnosis and suggesting that a cutoff of 30% of the CD4+CD26⁻ population provides a reliable tool when differentiating SS from inflammatory erythroderma.¹³ In our experience, the PE-CD26 clone L272 (BD Biosciences) is the most reliable of the antibodies available, in agreement with the Euroflow Consortium.³⁵ The right choice of antibody is critical to avoid misinterpretations.⁵ The use of an antibody that underestimates the CD26⁺ population could lead to a consequent increase of the CD4+CD26⁻ subset in both normal and reactive skin.

CD26/DPPIV, considered a T-cell activation antigen, is a characteristic transmembrane glycoprotein with multiple biological functions—it acts as a serine protease, receptor, costimulator, and adhesion molecule, and it induces apoptosis. Herein we discuss the role that the CpG hypermethylation of the CD26 gene promoter region plays in the loss of the CD26 surface marker, the same as that observed in adult T-cell leukemia (ATL).³⁶ Tsuji et al³⁶ demonstrated that CD26⁻ ATL cells had faintly detected transcripts of the gene that were aberrantly methylated. CpG methylation of various genes may be one of the most important molecular events in the late stages of leukemogenesis, and an analogous mechanism could be hypothesized also for other T-cell marker loss (eg, CD7).³⁷ The survival advantage observed in CD26⁺ or mixed SS cases, reported here for the first time, to our knowledge, might well be attributed to the tumorigenicity of CD26 loss. Since the SS cohort at our disposal was larger than the one we analyzed in 1998, we were able to confirm that all parameters related to tumor burden are significantly associated with poor survival, but not the prognostic value of CD7 negativity, in agreement with the findings from Vonderheid et al.²³

Considering the large panel of antibodies used, we would like to stress the importance of CD38 negativity in SS, something rarely described in the literature.⁴ CD38 is a glycoprotein involved in cellular adhesion, signal transduction, and intracellular calcium regulation, and its loss leads to a reduction in immune functions. Nowadays, it is considered an important prognostic factor used to stratify patients with B-cell chronic lymphocytic leukemia.³⁸ In our experience, the evaluation of a CD4+CD38⁻ population is a sensitive and reliable marker and can provide an additional parameter when defining the atypical population.

Another interesting observation that emerged from our retrospective analysis is that CD3 or CD5 loss is an extremely rare phenomenon in patients with SS³⁹: in our experience (data not shown), a CD3 loss can be found only in primary cutaneous CD30⁺ or follicular T-cell lymphomas. A “dim” T-cell marker expression in the atypical population represents the most frequent alteration in SS, as reported by other authors.^{4–6} In our opinion, the finding of a dim population, especially when coupled with the CD26 and/or TCR-V β analysis, represents a good tool for a reliable tumor burden assessment; nevertheless, the correct choice of the fluorochrome is important for a good resolution of the “dim” population.³⁵ Some molecules have recently been proposed as positive markers for SS. PD-1 plays a role in the maintenance of self-tolerance and prevention of autoimmunity, and its major goal is the inhibition of T-cell function, including proliferation and cytokine production. The present study confirms that PD-1^{40,41} is widely expressed in SCs. It is not suitable for diagnostic purposes, being also expressed in activated T and B cells, but its expression in SCs can be relevant from a functional point of view, defining the suppressor nature of these cells and representing a potential target for therapy. Although our experience is somewhat limited, our findings on CD158k-positive SCs are in disagreement with those of Poszepczynska-Guigné et al⁴² and Bahler et al,⁴³ who reported a very high percentage of CD158k-positive SCs (ie, from 82%–97%). Indeed, we observed CD158k expression in only 40% of cases. However, this bias may well be due to the different techniques employed: the PE-conjugated AZ158k Ab could be less sensitive than the CD158k/KIR3DL2 mAb

plus PE-coupled goat anti-mouse secondary mAb used by Poszepczynska-Guigné et al⁴² and the Q66 Ab used by Bahler et al.⁴³

The natural cytotoxicity receptor NKP46 was identified as the activating receptor that is mainly expressed by natural killer lymphocytes but also by the circulating SCs. For this reason, Bensussan et al⁴⁴ have recently proposed it as an additional marker on the circulating malignant T lymphocytes of patients with SS, in whom it functions as an inhibitory coreceptor that is capable of interfering with the processes governing their CD3-dependent proliferation. Again, our results differ from those of Bensussan et al,⁴⁴ showing a negativity of NKP46 in all but one patient with SS.

Phenotypical heterogeneity has already been described^{6,16,22} in a small series of patients with SS, and our finding confirms this observation on a wide series, in which a heterogeneous phenotype was detected in 32% of the study population. Notably, the review of our cases shows that in approximately 15% of cases, a subset of CD26+ cells can be present at diagnosis or arise during the follow-up. Moreover, we carried out evaluations to determine whether the positive and negative subsets for the same antigen belong to the same neoplastic clone at a molecular level. To this aim, two different approaches were used—the cytometric detection of TCR-V β regions and TCR GeneScan analysis—and a single clone was observed by both techniques. This strengthens the hypothesis that the neoplastic population possesses a plasticity that allows for heterogeneity at a phenotypical level.

A stable phenotype over time was documented in most patients (76.8%), including those with an aberrant mixed phenotype, in agreement with Washington et al,²⁴ who stated that immunophenotypic aberrancies were maintained over the course of disease in 95% of the 44 CTCLs analyzed despite varying treatments. The observation of an onset of new phenotypic subsets over time in a minority of our patients could be explained by the presence of several small undetectable populations since diagnosis: a different subset belonging to the minimal residual disease could proliferate after an effective treatment such as alemtuzumab.⁴⁵ However, on the basis of our findings, this phenomenon does not seem to bear prognostic relevance, since alemtuzumab was used in 21 (67.7%) patients in our series who achieved an almost complete response (data not shown) without any evidence of phenotypical changes.

In conclusion, we confirm the relevance that CD26 negativity has for a diagnostic purpose in SS. Nevertheless, due to the finding of stable phenotypical changes sometimes involving CD26, multiparameter flow cytometry is recommended. This should combine the assessment of routinely used T-cell markers and the cytometric TCR-V β analysis should the routine panel produce ambiguous results. In detail, we believe that the first-step panel for SS diagnosis should include the evaluation of CD26, CD27, CD28, CD38, CD45RO, and CD45RA, in addition to T-cell markers (CD2, CD4, CD7, CD8) and CD3 and CD45 as backbone markers, as suggested by the Euroflow Consortium.³⁵ As already suggested by other groups,^{6,46} multiparameter flow cytometry may also help the clinician in monitoring tumor burden in response to therapy, specifically alemtuzumab. At our institution, after evaluating the CD52 expression in basal conditions, this approach is used to guide alemtuzumab dose frequency to keep the side effects of this therapy to a minimum.

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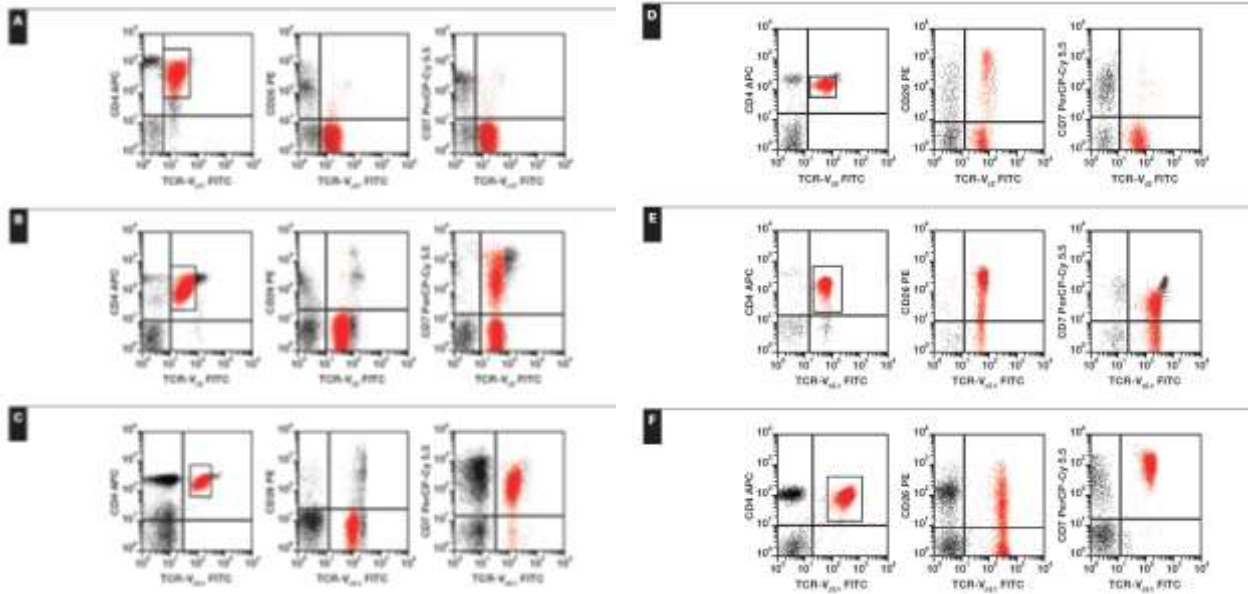


Figure 1 Six different Sézary syndrome cases in which the atypical Sézary cells population (in red) is identified at baseline by CD4+ T-cell receptor (TCR)-Vb positivity (A–F). Different CD26 expression: dot plots show CD26 negativity (A–C) and a CD26 mixed pattern with different amount of CD26 (D–F). CD7 expression in dot plots is negative (A, D), mixed (B, E), and positive (C, F).

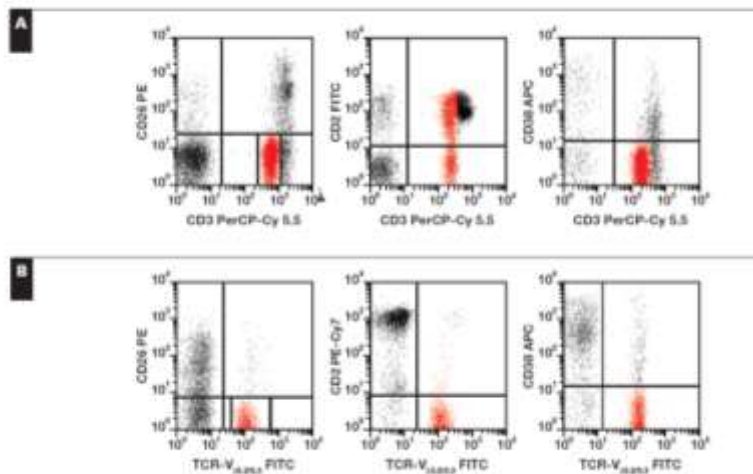


Figure 2 Aberrancies in CD2 and CD38 expression in two different Sézary syndrome (SS) cases. A, Patient with SS with CD3+dim CD26– phenotype, aberrant mixed CD2– and CD2+dim populations, and CD38 negativity. B, Patient with SS with T-cell receptor (TCR)-Vb 5.2/5.3+ CD26– phenotype, CD2 negativity, and CD38 negativity.

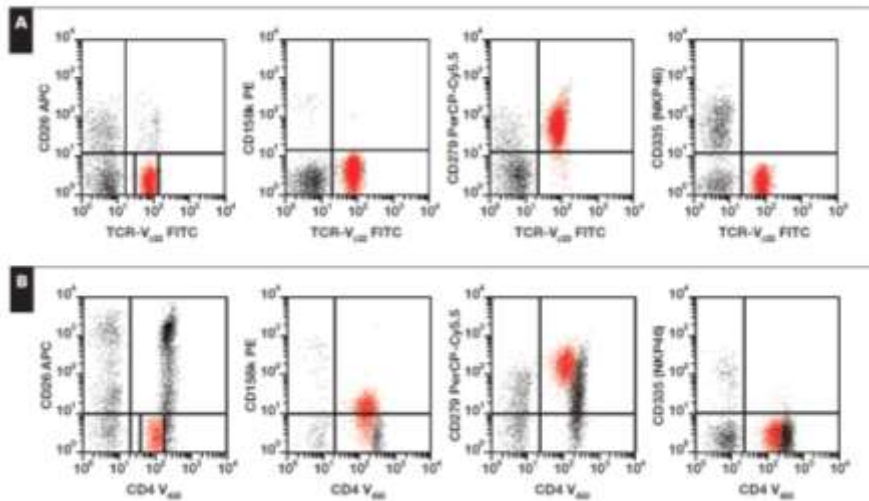


Figure 3 A, Patient with Sézary syndrome (SS) patient with high tumor burden: immunophenotype T-cell receptor (TCR)-Vb22+, CD26-, CD158k-, CD279+, NKP46- (red). B, Patient with SS with low tumor burden: immunophenotype CD4+dim, CD26-, CD158k+dim, CD279+, NKP46- (red). Residual normal CD4+ lymphocytes are CD26+, CD158k-, CD279±, NKP46- (black).

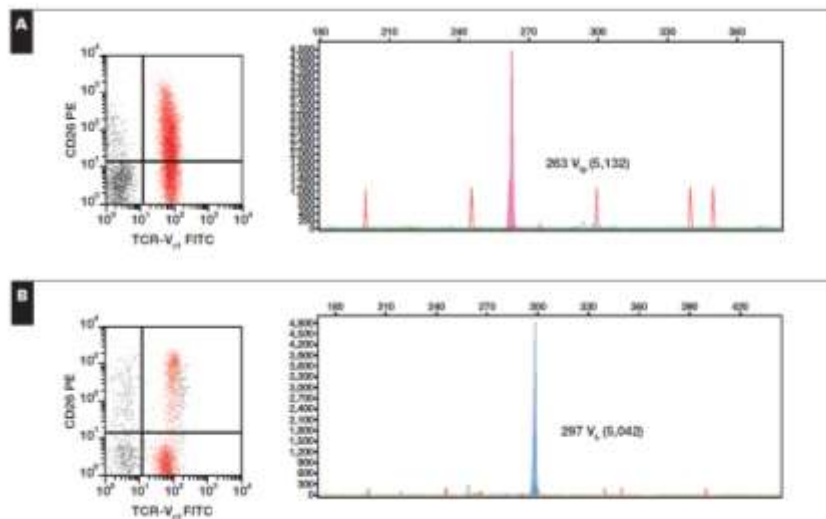


Figure 4 GeneScan identifies a single pathologic clone (filled peak), whereas cytometric analysis shows a continuous spectrum of CD26 expression from positive to negative (case A) and the coexistence of two CD26+ and CD26- subsets (case B), both expressing a single T-cell receptor (TCR)-Vb chain in the same peripheral blood lymphocyte sample.

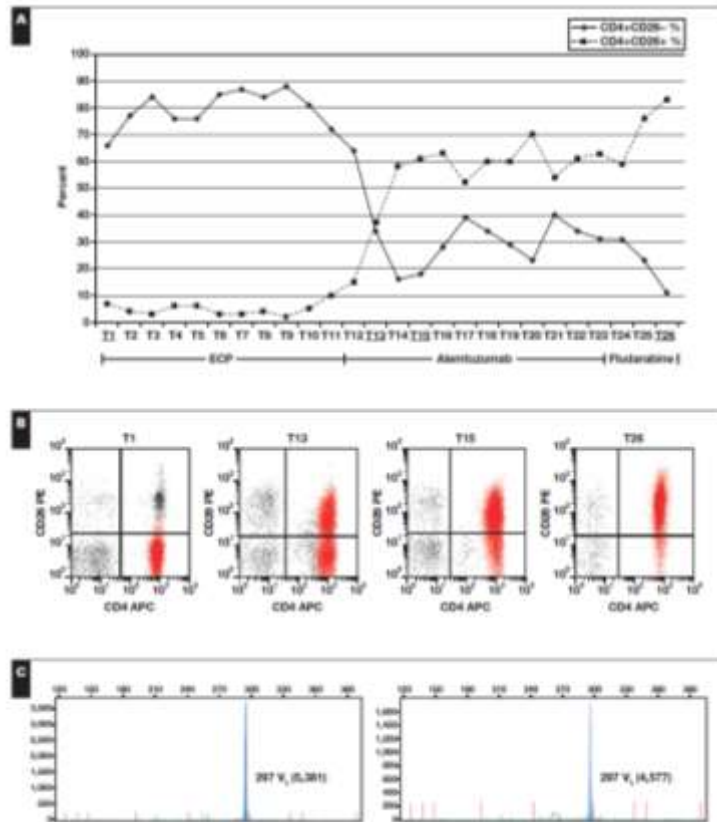


Figure 5 Phenotypical switch from CD4+CD26⁻ to CD4+CD26⁺ in a patient with Sézary syndrome. A, Fluctuation of the two subsets during follow-up according to treatment (extracorporeal photopheresis, ECP; alemtuzumab; fludarabine). B, Dot plots at time T1, T13, T15, and T26 show the progressive increase of the CD4+CD26⁺ population. C, GeneScan analysis of a peripheral blood lymphocyte before (left) and after (right) the phenotypical switch show an identical clone at the molecular level despite the phenotypical diversity.

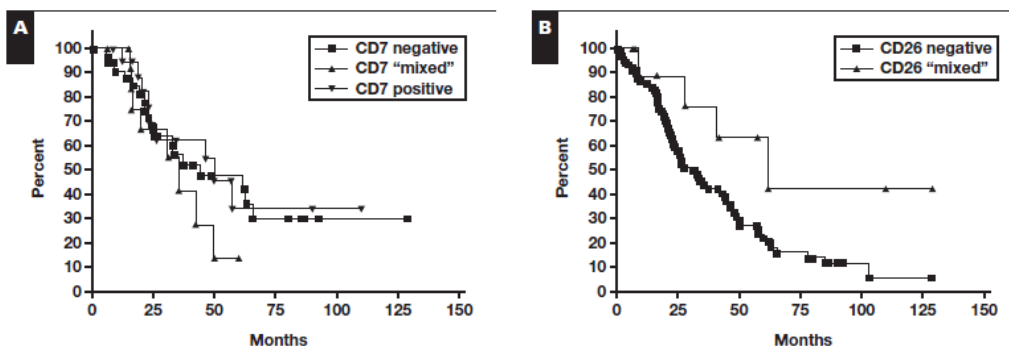


Figure 6 Survival according to CD7 (A; P = .4717) and CD26 (B; P = .0469) expression.

	Sezary cells	CD4+ lymph.	CD4+CD26- lymph.	Hypermethylated	Unmethylated
B.D. (SS CD4dim)	95	96	96	95.1	4.9
B.C. (SS CD4dim)	82	91	82	97.5	2.5
M.E. (SS TCR V β 13.2)	67	79	76	89.13	10.87
LR.G. (SS TCR V β 13.1)	91	95	93	98.7	0.83
D.A. (SS TCR V β 5.1)	91	93	15	6.67	93.33
Healthy donor 1	0	58	10	1.46	98.54
Healthy donor 2	0	60	6	0.07	99.93

Table 1 CD26 DNA methylation profile on CD4 purified lymphocytes from 5 SS cases and 2 healthy donors. All data are expressed as percentage values.

Characteristic	HR	HR (95% CI)	P Value
Gender	.88	(.542774 1.418286)	0.593
age at diagnosis	.99	(.9793775 1.018717)	0.909
B1/B2 blood stage	2.39	(1.3335 4.272529)	0.003
% of atypical cells	1.02	(1.010752 1.031901)	0.000
number of atypical cells	1.00	(1.000007 1.000049)	0.008
CD4/CD8 ratio	1.00	(1.000867 1.00464)	0.004
“dim” marker expression	2.59	(.3586435 18.78389)	0.345
Marker loss (CD2, CD4)	1.60	(.955714 2.680926)	0.074
CD7 expression	.89	(.551187 1.444637)	0.643
CD26 expression	2.70	(.9745076 7.474271)	0.050
Gender	.86	(.5234651 1.427505)	0.569
age at diagnosis	1.00	(.9850816 1.023853)	0.665
B1/B2 blood stage	2.14	(1.176012 3.904511)	0.013
CD4/CD8 ratio	1.00	(1.00021 1.004365)	0.031
CD26 expression	2.94	(1.025964 8.423034)	0.045

Table 2 Summary of a Univariate Analysis to Evaluate an Association With OS and a Multivariate Analysis to Identify Independent Predictors of OS

Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival..