Evaluation of Immunophenotypic and Molecular Biomarkers for Sezary Syndrome Using Standard Operating Procedures

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Differentiation between Sézary syndrome and erythrodermic inflammatory dermatoses can be challenging, and a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells that could be useful as additional diagnostic criteria. In this European multicenter study, the sensitivity and specificity of these immunophenotypic and recently proposed but unconfirmed molecular biomarkers in Sézary syndrome were investigated. Peripheral blood CD4\(^+\) T cells from 59 patients with Sézary syndrome and 19 patients with erythrodermic inflammatory dermatoses were analyzed for cell surface proteins by flow cytometry and for copy number alterations and differential gene expression using custom-made quantitative PCR plates. Experiments were performed in duplicate in two independent centers using standard operating procedures with almost identical results. Sézary cells showed MYC gain (40%) and MNT loss (66%); up-regulation of DNM3 (75%), TWIST1 (69%), EPHA4 (66%), and PLS3 (66%); and down-regulation of STAT4 (91%). Loss of CD26 (>80% CD4\(^+\) T cells) and/or CD7 (>40% CD4\(^+\) T cells) and combination of altered expression of STAT4, TWIST1, and DNM3 or PLS3 could distinguish, respectively, 83% and 98% of patients with Sézary syndrome from patients with erythrodermic inflammatory dermatoses with 100% specificity. These additional diagnostic panels will be useful adjuncts in the differential diagnosis of Sézary syndrome versus erythrodermic inflammatory dermatoses.

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INTRODUCTION

Sézary syndrome (SS) is a rare and aggressive type of cutaneous T-cell lymphoma that is derived from CD4\(^+\) skin-homing memory T cells and characterized by erythroderma, generalized lymphadenopathy, and neoplastic T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood (Wieselthier and Koh, 1990).

Differential between SS and erythrodermic inflammatory dermatoses (EID) may be extremely difficult, both clinically and histopathologically, but is very important from therapeutic and prognostic perspectives. For a long time the diagnosis was based on demonstration of atypical T cells, so-called Sézary cells, in blood smears (Sentis et al., 1986; Trotter et al., 1997). However, it was shown that Sézary cells can also be observed in the peripheral blood of patients with EID and even in healthy control subjects (Duncan and Winkelmann, 1978; Meijer et al., 1977). Demonstration of at least 1,000 Sézary cells per mm\(^3\) was often used as a decisive criterion, but this was not generally agreed on (Wieselthier and Koh, 1990). To prevent patients with EID...
being misclassified as having SS and being treated as such, in 1997 the European Organization for Research and Treatment of Cancer group proposed the demonstration of clonal T cells and the presence of an expanded CD4⁺ T-cell population resulting in a CD4/CD8 ratio above 10 as additional criteria for a diagnosis of SS (Willemze et al., 1997).

At present, the diagnosis of SS is based on clinical presentation (erythroderma and lymphadenopathy) and demonstration of a T-cell clone in the peripheral blood (preferably the same clone in the skin), in combination with one or more of the following criteria: an absolute Sézary cell count greater than 1,000 cells per mm³; loss of T-cell markers CD2, CD3, CD4, and/or CD5; and/or an expanding population of CD4⁺ T-cells leading to a CD4/CD8 ratio of more than 10 (Swerdlow et al., 2008; Willemze et al., 2005). However, distinction between SS and EID can still be difficult, because T-cell clonality can be observed in a substantial proportion of patients with EID as well, and not all SS patients have a CD4/CD8 ratio of greater than 10 at first presentation (Vonderheid, 2006; Vonderheid and Bernengo, 2003).

To solve this diagnostic problem, a number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells. Flow cytometry studies reported loss of CD7 and CD26 expression by Sézary cells and suggested CD4⁺CD7⁻ T-cells of at least 40% and CD4⁺CD26⁻ T-cells of at least 30% as tentative diagnostic criteria in those difficult cases (Bernengo et al., 2001; Fierro et al., 2008; Harmon et al., 1996; Jones et al., 2001; Kelemen et al., 2008; Klemke et al., 2008; Lima et al., 2003; Olsen et al., 2007; Rappl et al., 2001; Sokolowska-Wojdylo et al., 2005; Vonderheid et al., 2002; Vonderheid and Bernengo, 2003; Washington et al., 2002).

In addition, recent studies described expression of killer cell immunoglobulin-like receptors CD158a, CD158b, and CD158k and the “central memory” T-cell phenotype (CD27⁺, CD45RA⁺, CD45RO⁻) as characteristic features of Sézary cells (Bagot et al., 2001; Bahler et al., 2008; Campbell et al., 2010; Dummer et al., 1996; Fierro et al., 2008; Karenko et al., 2001; Klemke et al., 2008; Lima et al., 2003; Marie-Cardine et al., 2007; Michel et al., 2013; Poszepczynska-Guigne et al., 2004). Molecular investigations identified gain of JUNB, MYC, and loss of MYC antagonists MNT and MXI1 as recurrent genetic lesions in the SS genome (Mao et al., 2003; Mao et al., 2008; Vermeer et al., 2008). Gene expression studies showed increased expression of PLS3, DNM3, CDO1, TRAIL, CD1D, GATA3, JUNB, TWIST1, EPHA4, and MYC and decreased expression of STAT4 in Sézary cells (Booken et al., 2008; Goswami et al., 2012; Kari et al., 2003; Mao et al., 2008; Nebozhyn et al., 2006; Su et al., 2003; Van Doorn et al., 2004).

However, the diagnostic value of these biomarkers in diagnosing SS has not been investigated thoroughly. Moreover, most biomarkers were identified in small, single-center studies with a limited number of patients and controls and have not been confirmed in large independent studies. In addition, flow cytometry studies have used widely differing protocols, which impedes interpretation and comparison of results from different studies.

The goal of this European Organization for Research and Treatment of Cancer multicenter study was to investigate the sensitivity and specificity of these biomarkers for SS in a large group of well-defined SS patients compared with EID patients using standard operating procedures (SOPs).

### RESULTS

#### Patient characteristics

Clinical characteristics at diagnosis of the 59 SS patients and 19 EID patients are summarized in **Table 1**.

The patients with SS had a T-cell clone in the peripheral blood (59 of 59), a CD4/CD8 ratio above 10 (53 of 57), and/or a Sézary cell count above 1,000 per mm³ (34 of 43, including all four patients with a CD4/CD8 ratio lower than 10).

One EID patient showed a T-cell clone in the peripheral blood, and another EID patient had a CD4/CD8 ratio above 10 because of very low numbers of CD8⁺ T-cells, but none had a Sézary cell count above 1,000 mm³ (**Table 1**).

#### Flow cytometry

Flow cytometry experiments were performed both in Leiden and Paris for all 59 SS patients and 19 EID patients. Differences in flow cytometry results between Leiden and Paris for all 59 SS patients and 19 EID patients are summarized in **Table 1**.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SS Patients (n = 59)</th>
<th>EID Patients (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female ratio</td>
<td>37:22</td>
<td>16:3</td>
</tr>
<tr>
<td>Median age (range), years</td>
<td>65 (32–89)</td>
<td>67 (29–86)</td>
</tr>
<tr>
<td>Erythroderma</td>
<td>46/52 (88)</td>
<td>19/19 (100)</td>
</tr>
<tr>
<td>Pruritus</td>
<td>45/52 (87)</td>
<td>13/19 (68)</td>
</tr>
<tr>
<td>Ectropion</td>
<td>6/52 (12)</td>
<td>1/19 (5)</td>
</tr>
<tr>
<td>Hyperkeratosis hand/feet</td>
<td>21/52 (40)</td>
<td>3/19 (16)</td>
</tr>
<tr>
<td>Palpable lymphadenopathy</td>
<td>21/52 (40)</td>
<td>4/19 (21)</td>
</tr>
<tr>
<td>Lymphadenopathy confirmed by CT scan</td>
<td>17/41 (41)</td>
<td>1/7 (14)</td>
</tr>
<tr>
<td>Leukocytes ≥10.0 × 10⁹/L</td>
<td>40/56 (71)</td>
<td>5/14 (36)</td>
</tr>
<tr>
<td>CD4/CD8 ratio ≥10.0</td>
<td>53/57 (93)</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Absolute Sézary cell count ≥1,000 mm³</td>
<td>34/43 (79)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>T-cell clone in peripheral blood</td>
<td>59/59 (100)</td>
<td>1/17 (6)</td>
</tr>
<tr>
<td>Identical T-cell clone in blood and skin</td>
<td>32/38 (84)</td>
<td>0/3 (0)</td>
</tr>
</tbody>
</table>

Note. Values are given as n/total (%) unless otherwise noted. Abbreviations: CT, computed tomography; EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome.
Diagnostic Markers in Sézary Syndrome

never in EID patients (sensitivity = 54%, specificity = 100%). In addition, CD7dim was found in 2 of 59 (3%) SS patients, compared with none of the EID patients.

In the CD4+ T-cell population a percentage of 30% or more CD4+CD26- cells was found in 5 of 59 (86%) SS patients but also in 10 of 19 (53%) EID patients (sensitivity = 86%, specificity = 47%). When shifting the percentage to 80%, 39 of 59 (66%) SS patients but none of the EID patients had CD4+CD26- cells of 80% or more in the CD4+ T-cell population (sensitivity: 66%; specificity: 100%). In addition, CD26dim was found in 5 of 59 (8%) Sézary patients and in one of 19 (5%) EID patients.

Loss of CD26 by more than 80% and/ or loss of CD7 by more than 40% of CD4+ T cells was found in 49 of 59 (83%) SS patients but was never observed in the EID patients (sensitivity = 83%, specificity = 100%).

Investigations on CD158k expression showed that more than 5% of these CD4+ T cells expressed CD158k or CD158klow in 19 of 58 (33%) SS patients, compared with 1 of 19 (5%) EID patients (sensitivity = 33%; specificity = 95%). The results (including expression of CD158a and CD158b) are summarized in Table 2.

No major difference was observed in the expression of CD27, CD45RA, and CD45RO by CD4+ T cells between SS and EID patients (data not shown).

**Copy number variation**

Copy number variation (CNV) experiments were performed in 58 SS patients and 17 EID patients in Leiden. Duplicate experiments for 14 samples were performed in London, which gave identical results (see Supplementary Figure S1 online).

In 47 of 58 (81%) SS patients, alterations in copy number were found compared with none of the 17 EID patients. Gain of MYC was observed in 23 of 58 (40%) SS patients (sensitivity = 40%, specificity = 100%). MNT loss was found in 38 of 58 (66%) SS patients (sensitivity = 66%, specificity = 100%) and one (2%) patient showed gain of MNT (Figure 1). Gain of MYC and/or loss of MNT was found in 76% (44 of 58) of SS patients (sensitivity = 76%, specificity = 100%). Copy number alterations of JUNB and TWIST1 were found in only a minority of SS patients (Figure 1).

**Gene expression**

Gene expression (GE) analysis were performed on 55 SS, 19 EID, and 4 healthy control patients in Leiden. Thirty samples were also analyzed in London, with identical results in 28 samples (see Supplementary Figure S2 online); two samples could not be analyzed because of a technical error.

DNM3, TWIST1, EPHA4, PLS3, and STAT4 were the most differentially expressed genes in SS patients compared with EID patients and healthy controls with 100% specificity (P < 0.001) (Table 3, see Supplementary Figure S3 online). Up-regulation of DNM3, TWIST1, EPHA4, and PLS3 was found in 66–75% of SS patients, and STAT4 was down-regulated in 91% of SS patients (Figure 2). Up-regulation of DNM3, TWIST1, EPHA4, and PLS3 was found in 41 of 55 (75%), 38 of 55 (69%), 36 of 55 (66%), 36 of 55 (66%) of SS patients, respectively, and STAT4 was down-regulated in 50 of 55 (91%) of SS patients. Combining alterations in gene expression (STAT4, TWIST1, and DNM3 or STAT4, TWIST1, and PLS3), we could distinguish 54 of 55 (98%) SS patients from all EID patients (sensitivity = 98%, specificity = 100%).

Aberrant gene expression of CDO1, TRAIL, CD11D, GATA3, MYC, and JUNB was found in only a minority of SS patients (Table 3).

**DISCUSSION**

In the present multicenter study, we investigated the diagnostic sensitivity and specificity of immunophenotypic and molecular biomarkers in SS using SOPs. We show that by using SOPs, it is possible to obtain highly reproducible results for flow cytometry, CNV, and GE analysis and show that loss of CD7 and CD26 by CD4+ T cells; gain in copy number of MYC and loss of MNT; increased expression of DNM3, TWIST1, EPHA4, and PLS3; and decreased expression of STAT4 are highly characteristic for Sézary cells.

In the current study most SS patients have a CD4/CD8 ratio above 10, but we also show that a significant minority of patients (13%) does not fulfill this diagnostic criterion. For these patients additional immunophenotypic markers are clearly needed.

Comparison of results from previous flow cytometry studies is hindered by the use of different protocols and cell populations. In the present study we focused on CD4+ gated T cells because CD4 is rarely lost by Sézary cells, facilitating the comparison of expression levels of different immunophenotypic markers.

Previous studies reported that a CD4+CD26- cell population greater than 30% had a sensitivity of 97% and specificity of 100% in diagnosing peripheral blood involvement.

<table>
<thead>
<tr>
<th>Markers for SS Described in the Literature</th>
<th>SS Patients, n/total (n = 59)</th>
<th>EID Patients, n/total (n = 19)</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+CD8 ratio ≥10</td>
<td>46/53</td>
<td>1/12</td>
<td>87</td>
<td>92</td>
</tr>
<tr>
<td>CD4+CD7- ≥ 40%</td>
<td>32/59&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0/19</td>
<td>54</td>
<td>100</td>
</tr>
<tr>
<td>CD4+CD26+ ≥ 30%</td>
<td>51/59&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10/19</td>
<td>86</td>
<td>47</td>
</tr>
<tr>
<td>CD158a&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2/58</td>
<td>0/19</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>CD158b&lt;sup&gt;3&lt;/sup&gt;</td>
<td>13/59</td>
<td>1/19</td>
<td>22</td>
<td>95</td>
</tr>
<tr>
<td>CD158k&lt;sup&gt;3&lt;/sup&gt;</td>
<td>19/58</td>
<td>1/19</td>
<td>33</td>
<td>95</td>
</tr>
</tbody>
</table>

Abbreviations: EID, erythrodermic inflammatory dermatoses; SS, Sézary syndrome.
<sup>1</sup>Including four of seven SS patients with a CD4/CD8 ratio below 10.
<sup>2</sup>Including five of seven SS patients with a CD4/CD8 ratio below 10.
<sup>3</sup>Low expression and expression of 5% or more of the CD4+ lymphocytes.
and suggested this cut-off point as a tentative diagnostic criterion for SS (Bernengo et al., 2001; Vonderheid and Bernengo, 2003). Indeed, loss of CD26 in more than 30% of CD4\(^+\) T cells was found in 86% of SS patients but also in 53% of EID patients, resulting in a specificity of 47%. However, when using a percentage of 80% as the cut-off point, we found that 39 of 59 (66%) SS patients but none of the EID patients met this criterion. These discrepant results can be explained by different flow cytometry protocols. We evaluated CD26 expression on CD4\(^+\) gated T cells, whereas Bernengo et al. looked at CD4\(^+\)CD26\(^-\) cells on gated total lymphocytes. Indeed, when looking at the CD4\(^+\)CD26\(^-\) cells of 30% or more on total lymphocytes, similar results were found (sensitivity = 80%, specificity = 95%; data not shown).

A level of CD4\(^+\)CD7\(^-\) cells of more than 40% has also been suggested as a tentative criterion in the diagnosis of SS (Vonderheid et al., 2002). Consistent with the literature, we found that loss of CD7 above 40% of the CD4\(^+\) T cells is highly specific (specificity = 100%) but is not a sensitive marker (sensitivity = 54%) (Harmon et al., 1996; Nagler et al., 2012; Vonderheid and Bernengo, 2003). Similar results were found for 40% or more CD4\(^+\)CD7\(^-\) cells on total lymphocytes (sensitivity = 42%, specificity = 100%; data not shown).

Flow cytometry results show that in 83% of SS patients, CD4\(^+\) T cells display loss of CD26 by more than 80% and/or loss of CD7 by more than 40%, whereas this was never observed in EID patients. These observations are relevant because they can readily be included in immunophenotypic testing of erythrodermic patients.

Previous studies reported expression of killer cell immunoglobulin-like receptor CD158k in 65–97% of SS patients (Bahler et al., 2008; Klemke et al., 2008; Poszepczynska-Guigne et al., 2004). Flow cytometry analysis, performed in both Leiden and Paris, showed expression of CD158k in only 33% of SS patients (19 of 58), and in most of these SS patients (18 of 19) the CD158k antigen was expressed at low levels. This discrepancy can be explained by...
the fact that the present study was performed on frozen peripheral blood mononuclear cells (PBMCs) instead of freshly isolated PBMCs. Indeed, a recent study on freshly isolated PBMCs from SS patients showed high CD158k expression in Sézary cells (Moins-Teisserenc et al., 2015).

For CNV and GE analysis the use of SOPs and custom-made PCR platforms led to highly reproducible results as well. Gain of MYC and/or loss of its antagonist MNT was found in 76% of SS patients but never in EID patients. Gain of TWIST1 and JUNB was detected in only a small minority of SS patients.

In line with the literature, we found up-regulation of DNM3, TWIST1, EPHA4, and PLS3 and down-regulation of STAT4 in most SS patients (Booken et al., 2008; Goswami et al., 2012; Kari et al., 2003; Nebozhyn et al., 2006; Su et al., 2003; Van Doorn R. et al., 2004). In contrast, only a minority of SS patients showed up-regulation of GATA3, CD1D, TRAIL, CDO1, JUNB, and MYC, implying that these genes are not useful diagnostic markers. Why gain of MYC and loss of MNT, which is observed in most patients, does not lead to up-regulation of MYC expression is as yet unexplained.

Combined alterations in gene expression of STAT4, TWIST1, and DNM3 or STAT4, TWIST1, and PLS3 could distinguish 98% of SS patients from EID patients, suggesting that this diagnostic panel will be useful as an additional molecular criterion in the diagnostic differentiation between SS and EID.

In the present study 27 patients were diagnosed with SS before inclusion in the study. However, no significant differences were found in the prevalence of the previously described markers between the 27 patients already diagnosed with SS before inclusion in the study and the 32 SS patients newly diagnosed at time of inclusion.
(see Supplementary Table S1 online). Similarly, the prevalence of investigated markers was similar in the 36 SS patients who received treatment during sample collection at inclusion and the 23 SS patients who did not receive any form of treatment (see Supplementary Table S2 online). These observations argue that the observed immunophenotypic and molecular changes are stably expressed in Sézary cells.

We show that standardization of flow cytometry, CNV, and GE procedures leads to strong reproducibility of results. We argue that to facilitate comparison of results from different centers, it will be important to closely define the subset of cells that was investigated, and based on the present study we suggest gating on CD4+ T cells in future studies.

For patients in whom the distinction between SS and EID still cannot be made using the current diagnostic criteria, we propose that these two additional diagnostic panels can be used: (i) loss of CD26 (>80% CD4+ T cells) and/or loss of CD7 (>40% CD4+ T cells) for immunophenotypic analysis and (ii) combination of altered gene expression of STAT4, TWIST1, and DNM3 or STAT4, TWIST1, and PLS3 for molecular analysis.

MATERIALS AND METHODS

Design of the study

To achieve sufficient power for the study, a consortium of six European Organization for Research and Treatment of Cancer centers with extensive experience with SS was formed including centers from Helsinki, Finland; London, England; Leiden, The Netherlands; Mannheim, Germany; Turin, Italy; and Paris, France. At time of inclusion peripheral blood samples were collected for investigation of (i) expression of cell surface proteins by flow cytometry, (ii) CNV, and (iii) GE profiles. The markers were selected based on the literature and are presented in Supplementary Table S3 online.

To optimize standardization and to prevent interdepartmental differences, SOPs were produced for the workflow of blood sampling, isolation of PBMCs, and enrichment for CD4+ T cells (SOP 001), DNA isolation (SOP 002), RNA isolation (SOP 003), complementary DNA synthesis (SOP 004), CNV and GE assays (SOP 005), flow cytometry experiments (SOP 007), and the freezing and shipment of samples (SOP 008) (see Supplementary Figure S4 and Supplementary Materials online). Much effort was put into standardizing flow cytometry analysis, because this technique has been shown to have limited reproducibility in multicenter studies because of limited standardization of laboratory procedures, instrumental settings, and interpretation of results (van Dongen et al., 2012; Westers et al., 2012).

To test if the use of SOPs leads to increased reproducibility, the flow cytometry experiments were performed in duplicate in Leiden and Paris on all samples, and assays for CNV and GE were performed in Leiden and repeated in London for a selected number of samples.

In all participating centers the study was approved by the local institutional ethical review boards, and written informed patient consent was obtained. Consensus meetings to compare experimental results were held on August 31, 2012, in Paris and October 31, 2013, in Leiden.

Patient selection and clinical characteristics

Between September 2009 and October 2013 a total of 103 subjects were enrolled with the following diagnosis: SS (n = 72), EID (n = 27), and healthy controls (n = 4).

Inclusion criteria for the SS patients were diagnosis of SS based on the recent World Health Organization-European Organization for Research and Treatment of Cancer criteria and available complete clinical data. Inclusion criteria for patients with EID were presentation with erythroderma and blood test results not meeting the SS blood criteria.

From the initial 72 patients with SS 13 were excluded because of inferior sample quality (n = 10) or insufficient clinical data (n = 3). From the initial 27 EID patients eight were excluded because of inferior sample quality (n = 3) or insufficient clinical data (n = 5).

The final study group consisted of 59 patients with SS, 19 patients with EID, and four healthy controls. The SS group consisted of 32 patients with newly diagnosed SS and 27 patients with known SS. Thirty-six SS patients received treatment at the time of blood sampling (10 newly diagnosed with SS and 26 with known SS). The treatment consisted of psoralen plus UVA therapy (n = 2), extracorporeal photopheresis as monotherapy or combined with immunomodulatory agents (n = 12), prednisone alone or in combination with chlorambucil (n = 9), monotherapy with interferon alfa, bexarotene, methotrexate, or acitretin (n = 11), and polychemotherapy (n = 2).

The EID group included nine patients with atopic erythroderma, five patients with erythrodermic psoriasis, two patients with erythrodermic drug eruption, two patients with idiopathic erythroderma, and one patient with paraneoplastic erythroderma secondary to a cholangiocarcinoma. None of the EID patients developed a lymphoma during follow-up study (median follow-up = 22 months, range = 8–38 months).

Workup blood samples

PBMCs were isolated from peripheral blood and stored in liquid- or vapor-phase nitrogen. Part of the fresh PBMCs were enriched for CD4+ T helper cells by depletion of non-CD4+ T cells, resulting in greater than 95% purity for the CD4+ T-cell population, using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) for CNV and GE assays.

Genomic DNA was extracted using the QiAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at −20°C.

DNA was isolated using the RNeasy Mini Kit (Qiagen), which included on-column DNase digestion. Two µg of RNA was reverse-transcribed in triplicate with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA), using random priming in a final volume of 20 µL. After synthesis, complementary DNA samples were stored at −20°C. A detailed description of this workup of blood samples is found in the Supplementary Materials (SOPs 001–004 and 008).

Flow cytometry

In Leiden and Paris flow cytometry was performed for the following antigens: CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD27, CD45RA, CD45RO, CD158a, CD158b, and CD158k, including isotype-specific controls (see Supplementary Table S4 online and SOP 007 in the Supplementary Materials). Lymphocytes were gated from forward and sideward scatter patterns; next, antigen expression was assayed from CD4+ gated lymphocytes. Specific antigen expression was observed relative to autofluorescence and (non-) specific signals obtained from each individual patient and a PBMC control sample. Specific antigen expression was observed relative to autofluorescence and (non-) specific signals, as determined with unlabelled cells, isotype-specific control and epitope-specific labelling relatively, from each individual patient and a PBMC control.
sample. This control sample was derived from two healthy donors and functioned as an internal reference sample for each flow cytometry session. Samples were analyzed in a blinded setting.

Specific antigen expression in the population of gated cells (expression or loss) was displayed in percentages (Figure 3a and b). Antigen expression was considered dim if all gated cells showed diminished expression around the determined threshold (Figure 3c). For CD158a, CD158b, and CD158k, expression below 5% of the gated cells was considered as no expression, but when intermediate expression of a single population of gated cells, surrounding a determined threshold, was found, this was characterized as low-expressing antigen (for example, CD158klow) (Figure 3d).

**CNV assay**

Quantitative PCR assays with 6-Carboxyfluorescein-labeled hydrolysis Minor Groove Binder probes (Life Technologies, Carlsbad, CA) were developed for target genes JUNB, TWIST1, MYC, and MNT and reference genes ABT1, ARG2, and DNM3 (see Supplementary Table S5 online). Reference genes were selected from different large copy number-stable chromosomal regions in SS, selected from array-based comparative genomic hybridization experiments on 20 SS samples (Vermeer et al., 2008). Amplification efficiency was evaluated in triplicate, using eight 4-fold serial dilution points ranging from 3 ng/μL to 183 fg/μL DNA concentration, under optimized primer and hydrolysis probe concentrations. Assays with amplification efficiency value between 90% and 100% and a correlation coefficient above 0.98 were accepted for CNV analysis. Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005 (see Supplementary Materials).

Data were normalized against reference genes and relative to the common reference using the ΔΔCq method and are presented as relative copy number, where 2 stands for diploid DNA (Livak and Schmittgen, 2001). The following thresholds were maintained for the CNV data: 1.5–2.5 was considered as diploid (normal) DNA, greater than 2.5 as gain in copy number, and less than 1.5 as loss in copy number.

**GE assay**

GE quantitative PCR assays with FAM-labelled hydrolysis MGB probes (Life Technologies) were developed and validated, as described for CNV quantitative PCR assays, for target genes PLS3, DNM3, CDO1, TRAIL, CD1D, GATA3, MYC, JUNB, TWIST1, EPHA4, and STAT4 and reference genes ARF5, ERCC3, and TMEM87A (see Supplementary Table S5). Stably expressed reference genes were selected from microarray experiments on SS samples and validated in SS and EID samples according to the GeNorm method (Booken et al., 2008; Vandesompele et al., 2002; Van Doorn R. et al., 2004). Assays were performed on custom-made PCR plates (Life Technologies) following SOP 005. Receiver operating characteristic curve analyses were used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. A one-tailed
Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. P-values below 0.05 were regarded as statistically significant.

**CONFLICT OF INTEREST**
MF received travel grants from TEVA and ICON.

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**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.01.038.

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