CD158k Is a Reliable Marker for Diagnosis of Sézary Syndrome and Reveals an Unprecedented Heterogeneity of Circulating Malignant Cells

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The diverse aspects of cutaneous T-cell lymphomas may impede the diagnosis of Sézary syndrome (SS) and mycosis fungoides (MF), in particular, at early stages of the disease. We defined the CD158k/KIR3DL2 molecule as a first positive cell surface marker for Sézary cells (SCs). Here, we designed an optimized flow cytometry gating strategy, allowing the definition of lymphocytes of different sizes and defects of cell surface markers. Quantification by cytomorphology, flow cytometry, or clonal evaluation, gave similar results at initial time points and during the evolution in a prospective study involving 64 consecutive cutaneous T-cell lymphoma or erythrodermic patients. We found that CD158k+ T cells and circulating CD4+ T cells from MF patients exhibited unexpected patterns of cell surface expression with a marked heterogeneity of circulating lymphocytes even at initial diagnosis. Taken together, our results show that a multistep gating of CD158k+ cells is reliable to assess tumor burden in case of SS and suggest that both circulating MF CD4+ T cells and CD158k+ T cells are not homogeneous distinct memory populations. Further phenotypic and functional characterizations of such subsets are needed to better understand the underlying molecular mechanisms leading to the development of these diseases.

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INTRODUCTION

Cutaneous T-cell lymphomas (CTCL) are a heterogeneous group of lymphomas primarily involving the skin (Willemze *et al.*, 2005). They encompass diverse presentations such as mycosis fungoides (MF), in which malignant cells reside primarily in infiltrated skin lesions. Sézary syndrome (SS) is a disease corresponding to an aggressive and leukemic form of CTCL characterized by erythroderma, lymphadenopathy, and high numbers of atypical lymphocytes with cerebriform-like nuclear contours (Sézary cells, SCs) in peripheral blood and skin. Erythrodermic MF (EMF) is distinguished from SS by absent or minimal blood involvement. In the clinical setting of erythoderma, the distinction between SS, MF, EMF, and benign erythrodermic inflammatory diseases is difficult to determine as clinical and histopathological aspects may not be specific, in particular at early stages of the disease.

The criteria recommended by the International Society for Cutaneous Lymphomas to distinguish SS from non-leukemic expressions of erythrodermic CTCL and erythrodermic inflammatory diseases consist of high blood burden with blood smears Sézary cell (bsSC) count $\geqslant$1,000 cells μl^{-1} and a positive T-cell clone. One of the following can be substituted for bsSCs: a CD4/CD8 ratio $\geqslant$10, CD4+CD7− cells $\geqslant$40%, or CD4+CD26− cells $\geqslant$30% (Olsen *et al.*, 2011b). Because the detection of SCs is not specific enough to allow an unequivocal detection of tumoral cells and given that the significance of clonal T-cell expansion in terms of malignancy is not clear (Ortonne *et al.*, 2006), the identification of a specific marker of malignant SCs has been a challenging issue for many years (Delfau-Larue *et al.*, 2000; Vonderheid, 2006). The use of flow cytometry is mainly based on the loss or the lack of T-cell markers such as CD2, CD3, CD5, CD7, and CD26, which is not constant and may be observed in some benign dermatoses (Harmon *et al.*, 1996; Edelman and Meyerson, 2000; Bernengo *et al.*, 2001; Bahler *et al.*, 2008).

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Abbreviations: KIR, killer cell Ig-like receptors; MF, mycosis fungoides; SC, Sézary cell; SS, Sézary syndrome

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Another approach to identify clonal neoplastic T cells in SS is the use of anti-TCR-Vβ chain antibodies (Schwab *et al.*, 2002; Morice *et al.*, 2006), which is able to detect only about 70% of the occurring TCR-Vβ chain antigens (Schwab *et al.*, 2002).

By using the AZ158 mAb and PCR analysis with the specific probes followed by subsequent sequencing, we identified CD158k/KIR3DL2, a member of the killer cell Ig-like receptor family, as a specific marker for the evaluation of circulating malignant T cells by flow cytometry (Bagot *et al.*, 2001; Poszepczynska-Guigne *et al.*, 2004; Bagot and Bensussan, 2006). In healthy donors (HD), CD158k expression is highly restricted and is detected only on a minor NK-cell subset and on rare CD3+CD8+ T cells (Moretta *et al.*, 1997). In SS patients, the percentage and absolute numbers of CD158k+ cells are strongly correlated to the percentage and absolute numbers of atypical circulating bsSCs (Poszepczynska-Guigne *et al.*, 2004; Klemke *et al.*, 2008; Bouaziz *et al.*, 2010) and CD4+CD158k+ lymphocytes correspond to the malignant clonal cell population (Poszepczynska-Guigne *et al.*, 2004). Ortonne *et al.* (2008) demonstrated that the overexpression of CD158k transcripts in lesional skin from erythrodermic patients may be used as a reliable early marker for SS . In addition, immunohistological staining with CD158k allows the distinction between biopsies from MF and SS (Wechsler *et al.*, 2003).

Several reports demonstrate that lymphocytes from MF and SS patients exhibit distinct molecular patterns of cell surface expression. On antigenic stimulation, naïve T cells enter a multistep process of expansion and differentiation into memory cells with distinct homing characteristics and effector functions (Sallusto *et al.*, 1999). Additional memory subset characterization using a combination of cell surface markers such as CD45RA, CCR7, and CD27, was reported with the definition of central memory (TCM), transitional memory (TTM), effector memory (TEM), and terminally effector memory (TEMRA) lymphocytes, leading to a more accurate definition of memory cells (Sallusto *et al.*, 2004; Fritsch *et al.*, 2005; Riou *et al.*, 2007).

It has been shown that malignant SCs usually have a central memory phenotype, whereas T cells from MF skin lesions are polarized TEM (Campbell *et al.*, 2010; Clark *et al.*, 2011). Skin-homing receptors such as cutaneous lymphocyte antigen (CLA) and CCR4 seem to be increased in SS patients, defining a general phenotype of skin-homing TCM lymphocytes (Ferenczi *et al.*, 2002; Sokolowska-Wojdylo *et al.*, 2005).

Our goal was to assess the reliability of CD158k to make the diagnosis of SS in a prospective study of 55 patients with CTCL and nine patients with skin inflammatory disease from other origin. CD158k expression and a combination of cell surface markers on peripheral blood mononuclear cells (PBMCs) were evaluated by flow cytometry. Our gating strategy allowed a precise characterization of circulating CD158k+ T cells at initial diagnosis, including large- and small-sized lymphocytes, with or without defects of CD3 and/or CD4 expression. Quantification by using cytomorphology, flow cytometry, or clonal evaluation by immunoscope, gave similar results at initial time points and during the evolution. Unexpectedly, we did not find a homogeneous distribution of TCM CD158k+ cells at initial diagnosis and during the course of the disease and circulating CD4+ T cells from MF patients were not uniformly of the TEM phenotype. Expression of cutaneous addressin molecules was also very heterogeneous within "naïve" and memory subsets of CD158k+ T cells and CD4+ T cells from MF patients.

RESULTS

Optimization of CD158k staining with AZ158 mAb to establish the diagnosis of SS

At initial diagnosis, SS patients displayed at least one T-cell clone expansion as assessed by either the size of the β-chain CDR3 or TCR gene analysis (Table 1). Detection of SCs above $1,000\,\mu l^{-1}$ on blood smears was found in 10 out of 12 SS patients. MF patients' BsSC counts (five available results at initial diagnosis) were all below $50\,\mu l^{-1}$. At the time of diagnosis, a CD4/CD8 ratio >10 was reported in 10 out of 12 SS, whereas none of the 13 remaining patients with MF reached this ratio (Table 1). The median percentage of CD4+ CD26− blood lymphocytes was higher in SS patients (67%) than in MF (19%), EMF (21%), or HD (17%) individuals (Table 1).

Given that SC may display different morphological features in terms of size and that phenotypic aberrancies of clonal cells are often described, flow cytometry evaluation of CD158k+ T cells required multistep analyses. Distinction between large lymphocytes and monocytes was done by the exclusion of CD14+ cells. As CD158k may be expressed on normal CD3+CD8+ T cells and NK cells, these subsets were eliminated from total CD4+/−CD158k+ cells (Figure 1a). Using this gating strategy, we show in Figure 1b that 92% of circulating lymphocytes express both TCRVβ17 and CD158k in patient LESC45. We then performed cell sorting on five SS patients with various tumor burden. TCRVβ repertoire from sorted CD158k+ T cells and their negative counterpart CD3+CD4+CD158k− cells was investigated. In all cases, CD158k+ T cells displayed an expansion of one major Vβ family (ranging from 77 to 96% of total Vβ families), whereas almost all Vβ families were present in CD3+CD4+ CD158k− cells (three representative patients are shown in Figure 1c). The CDR3 size was analyzed for each expanded Vβ family detected in the five patients. Data show a unique TCRVβ junctional region size in CD158k+ T cells, whereas TCRVβ was polyclonal in CD3+CD4+CD158k− cells. Figure 1d shows the representative results obtained with GOLM47, where only one TCRVβ6 clone with a unique CDR3 size is obtained within CD158k+ T cells. Multiple clones with distinct TCRVβ6 CDR3 sizes are found within the negative fraction including a slight expansion of the same TCRVβ6 clone detected in CD158k+ T cells, which accounts for less than 5% of total TCRVβ families. This may represent a very small fraction of SCs, which are not CD158k as previously described (Ortonne *et al.*, 2006) or reflects the limitation of the cell sorting procedure.

At initial diagnosis, absolute count of CD158k+ T cells was positively correlated to the absolute count of atypical circulating BsSC ($R^2 = 0.906$, $P < 0.0001$) in SS patients.

Table 1. Patients' biological characteristics and diagnosis at first sampling

Patient	CD4/CD8 ratio	bsSC, μl^{-1}	CD158k+ T cells, μl^{-1}	CD4+ CD26−, %	CD4+ CD7−, %	Clonality TCRG rearrangement		TCR-Vβ	% Vβ	Diagnostic	Time of sampling
						Blood	Skin				
AHHO39	4	0	22	19	8	ND	ND	ND	NA	Eczema	Initial Dg
FONS16	7	0	5	ND	ND	ND	ND	Polyclonal	NA	Eczema	Initial Dg
KORA67	1	0	19	ND	ND	ND	ND	Vβ14	60	Eczema	Initial Dg
MARC42	4	0	8	ND	ND	ND	ND	Polyclonal	NA	EID	Initial Dg
PORJ 28	4	0	10	ND	ND	ND	ND	Polyclonal	NA	EID	Initial Dg
ATIY85	1	0	39	ND	ND	ND	ND	Vβ6-Vβ13a	15–16	Psoriasis	Initial Dg
BELM43	6	0	21	30	28	ND	ND	ND	NA	Psoriasis	Initial Dg
BELG33	2	0	16	ND	ND	ND	ND	ND	ND	Psoriasis	Initial Dg
VIDG46	1	0	46	20	14	ND	ND	ND	NA	Psoriasis	Initial Dg
ARRL59	1	ND	15	13	ND	ND	ND	Polyclonal	NA	MF	Initial Dg
BESG36	2	0	41	ND	ND	Polyclonal	ND	Vβ6	20	MF	Initial Dg
BEYC32	2	0	8	19	ND	Oligoclonal	ND	Vβ2–Vβ6	39–14	MF	Initial Dg
DAGJ97	2	ND	26	15	ND	Polyclonal	ND	Polyclonal	NA	MF	Initial Dg
ELKS48	1	0	31	64	ND	Clonal	Clonal	Vβ3–Vβ4	16–16	MF	Initial Dg
FOUR48	1	0	2	26	ND	Polyclonal	ND	Polyclonal	NA	MF	Initial Dg
LEPL46	2	0	2	20	ND	Polyclonal	Clonal	Polyclonal	NA	MF	Initial Dg
BREE41	2	0	58	8	ND	ND	ND	Polyclonal	NA	MF	TT
DERF31	1	ND	9	ND	ND	Clonal	Clonal	Polyclonal	NA	MF	TT
LENM54	2	121	258	9	ND	ND	ND	Vβ4	41	MF	TT
DAUP57	2	0	14	12	ND	ND	ND	Polyclonal	NA	TMF	TT
POLG 47	2	38	8	20	ND	Polyclonal	ND	Vβ6	25	TMF	TT
CHAM28	1	0	0	ND	ND	ND	ND	Oligoclonal	NA	TMF + B-CLL	TT
ANNJ56	2	455	487	13	ND	Clonal	Clonal	Vβ8	48	EMF	Initial Dg
CHAG62	3	0	810	ND	ND	Polyclonal	ND	Polyclonal	NA	EMF	Initial Dg
GACS43	7	0	17	8	ND	Polyclonal	ND	Oligoclonal	NA	EMF	Initial Dg
GAIY67	8	320	621	77	33	Clonal	Clonal	Vβ5a	60	EMF	Initial Dg
LEVJ 38	1	0	6	25	ND	Polyclonal	ND	Polyclonal	NA	EMF	Initial Dg
STAA22	2	0	3	12	ND	ND	ND	Polyclonal	NA	EMF	Initial Dg
BRAM39	15	0	ND	24	ND	Polyclonal	ND	Polyclonal	NA	EMF	TT
FLED50	4	0	24	39	ND	ND	ND	Polyclonal	NA	EMF	TT
GAUC50	3	0	4	7	ND	Clonal	ND	ND	ND	EMF	TT
ILNV63	5	472	933	18	ND	ND	ND	Vβ2	62	EMF	TT
ARGP38	30	5,428	3,171	96	11	Clonal	ND	ND	ND	SS	Initial Dg
CHAS65	32	2,265	2,241	2	ND	Clonal	Clonal	ND	NA	SS	Initial Dg

Table 1. (Continued)

Patient	CD4/CD8 ratio	bsSC, μl^{-1}	CD158k+ T cells, μl^{-1}	CD4+ CD26−, %	CD4+ CD7−, %	Clonality TCRG rearrangement		TCR-Vβ	% Vβ	Diagnostic	Time of sampling
						Blood	Skin				
COIJ47	5	2,966	2,808	80	5	Clonal	Clonal	Vβ13a	92	SS	Initial Dg
DARM33	6	626	508	78	64	Clonal	ND	ND	ND	SS	Initial Dg
DORM62	289	6,256	5,211	43	50	ND	ND	Vβ2	85	SS	Initial Dg
ESTA33	16	106	74	54	ND	ND	ND	Vβ6	78	SS	Initial Dg
FIAJ21	12	3,475	1,628	60	ND	Clonal	Clonal	Vβ5a	77	SS	Initial Dg
KOUA40	17	8,313	4,406	97	82	Clonal	Clonal	Vβ8	95	SS	Initial Dg
RUBL38	10	1,490	1,743	73	92	Clonal	Clonal	Vβ6	99	SS	Initial Dg
SAMP30	15	1,678	1,598	82	68	ND	ND	Vβ14	57	SS	Initial Dg
SEMF36	57	19,062	28,520	35	0	Clonal	ND	ND	ND	SS	Initial Dg
STAR23	250	4,158	7,012	73	ND	ND	ND	Vß21	93	SS	Initial Dg
AYET29	23	939	835.5	50	ND	Clonal	Clonal	Vβ12–Vβ13	51–43	SS	TT
BERP36	65	3,074	2,112	100	100	Clonal	Clonal	Vβ3–Vβ13a	50–40	SS	TT
CARE49	2	676	477	82	2	Clonal	Clonal	Vβ6	94	SS	TT
CERA45	23	2,853.2	2,391	88	10	Clonal	Clonal	Vβ22	94	SS	TT
CHAP25	107	3,726	5,345	20	ND	Clonal	Clonal	Vβ13a	69	SS	TT
GASM45	21	0	0	18	2	ND	ND	ND	ND	SS	TT
GOLM47	12	385	1,656	82	6	Clonal	ND	Vβ6	96	SS	TT
GRIM40	4	424	2	21	ND	Clonal	Clonal	Vβ17	44	SS	TT
KLUF25	11	1,328	676	50	ND	Clonal	Clonal	Vβ9	87	SS	TT
LEGI64	96	1,273	1,200	ND		ND	Oligoclonal	Vβ22	95	SS	TT
LESC45	5	917	1,300	87	11	Clonal	ND	Vβ17	77	SS	TT
MANV26	19	0	526.0	ND	ND	Polyclonal	ND	Polyclonal	NA	SS	TT
MOTM35	1	4,496	6,316	91	97	Clonal	Clonal	Vβ22	81	SS	TT
OUAA37	4	2,244	3,049	95	68	Clonal	Clonal	Vβ4–Vβ5b	21–76	SS	TT
TETC49	33	780	924	91	88	ND	ND	Vβ13a	84	SS	TT
THOJ40	23	5,367	1,938	92	87	Clonal	Clonal	Vβ21	15–76	SS	TT
TIRD53	12	123	791	75	4	Clonal	Clonal	Vβ2	65	SS	TT
TROB52	57	8,965	5,253	27	ND	ND	ND	Vβ2	66	SS	TT
VACH58	89	2,052	5,897	7	4	ND	ND	Vβ6	78	SS	TT
WALP44	22	4,046	8,838	86	99	ND	ND	Vβ7	99	SS	TT

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; bsSC, Sézary cells enumerated on blood smears; EID, erythrodermic inflammatory disease, EMF, erythrodermic MF; MF, mycosis fungoides; NA, not applicable; ND, not done; SS, Sézary syndrome; TMF, transformed MF; TCRG, TCR gamma rearrangement; TT, under therapy.

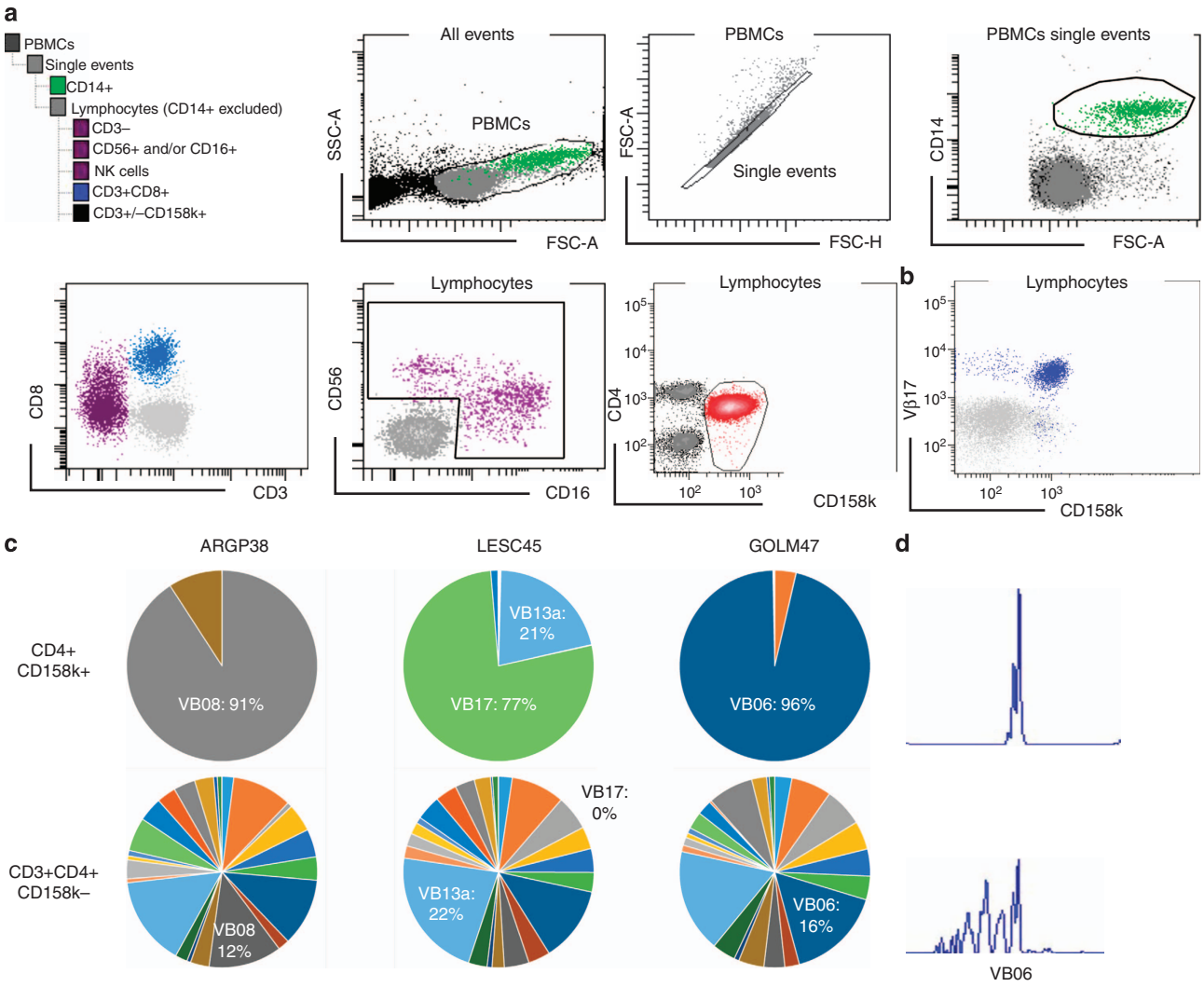

Figure 1. Identification of CD158k+ T cells. (**a**) Gating strategy: PBMCs are isolated according to their morphological features. FSC-A/FSC-H gating is used to consider only single events. Monocytes are characterized as CD14+ PBMCs (green) and then excluded to define lymphocytes as single CD14− PBMCs. NK cells are considered as CD3− lymphocytes expressing CD56 and/or CD16 molecules (purple). CD158k+ T cells are then defined within the lymphocyte subset containing CD158k+ cells expressing or not CD4 from which NK and CD3+CD8+ T cells are excluded. (**b**) Co-expression of TCR-Vβ17 and CD158k in a SS patient with a major clonal expansion. (**c**) TCRVβ family distribution within CD158k+ T cells (upper panel) and CD3+CD4+CD158k− sorted lymphocytes (lower panel): the relative percentage of the main families is indicated. (**d**) Sorted CD158k+ T cells from patient GOLM47 exhibit a unique TCRVβ6 junctional region size whereas ''normal'' CD3+CD4+CD158k− cells display a polyclonal profile. NK, natural killer; PBMC, peripheral blood mononuclear cell.

Immunoscope did not reveal any clonal Vβ expansion in Patient CHAS65 but a clonal rearrangement of the VβTCR locus was present in the blood and the skin. Three out of six EMF patients had relatively high CD158k+ T-cell counts (ANNJ56 = 487 μl^{-1}, CHAG62 = 810 μl^{-1}, and GAY67 = 621 μl^{-1}). None of the remaining patients (MF or skin inflammatory disease) had CD158k+CD4+ counts above 150 μl^{-1}.

Twenty SS patients were evaluated for SC counts after introduction of treatment. Among seven patients with both BsSC and CD158k+ T cells below 1,000 μl^{-1}, two were in complete remission (GASM45, MANV26), one in partial remission (TIRD53), one with a stable disease (TETC49), and two were investigated at the beginning of clinical relapse with less than 25% of skin invasion (CARE49, GRIM40).

CD158k+ T-cell count correlated with absolute T-cell clone count (Supplementary Figure S1a online), ($R^2 = 0.78$). The correlations between CD3+CD4+CD26− T-cell counts and bsSC, CD158k+ T cell or absolute T-cell clone counts were slightly weaker ($R^2 = 0.56$, $= 0.46$, and $= 0.66$, respectively).

Numerations of malignant cells using the three methods (blood smear, CD158k staining, and clonality) gave similar results during the course of the disease (Supplementary Figure S1b online). A receiver operating characteristic analysis was done to evaluate the respective performance of cytomorphology and flow cytometry methods for the diagnosis

of SS. According to the established diagnosis of SS, we determined true positive and false positive data from both methods at various threshold settings. These two methods gave similar results in terms of sensitivity and specificity (Supplementary Figure S2 online) with the definition of a threshold value of CD158k+ T cells = 1,200 μl^{-1} allowing a specificity of 100% and a sensitivity of 70% for the diagnosis of SS (Supplementary Table S1 online).

Phenotypic heterogeneity of Sézary cells

Naïve and memory T-cell subsets from CD158k+ T cells (SS patients), and from total CD4+T cells (MF patients and HD) were identified based on CD45RA and CCR7 expression. Naïve cells (TN) were characterized as CD45RA+CCR7+, TCM had the CD45RA-CCR7+ phenotype, TEM were defined by the lack of expression of these markers, and TEMRA exhibited CD45RA+CCR7− phenotype. The use of a subtractive gating strategy, excluding CD158k+ T cells from total CD3+CD4+ T cells in SS patients allowed the characterization of a ''normal'' TCD4+ subset (CD158k−TCD4+). Importantly, although we demonstrated that CD158k− CD3+CD4+ non-malignant lymphocytes exhibited a distinct TCR-Vβ repertoire as compared with the malignant clonal population (CD158k+ T cells) in the same SS patient, we found that the relative distribution of naïve and memory cells in both populations was very similar (Figure 2). Naïve T cells were significantly lower in MF and SS patients (Figure 2a) as compared with HD (median = 5.7, 20.3, and 42.2%, respectively). The majority of circulating MF CD4+ T cells was of the TEM phenotype (median = 60.9%), whereas the distribution of TEM in HD and SS was similar (median = 24.6 and 24.4%, respectively; Figure 2b). Percentages of central memory cells were higher in SS than in MF (median = 20.7 vs. 8.1%) but did not differ from HD (24.5%). However, the distribution of TCM in SS patients was extremely heterogeneous (Figure 2c). We found an important increase of the TEMRA subset in MF and SS patients (24 and 12%, respectively), whereas it represented a very small fraction of total CD4+ T cells in HD (median = 1.3%; Figure 2d).

We then studied the pattern expression of the skin-homing addressins CCR4 and CLA. The median percentages of CCR4 were similar in HD TCD4+ (45%), MF TCD4+ (43%), CD158k-TCD4+ (52%), and CD158k+ T cells (44%; Figure 2e). We observed the same findings for CLA: HD TCD4+ (15%), MF TCD4+ (23%), CD158k-TCD4+ (16%), and CD158k+ T cells (22%) (Figure 2f). However, an extreme heterogeneity of CCR4 and CLA expression was found in SS patients, i.e., percentage ranges of CCR4+CD158k+ T cells were 0.5–90% (HD CCR4+CD4+ T cells = 35–52%) and % ranges of CLA+CD158k+ T cells were 0.3–73% (HD CLA+ CD4+ T cells = 9–31%). We analyzed CCR4 expression on naïve (or ''naïve-like'') and memory subsets from HD

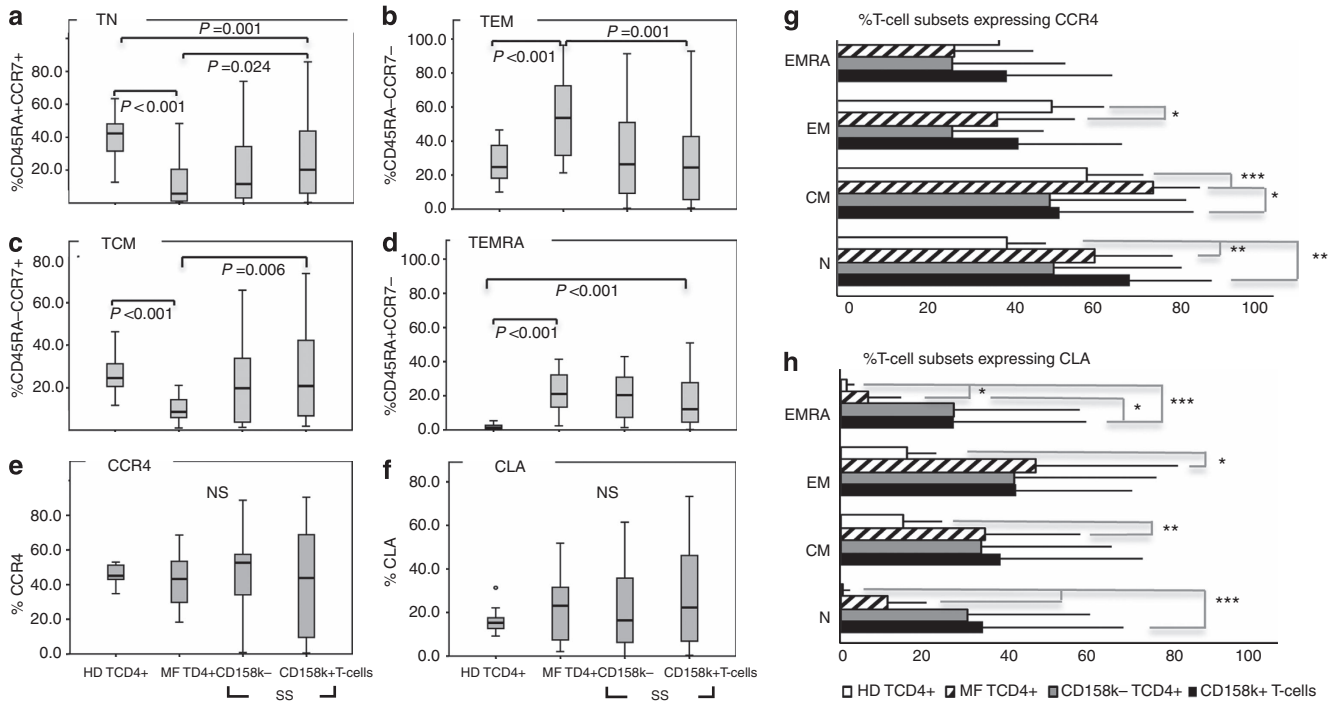

Figure 2. Heterogeneity of blood-derived CD158k+ T cells and CD4+ T cells from HD, MF, and SS patients. Subsets of CD4+ T cells from HD, MF patients, as well as CD158k+ T cells and their ''normal'' CD4+ T-cell counterpart from SS patients were analyzed for their phenotype. (**a**) Naïve cells or ''naïve-like'' cells are decreased in MF and SS patients; (**b**) MF CD4+ T cells are mainly effector memory cells. (**c**) Percentages of central memory cells are similar between SS and HD; (**d**) MF CD4+ T cells and CD158k+ T-cell TEMRA subsets are significantly increased compared with HD. (**e**) CLA and (**f**) CCR4 expression is markedly heterogeneous on MF CD4+ T cells and CD158k+ T cells with significant differences in the distribution of T-cell subsets expressing (**g**) CCR4 and (**h**) CLA. Results are expressed as mean ± SD. *P<0. 05, **P<0.01, ***P<0.001. CLA, cutaneous lymphocyte antigen; HD, healthy donors; MF, mycosis fungoides; SS, Sézary syndrome; TEM, effector memory cells; TEMRA, terminally effector memory cells.

TCD4+, MF TCD4+, CD158k−TCD4+, and CD158k+ T cells (Figure 2g). The majority of MF CD4+ T cells expressing CCR4 were TCM (HD vs. MF, $P<0.001$, SS vs. MF, $P=0.04$). Percentages of ''naïve-like'' T cells expressing CCR4+ were higher in MF and SS patients when compared with naïve HD CD4+CCR4+ T cells ($P=0.001$ and $P<0.001$, respectively). CLA expression was significantly higher in overall lymphocyte subsets from MF and SS patients than in HD (Figure 2h). CLA expression within the TEMRA and the ''naïve-like'' subsets was increased in MF CD4+ T cells and SCs than in HD ($P=0.003$ and $P<0.001$, respectively for TEMRA and $P<0.001$ and $P<0.001$, respectively for naive). Interestingly, the pattern of CCR4 and CLA expression was similar between CD158k−CD4+ T cells and CD158k+ T cells from SS patients (Figures 2g and h).

Sézary cells and circulating MF TCD4+ display distinct combinations of CD45RA, CCR7, and CD27 cell surface expression.

To further characterize memory CD158k+ T cells, we defined additional subsets, comprising of TTM (Riou *et al.*, 2007). Commercially, anti-TCR-Vβ mAbs were available for 18 patients. Clonal Vβ+ malignant cells were tested in parallel (Figure 3 and Supplementary Figure S3 online). CD45RA− and CD45RA+ subsets were analyzed according to the respective expression of CCR7 and CD27. Within the CD45RA− subset, TCM were defined as CD27+CCR7+, TTM as CD27+CCR7−, TEM as CD27−CCR7−, and a minor subset was CD27−CCR7+ (CD27− TCM). Within the CD45RA+ subset, the majority of TN was CD27+ CCR7+, with a small subset of CD27− TN and TEMRA were subdivided into CD27+ and CD27− TEMRA (Figure 3). Subset analyses using either CD158k or TCR-Vβ mAbs gave similar results (Figures 3a and b). Figures 3c–e show representative data illustrating the heterogeneity of CD158k+ T cells. Therefore, the combined staining with anti-CD45RA, -CD27, and -CCR7 mAbs discriminates 1/naive and TCM within CD27+CCR7+ cells, 2/TEM and TTM within CD45RA-CCR7-cells, and 3/TTM and CD27+TEMRA within CD27+CCR7− cells. We performed the same analysis on CD4+ T cells from MF patients and HD (Figure 3f). Unexpectedly, an important part of MF CD4+ T cells and CD158k+ T cells displayed a CD27+ TEMRA phenotype (range in MF = 4–63%, SS = 3–86%) as compared with HD (range = 0–15%). MF CD4+ T cells were uniformly distributed within CD27+ TEMRA, TTM, and TEM subsets, whereas CD158k+ T cells were heterogeneous with a predominance of CD27+ TEMRA, CD27+ TN, TTM, and CD27+ TCM subsets.

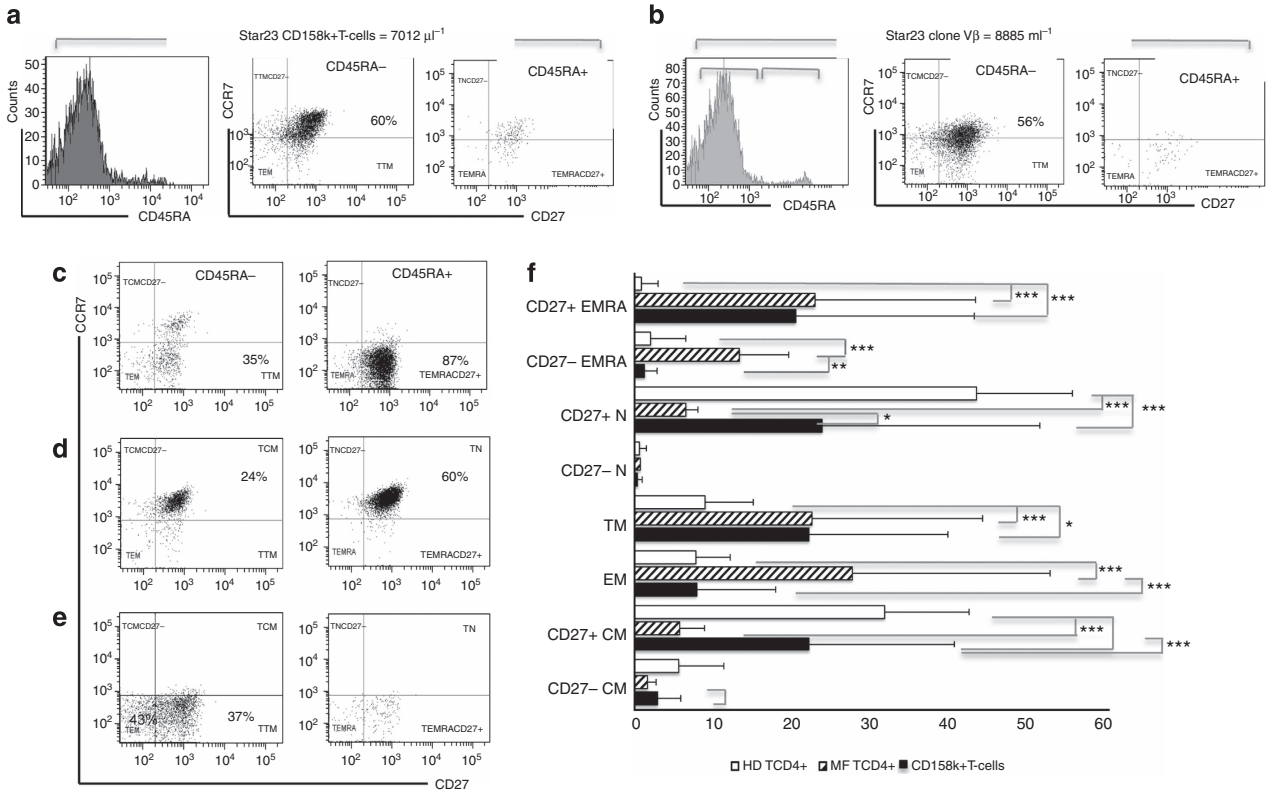

Figure 3. The combinatory expression of CD45RA, CCR7, and CD27 allows a precise characterization of circulating cells in SS and MF patients. (**a**) Gating strategy to characterize naïve and memory subsets in CD158k+ T cells: CD45RA− and CD45RA+ were first isolated within CD158k+ T cells to define subsets of naïve and memory cells according to the respective expression of CCR7 and CD27 molecules on CD45RA− and CD45RA+ cells. Percentages of dominant subsets within CD158k+ T cells are indicated in each plot. (**b**) The same gating strategy gives similar results using anti-TCR-Vβ mAbs. (**c**) Patient with a very unusual population of CD27+ TEMRA. (**d**) Patient with a prominent subset of naïve CD158k+ T cells and a minor population of TCM. (**e**) Patient with CD158k+ T cells of the TTM phenotype. (**f**) Distribution of naïve, transitional, and memory subsets of CD4+ T cells (HD and MF) and CD158k+ T cells. HD, healthy donors; MF, mycosis fungoides; SS, Sézary syndrome; TCM, central memory cells.

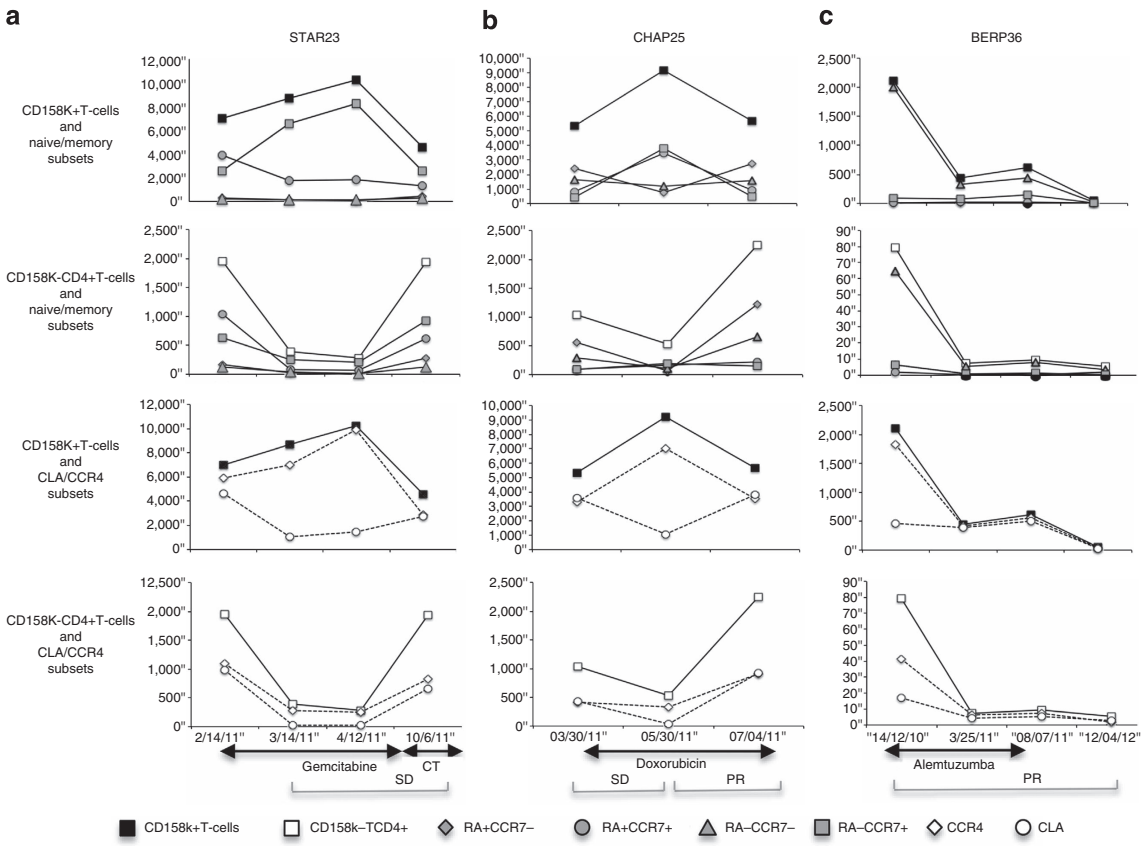

Figure 4. Longitudinal evolution of CD158k+ T-cell subsets in SS patients under therapy. (**a**, **b**) Patients under chemotherapy; (**c**) patient treated with immune modulatory therapies. PR, partial remission; SD, stable disease; SS, Sézary syndrome.

Longitudinal evolution of CD158k+ T cells and CD158k−CD4+ subsets

Absolute counts of circulating CD158k+ T cells, CD158k−CD4+ T cells, and naïve/memory subsets were evaluated during the course of the disease in 12 SS patients. Figure 4 shows three representative data from patients treated with chemotherapy, corticoids, and/or alemtuzumab. The decrease of CD158k+ T cells was associated with a response to chemotherapy and/or corticotherapy with stable disease and partial response in Patients STAR23 and CHAP25, respectively, whereas CD158k−CD4+ T cells had an inverse profile (Figures 4a and b, four upper panels). The distribution of naïve-like and memory subset was extremely heterogeneous between patients and during the follow-up. BERP36, treated with alentuzumab, experienced a rapid deletion of both CD158k+ T cells and CD158k−CD4+ T-cell subsets associated with a 6-month partial remission (Figure 4c).

Of note, Figure 4 shows two examples of circulating CD158k+ T cells predominantly expressing CCR4 during the time course of the disease, with low levels of circulating CD158k+CLA+ T cells, whereas absolute count of CD158k−CLA+CD4+ T cells paralleled those of CD158k−CCR4+CD4+ T cells (Figure 4, the six lower panels).

DISCUSSION

The first aim of this study was to determine a flow cytometric gating strategy to optimize the identification of CD158k+ T cells from total PBMCs, including lymphocyte subsets of different sizes with or without defects of either CD3 or CD4 expression. We applied this strategy to evaluate the ability of CD158k mAbs to make the diagnosis of SS. Our data showed that the respective performance of cytomorphology and flow cytometry methods was similar at initial diagnosis, with a threshold value of CD158k+ T-cell count = 1,200 μl^{-1} allowing a specificity of 100%. These data confirm and extend previous reports from our group and others (Poszepczynska-Guigne *et al.*, 2004; Bahler *et al.*, 2008; Klemke *et al.*, 2008; Bouaziz *et al.*, 2010). When considering the total group of 55 CTCL patients, we found a strong correlation between absolute T-cell clone, bsSC, and CD158k+ T-cell counts. The usefulness of flow cytometry in tracking tumor burden and response to therapy relies on the assumption that at least one marker remains relatively constant over time. As SCs often have an aberrant immunophenotype, such as a loss of T-cell antigens, the CD158k molecule constitutes a positive marker for disease monitoring, as evidenced by the repertoire analysis of CD158k+ T cells as compared with CD158k−CD3+CD4+ T cells (Figures 1c and d). We confirm the extreme heterogeneity of circulating CD158k+ T cells

between patients, in terms of morphology and phenotypic aberrancies (Bouaziz *et al.*, 2010; Clark *et al.*, 2011; Vaughan *et al.*, 2012). Serial flow cytometric analyses performed in 12 patients revealed changes involving CD3, CD4, and CLA expression on CD158k+ T cells, particularly in individuals under chemotherapy.

We found an unexpected heterogeneity of CD45RA and CCR7 expression on CD158k+ T cells, with elevated numbers of ''naïve-like'' or TEMRA CD158k+ T cells in some patients, even at initial diagnosis. Central memory CD45RA− CCR7+ cells were well represented within CD158k+ T cells, but at the same level as for CD4+ T cells from HD. The majority of blood-derived CD4+ T cells from MF patients was CD45RA−CCR7−, a phenotype consistent with TEM, reported to be predominant in blood and within skin lesions (Campbell *et al.*, 2010; Clark *et al.*, 2012). However, significant high percentages of TEMRA CD4+ T cells were also found in MF patients. The apparent discrepancies between our results and results from prior studies may reside on the cell surface molecules used to define naïve and memory T-cell subsets. Some reports are based on the respective expression of CD45RA and CD27, others on CCR7/CD62L and CD27 (Fierro *et al.*, 2008; Campbell *et al.*, 2010). In our hands, CD45RA was not an informative marker by itself to distinguish HD, MF CD4+ T cells, and CD158k+ T cells. CD27 expression was significantly lower on CD4+ T cells from MF patients ($P<0.001$ when compared with either HD or SS patients) and CCR7 alone was the most discriminative marker. Although CCR7 expression overlaps that of CD27 in HD, this was not the case in MF and SS patients. This confirms previous data demonstrating that the loss of CD27 expression does not necessarily follow the expression of CCR7 during the stepwise differentiation of memory CD4+ T cells (Fritsch *et al.*, 2005). A singular subset of CD27+ TEMRA lymphocytes was present in more than 50% of MF and SS patients, whereas this subset barely exists in HD. CD27 is a member of the TNF-R family, which is transiently upregulated on normal T cells on TCR engagement and irreversibly lost after repeated antigenic stimulation (Sallusto *et al.*, 2004; Fritsch *et al.*, 2005; Riou *et al.*, 2007). The sequential loss of CCR7 and CD27 is associated with a differential functional capacity of these subsets: high production of IL-10 in TCM (CD45RA-CCR7+CD27+), IFN-γ in TTM, and IL-4 production correlating with CD27 loss (Fritsch *et al.*, 2005). As SS is associated with significant immune abnormalities characterized by dysregulation of both innate and adaptive immunity and a Th2 profile (Olsen *et al.*, 2011a), and giving that many therapies may interfere with the host immune response, the biological significance of atypical expansions of lymphocyte subsets such as CD27+ TEMRA needs to be explored at the functional level. Interestingly, the naïve/memory profiles of CD158k−CD3+CD4+ T cells paralleled those of CD158k+ T cells. Thus, the ''normal'' compartment of CD4+ T cells in SS patients may be altered by the general immune disorder, which is correlated with tumor burden (Wysocka *et al.*, 2002), leading to the development of opportunistic infections.

The first step in the process of T-cell skin homing is mediated through the attraction of CCR4+ lymphocytes by a chemokine gradient, followed by the extravasation to the skin involving CLA interaction with its ligand, E-selectin (Picker *et al.*, 1990). As previously reported, CLA was not universally expressed on MF CD4+ T cells and CD158k+ T cells, but, surprisingly, we found an extreme heterogeneity of CCR4 expression on CD158k+ T cells (Ferenczi *et al.*, 2002; Fierro *et al.*, 2006; Clark *et al.*, 2012). The increase of tumor burden during SS evolution was found associated with less circulating CLA+CCR4+ and more CLA−CCR4+, specifically on the CD158k+ T-cell subset, suggesting an enhanced CD158k+CLA+ T-cell migration toward skin.

The physiopathogenesis of SS still remains unclear, several studies have pointed out the possibility of a chronic superantigen stimulation leading to the expansion of both malignant and non-malignant cells. Still, the absence of a homogeneous expansion of memory CD158k+ T cells at initial diagnosis is challenging. It may reflect either intrinsic phenotypic aberrancies of malignant cells, a transitional state of differentiation during disease progression, or the impact of the cytokinic environment. Of note, it was reported that most, if not all, of the described immune abnormalities tend to normalize on clearance of SCs and clinical remission (Yoo *et al.*, 2001).

In conclusion, our gating strategy, using multiple color flow cytometry enables the optimization of CD158k as a positive marker of SCs, for the diagnosis and the follow-up of SS in routine practice. We found that CD158k+ T cells and circulating CD4+ T cells from SS and MF patients exhibit distinct patterns of cell surface expression. We are currently monitoring CD158k+ T-cell subsets expressing naïve and/or memory phenotypes together with skin-homing addressins to evaluate phenotypic and functional changes under chemotherapeutic regimens and/or immune modulatory therapies. Further, the specificity of this marker prompted us to develop chimeric CD158k mAbs for a targeted immunotherapy, as we previously demonstrated that malignant Sézary cells are sensitive to antibody-dependent cellular cytotoxicity mediated by anti-KIR3DL2 mAbs (Bouaziz *et al.*, 2005).

MATERIALS AND METHODS

Patients and HDs

Fifty-five CTCL patients were consecutively included in this prospective study at the Hôpital Saint-Louis (Paris, France) between November 2010 and June 2012. The age ranged from 48 to 90 years (median 68 years). In 32 patients, a diagnosis of SS was established, according to the WHO-EORTC classification of CTCL (Willemze *et al.*, 2005) and the criteria of the International Society for Cutaneous Lymphomas (Olsen *et al.*, 2011b), based on clinical features, the presence of an identical T-cell clone evidenced in blood and/or skin, bsSC cell count detected by cytomorphology, CD4/CD8 ratio, CD26 and/or CD7 loss, and cutaneous histology. In addition, nine patients with inflammatory diseases from other origins were investigated. Twenty-five patients were investigated before diagnosis (12 SS and 13 MF or EMF), and a longitudinal follow-up was performed for 12 patients. All patients

were included after the informed and written consents were obtained (CPP, Hôpital Saint-Louis, Paris, France). Forty-nine samples from HD were collected from the blood donor center (Etablissement Français du Sang, Hôpital Saint-Louis). The type of CTCL was disclosed to the experimenter only after completion of analyses.

Blood samples

Samplings were performed before diagnosis and/or at sequential time points during the follow-up. bsSC absolute cell counts (μl^{-1}) were calculated as previously described (Bouaziz *et al.*, 2010). PBMCs were isolated by density-gradient centrifugation using lymphocyte separation medium (Eurobio, Les Ulis, France) and stored in liquid nitrogen. Genomic DNA and RNA were purified using TRI Reagent (Molecular Research Center, Cincinnati, OH).

Flow cytometry

Absolute lymphocyte count was calculated from freshly collected blood using the TruCount system (Becton Dickinson, le Pont de la Claix, France) with CD3-APC, CD45-PerCP-Cy5.5, CD8FITC, and CD4PE mAbs.

Eight-color labeling was performed on washed PBMCs with the following mAbs: anti-CD3-V450, -CD4-V500, -CD8-PerCP, -CD16-FITC, -CD26-FITC, -CD7-APC, -CD56P-ECy7, -CD14-APC-H7, -CLA-FITC, -CCR4-PerCPCy5.5, -CCR7-PECy7, CD45RA-APC, -CD158k-PE (BD Bioscience, Le Pont-de-Claix, France). The anti-CD158k (IgG2a) termed AZ158 was kindly provided by Innate Pharma (Marseille, France). Anti-TCR-Vβ mAbs were used for subsequent analyses (Beckman Coulter, Villepinte, France). Acquisition was performed using a FACSCanto II flow cytometer. Results were expressed according to the « fluorescence minus one » control, allowing the definition of the background signal (Perfetto *et al.*, 2004) and data were analyzed using FACS Diva (BD Biosciences)

T-cell repertoire and clonality

Determination of Vβ usage was made from total RNA using quantitative ''Immunoscope'' as previously described (Clave *et al.*, 2009). Absolute count of T-cell clones was calculated according to the numbers of CD3+ T cells. Cell sorting was performed for five representative patients (FACS ARIA III, Becton Dickinson). CD158k+ percentages were above 98% within the positive fraction and below 4% within the negative fraction. Repertoire analysis was done on purified CD158K+ T cells and CD158k−CD4+ T cells. TCRVβ usage and size polymorphism were analyzed.

TCR gamma (TCRG) rearrangement was analyzed by PCR as previously described (Senechal *et al.*, 2007).

Statistical analysis

Non-parametric Mann–Whitney test was used to compare T-cell populations between Sézary and MF patients or HDs. All statistical analyses were performed with SPSS20 software (Barcelona, Spain).

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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