The role of 9-O-acetylated ganglioside D3 (CD60) and α4β1 (CD49d) expression in predicting the survival of patients with Sézary syndrome

Enrico Scala,[1] Damiano Abeni,[2] Debora Pomponi,[1] Maria Grazia Narducci,[3] Giuseppe Alfonso Lombardo,[4] Adriano Mari,[1] Marina Frontani,[4] Maria Cristina Picchio,[3] Maria Antonietta Pilla,[4] Elisabetta Caprini[3] and Giandomenico Russo[3]

[1]Center for Molecular Allergology, Istituto Dermopatico dell'Immacolata (IDI-IRCCS), Rome, Italy; [2]Health Services Research Unit, Istituto Dermopatico dell'Immacolata (IDI-IRCCS), Rome, Italy; [3]Laboratory of Molecular Oncology, Istituto Dermopatico dell'Immacolata (IDI-IRCCS), Rome, Italy and [4]III Division of Dermatology, Istituto Dermopatico dell'Immacolata (IDI-IRCCS), Rome, Italy

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Correspondence: Enrico Scala, Center for Molecular Allergology [IDI-IRCCS] Via dei Monti di Creta 104, I-00167, Rome, Italy. E-mail: e.scala@idi.it

ABSTRACT

Background

Sézary syndrome is a rare and very aggressive leukemic variant of cutaneous T-cell lymphoma characterized by extensive skin involvement and a malignant circulating $CD4^+$ T-cell clone which homes to the skin, over-expresses CD60, and lacks CD7, CD26 and CD49d. So far prognostic markers in this disease are limited to treatment with systemic steroids, age, serum lactate dehydrogenase, and a white blood cell count of 20×10^9/L or higher: no other biological marker with prognostic value, especially related to malignant cells, has been described.

Design and Methods

We used flow activated cell sorting analysis to compare the distribution of the T-cell receptor-Vβ repertoire and several surface molecules (CD7, CD26, CD49d and CD60) within the circulating $CD4^+$ T-cell population in 62 patients with Sézary syndrome, 180 with mycosis fungoides, 6 with B-cell lymphomas, and 19 with chronic eczema. We calculated the 5-year overall survival of patients with Sézary syndrome after first hospital admission using Kaplan–Meier product–limit estimates and hazard ratios from the Cox proportional hazards model.

Results

We found that both higher number of $CD60^+$ and lower number of $CD49d^+$ cells within circulating $CD4^+$ T cells at disease presentation were significantly associated with a lower probability of survival. An exceedingly high risk of death was observed for patients with a combination of a high proportion of $CD4^+CD60^+$ cells ($\geq 0.5\times10^9$/L) and low proportion of $CD4^+CD49d^+$ cells ($<0.5\times10^9$/L) (hazard ratio = 12.303, 95% confidence interval 1.5–95.9; $P<0.02$). In addition, a skewed usage of T-cell receptor-Vβ subfamilies was observed in the circulating T-cell clone for 61.9% of all patients with Sézary syndrome, T-cell receptor-Vβ 2 and 5.1 subfamilies being the most frequently represented (42.8%), followed by T-cell receptor-Vβ 12 and 13.1.

Conclusions

In this study we showed that up-regulation of CD60 and down-regulation of CD49d on circulating $CD4^+$ T cells are two useful markers for predicting a very poor outcome in patients with Sézary syndrome.

Key words: TCR-Vβ repertoire, CD60, CD49d, survival rate, Sézary syndrome.

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Introduction

The presence of a measurable circulating clonal T-cell population is one of the hallmarks of Sézary syndrome (SS), an extra-nodal variant of non-Hodgkin's lymphoma of T-cell origin with primary cutaneous involvement.[1,2] The morphological and immunological phenotype of the SS circulating T-cell clone has been described by several authors.[3-6] Down-regulation of CD7 expression was originally suggested as a typical attribute of SS circulating $CD4^+$ T cells,[7] but the observation that $CD7^-$ T cells constitute a subset of normal human blood lymphocytes progressively increasing with age[8] prompted the search for more reliable and unequivocal markers. Over-expression of CD60 and loss of CD26 and CD49d appeared to be reliable features of SS circulating T cells.[4-6,9] We also noted that less than 5% of cells from normal individuals showed a similar phenotype ($CD7^-CD26^-CD49d^-CD60^+$).[9] Evaluation of the T-cell receptor (TCR)-Vβ repertoire in the peripheral blood of patients with SS might provide additional information for the assessment of the circulating T-cell clone and quantification of tumor burden.[9,10] Combined assessment of these markers is considered a reliable approach for the evaluation of circulating clonal T cells in SS.[4,5,11,12]

Previous studies of prognostic indicators in SS showed that circulating Sézary cell count, high CD4/CD8 ratio, advanced age, high lactate dehydrogenase serum level and a high white blood cell count were associated with an unfavorable outcome.[13-20] Up to now, scanty data have been provided concerning possible associations between the immunophenotype of circulating $CD4^+$ T cells and survival in SS. As for other hematologic malignancies,[21,22] it is important to clarify the prognostic relevance of activation of homing receptors expressed by circulating $CD4^+$ T cells in SS, since these markers might be directly linked to the pathogenesis and outcome of the disorder.

Consequently, taking advantage of having followed a large group of patients with SS for 11 years, we investigated whether the TCR-Vβ repertoire distribution and the expression of CD26, CD49d and CD60 on $CD4^+$ T cells, measured at the time of first clinical observation, were associated with a higher risk of mortality in an Italian cohort of 62 patients with SS.

Design and Methods

Patients

We enrolled 242 consecutive patients with cutaneous T-cell lymphomas (62 with SS, 180 with mycosis fungoides; mean age, 66±12 and 60±17 years, respectively), followed in the *Istituto Dermopatico dell'Immacolata* (IDI-IRCCS, Rome, Italy) from 1998 to 2009. Six cases of B-cell lymphoma (mean age, 60±11 years), 19 patients with chronic eczema (mean age, 62±15 years) and 20 normal individuals (mean age, 58±17 years) were chosen as diseased and healthy control groups. Demographic (age and gender) and clinical data were recorded for all patients in an informatics database. This database was created to collect, in a standardized fashion, all information available on patients with cutaneous lymphoma hospitalized at our institute. Informed consent for blood sampling was obtained from patients or caregivers during clinical examinations as a normal routine procedure during the dermatological diagnostic process. The database contains patients' demographic variables and clinical history, as well as their clinical, histological, hematologic and immunological information since 1998. For some cases clinical information was also available since 1983. The database is regularly updated twice a year for each patient, or on the occasion of a major event. Disease status was assessed at first admission to hospital, when all personal and clinical information was collected. Although patients were seen regularly at our institute for therapy and check-up visits, we performed an active follow-up around mid 2009 to ensure a standardized ascertainment of survival time. For patients who had died, the date of death was recorded, while surviving patients were censored at the date of the last contact. All-cause mortality was considered the outcome of interest. As of December 31th, 2009, we had information on 62 SS patients (42 males and 20 females) with a mean age of 61.4±13.5 years. The diagnosis of SS was established according to the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues[1] on the basis of characteristic erythroderma, diagnostic skin biopsy specimens and blood Sézary cell count. Twenty-nine patients died (20 males and 9 females) during the study period.

All subjects were enrolled in clinical protocols approved by the Ethical Committee of the IDI-IRCCS and informed consent was obtained in accordance with the Declaration of Helsinki.

Immunophenotyping and analysis of Vβ domains of expressed T-cell receptor β chains

The following monoclonal antibodies were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-CD7, CD45R0 and CD60; R-phycoerythrin (PE) conjugated anti-CD26, CD45RA, and CD49d (VLA4), FITC-, PE-, peridin chlorophyll protein (PCP)-, allophycocyanin (APC)- or Pacific blue (PB)-conjugated anti-CD3, CD4, or CD8. All antibodies were acquired from BD Biosciences (Becton Dickinson Immunocytometry System, Mountain View, CA, USA) except IgM anti-CD60, which was obtained from Ancell Corporation (Bayport, MN, USA). Multiple-color flow cytometry analysis was performed as previously described.[9] Fresh peripheral venous blood samples (all specimens studied by TCR-Vβ flow cytometric analysis were less than 4 h old) from patients with cutaneous T-cell lymphomas and controls were collected in tubes containing 1.5 mg of ethylenediamine tetraacetic acid-K2 per mL of blood. One hundred microliters of well-mixed whole blood was delivered into the test-tube and placed in an ice-water bath. Erythrocytes were lysed using ammonium chloride lysing solution (Ortho-mune lysing solution; Ortho Diagnostic System, Raritan, NJ, USA) for 10 min at room temperature according to the manufacturer's instructions. Peripheral blood lymphocytes were washed and resuspended in cold phosphate-buffered saline containing 2% fetal bovine serum (GIBCO, Paisley, Scotland, UK) and 0.02% natrium azide (NaN_3) at 2×10^6/mL. All the subsequent steps were completed while keeping the cells in an ice bath. A total of 100 μL of cell suspension was mixed with 10 μL of each monoclonal antibody of interest or isotype-matched control mouse immunoglobulin that had been conjugated directly with FITC, PE, APC, PCP or PB (all from Becton Dickinson) and incubated in the dark for 30 min at 4°C. After 30 min, cells were washed twice in cold phosphate-buffered saline and then resuspended in 400 μL of phosphate-buffered saline containing fetal bovine serum and NaN_3 for immediate flow cytometric analysis. For each patient, five different multiparametric stains were performed, constantly including CD3, CD4 or CD8 combined with two other markers. These doublets of markers were CD60 with CD49d, CD7 with CD26, CD45RA with CD45R0, CD7 with CD49d and CD60 with CD26. Isotype control staining was matched to these combinations. Further background gating controls were added, leaving out of the staining cocktail one fluorochrome at a time (fluorescence-minus-one analysis) in order to define background fluorescence for each sample, ruling out the possibility of reciprocal interference. Unstained

controls confirmed that cell autofluorescence fell far below the thresholds defined by the fluorescence-minus-one analysis. Experiments using cell lines negative for CD7, CD26, CD49d and CD60 demonstrated minimal non-specific labeling with the antibodies used.

The samples were studied in more detail for TCR-Vβ domain expression using a comprehensive panel of 25 monoclonal antibodies, 24 of which (IO Test Beta Mark TCR Vβ Repertoire Kit) were obtained from Beckman Coulter (Fullerton, CA, USA): Vβ1 (BL37.2), Vβ2 (MPB2D5), Vβ3 (CH92), Vβ4 (WJF24), Vβ5.1 (IMMU157), Vβ5.2 (36213), Vβ5.3 (3D11), Vβ7.1 (ZOE), Vβ7.2 (ZIZOU4) Vβ8 (56C5.2), Vβ9 (FIN9), Vβ11 (C21), Vβ12 (VER2.32), Vβ13.1 (IMMU222), Vβ13.2 (H132), Vβ13.6 (JU-74.3), Vβ14 (CAS1.1.3), Vβ16 (TAMAYA 1.2), Vβ17 (E17.5F3), Vβ18 (BA62.6), Vβ20 (ELL 1.4), Vβ21.3 (IG125), Vβ22 (IMMU 546) and Vβ23 (AF 23). The anti-TCR-Vβ6.7 (OT145) antibody was obtained from T Cell Diagnostics (Woburn, MA, USA). A consecutive gating strategy was applied in order to examine the Vβ repertoire within the total $CD3^+CD4^+$ population. Briefly, the Vβ repertoire of total $CD4^+$ T cells was investigated by initially gating live lymphocytes in a forward *versus* side scatter histogram and then passing these gated events into a side scatter *versus* CD3 histogram. $CD4^+$ positive events, within the total $CD3^+$ cells, were then further gated to display the expression of the three Vβ specificities that are provided by each Beta Mark reagent. To define single Vβ expression, the mean normal Vβ values was used. Clonality was defined as the expression of a single TCR-Vβ either at a level 10-fold above its normal range or by greater than 50% of the $CD4^+$ T cells.[23] TCR-Vβ monoclonal antibodies were then used in combination with CD7, CD26, CD49d and CD60 to further define the immunophenotype of the expanded T-cell population. Quantitative analysis for five-color flow cytometry was carried out using a FACS-Aria instrument (Becton Dickinson). At least 10,000 events were acquired in list mode and all the data were analyzed by Diva software (Becton Dickinson). Monoclonal antibodies currently available for the analysis of the TCR-Vβ repertoire allow the evaluation of 70-80% of the entire repertoire.[24] As a consequence, in several cases, the circulating T-cell clone could not be properly identified by means of flow cytometry. In those cases all the monoclonal antibodies against TCR-Vβ currently available, once mixed together, can be probed in a single reaction: the presence of more than 70% of TCR-Vβ unreactive T $CD4^+$ cells in the peripheral blood may be considered indirect proof of clonality.[23] Furthermore, we demonstrated that also the TCR-Vβ unreactive T $CD4^+$ cells showed the same features of expanded TCR-Vβ-positive T cells, such as down-regulation of CD7, CD26 and CD49d and over-expression of CD60.[9]

Statistical analyses

A time-to event analysis was performed using non-parametric Kaplan-Meier product-limit survival estimates. Differences between Kaplan-Meier survival curves were analyzed using the Mantel-Haenszel log-rank test. Continuous variables (white blood cell count, lactate dehydrogenase concentration, etc.) were categorized on the basis of receiver operating characteristic (ROC) curves, produced using vital status as the outcome measure. Using the ROC curve, we plotted sensitivity *versus* 1-specificity for all the values of the surface molecule expression, as well as for all the other continuous variables measured. The ideal cut-off was indicated by the point of the curve closest to the upper left corner of the plot.[25] Survival functions and probabilities of surviving at 5 years after first observation were computed for all levels of all variables.[26] Statistical analyses were performed using Statistical Package SPSS v. 13.0.

Table 1. Expression of T-cell markers in patients with cutaneous T-cell lymphoma and in normal controls. The results are based on two-sided tests assuming equal variances with a significance level 0.05. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

	Healthy donors (n=20)	Sézary syndrome (n=62)	Mycosis fungoides (n=180)	Chronic eczema (n=19)	B-cell lymphoma (n=6)
$CD3^+CD4^+$ (cells/μL)	47.2±9% (1004±241)	76.1±18%* (4402±4758)*	45.6±15.7% (844±671)	47.25±16.4% (865±430)	33.8±21.6% (727±557)
$CD3^+CD8^+$ (cells/μL)	24.7±9.8%** (556±301)**	8.2±9.05% (279±266)	23.8±13.6%** (419±341)	29.7±18.3%** (610±626)**	30.3±20.8%** (898±846)**
Within $CD3^+CD4^+$ cells					
$CD7^{positive}$ (cells/μL)	74.5±17.5%** (631±212)	31.9±31.8% (1102±1816)§	70.7±21.4%** (607±421)	71.9±19.4%** (620±323)	83.2±8.2%** (772±466)
$CD7^{negative}$ (cells/μL)	25.4±17.5% (249±185)	68.1±31.8%* (3300±4232)***	29.2±21.5% (276±592)	28.1±19.4% (244±210)	16.7±8.2% (143±67)
$CD26^{positive}$ (cells/μL)	76.5±16.8%** (756±229)**	13.8±19.2% (295±387)	68.7±25.1%** (588±439)**	66.25±26.4%** (547±343)	86±4.9%** (785±430)
$CD26^{negative}$ (cells/μL)	23.5±16.8 (248±216)	86.2±19.1* (4107±4688)***	31.2±25.2 (300±587)	33.7±26.4 (317±308)	13.9±4.9 (130±79)
$CD49d^{positive}$ (cells/μL)	66.1±11.6%** (669±217)	26.1±27.5% (914±1484)§	60.7±21.8%** (510±398)	62.9±27.7%** (502±378)	76.2±7.4%** (431±517)
$CD49d^{negative}$ (cells/μL)	33.8±11.7 (335±144)	73.9±27.5* (3512±4457)***	39.1±21.9 (329±407)	37.0±27.7 (334±331)	23.7±7.4 (197±66)
$CD60^{positive}$ (cells/μL)	14.8±10.5% (158±127)	39.38±31.84%* (1750±3009)***	14.5±15.4% (126±175)	27.2±29.6% (208±282)	14.1±8.7% (145±148)
$CD60^{negative}$ (cells/μL)	85.2±10.5** (846±187)	60.6±31.8 (2652±3706)***	85.7±15.3** (767±670)	72.7±29.6 (657±492)	85.9±8.7 (771±368)
CD4:CD8 ratio	2.5±1.8	33.9±51.5*	3.3±4.8	2.3±1.7	2.1±1.8

**Significantly higher when compared to all the other groups; **Significantly higher when compared to SS; ***Significantly higher when compared to healthy donors, mycosis fungoides and chronic eczema; §Significantly higher when compared to mycosis fungoides.*

In order to estimate the possible independent role of the different factors of interest, while simultaneously adjusting for relevant variables, we also applied the Cox proportional hazards method.[27] Graphs of the log[–logS(t)] *versus* time were generated to verify the necessary assumption of proportionality of risks.

Results

Surface analysis of CD4, CD7, CD8, CD26, CD49d and CD60

We studied and compared, in different ways, peripheral blood lymphocytes from patients with SS, mycosis fungoides, B-cell lymphoma, and chronic eczema and from healthy controls.

As expected the total lymphocyte count was higher in patients with SS than in those with mycosis fungoides or chronic eczema; both the percentages and absolute numbers of $CD4^+$ T lymphocyte counts were significantly higher in patients with SS than in all the other groups (Table 1). The percentages of $CD8^+$ T cells and $CD4^+$ T cells expressing CD7, CD26 or CD49d were significantly reduced (P= 0.001), thus representing clear-cut features of circulating SS T cells, as already extensively described.[4-6,9,28] Similarly, the levels of $CD4^+$ T cells expressing CD60 were significantly higher in patients with SS than in all the other groups. Interestingly, the absolute number of $CD4^+$ T cells expressing CD7 or CD49d was similar in the other groups, but significantly higher than in the patients with mycosis fungoides, suggesting an expansion of $CD4^+$ cells lacking CD7 and CD49d, rather than a loss of $CD4^+CD7^+$ and $CD4^+CD49d^+$ cells (Table 1). A close relationship between diagnostic skin biopsy and down-regulation of CD49d (Pearson's χ^2 test, P<0.0001) was observed, while a weaker association was observed in the case of up-regulation of CD60 (P= 0.044).

Analysis of T-cell receptor-Vβ families in circulating Sézary syndrome cells

A clonal expansion was observed by means of TCR-Vβ repertoire analysis in 42 patients. Every single clonal expansion was confirmed by serial evaluations of the TCR-Vβ repertoire, performed every 6 months in each patient. In the present report only the values measured during the first evaluation at presentation are taken into consideration. A skewed usage of several TCR-Vβ families, associated with down-regulation of the other families, was observed. A clonal expansion of TCR-Vβ5.1 and TCR-Vβ2 was present in ten and eight cases, respectively, representing 42% of TCR-Vβ-positive individuals. TCR-Vβ13.1 and TCR-Vβ12 families were also found frequently in our Italian cohort (4 patients each). As a result, in 61.9% of cases only four out of the 25 sub-families studied were involved in the T-cell clonal expansion. On the other hand, significantly lower levels of TCR-Vβ3, TCR-Vβ4, TCR-Vβ9, TCR-Vβ14, TCR-Vβ16, TCR-Vβ18 and TCR-Vβ21.3 families were observed when compared to all the other control groups, thus representing another distinctive feature of patients with SS (*data not shown*).

Twenty patients (32.26% of all studied) did not recognize any of the TCR-Vβ monoclonal antibodies currently available and were thus considered as "TCR-Vβ null".[9] No phenotypic differences were recorded between these patients and the TCR-Vβ positive population. These TCR-Vβ null patients, in fact, showed the same characteristics already described in TCR-Vβ positive population (presence of circulating $CD4^+$ T lymphocytes with over-expression of CD60 and down-regulation of CD7, CD26 and CD49d). As already mentioned in the *Design and Methods* section, this is considered by us and others as indirect proof of clonality.[23] Incidentally, no difference in the survival rate between TCR-Vβ positive and TCR-Vβ null patients was noted (see below). No difference in TCR-Vβ repertoire was observed comparing patients with other dermatoses (mycosis fungoides, chronic eczema and B-cell lymphoma) to healthy donors. These results indicate that the preferential usage of several families (TCR-Vβ2, TCR-Vβ5,1 , TCR-Vβ12 and TCR-Vβ13.1) and underusage of TCR-Vβ3, TCR-Vβ4, TCR-Vβ9, TCR-Vβ14, TCR-Vβ16, TCR-Vβ18 and TCR-Vβ21.3 families may represent a distinctive feature of Italian SS patients.

CD60 and CD49d as independent markers of prognosis in Sézary syndrome

We evaluated our cohort of SS patients for the levels of expression of CD60 and CD49d on circulating $CD4^+$ T cells. Figure 1 shows the results of representative patients

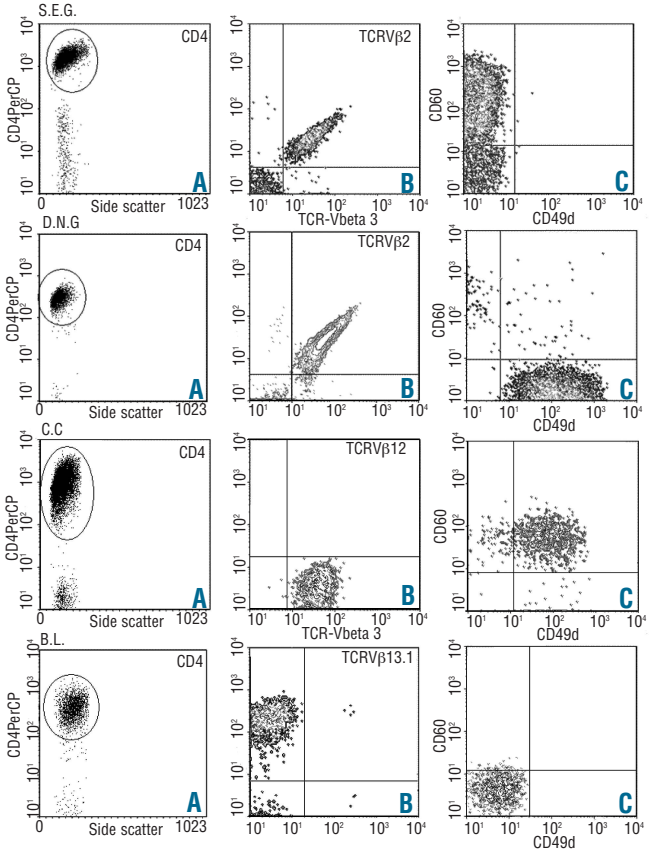

Figure 1. Four representative samples from patients with different CD49d/CD60 phenotypes at the time of diagnosis. (A) Gating strategy of live lymphocytes expressing CD4 selection. (B) and (C) The three-color staining of the TCR-Vβ family expanded or the double-color stainig of CD60 and CD49d on electronically gated $CD4^+$ T cells, respectively. In all cytograms, the lower-left quadrants delimit the fluorescence intensities obtained with appropriate isotype and fluorochrome-matched negative control antibodies. Quadrant gates in (C) are derived from appropriate fluorescence-minus-one controls.

Table 2. Five-year survival since first hospitalization for patients with Sézary syndrome. Kaplan–Meier product–limit estimates for relevant variables.

Variable/ Level	N.	% surviving 5 years	*P* value (long rank)
Overall	62	53.5±7.8	-
Sex			
Male	42	50.0±9.6	0.988
Female	20	61.8±12.6	
Age at first admission			
<60	38	73.9±13.3	0.415
≥60	24	46.4±9.0	
Lactate dehydrogenase (units/L)			
<270	33	60.5±10.1	0.15
≥270	18	30.4±12.4	
Lymphocytes (cells/μL)			
<3000	25	47.8±13.3	0.706
≥3000	37	56.1±9.8	
$CD4^+$ (%)			
<85	37	60.4±10.4	0.17
≥85	25	46.9±11.3	
$CD4^+$ (cells/μL)			
<1200	15	46.3±15.6	0.84
≥1200	47	56.0±8.9	
$CD8^+$ (cells/μL)			
<150	26	43.5±11.0	0.13
≥150	36	67.1±9.9	
CD4/CD8 ratio			
<7.00	18	68.4±15.8	0.56
≥7.00	44	48.4±8.8	
TCRVβ clone (cells/μL)			
<2000	29	61.2±11.3	0.657
≥2000	33	47.1±10.5	
$CD4^+CD26^+$ (%)			
<5.00	35	45.9±10.2	0.42
≥5.00	27	65.1±11.5	
$CD4^+CD26^+$ (cells/μL)			
<199	32	43.5±9.7	0.053
≥200	30	70.0±11.7	
$CD4^+CD60^+$ (%)			
low (<37.5)	33	81.4±7.6	0.004
high (>37.5)	29	34.1±9.9	
$CD4^+CD60^+$ (cells/μL)			
low (<500)	26	72.9±11.3	0.035
high (>500)	36	41.1±9.7	
$CD4^+CD49d^+$ (%)			
low (<7.50)	40	33.3±10.3	0.04
high (>7.5)	22	72.4±9.7	
$CD4^+CD49d^+$ (cells/μL)			
low (<500)	37	42.0±9.1	0.012
high (>500)	25	76.7±12.6	
$CD4^+CD60^+$ and $CD49d^+$ combination (%)			
$CD60^{low}$ AND $CD49d^{high}$	27	86.5±7.3	
$CD60^{high}$ OR $CD49d^{low}$	19	56.0±13.9	0.002
$CD60^{high}$ AND $CD49d^{low}$	16	25.0±10.8	
$CD4^+CD60^+$ and $CD49d^+$ combination (cells/μL)			
$CD60^{low}$ AND $CD49d^{high}$	12	90.9±8.7	
$CD60^{high}$ OR $CD49d^{low}$	27	62.7±12.9	0.012
$CD60^{high}$ AND $CD49d^{low}$	23	33.6±10.3	

analyzed from our cohort with different CD49d/CD60 phenotypes at the time of diagnosis. We noted that subjects still alive at the present observation had significantly higher levels of $CD49d^+$ and lower levels of $CD60^+$ $CD4^+$ T cells ($P<0.001$) at the time of their first hospital admission (Figure 2) when compared to patients who had a poorer outcome (patient B and A in Figure 1, respectively). Secondly, there was an inverse relationship between CD49d and CD60 expression (Pearson's correlation -0.333, $P=0.008$). These observations prompted us to evaluate whether clinical outcome could be related to surface molecule expression on circulating $CD4^+$ T cells at the time of diagnosis.

As shown in Table 2, the 5-year overall survival rate in the 62 patients was 53.5%, with the median survival being 35 months. High levels of $CD4^+CD60^+$ and low levels of $CD4^+CD49d^+$ T cells (both in terms of percentages and absolute numbers) in the peripheral blood were significantly associated with a less favorable prognosis (Table 2, Figure 3A and 3B). In addition, a lower absolute number of $CD4^+CD26^+$ T cells, $CD8^+$ T cells, and higher levels of lactate dehydrogenase were associated with higher mortality, although differences were not statistically significant (Table 2).

After inspection of the survival curves of patients divided according to levels of expression of $CD4^+CD60^+$ and $CD4^+CD49d^+$, we created a new parameter combining these two variables: patients who had both high $CD4^+CD60^+$ expression (above 37.5%) and low $CD4^+CD49d^+$ (below 7.5%) had a very low probability of survival (25% at 5 years), while patients with the opposite phenotype had a 5-year survival rate of 86.5% (Table 2). Similar results were obtained evaluating the absolute numbers of the above mentioned subsets and are illustrated in Figure 3.

Next, we applied Cox regression models to investigate whether, when simultaneously controlling for established prognostic factors for SS (i.e. CD4/CD8 ratio and lactate dehydrogenase serum levels), the levels of CD60 and CD49d expression on $CD4^+$ T lymphocytes were still sig-

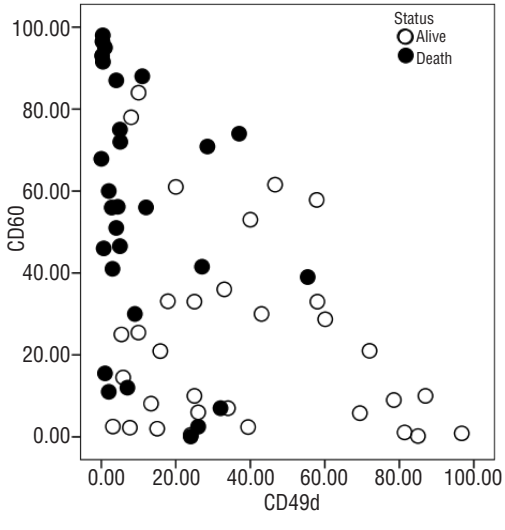

Figure 2. Percentages of CD60 or CD49d expression on $CD4^+$ T cells at the time of diagnosis. Open circles represent patients still alive, black circles indicate patients who had died before the current observation.

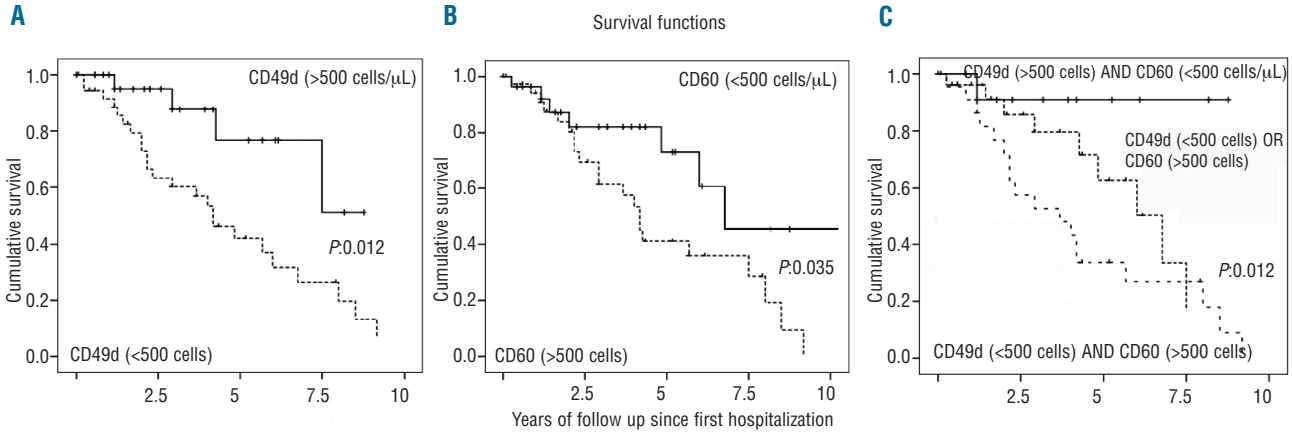

Figure 3. Cumulative survival of 62 patients with SS according to (A) CD49d number, (B) CD60 number and (C) the CD49d and CD60 combination.

nificantly associated with survival (Table 3). However, high lactate dehydrogenase (>270 U/L) showed a hazard ratio of 2.042 with a *P* value of 0.08, i.e. very close to the 0.05 conventional level of significance. In addition, the multivariate analysis confirmed an exceedingly high risk of death for patients with a combination of $CD4^{+}CD60^{+,high}$ cells ($\geq 0.5\times10^{9}$/L) and $CD4^{+}CD49d^{+,low}$ cells ($<500\times10^{9}$/L). In all multivariate models, CD60 remained significantly associated with the outcome. Several variables were excluded from this analysis because they violated the proportionality assumption of the Cox model (e.g. for CD8, percentages below 7.5% had a better 5-year survival, but a poorer 10-year survival compared with the opposite phenotype).

Table 3. Multivariate analysis of prognostic factors for survival from first hospitalization for patients with Sézary syndrome, at the IDI-IRCCS, Rome. Hazard ratios from the Cox proportional model.

Variable/level	Hazard ratio[a]	95% CI	*P* value
CD4/CD8 ratio			
≥ 10	1,046	0.422-2.590	0.923
TCRVβ clone (cells/μL)			
≥ 2000	1,818	0.662-4.220	0.3
CD8+ (cells/μL)			
< 149	1,576	0.720-3.450	0.255
LDH (units/L)			
< 269	2,042	0.909-4.592	0.084
CD4+CD60+ (cells/μL)			
≥ 500	2,895	1.111-7.546	0.03
CD4+CD49d+ (cells/μL)			
< 499	4,102	1.378-12.213	0.011
$CD4^{+}CD60^{+}$ and $CD49d^{+}$ combination (cells/μL)			
$CD60^{high}$ OR $CD49d^{low}$	4.895	0.596-40.168	0.139
$CD60^{high}$ AND $CD49d^{low}$	12.303	1.577-95.972	0.017

[a]*All hazard ratios are adjusted for gender, age at first hospital admission and lactate dehydrogenase (LDH) level. The reported estimate for LDH is from the model including CD60.*

Discussion

We evaluated the diagnostic and prognostic relevance of CD60 and CD49d expression on circulating $CD4^{+}$ T cells in 62 patients with SS. We found that CD60 over-expression and CD49d down-regulation appear to predict the survival rate of patients affected by SS.

A number of studies have identified several prognostic factors in SS, such as circulating Sézary cell count, CD4/CD8 ratio, patient's age, lactate dehydrogenase concentration and high number of white blood cells.[13-20] However, none of these markers is directly associated with the tumor phenotype of the SS cells and they seem to be mere markers of risk or, at best, the result of some deregulated mechanisms. Here we describe, for the first time, the prognostic significance of two antigens directly related to T-cell function, and thus possibly involved in the pathogenesis of SS.

CD60 is a 9-O-acetylated form of the GD3 ganglioside. It is typically expressed by only a tiny proportion of circulating human T cells, mainly in the memory effector subset ($CD45RO^{+}$).[29] The ganglioside GD3 (Neu5Acα8Neu5Acα3Galβ4GlcCer) is an intracellular second lipid messenger that has a pro-apoptotic effect, through FAS/FAS ligand, tumor necrosis factor-α or β-amyloid pathways, in several lymphoid and myeloid cell types.[30] The presence of the "acetylated form" of GD3 (9-O-acetyl GD3), i.e. CD60, had already been observed in several cancers such as melanoma and breast cancer, and in a number of tumor cell lines.[31-33] The O-acetylation may reduce or totally abolish apoptosis, irrespectively of apoptosis-inducing agents.[30] Furthermore, O-acetylation of GD3 could protect lymphoblasts from GD3-induced apoptosis and has been suggested to be a common strategy adopted by leukemic blasts towards survival in acute lymphoblastic leukemia.[34] 9-O-acetyl GD3 can be removed by de-O-acetylation, leading to increased receptivity to apoptosis.[30] In consequence, de-O-acetylation of GD3 may represent a potential strategy for inducing sensitivity to apoptosis in the circulating malignant T-cell clone in SS, opening new interesting therapeutic perspectives.

Integrin CD49d, also known as very late antigen-4 (VLA4) or α4β1, mediates the migration of lymphocytes throughout the vessel wall after its binding to vascular cell adhesion molecule-1 (VCAM-1), a cell adhesion molecule expressed on the activated endothelium. In normal individuals, circulating lymphocytes maintain α4β1 integrin in a low affinity state, unable to interact to VCAM-1 or

fibronectin. In the intermediate affinity state, VLA4 binds VCAM-1 but not fibronectin, while, *in situ*, the molecule is modulated to develop a high avidity for fibronectin to establish shear-resistant adhesion and firm linkage to the sub-endothelial extracellular matrix.[35] Consequently, the absence of VLA4 on the circulating T-cell clone in SS cells, may lead us to assume that T cells in peripheral blood of patients with cutaneous T-cell lymphomas, although committed to the skin, are unable to migrate through the vessel wall and firmly adhere to the extracellular matrix because of the defective expression of VLA4. The *CD49D* gene is located on chromosome 2q31-q32, an area not involved by major abnormalities described in SS, to date. The presence of a circulating T-cell clone over-expressing CD60 and down-regulating CD49d constitutes a distinctive and exclusive characteristic of SS. Furthermore, we observed that the more CD49d is down-regulated and CD60 over-expressed, the worse the prognosis.

Finally in this study we also observed a skewed usage of the TCR-Vβ families in Italian patients affected by SS. A loss of T-cell diversity in cutaneous T-cell lymphomas (mainly mycosis fungoides) was demonstrated by Yawalkar *et al.*[36] by polymerase chain reaction-based methods and was also noted after infection with specific viruses such as human immunodeficiency virus-1.[37] About one third of the SS patients in our cohort with circulating T-cell clones expressed TCR-Vβ5.1 (10 cases) or TCR-Vβ2 (8 cases). Other frequently found TCR families were Vβ12 and Vβ13.1, while 11 TCR-Vβ families were never used in the group of patients studied. Moreover, several TCR Vβ families were constantly under-represented in SS (TCR-Vβ 3, 4, 9, 14, 16, 18 and 21.3), thus defining another distinctive marker of this extranodal T-cell lymphoma. Similar results were observed by Vonderheid *et al.*[38] although there were no TCR-Vβ2 positive cases in their cohort. There are at least 65Vβ segments in the human TCR beta V (*TRVB*) locus located on chromosome 7 at band 7q34.[39] These segments can be grouped into 25 subfamilies, more (Vβ2, Vβ4, Vβ6, and Vβ13) or less (Vβ10 and Vβ18) represented in normal peripheral blood.[40,41] There are several possible explanations for the increased usage of Vβ2 and Vβ5.1 families in our cohort of patients with SS. The first is that the more frequent usage of these families may simply reflect their high frequency of expression within the $CD4^+$ subset in the peripheral blood of healthy individuals.[42] Another intriguing explanation of this preferential usage derives from the observation that some cases of cutaneous T-cell lymphoma may follow a chronic stimulus, such as those observed in atopic dermatitis.[43-45] It is widely known that the skin in atopic dermatitis is often colonized by *Staphylococcus aureus* strains that may generate a number of enterotoxins with super-antigenic capacity, such as Staphylococcal enterotoxins (SE)-A, SE-B , SE-C, SE-D, SE-E and toxic shock toxin 1 (TSST-1).[46-52] All these super-antigens may amplify the expression of skin-homing receptor (CLA) on T cells and induce the appearance of skewed T-cell clones.[47,48,52] For example, TSST-1 may stimulate selectively TCR-Vβ2-bearing cells, SE-C may bind both Vβ5.1 and Vβ13.1, SE-B and SE-C Vβ17 and SE-E Vβ5.1.[53-56] A repeated super-antigenic stimulation of the TCR would cause activation-induced cell death.[57] Aberrant over-expression of CD60, which confers anti-apoptotic properties, could explain why repetitive stimulation by a super-antigen may eventually result in T-cell oligoclonality.[58] Consequently, it may be hypothesized that the preferential usage of Vβ2 and Vβ5.1 observed in Italian patients with SS may be the consequence of chronic stimuli of cells by super-antigenic enterotoxins that do not undergo activation-induced apoptosis.

In conclusion we have provided evidence that there was skewed usage of several TCR-Vβ families, particularly Vβ2 and Vβ5.1, over-expression of CD60 and down-regulation of CD49d on circulating T cells in our Italian cohort of patients with SS, and that this specific signature was strictly associated with a significantly lower survival rate. These observations appear to be clinically relevant, thus raising new pathogenic hypotheses regarding SS.

Authorship and Disclosures

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