Differences in anti-apoptotic and multidrug resistance phenotypes in elderly and young acute myeloid leukemia patients are related to the maturation of blast cells

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Background and Objectives. Elderly patients with acute myeloid leukemia (AML) have a less favorable outcome, which has been related, among other factors, to multidrug resistance (MDR) phenotypes.

Design and Methods. Freshly obtained erythrocyte-lysed bone marrow samples from 150 elderly patients (> 65 years) with *de novo* AML and 30 younger AML patients were analyzed using a 4-color immunofluorescence technique for quantitative expression of proteins associated with apoptosis (bcl-2, bax, APO2.7) and MDR (P-gp, MRP, LRP) in 3 blast cell subpopulations, defined according to their maturation stage.

Results. Although a homogeneous CD34$^+$ blast cell population was more frequent in the elderly patients, (25% vs 7%, p=0.02), no statistically significant differences were detected between the two age groups in the expression of either apoptosis- or MDR-associated proteins, except for slightly higher quantities of LRP protein in the more immature CD34$^+$ blast cell subset in the elderly AML cases (p=0.04). Interestingly, when different blast cell populations were compared, immature (CD34$^+$) blast cells were characterized by higher levels of bcl-2 in both age groups and lower levels of APO2.7 in the elderly group. In addition, higher P-gp levels were found in CD34$^+$ blast cells than in CD34$^-$ ones in elderly AML patients. Reactivity for LRP was low in both elderly and younger patients.

Interpretation and Conclusions. In summary, our results suggest that the higher resistance to chemotherapy observed in elderly AML patients could be related to a higher incidence of cases with a CD34$^+$ homogeneous blast cell population, since these blast cells frequently display a more pronounced anti-apoptotic and MDR1 phenotype.

Key words: acute myeloid leukemia, elderly and younger patients, apoptosis, multidrug resistance, blast cells.

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In recent years important advances have been achieved in the treatment of patients with acute myeloid leukemia (AML). However, most of these advances have applied to young AML patients while elderly AML patients continue to face a dismal outcome.[1,2] Several studies[3-10] have attempted to explain the worse prognosis in elderly AML patients on biological grounds. It has also been suggested that blast cells from elderly AML patients frequently show unfavorable karyotypes[1-6,8] in association with both a high expression of multidrug resistance 1 (MDR1) and a great drug extrusion potential.[1,3,6] In turn, high MDR1 expression[3,8,11-14] as well as an increased resistance to apoptosis[15,16] have been associated with a CD34$^+$ phenotype in AML and it has been shown that within elderly AML patients, those cases with MDR1$^-$ blast cells and intermediate/favorable cytogenetics have complete remission rates similar to those in younger AML patients.[3,11] Altogether, these results would suggest that biological features more than chronological age may determine the poor clinical outcome of elderly patients with AML.[2] However, several studies in which the age-associated incidence of unfavorable cytogenetics was confirmed did not find significant differences in the frequency of MDR1 and CD34 expression in elderly AML patients as compared to in younger patients.[4-6] These discrepancies concerning the frequency and clinical relevance of CD34[17] and/or p-glycoprotein (P-gp)[18] expression in AML could be explained, at least to a certain extent, by biological and methodological factors such as the different ages used to define elderly patients,[2,5] the analysis of heterogeneous panels of multidrug resistance (MDR) proteins,

including P-gp, multidrug resistance protein (MRP) and lung resistance-associated protein (LRP), and different methods for their detection.[19-22] In studies of resistance to drugs it is also important to carry out a parallel investigation of apoptotic markers since an increased resistance to apoptosis may also confer resistance to multiple chemotherapeutic drugs in AML.[7,23-26] In fact overexpression of bcl-2 has been associated with both resistance to chemotherapy and shorter survival rates,[14,23,24] while a high expression of bax and a greater bax/bcl-2 ratio have been reported to be good prognostic indicators in AML.[24-26] Despite this, to the best of our knowledge, no study has been reported so far in which the expression of the P-gp, MRP and LRP MDR-related proteins and the APO2.7, bcl-2 and bax apoptosis-associated proteins have been simultaneously measured in fresh blast cells from elderly patients with *de novo* AML and analyzed according to the stage of maturation of the blast cells, and then this expression compared with that observed in younger patients. In this study we have analyzed the expression of proteins associated with both apoptosis (APO2.7, bax and bcl-2) and MDR (P-gp, MRP and LRP) in blast cell subsets defined according to their maturation stage, in a large series of 150 elderly AML patients and compared the results with those obtained in a control group of younger AML patients.

Design and Methods

Patients and samples

One hundred and eighty previously untreated patients with *de novo* AML other than an acute promyelocytic leukemia were included in the present study. Of these patients, 150 were categorized as elderly (> 65 years old) (98 males and 52 females; mean age 71±4 years) and 30 constituted the control group of younger AML individuals (≤65 years old; 19 males and 11 females with a mean age of 46±13 years). In addition, 12 bone marrow (BM) samples from healthy age-matched adult donors were included in the study. According to the French-American-British (FAB) classification,[27] the distribution of the types of *de novo* AML among the elderly patients was as follows: M0, 13%; M1, 17%; M2, 31%; M4, 15%; M5, 19%; M6, 3%; M7, 2%. For the younger cases, FAB subtypes were distributed as follows: M1, 24%; M2, 28%; M4, 19%; M5, 24%; M6, 5%. In all cases, bone marrow (BM) samples were collected in EDTA anticoagulant prior to the initiation of therapy and processed within the first 24 hours after collection.

From the total group of elderly AML patients, 117 were eligible for treatment and received chemotherapy according to the Spanish Pethema Co-operative Group protocol LAM99 for elderly patients, which included one or two 3/7 courses of idarubicin (12 mg/m^2) and cytosine arabinoside (ara-C) (100 mg/m^2); 53 patients achieved complete remission (CR) with one course, 10 achieved CR with two courses, while the remaining 54 patients did not achieve CR. Subsequently, patients who reached CR received one identical consolidation cycle, followed by intensification therapy with one course of ara-C (500 mg/m^2/12 h for a total of 8 doses) and daunorubicin (45 mg/m^2) for 3 days. All 30 young (< 65 years old) AML patients were treated according to the Spanish Pethema Co-operative Group protocol LAM99 for younger patients, which included one or two 3/7 courses of idarubicin (12 mg/m^2) and ara-C (200 mg/m^2); 20 patients achieved CR with one course, 6 achieved CR with two courses, while the remaining 4 patients did not achieve CR. Subsequently, patients in CR received one identical consolidation cycle, followed by intensification therapy with one course of ara-C (1g/m^2/12 h for a total of 8 doses) and mitoxantrone (12 mg/m^2) for 3 days (8 cases at this point of follow-up) followed by an autologous stem cell transplant (14 cases at this point of follow-up).

Immunophenotypic studies

Freshly obtained erythrocyte-lysed BM samples, processed within the first 24 hours after collection, were analyzed according to well-established methods,[13,27] using a four-color immunofluorescence technique for the simultaneous staining of surface and cytoplasmic (cyt) antigens. The following combinations of monoclonal antibodies (MAb) – fluorescein isothiocyanate (FITC)/ phycoerythrin (PE)/ PE-cyanine 5 (Cy5)/ allophycocyanine (APC) – were used: $_{cyt}$bcl-2/ CD32/CD34/CD45, $_{cyt}$bax/CD32/CD34/CD45, CD32/$_{cyt}$APO2.7/CD34/CD45, CD19/P-gp/ CD34/ CD33,$_{cyt}$MRP/CD34/CD19/CD33, $_{cyt}$LRP/ CD34/ CD19/CD33. The specific MAb reagent clones used and their source was as follows: anti-APO2.7-PE (clone 2.7A6A3), anti-bax (clone 4F11), CD19-PECy5 (clone J4.119) and CD34-PECy5 (clone 581) were purchased from Beckmann-Coulter (Miami, FL, USA); anti-Bcl-2-FITC (clone 124) was obtained from DAKOCYTOMATION (Glostrup, Denmark); CD32-FITC and CD32-PE (clone AT 10) were obtained from SEROTEC (Oxford, UK); CD19-FITC (clone 4G7), CD34-PE (clone 8G12), CD33-APC (clone p67.6) and anti-P-gp (clone 15D3) were purchased from Becton Dickinson Biosciences (BDB; San José, CA, USA); anti-MRP (clone MRPm6) and anti-LRP (clone LRP-56) were obtained from CHEMICON (Temeluca, CA, USA) and; CD45-APC (clone HI30) was purchased from Caltag Laboratories (San Francisco, CA, USA). In all cases, appropriate isotype-matched negative controls were analyzed in parallel. Data acquisition was performed in a FACScalibur flow cytometer (BDB)

using the CellQuest software program (BDB). In all cases analyzed, a minimum of 5×10^4 bone marrow events corresponding to the whole bone marrow cellularity, was acquired for each 4-color staining. The PAINT-A-GATE software program (BDB) was used for data analysis. Myeloid blast cells from each patient were phenotypically classified into one or more of the following three maturation-associated subsets according to their reactivity for the CD34, CD32 and CD33 antigens: $CD34^+$ cells (subpopulation I), $CD34^-/CD32^{-/+}/CD33^+$ myeloblasts (subpopulation II) and $CD34^-/CD32^{+/++}/CD33^{++}$ (subpopulation III). Dim antigen expression for CD32 and CD33 ($^+$) was defined as a mean fluorescence intensity (MFI) ≤ 50 on a relative linear fluorescence channel (arbitrary units scaled from 0 to 10^4) while high antigen expression ($^{++}$) was considered as an MFI ≥ 50; $CD32^{-/+}$ and $CD32^{+/++}$ reflect heterogeneous CD32 antigen expression with or without CD32-negative blast cells, respectively. A case was considered to have a specific subpopulation of blast cells if ≥ 5% of all blast cells displayed that specific immunophenotype. The *term $CD34^+$ homogeneous blast cell population* refers to AML cases in which the tumor cell compartment consisted of a single subpopulation of $CD34^+$ blast cells. The MFI obtained for each individual apoptosis- and MDR-associated marker and the corresponding isotype-matched negative control was recorded for each subset of blast cells under analysis. For each marker analyzed within each blast cell subset, results were reported as their relative fluorescence intensity (RFI) calculated as the ratio between the MFI of the protein analyzed and the MFI of the corresponding isotype-matched negative control. For statistical analyses RFI was used as a continuous variable. In all cases, the consensus recommendations for staining and analysis of multidrug-resistance associated proteins were strictly followed.[29-31]

Statistical methods

For all phenotypic variables analyzed, median and mean values as well as their standard deviation and range were calculated using the SPSS software program (SPSS 10.0. Inc. Chicago, IL, USA). The Mann-Whitney U and Kruskal-Wallis tests were used to estimate the statistical significance of the differences observed between groups. *p* values lower than 0.05 were considered to be statistically significant.

Results

Table 1 shows the distribution of both elderly and young adult AML patients according to the immunophenotype of their bone marrow blast cells. As shown in the table, there were no major differences between the two groups of patients, except for a higher frequency of cases showing a homogeneous immature $CD34^+/CD32^{-/+}/CD33^+$ blast cell population (subpopulation I alone) found among elderly patienst with AML (25% versus 7%; p= 0.02). Table 2 shows the relative distribution of each blast cell subpopulation referring to the total blast cell compartment in both groups of AML patients.

Interestingly, once the expression of the bcl-2, bax and APO2.7 apoptosis-associated proteins was specifically analyzed within each immunophenotypically defined subpopulation of blast cells (Table 3), no major differences were observed between elderly and younger AML patients. Despite this, in both groups of patients blast cells from subpopulation I displayed significantly higher levels of bcl-2 than did the more mature subpopulations of blast cells (Table 4). In addition, in elderly AML, but not in younger patients, blast cells from subset I also had lower expression of APO2.7. No statistically significant differences were found in the expression of bax in the individual sub-

Table 1. Distribution of elderly versus younger AML patients according to the immunophenotypic characteristics of blast cells.

Blast cell subpopulation	*Elderly AML n=150*	*Younger AML n=30*	*p value*
I	38 (25)	2 (7)	0.02
II	16 (11)	1 (3)	NS
III	4 (3)	5 (17)	NS
I + II	42 (28)	9 (30)	NS
I + III	21 (14)	4 (13)	NS
II + III	18 (12)	5 (17)	NS
I + II + III	11 (7)	4 (13)	NS

Results expressed as number of cases and percentage values in brackets. NS: statistically not significant. AML: acute myeloid leukemia. Overall in elderly AML patients, type I blast cells were detected in a total of 112 cases (either as an isolated population – 38 cases, or mixed with another one – 74 cases); type II blast cells were detected in a total of 87 cases (isolated in 16 cases , mixed in 71 cases; and type II blast cells were detected in a total of 54 cases (isolated in 4 cases and mixed in 50 cases. In young AML patient type I blast cells were detected in 19 cases (isolated in 2 cases and mixed in 17 cases); type II blast cells were detected in 19 cases (isolated in 1 case and mixed in 18 cases), and type III blast cells were detected in 18 cases (isolated in 5 cases and mixed in 13 cases).

Table 2. Relative distribution of each maturation-associated subpopulation of blast cells within the total blast cell population in elderly versus younger AML cases.

	% of cells in each subpopulation		
Diagnostic group	*Subpopulation I*	*Subpopulation II*	*Subpopulation III*
Elderly AML	65±32 5-100 n=112	59±30 7-100 n=87	49±27 5-100 n=54
Younger AML	52±31 6-100 n=19	55±24 25-100 n=19	54±35 10-100 n=18

Results expressed as mean ± standard deviation and range.

Table 3. Expression of the bcl-2, bax and APO2.7 apoptosis-related proteins in different maturation-defined subpopulations of blast cells in elderly and younger AML patients.

Diagnostic group	Blast cell subpopulation	bcl-2	bax	APO 2.7
Elderly AML	I:	13.2±6.0*	1.4±0.5	2.6±1.9#
	n=109	12.9	1.1	2.3
	II:	8.2±5.5	1.4±0.7	3.1±2.2
	n=84	6.8	1.2	2.6
	III:	4.8±3.2	1.8±1.4	3.5±2.1
	n=53	3.8	1.4	3.0
Younger AML	I:	12.5±9.2°	1.3±0.5	2.9±0.9
	n=19	10.6	1.2	2.6
	II:	10.9±10.8	1.5±0.5	3.3±1.0
	n=19	7.9	1.4	3.4
	III:	4.8±2.3	1.9±1.2	3.6±1.7
	n=18	4.1	1.5	3.3

*Results expressed as mean ± standard deviation and median relative fluorescence intensity (RFI). The RFI is calculated by the mean fluorescence intensity (MFI) of the stained cells divided by the MFI of the corresponding negative control. *p<0.0001 and #p= 0.003 comparing subpopulation I with II and III, respectively, in elderly AML patients. °p= 0.009 comparing subpopulation I with both subpopulations II and III in younger AML patients.*

Table 4. Expression of the P-gp, MRP and LRP multidrug-associated proteins in different maturation-defined blast cell subsets from elderly versus younger AML patients.

Diagnostic group	Blast cell subpopulation	P-gp	MRP	LRP
Elderly AML	I:	3.4±3.4*	2.4±1.8	6.0±4.9+°
	n=108	2.3	1.9	4.5
	II:	2.6±2.2	2.2±1.2	7.9±5.4
	n=76	2.0	1.9	6.0
	III:	1.9±0.9	2.7±1.9	12.2±8.9
	n=46	1.6	1.8	8.8
Younger AML	I:	2.3±0.9	2.1±0.8	4.3±2.8#
	n=16	2.2	1.9	3.5
	II:	2.3±0.8	2.6±1.2	6.8±5.3
	n=13	2.0	2.1	5.5
	III:	2.1±1.2	3.4±2.6	11.3±8.9
	n=12	1.7	2.4	9.1

*Results expressed as mean ± standard deviation and median relative fluorescence intensity (RFI). The RFI is calculated from the mean fluorescence intensity (MFI) of the stained cells divided by the MFI of the corresponding negative control). +p= 0.0001 for older AML subjects compared to younger AML patients. *p= 0.005 and °p< 0.0001 comparing subpopulation I with subpopulations II and III, respectively, in elderly AML patients. #p= 0.005 comparing subpopulation I with both subpopulations II and III in younger AML patients.*

sets of blast cells in either group of patients. The expression of P-gp, MRP and LRP in the three subpopulations of blast cells was not statistically significantly different between older and younger adult AML patients, except for higher amounts of LRP found in the more immature CD34+ blast cell subpopulation in elderly AML patients (mean RFI of 6.0±4.9 versus 4.3±2.8, respectively; p= 0.04) (Table 4).

As for the apoptosis-related proteins, statistically significant differences were found in the expression of MDR-associated proteins between subsets of blast cells displaying a different phenotype, both in elderly and younger adult AML patients. In elderly AML patients CD34+ blast cells (subpopulation I) showed higher expression of P-gp than did CD34- leukemia cells (subpopulations II and III) (p=0.005). Lower reactivity for LRP occurred in CD34+ cells (subpopulation I) than in subpopulations II and III in both elderly AML patients (p< 0.0001). No statistically significant differences were found in the expression of MRP between different blast cell subsets in either of the two groups of AML patients analyzed (Table 4).

Table 5 summarizes the results after comparing expression of apoptosis and MDR-associated proteins in AML blast cells and in normal bone marrow cells. As the table shows, CD34+ and CD34- AML blast cells displayed higher levels of bcl-2 than did normal bone marrow CD34+ and CD34- granulomonocytic precursors (p=0.005 and p<0.001, respectively); in addition, the more mature CD34- blast cells (subpopulations II and III) from elderly and younger AML patients also showed a decreased reactivity for APO2.7 (p<0.001) with respect to granulomonocytic cells from healthy controls. In turn, no significant differences were observed between CD34+ blast cells from AML patients and normal bone marrow CD34+ cells as regards the expression of the drug resistance-associated proteins analyzed; in contrast, more mature CD34- blast cells (subpopulations II and III) from both elderly and younger AML patients showed lower levels of LRP (p= 0.007) and MRP (p=0.005) than did normal granulomonocytic bone marrow cells from healthy individuals.

From the clinical point of view, the CR rates were significantly lower in the 117 elderly patients receiving intensive chemotherapy than they were in the younger patients (54% vs 87%; p= 0.02), and this was also associated with a shorter median relapse-free survival (9 months vs not reached; p= 0.01) and overall survival (8 months vs not reached) for the older group. Among elderly AML patients, expression of CD34 on blast cells was associated with a lower CR (46% vs 81%; p=0.005) and a slightly higher relapse rate (59% vs 43%; p= 0.07); similarly, among the younger AML patients, all new relapses which have occurred so far have been in CD34+ patients (59% vs 0% among CD34- cases; p= 0.20). Despite this, no statistically significant differences were observed in the relapse-free and overall survival of either AML group according to CD34 expression.

Discussion

Despite the development of more effective treatment strategies for AML, the outcome of elderly patients with this disease, even those with a good per-

Table 5. Expression of apoptosis and multidrug resistance-related proteins in the CD34⁻ cell compartment from bone marrow of healthy donors, elderly AML patients and younger AML patients.

	CD34+ cells (Subpopulation I)				*Granulomonocytic precursors (Subpopulations II+III)*			
	Normal BM	*Younger AML*	*Elderly AML*	*p*	*Normal BM*	*Younger AML*	*Elderly AML*	*P*
bcl-2 (RFI)	7.4±2.2 (7.0)	12.5±9.2 (10.6)	13.2±6.0 (12.9)	0.005	2.0±0.4 (2.0)	8.0±8.4 (5.9)	6.8±5.0 (5.4)	< 0.0001
Bax (RFI)	1.1±0.2 (1.1)	1.3±0.5 (1.2)	1.4±0.7 (1.1)	NS	1.4±0.4 (1.3)	1.7±0.9 (1.4)	1.6±1.0 (1.3)	NS
APO2.7 (RFI)	2.8±0.6 (2.9)	2.9±0.9 (2.6)	2.6±1.9 (2.3)	NS	6.7±3.2 (6.1)	3.5±1.4 (3.3)	3.2±2.2 (2.7)	< 0.0001
P-gp (RFI)	4.1±2.6 (3.1)	2.3±0.9 (2.2)	3.4±3.4 (2.3)	NS	2.5±1.0 (2.4)	2.2±1.0 (1.9)	2.3±1.8 (1.8)	NS
MRP (RFI)	2.5±0.9 (2.4)	2.1±0.8 (1.9)	2.4±1.8 (1.9)	NS	5.7±4.2 (4.4)	3.0±2.0 (2.2)	2.4±1.6 (1.9)	0.005
LRP (RFI)	4.2±1.6 (3.6)	4.3±2.8 (3.5)	6.0±4.9 (4.5)	NS	15.1±6.2 (15.1)	8.9±7.4 (6.0)	9.5±7.2 (7.3)	0.007

Results expressed as mean ± standard deviation and median relative fluorescence intensity (RFI). The RFI is calculated from the mean fluorescence intensity (MFI) of the stained cells divided by the MFI of the corresponding negative control).

formance status, is significantly worse than that of young AML patients.[1,2] Several studies[1-6,8] have pointed out that an increased frequency of unfavorable cytogenetics is one of several factors that could explain the poor response of elderly AML patients to therapy. Other factors unrelated to age that could also be involved in the greater resistance of the elderly AML patients to chemotherapy are both overexpression of MDR-associated proteins and anti-apoptotic mechanisms.

In the present study, no major differences were observed in the expression of either the bcl-2, bax and APO2.7 apoptosis-associated proteins or the P-gp, MRP, LRP MDR-related proteins, once the blast cell subsets displaying a similar phenotype were compared between the two age groups. The only exception was the expression of LRP which was found to be slightly higher in immature CD34⁺ blast cells from elderly AML patients than in the corresponding blasts from younger AML patients. These results are in line with previously reported data, which showed no differences in the MDR phenotype between the two age groups of AML.[4-6] In contrast, Garrido *et al.*[7] reported that elderly AML patients showed lower levels of apoptosis, but the authors did not correlate apoptotic data with the maturation stage of blast cells. A detailed analysis of the pattern of expression of the apoptosis- and MDR-associated proteins in distinct immunophenotypic subsets of blast cells confirmed the existence of important maturation-associated differences in both groups of AML patients. Accordingly, CD34⁺ blast cells constantly showed higher expression of the anti-apoptotic bcl-2 protein in both groups of patients together with greater amounts of P-gp among the older AML cases. Interestingly, while each subpopulation of myeloid blast cells showed a similar apoptosis- and MDR-associated phenotype in elderly and younger patients, a high percentage of the elderly patients had leukemic blast cells showing a homogenous CD34⁺ phenotype. These results suggest that differences between elderly and younger AML patients could be based mainly on the frequency of cases showing a homogenous immature CD34⁺ blast cell phenotype; it should be noted that evaluation of the frequency of positivity for CD34 in the context of the whole blast population using arbitrary cut-off values would not allow the identification of major differences between the two groups, as reported in other studies,[4-6] and confirmed by our own findings. In line with these findings, in the present series, expression of CD34 had a negative impact on the achievement of CR in the elderly AML group, although this only translated into a statistically non-significant tendency towards a lower relapse-free survival and overall survival for these elderly CD34⁺ patients.

In contrast to P-gp, LRP expression was lower among blast cells showing a more immature phenotype (CD34⁺), not only in elderly AML patients but also in the younger cases. In line with these findings, it should be noted that up-regulation of vault-protein expression has been observed during differentiation and maturation of human monocyte-derived dendritic cells.[32] This could explain the higher amount of LRP expression observed in blast cells from AML cases with a more differentiated morphology (FAB M4 or M5),[33-35] in contrast to the low amounts of this protein detected by us in more immature bone marrow CD34⁺ blast cells.[13] It has been pointed out that elevated LRP expression is associated with poor outcome in AML patients after intensive chemotherapy.[10,35,37]

Interestingly, the CD34⁺ blast cell subpopulation from elderly AML had higher LRP levels than those observed in the control group of young AML patients; this finding might indicate that the protein could also partially contribute to the poorer outcome of elderly AML patients. In contrast, our results suggest that MRP protein does not play a key role in determining the behavior of AML in elderly patients. This conclusion concurs with previous reports showing that blast cells from AML patients studied at diagnosis show low

MRP antigen expression[22,34,38] with no significant impact on the patients' outcome.[9,10,22,34,38,39]

In summary, our results suggest that the greater resistance to chemotherapy observed in elderly patients with AML could be related in part to a higher incidence of cases showing a homogeneous CD34+ blast cell population, since blast cells displaying this phenotype frequently show a more pronounced anti-apoptotic and MDR phenotype.

JFSM and AO designed and supervised the study. LS, MBV and AO analyzed the data and wrote the manuscript. LS, AL, MCLB, and MS performed the immunophenotypic studies. MJM, JGL, CPL, MT, and EL recruited patients and compiled databases. AO and JFSM supervised the flow cytometry data analysis and critically reviewed the manuscript. The authors declare that they have no potential conflicts of interest.

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