The presence of STAT1-positive tumor-associated macrophages and their relation to outcome in patients with follicular lymphoma

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Background and Objectives. The presence of tumor-associated macrophages (TAM) is a prognostic factor for survival in follicular lymphoma (FL). Overexpression and/or activation of the signal transducer and activator of transcription 1 (STAT1) in these TAM have also been observed. The aim of this study was to determine the extent to which macrophages are present in FL and to investigate the expression of STAT1 in these cells.

Design and Methods. We retrospectively analyzed 211 patients with distinct stages and grades of FL. Expression of the CD68 proteins, chosen as a marker for macrophages, and STAT1 was quantified by immunohistochemistry and double immunofluorescence.

Results. Automated determinations revealed the presence of CD68-positive macrophages in all FL tissues studied (mean 57.6±45.1 cells/field), while STAT1 protein was expressed in 29.94% of cases. Double-fluorescence staining confirmed that STAT1 protein co-localized exclusively with CD68, indicating the presence of a subset of STAT1-expressing TAM localized principally in the vicinity of tumor cells. Multivariate analysis showed that, besides the Follicular Lymphoma International Prognostic Index (FLIPI) classification, expression of STAT1 was an important independent prognostic factor for shorter overall survival in FL.

Interpretation and Conclusions. These results demonstrate the presence of STAT1-expressing TAM in FL and their association with an adverse outcome, thus emphasizing the relevance of non-tumor cells in the control of the growth and survival of lymphoma cells.

Key words: follicular lymphoma, tumor-associated macrophages, CD68, STAT1, survival.

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The molecular events involved in the pathogenesis of follicular lymphoma (FL) appear to consist of a combination of microenvironmental changes and a series of hazardous events that contribute to clonal evolution.[1,2] Although imperfect, the main predictors of clinical course described to date are the International Prognostic Index (IPI), which classifies a relatively small percentage of patients into a high-risk category,[3,4] and the Follicular Lymphoma International Prognostic Index (FLIPI), which incorporates additional factors, such as hemoglobin level and the number of affected nodal sites, permitting the classification of FL patients into three, more equally distributed, risk groups.[5-8] The growth and survival of tumoral cells in Hodgkin's lymphoma (HL) and FL depend on interactions between the tumoral and tumor-infiltrating cells, specifically cytotoxic and regulatory T cells.[9-11] Recently, differences in the biology of the host immune/inflammatory microenvironment have been suggested to be the cause of different clinical courses and outcomes in FL. A primary signature includes a complex mixture of T-cell and macrophage markers specifically associated with long survival in FL, while a secondary signature includes genes coding for markers restricted to macrophages and dendritic cells specifically associated with short survival.[12] Whereas the precise location of the immune cells expressing these genes has not been defined,[13,14] an immunohistochemical study in a limited cohort of FL patients described the presence of CD68-positive lymphoma-associated macrophages (LAM) within the population of non-neoplastic infiltrating cells, and drew attention to their potential to predict overall survival in patients with FL.[15]

Macrophages that migrate into the tumor stroma have complex dual functions in their interaction with neoplastic cells.[16-18] The cells defined as tumor-associated macrophages (TAM) are pivotal types of inflammatory cells that have an important role in the inhibition of the immune response against tumor cells and can promote tumor progression. The available evidence suggests that in established, progressively growing solid tumors, TAM are reprogrammed to induce immune suppression of host defenses *in situ* through the release of specific cytokines, prostanoids and other humoral mediators. This disordered response results in the inhi-

bition of effective anti-cancer cell-mediated immune mechanisms.[18] The signal transducer and activator of transcription 1 (STAT1) is essential for interferon-mediated signaling and is involved in the regulation of macrophage functions, suggesting that the STAT1 pathway is an important mediator of immune anti-tumor signals.[19] In fact, a recent study in experimental tumor models demonstrated a significant increase in the level of activated STAT1 in the F4/80$^+$ macrophages isolated from the tumors.[20] *In vitro* culture demonstrated that F4/80$^+$ TAM isolated from wild-type STAT1$^{+/+}$ mice drastically inhibited T-cell proliferation, in contrast to those isolated from STAT1$^{-/-}$ mice. These results suggest that STAT1 may be involved in TAM-mediated T-cell inhibition in tumors. Taking into account these previous observations, we evaluated the presence and tissue distribution of macrophages and the expression of STAT1 in a group of 211 patients with all clinical stages and histological grades of FL, and the possible relation between the presence of these components and the outcome of the disease.

Design and Methods

Patients and samples

Two hundred and eleven patients with FL were diagnosed in different Spanish clinical institutions and randomly registered by collaborating members of the Spanish Lymphoma Study Group. All patients were clinically staged according to standard protocols and tumors were graded according to the criteria in the WHO classification of hematopoietic neoplasms.[21] Treatment modalities varied over time and were administered according to local protocols at the time of diagnosis. Treatment regimens included principally: cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP: 44%); cyclophosphamide, vincristine, prednisone (CVP: 15%); and a variety of other monotherapies, with or without adjuvant radiotherapy and/or surgery (41%). Only five patients received CHOP or CVP chemotherapy complemented by rituximab.

The patients' clinical records included all those variables that were already known to be of prognostic relevance, such as age, sex, Ann Arbor stage, performance status, grade, bone-marrow involvement, B symptoms, nodal sites, lactate dehydrogenase (LDH) levels, hemoglobin levels, and FLIPI, for overall survival (OS) and progression-free survival (PFS). The FLIPI grouped patients with respect to age (≤60 years vs. >60 years), Ann Arbor stage III or IV, hemoglobin level <120 g/L, elevated LDH, and more than four nodal sites.[5] OS is defined as the interval between the date of diagnosis and death from any cause. PFS is defined as the interval between diagnosis and death or lymphoma progression, whichever occurred first. The diagnosis of FL was reviewed by FC, CB and MP. Only cases with a consensual diagnosis were included.

Immunohistochemistry

Available diagnostic biopsy tissue from FL cases was incorporated into tissue microarrays, as previously described.[22] Selected 1-mm diameter cores from two different representative areas were included for each case, along with different controls, to ensure adequate scoring of the tumoral cells, independently of the tissue heterogeneity of the tumor. Several studies have shown that data obtained using this procedure are comparable to those obtained from analyzing whole tissue sections in series of non-Hodgkin's and Hodgkin's lymphoma.[22-24] Eight tissue microarray blocks containing between 90 and 140 cylinders (including duplicate sections and control sections) were constructed. Sections of reactive lymphoid tissue, including 14 samples of tonsil and 11 samples of spleen, as well as non-Hodgkin's lymphoma-derived cell lines (KARPAS-422 and WSU-NHL) were used as positive controls.

Immunohistochemical detection was performed as described previously.[9,10] Briefly, sections were deparaffinized and, after heat-induced epitope retrieval, incubated with the appropriate primary antibody: macrophages were detected using anti-CD68 (clone KP1, Dako, Carpinteria, USA) and STAT1 was detected using the Santa Cruz C-136 clone (Santa Cruz Biotechnology, Santa Cruz, USA). Final detection using the EnVision™ method (Dako, Carpinteria, California, USA) was carried out according to the manufacturer's instructions.

Double staining

For double immunofluorescence staining, sections from reactive lymphoid tissues (tonsils) and representative FL specimens were incubated with the following antibodies: CD68 clone PG-M1 IgG3 (1:10 dilution; DakoCytomation, Glostrup, Denmark) and STAT1 p84/p91 IgG1 (1:10 dilution; Santa Cruz Biotechnology, Santa Cruz, USA). After simultaneous 30-min incubation at room temperature with the primary antibodies, sections were washed in phosphate-buffered saline and incubated with secondary antibodies for 1 hour at room temperature: Alexa 488-conjugated goat anti-mouse IgG1 (1:200, Molecular Probes, Eugene, USA) and Alexa 599-conjugated Goat anti-Mouse IgG3 (1:200, Molecular Probes, Eugene, USA). Special consideration was given to primary antibody isotypes to avoid artifactual co-staining of antigens. In the conditions we used, previous staining in normal tonsils indicated the absence of cross-reactivity with the secondary monoclonal antibodies. For double histochemical and fluorescence staining, sections were incubated with STAT1 (1:10 dilution) and CD21 clone 1F8 IgG1 (1:5 dilution, DakoCytomation) for 30 min at room temperature. CD21 immunodetec-

tion was performed with biotinylated anti-mouse, followed by peroxidase-labeled streptavidin LSAB (DakoCytomation) with diaminobenzidine chromogen as the substrate. STAT1 was incubated with the secondary antibodies Alexa 488-conjugated goat anti-mouse IgG1 for 1 hour at room temperature. All tissue sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) and directly visualized under a DMRA microscope (Leica Microsystems, Wetzlar, Germany) fitted with appropriate fluorescence filters. Series of images were processed and analyzed using the accompanying software (Leica Microsystems) and the Adobe Photoshop 5.5 image-processing program.

Scoring methods

The immunostained tissue microarray slides were scored for architectural pattern and positivity of immunostained cells. Firstly, the whole area of each tissue was observed under a Leica DM LB2 bright-field microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 2.5 X magnification for a qualitative determination of the distribution of the infiltrating cells within and outside the neoplastic follicles. Two representative areas with the most abundant immune infiltration were examined at the same magnification and then the representative areas were scanned at a 40 X magnification with a Leica DFC320 digital camera (Leica Microsystems GmbH). The area of each analyzed field was 9.77 mm^2. CD68-positive stained cells were scored automatically with the Image-Pro Plus® 5.0 program (MediaCybernetics Inc., Silver Spring, MD, USA), using a specific macro previously designed in our laboratory.[10] The results from the different representative fields of the same tissue were evaluated and scoring consistency between the two cores was considered necessary for the sample to be suitable for inclusion in the study (average calculated). In order to evaluate STAT1 immunostaining (expressed in few cores), non-neoplastic cells showing cytoplasmic and/or nuclear staining were regarded as positive. The consistency of results from the duplicate cores was also analyzed. Tumoral cells in FL did not express STAT1.

Statistical analyses

All statistical analyses were carried out using SPSS version 11.0 (SPSS Inc, Chicago, IL, USA). Frequencies and associations between the different clinicopathological and immune variables were compared and estimated using χ^2 contingency analysis. Student's t-test for independent samples was used to compare normally distributed variables, and the Mann-Whitney U-test and Spearman's rho correlation were used to compare non-normally distributed variables. The OS and PFS probabilities were determined using the Kaplan-Meier method and the survival curves were compared using the log-rank test. Cox's proportional hazards regression method was used to assess the simultaneous distribution of variables of interest (immune parameters and FLIPI) with respect to OS and PFS. All estimates of effects on OS and PFS are expressed as hazard ratios (HR), each with an associated 95% confidence interval (CI) and p-value. The nominal level of statistical significance for the end-points was 5% (two-sided test).

Results

Patient's clinical and tumoral characteristics

The clinical and tumoral data of the patients included in the study are summarized in Table 1. The overall median follow-up was 74 months and the median follow-up time among patients alive at last follow-up was 83 months. Survival times, illustrated in Figure 1, were from date of diagnosis to the date of death (OS) and from the date of diagnosis to the date of death or lymphoma progression (PFS). The probability curves for PFS do not start at 100% at time zero because of failures during the initial treatment.[25] The median OS was 159 months and the median PFS was 41 months. The 5- and 10-year OS rates were 79% and 58%, and the 5- and 10-year PFS rates were 42% and 32%, respectively. By the end of the follow-up, 33% of the patients had died. Survival analysis (Kaplan-Meier method) indicated that an unfavorable OS was more strongly associated with male gender (p=0.014), age above 60 years (p<0.000), bone marrow involvement (p=0.016), performance status ≥1 (p=0.001), and advanced stage of disease at diagnosis (p=0.006), whereas an unfavorable PFS was more strongly associated with bone marrow involvement (p=0.001), performance status ≥1 (p=0.015), and advanced stage of the disease at diagnosis (p=0.004). Consistent with the findings of previous studies, the distribution of patients according to the FLIPI was as follows: 39% with a low score (0/1), 38% with an intermediate score (2), and 23% with a high score (3-5). As illustrated in Figure 2, patients included in the intermediate- and high-risk FLIPI groups had significantly lower OS and PFS (p<0.000 and p=0.001, respectively).

Distribution of CD68- and STAT1-positive cells and clinical association

Expression of CD68 protein was detected in the cytoplasm of macrophages in normal lymph nodes. STAT1 protein expression was detected in both the cytoplasm and the nucleus of scattered macrophages in the follicles and parafollicular area.[26] Both cytoplasmic and nuclear staining were associated, since cases with purely nuclear or cytoplasmatic staining were not found. The pattern of distribution of CD68 and STAT1 staining in the immune infiltrate of FL is shown in Figure 3. The automated scoring of CD68 staining indicated a mean number of CD68-positive cells/field of 57.6±45.1 (range, 2-219). Since

Table 1. Clinico-biological characteristics and prognostic factors for overall survival (OS) and progression-free survival (PFS) in 211 patients with follicular lymphoma.

Variable	N. of cases at diagnosis (%)	10-year OS % alive	p[a]	10-year PFS % alive	p[a]
Sex			*0.014*		*NS*
Female	116 (55.5)	68.06		36.45	
Male	93 (44.5)	48.30		27.33	
Age			*<0.000*		*NS*
≤60 years	127 (62.0)	71.40		36.50	
>60 years	78 (38.0)	38.33		26.07	
Number of nodal sites			*NS*		*0.051*
≤4	143 (68.8)	66.54		40.29	
>4	65 (31.2)	45.98		15.03	
Bone-marrow involvement			*0.016*		*0.001*
Absence	98 (48.5)	71.98		42.08	
Presence	104 (51.5)	47.59		24.82	
B symptoms			*NS*		*NS*
Absence	144 (74.6)	61.12		34.56	
Presence	49 (25.4)	53.21		20.17	
Performance status (ECOG)			*0.001*		*0.015*
0	143 (71.1)	65.55		38.07	
1	47 (23.4)	42.86		14.79	
2	11 (5.5)	45.45		27.27	
Ann Arbor stage			*0.006*		*0.004*
I-II	41 (20.8)	88.54		54.69	
III-IV	145 (79.2)	51.41		25.13	
Grade			*NS*		*NS*
1	87 (41.2)	60.25		31.61	
2	66 (31.3)	61.33		31.00	
3a	39 (18.5)	57.85		38.16	
3b	19 (9.0)	56.56		25.97	
Serum LDH			*NS*		*NS*
ULN[b]	154 (82.4)	58.13		34.23	
>ULN	33 (17.6)	57.26		24.57	
Hemoglobin level			*NS*		*NS*
<12 g/dL	21 (12.1)	60.37		28.26	
≥12 g/dL	152 (87.9)	55.13		33.04	
FLIPI score					
0-1	61 (38.9)	85.65	<0.000	54.50	0.001
2	60 (38.2)	44.88		27.01	
3-5	36 (22.9)	36.92		10.32	

[a]Results of univariate analysis (log-rank test); [b]ULN; upper limit of normal.

most cases had few or no STAT1⁺ cells and only a small number of cases revealed many positive cells, STAT1⁺ cells were scored qualitatively: cases were considered to be positive when there were more than 20 positive cells/field. Under these conditions, 30% of the cases stained positively for STAT1. CD68-positive macrophages were mainly present outside the neoplastic follicles, fewer cells being detected within the follicles. Cells expressing STAT1 protein, principally in the

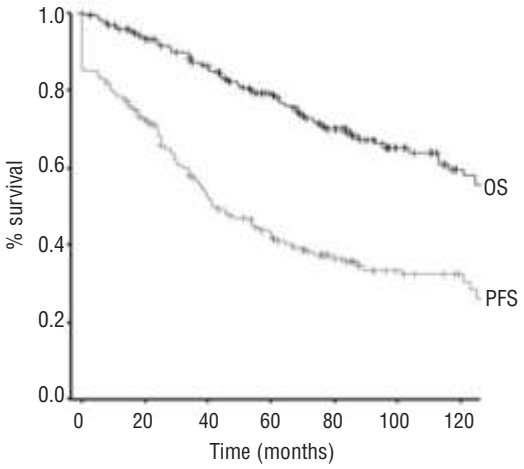

Figure 1. Overall survival (OS) and progression-free survival (PFS) of the study population.

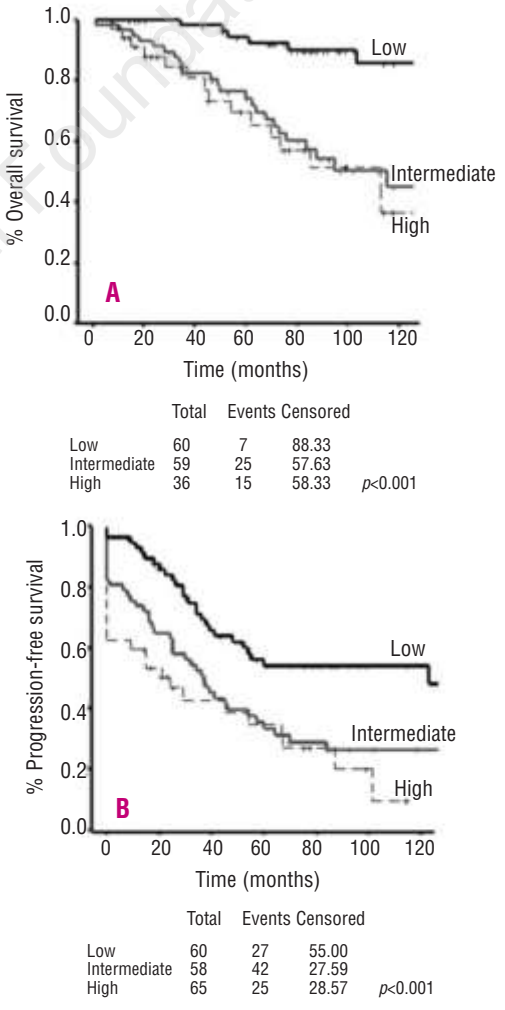

Figure 2. (A) Overall survival and (B) progression-free survival of FL patients divided into three risk groups defined by the Follicular Lymphoma International Prognostic Index.

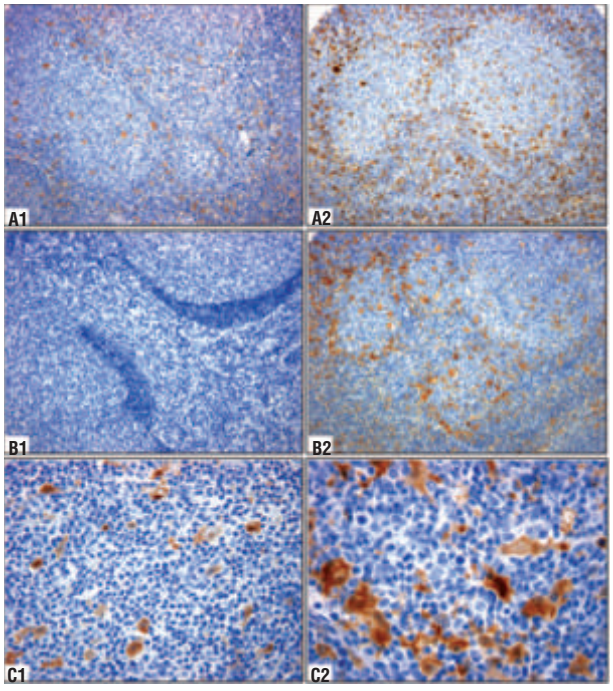

Figure 3. Selected immunohistochemical features of FL lymph-node samples. A1. Scarce CD68-positive cells. A2. Abundant CD68-positive cells. In both cases, the majority of the CD68-positive cells are located outside the neoplastic follicles. B1. A STAT1-negative case. B2. A STAT1-positive case. The positive cells are mainly distributed within the neoplastic follicles but some cells are also present outside them, either at the edge or in the interfollicular area. Tumor cells are negative. C1. Cytological detail of the STAT1-positive cells in the interfollicular area, or (C2) inside the tumoral follicles. Positive cells had a larger nucleus and cytoplasm and more prominent cytoplasmic prolongations.

cytoplasm but also with nuclear staining, had a different distribution in that there were more positive cells inside the follicles, particularly at the germinal-center edge of the tumoral follicles. Cytological observations indicated that the majority of STAT1-positive cells outside the follicles were smaller and more regularly shaped than those present inside the tumoral follicles, where the cells had a more voluminous nucleus and cytoplasm and longer cytoplasmic prolongations.

To identify which types of cell express STAT1 protein, double staining for CD68/STAT1 and CD21/STAT1 was performed on representative FL cases, which yielded information on the presence of macrophages and follicular dendritic cells, respectively. As can be seen in Figure 4, STAT1 was expressed by CD68-positive macrophages but not by CD21-positive dendritic cells. This finding was also confirmed by the existence of a statistically significant association between the number of CD68-positive macrophages and the degree of STAT1 expression (Mann-Whitney U=1440.0, $p<0.001$).

The presence of STAT1-positive macrophages was not restricted to neoplastic follicles. In the light of this finding, we evaluated the presence and localization of STAT1 in tonsils and lymph nodes with reactive follicular hyperplasia. Immunohistochemical staining revealed the occasional presence of STAT1-positive macrophages.

These were mainly located in interfollicular areas, with STAT1-positive macrophages being only rarely present within germinal centers (*data not shown*).

Regarding the individual clinical and tumoral features in this FL series, significantly more CD68-positive macrophages were detected in patients without bone marrow involvement (mean 64.7±48.3 vs. 48.6±40.1 cells/field; $p=0.021$), with a low Ann Arbor stage (mean 73.0±45.7 vs. 53.3±44.1 cells/field; $p=0.004$) and also with histological grade 3 disease (mean 71.5±44.7 vs. 52.0±44.3 cells/field; $p=0.002$). No significant differences

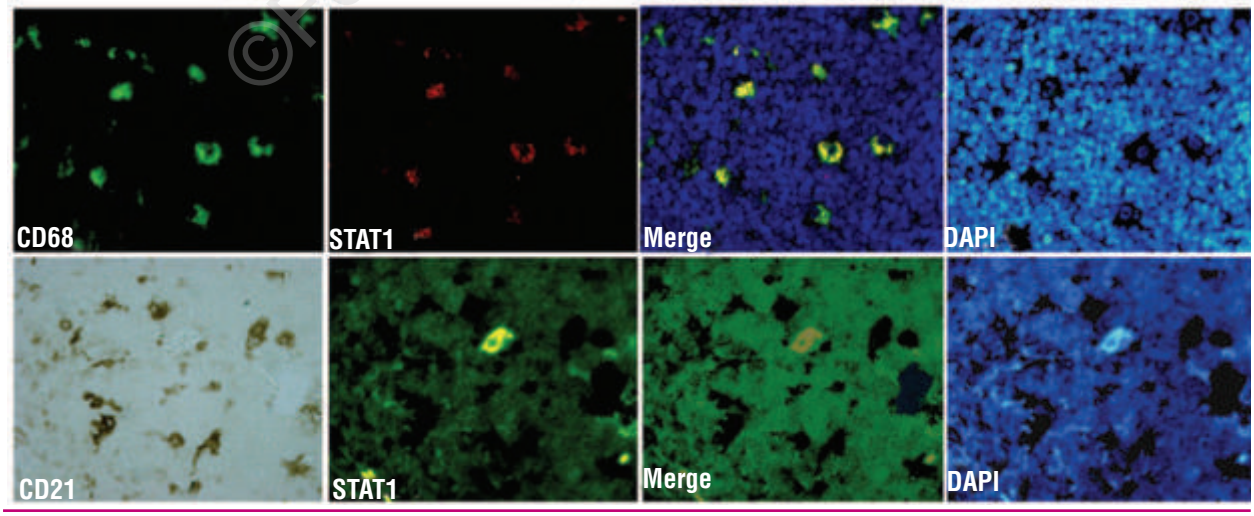

Figure 4. Double staining of CD68/STAT1 and CD21/STAT1 in paraffin-embedded FL-tissue sections. *Upper panel*: double immunofluorescence analyses demonstrate co-localization of CD68 staining (in green) with STAT1 staining (in red). *Lower panel*: double histochemical and fluorescence analyses demonstrate the absence of co-localization of CD21 DAB staining (in brown) with STAT1 (in green).

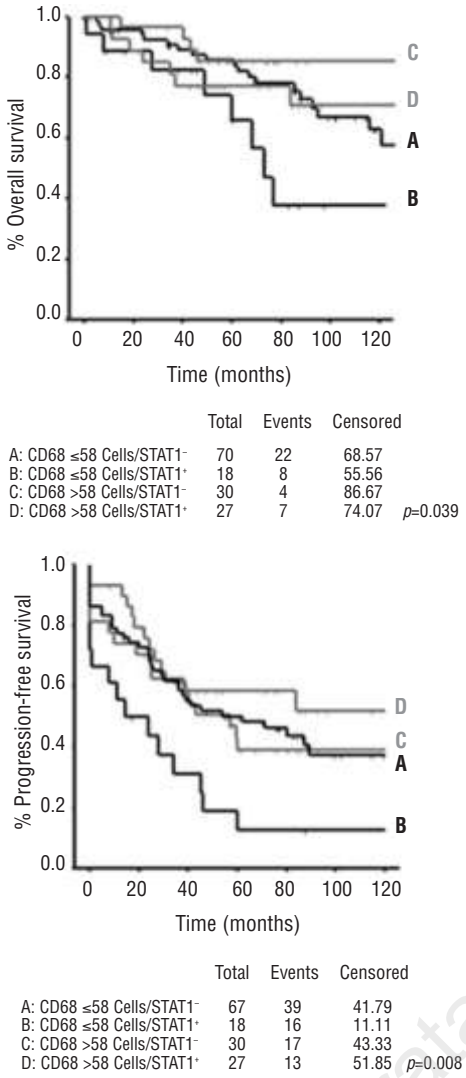

Figure 5. Overall survival and progression-free survival in patients with FL in relation to the composite CD68/STAT1 phenotype. The Kaplan-Meier survival curves indicate the significant effect of STAT1 expression on OS and PFS, in relation to the level of infiltrated CD68-positive macrophages of FL. The significance of the difference in survival of the groups was determined by the log-rank test.

in the number of infiltrating STAT1-positive cells were detected in the different groups of patients.

Prognostic significance of STAT1 expression combined with the presence of CD68-positive macrophages and the FLIPI

In order to analyze survival, the clinical and immunohistochemical factors were considered according to established dichotomized values. According to Kaplan Meier curve analyses, the optimal cut-off value for the level of CD68-positive macrophage was the mean value (58 cells/field). When the two immunohistochemical markers for macrophages level and STAT1 expression were analyzed separately, no statistically significant differences in the survival of patients was observed, although cases with few infiltrated macrophages (≤58 cells/field) and those that expressed STAT1 protein tended to have shorter OS and PFS (*data not shown*). Considering combinations of these two markers gave a more accurate classification of FL patients into four groups with statistically significant differences in their survival. Univariate Kaplan–Meier analyses of OS and PFS curves indicated that STAT1-positive cases generally had a poorer survival (p=0.039 and p=0.008 for OS and PFS, respectively). When these four groups were analyzed separately, a statistical difference was observed only in patients with a low infiltration of CD68-positive macrophages (Figure 5; A versus B; OS, p=0.028 and PFS, p=0.004). Additionally, the combined influence of FLIPI with the expression of STAT1 enabled differences in the global outcome of patients to be distinguished (p<0.000 and p=0.026 for OS and PFS, respectively).

In particular, cases expressing STAT1 and with an intermediate FLIPI score had a significantly lower OS than those not expressing STAT1 (p=0.011). No significant differences in PFS were observed in relation to the individual parameters of FLIPI and STAT1 expression.

A multivariate Cox model including CD68-positive cells (a dichotomized variable with ≤58 or >58 positive cells/field), STAT1 protein expression (positive or negative), and the FLIPI was fitted. Positive STAT1 expression was shown to be a strong predictor of adverse clinical course, given the OS (RR=3.47, 95% CI=1.43-8.39, p=0.006), and intermediate- and high-risk FLIPI groups were shown to be negative prognostic predictors for OS (RR=4.53, 95% CI=1.46-14.10, p=0.009 and RR=7.09, 95% CI=1.90-26.45, p=0.004, respectively), and also for PFS (RR=2.05, 95% CI=1.14-3.70, p=0.017 and RR=1.49, 95% CI=1.02-4.67, p=0.043, respectively).

Discussion

In this study, we analyzed the presence of macrophages and the expression of STAT1 protein in a large number of FL cases. Our results confirm the presence of CD68-positive macrophages in this disease and indicate that approximately 30% of the cases express STAT1 protein. The survival rates in patients whose tumors contained STAT1-positive macrophages tended to be significantly lower. The clinicopathological data of our cohort of FL patients are consistent with those in the literature, although patients with intermediate and high risk, as determined by the FLIPI, have virtually identical survival. There appears to be a slight discrepancy between our results and those of the two previous studies on tumor-infiltrating immune cells in FL patients.[12,15] This may reflect a step forward in the identification of functionally relevant subsets of macrophages that play a

role in the control of immune surveillance. Indeed, in the gene-profiling study by Dave and colleagues, genes that were highly and/or preferentially expressed in macrophages were present in both the immune response 1 signature and the immune response 2 signature, which predicted, respectively, a favorable and unfavorable outcome in FL patients.[12] In a more recent immunohistochemical study by Farinha and colleagues, the presence of a large number of macrophages predicted an unfavorable outcome for FL patients,[15] although specific subsets of macrophages were not investigated. The discrepancy with our results could additionally have derived from the restrictive criteria used for case inclusion: patients older than 60 years were excluded and relatively few high-grade cases were included. An additional source of variability could have been the different cut-offs used in the studies; the cut-off in our study was the mean of the expression, while Farinha *et al*. used a cut-off that separated the series into two groups composed of 12 and 87 patients.[15] The STAT1 pathway is known to play a key role in immune anti-tumor signaling.[19] The cytological characteristics of cells expressing STAT1, which is both a nuclear and cytoplasmic mediator, with prolongations and a dendritic-like morphology raise the possibility that these cells are macrophages, dendritic cells, or both. Double-staining analyses for both STAT1 and CD21, as a marker of follicular dendritic cells, and for both STAT1 and CD68, as a marker of macrophages, confirmed the co-localization of STAT1 with CD68 (but not with CD21), and subsequently identified these cells as macrophages. Regardless of their position in the tissue, CD68-positive macrophages that did not co-express STAT1 protein appeared to be separated from the tumor and were evenly distributed in the stroma, whereas CD68-positive macrophages that co-expressed STAT1 protein were located more specifically inside the neoplastic follicles and characteristically identified the edge of the tumoral germinal centers. It is possible that gene-expression data derived from these STAT1-positive macrophages were included in the immune response 2 signature defined in the study by Dave and colleagues.[12]

These observations suggest that STAT1 is differentially expressed in macrophages, which may merely reflect an activation stage that distinguishes a subset of TAM or a specific differentiation towards immunomodulatory cells. The preliminary results obtained with some reactive lymph-node cases indicated the presence of only scattered STAT1-positive cells preferentially located outside the reactive follicles. In the context of tumors, available evidence suggests that macrophages recruited at tumor sites and defined as TAM are reprogrammed to induce immune suppression of host *in situ* defenses.[16,18,27] The presence and clinical relevance of the STAT1-expressing macrophages, as observed in our study, suggest that these cells could represent reprogrammed TAM, also described as polarized M2 phenotype macrophages.[27-32] The various cytokines, interleukins and chemokines present in the microenvironment of the tumor appear to be crucial determinants of the process of differentiation towards TAM or dendritic cells and their relative distribution.[27,33]

In the case of FL, a specific Th2-like inflammatory environment might be responsible for the appearance and concentration of TAM in the tumor. Several groups have reported the important role that STAT1 plays in the regulation of inducible nitric oxide synthase activity, an important mediator of cytotoxic functions of TAM.[34] The factors or mechanisms responsible for STAT1 upregulation in TAM remain uncertain because the regulation of interferon signaling through the Jak-STAT pathway is complex and occurs at several levels within the cells.[35,36] Together with our results, these findings suggest that STAT1 expression is a possible marker for increased immune-suppressor activity. The importance of STAT1 expression by TAM is underscored by its prognostic significance in different diseases.[37-42] In FL, the prognostic value of STAT1 is highlighted when its expression is combined with the level of CD68-positive macrophages and with the FLIPI. Kaplan-Meier curves clearly demonstrated that patients with STAT1 expression and low infiltration of macrophages have reduced OS and more frequent disease progression than do patients with STAT1-negative tumors.

Although STAT1 expression alone did not prove to be of prognostic significance for survival in the Kaplan Meier analysis, it aquired a highly significant prognostic value in the multivariate analysis in combination with the FLIPI. Although infrequent, this situation could arise and demonstrates that the unfavorable prognostic impact of STAT1 expression can be regarded as independent of the conventional FLIPI. Thus, these results support the interpretation that STAT1-expressing TAM play a role in determining the outcome of FL patients.

In conclusion, this study has demonstrated the presence of STAT1-positive cells in the microenvironment of FL samples, which turned out to be a subset of TAM. The presence of STAT1-positive TAM was associated with an adverse outcome, indicating the importance of non-tumoral cells in the control of the growth and survival of lymphoma cells. Further studies on the functional relevance and the mechanisms of action of these STAT1-positive cells could help us to understand the cross-talk between FL and the microenvironment, and to design new therapeutic strategies.

TÁ: conceived, designed, and supervised the study and reviewed successive drafts of the manuscript; ML was responsible for the immunohistochemical validation of the immune markers, data acquisition, and prepared the final manuscript; FIC was responsible for the construction of the tissue microarrays (TMA) of the FL samples; LS was responsible for the double-immunofluorescence technique; MTS was responsible for producing the final database and for statistical analyses; CL was responsible for quantifying the immune markers of all the TMA; JFG and MAP

contributed to the design of the project, data analysis and revision of the manuscript; RB, JJ and LP participated in the development of the study protocol, and played a role in the immunohistochemical techniques; CB, CM and RA were involved in the design of the project, case revision and clinical data collection. All authors reviewed and approved the final version. The authors declare that they have no potential conflict of interest.

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