VOLUME 27 · NUMBER 9 · MARCH 20 2009

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

High Numbers of Tumor-Infiltrating Programmed Cell Death 1–Positive Regulatory Lymphocytes Are Associated With Improved Overall Survival in Follicular Lymphoma

Joaquim Carreras, Armando Lopez-Guillermo, Giovanna Roncador, Neus Villamor, Lluis Colomo, Antonio Martinez, Rifat Hamoudi, William J. Howat, Emili Montserrat, and Elias Campo

From the Hematopathology Section, Departments of Pathology and Hematology, Hospital Clinic, Institut d'Investigacions Biomèdiques August Pi-Sunyer, University of Barcelona, Barcelona; Centro Nacional de Investigaciones Oncológicas, Madrid, Spain; Division of Molecular Histopathology, Department of Pathology, University of Cambridge, Cambridge; and Histopathology/In Situ Hybridization Facility, Cancer Research UK, Cambridge Research Institute, Li Ka Shing Centre, Cambridge, United Kingdom.

Submitted May 11, 2008; accepted October 27, 2008; published online ahead of print at www.jco.org on February 17, 2009.

Supported by the Spanish Ministry of Education and Science (Grant No. SAF 05/5855), Spanish Ministry of Health (Grant No. PI070409), and Instituto de Salud Carlos III, Red Temática de Investigación Cooperativa en Cáncer (Grant No. 2006-RET2039-O).

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Armando López-Guillermo, MD, Department of Hematology, Hospital Clinic, IDIBAPS, Villarroel 170, 08036-Barcelona, Spain; e-mail: alopezg@clinic.ub.es.

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

0732-183X/09/2709-1470/$20.00

DOI: 10.1200/JCO.2008.18.0513

ABSTRACT

Purpose
Tumor microenvironment influences the behavior of follicular lymphoma (FL), although the specific cell subsets involved are not well known. The aim of this study was to determine the impact of programmed cell death 1 (PD-1) –positive inhibitory immunoregulatory lymphoid cells in the clinicobiologic features and outcome of patients with FL.

Patients and Methods
We examined samples from 100 patients (53 men and 47 women; median age, 54 years) at diagnosis, as well as in 32 patients at first relapse, with a recently generated monoclonal antibody against PD-1. The cells were quantified using computerized image analysis. Additional analysis consisted of double immunofluorescence and flow cytometry.

Results
PD-1 expression was alternative to FOXP3 in lymphoid cells from both reactive tonsils and FL. At diagnosis, the median percentage of PD-1–positive cells was 14% (range, 0.1% to 74%). Patients with grade 3 FL, poor performance status, and high serum lactate dehydrogenase showed lower numbers of PD-1–positive cells. After a median follow-up of 6.2 years, patients with PD-1–positive cells ≤ 5% (n = 25), 6% to 33% (n = 50), and more than 33% (n = 25) had a 5-year progression-free survival rate of 20%, 46%, and 48% ($P = .038$) and overall survival (OS) of 50%, 77%, and 95% ($P = .004$), respectively. PD-1 and FL International Prognostic Index maintained prognostic value for OS in multivariate analysis. Patients with PD-1–positive cells ≤ 5% showed a higher risk of histologic transformation. At that time, transformed diffuse large B-cell lymphomas had lower percentage of PD-1–positive cells than FL.

Conclusion
A high content of PD-1–positive cells predicted favorable outcome of FL patients, whereas a marked reduction is observed in transformation.

J Clin Oncol 27:1470-1476. © 2009 by American Society of Clinical Oncology

INTRODUCTION

Follicular lymphoma (FL) follows an indolent clinical evolution, with 7 to 10 years median survival, although it is individually heterogeneous, with alternating relapses and remissions. In 10% to 15% of the patients, it develops aggressively, with poor therapy response or transformation to diffuse large B-cell lymphoma (DLBCL), leading to short survival.[1] The FL International Prognostic Index (FLIPI)[2,3] has helped to stratify patients in several risk groups with different clinical evolution.

Gene expression profile studies and immunophenotyping characterization of nonneoplastic cells in FL have emphasized the role of microenvironment in clinical behavior and therapy response.[4-12] Expression data identified two immune response (IR) signatures: IR-1, enriched in T-cell and monocyte-restricted genes, conferred favorable prognosis, whereas IR-2 predicted poor outcome and included activated macrophages-dendritic genes.[7] Immunophenotypic studies showed that high numbers of macrophages may confer unfavorable evolution,[8] whereas high content of $CD8^+$ and $CD4^+$ lymphocytes endows more limited disease and prolonged survival.[4,10,12] Conversely, high numbers and follicular localization of FOXP3-positive regulatory T cells (Tregs) are correlated with an improved outcome in FL.[6,11]

In addition to Tregs, other mechanisms may regulate the germinal center (GC) microenvironment and FL pathogenesis. Programmed cell death 1

(PD-1), a member of the CD28 receptor family expressed in lymphoid cells, has an important function in inhibitory pathways, attenuating T-cell responses.[13,14] The role of cells expressing this receptor in tumor immunity is not well known, but it seems that they may facilitate cancer immune evasion.[14] PD-1 expression in lymphoid tissues is preferentially identified in follicular GC T cells, suggesting an involvement in the FL pathogenesis.[15,16] In this study, we have examined a series of patients with FL at diagnosis and relapse to determine the role of PD-1 in its progression and outcome.

PATIENTS AND METHODS

Patients and Samples

One hundred available diagnostic biopsies from 225 patients with FL diagnosed in a single institution between 1988 and 2005 were included, reviewed, and reclassified according to the WHO classification.[17] The median age of the patients was 54 years (range, 26 to 88 years); 53 patients were men and 47 patients were women. The main initial features of the patients are listed in Table 1. FLIPI[3] was retrospectively assessed in 89 patients: low risk, 35 cases (39%); intermediate risk, 23 cases (26%); and high risk, 31 cases (35%).

Staging was performed according to standard methods. Eighty patients received combination chemotherapy (62 patients received cyclophosphamide, doxorubicin, vincristine, and prednisone or similar regimens, and 18 patients received fludarabine, cyclophosphamide, and mitoxantrone), six patients received alkylating monotherapy, and three patients received radiotherapy. In 11 cases (patients $\geq$ 65 years with no high tumor burden), a watchful waiting policy was established. Twenty-two patients received also rituximab. Post-therapy restaging consisted of the repetition of the previously abnormal tests and/or biopsies.

Response was assessed according to conventional criteria.[18] Among the 89 patients with assessable response, 51 patients (57%) achieved a complete response, 31 patients (35%) achieved a partial response, and seven patients (8%) did not respond to treatment. After a median follow-up of 6.2 years (range, 0.4 to 15.7 years) for surviving patients, 36 patients had died. The 5- and 10-year overall survival rates (OS) were 76% (95% CI, 66% to 86%) and 52% (95% CI, 39% to 65%), respectively (Appendix Fig A1, online only). Eleven patients experienced histologic transformation during the follow-up, with a risk of transformation of 12% (95% CI, 4% to 20%) at 5 years.

Sequential biopsies at relapse were available in 15 patients. Moreover, samples at relapse from another 17 patients with FL were also examined.

The study was approved following the institutional guidelines of the ethical committee.

Table 1. Main Clinical and Histopathologic Features of 100 Patients With Follicular Lymphoma at Diagnosis

Characteristic	No.	%
Age older than 60 years	38	38
Male sex	53	53
Histologic grade		
1	20	20
2	59	59
3	21	21
Architectural pattern		
Follicular	93	93
Diffuse	7	7
"B" symptoms	17	20
Nonambulatory performance status (ECOG > 1)	15	16
Bulky disease	13	14
Stage IV disease	71	73
Bone marrow involvement	61	65
High serum LDH*	19	21
High serum β_2-microglobulin*	28	35
FLIPI*		
Low risk	35	39
Intermediate risk	23	26
High risk	31	35

Abbreviations: ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; FLIPI, Follicular Lymphoma International Prognostic Index.
*LDH, β_2-microglobulin, and FLIPI were available in 91, 81, and 89 patients, respectively.

Immunohistochemistry and Quantitative Assessment, Immunofluorescence, and Flow Cytometry

Paraffin-embedded whole-tissue sections were immunostained using undiluted hybridoma supernatant of anti–PD-1 (NAT105) murine monoclonal antibody as previously described.[16] Eighty-seven cases previously studied for FOXP3 were included in this series.[6] Follicular PD-1–positive cells were quantified using an automated scanning microscope and computerized image analysis system, always under pathologist visual supervision, as previously described[6] (Ariol SL-50; Genetix Ltd, Queensway, New Milton, Hampshire, United Kingdom).

Double immunofluorescence labeling of tissue sections for PD-1 in combination with FOXP3 was performed as previously described[19] in five reactive tonsils and 10 FLs. PD-1 expression was also assessed by flow cytometry in cell suspensions from five reactive tonsils and seven FLs using unlabeled anti–PD-1 monoclonal antibody, antimouse immunoglobulins coupled to phycoerithrin or allophycocyanin, and directly labeled monoclonal antibodies for different lymphocyte subpopulations, including CD57, CD4, CD8, CD25, CD56, and CD20. At least 50,000 cells were acquired in a FACScalibur flow cytometer, and the data were analyzed using the FACSDiva software (Pharmingen, Becton Dickinson, San Jose, CA). A more detailed methodology is provided in the Appendix (online only).

The expression of the PD-1 ligand PD-L1 (CD274) was assessed by flow cytometry in B and T cells using a phycoerythrin-labeled anti–PD-L1 antibody (Pharmingen) in cell suspensions from seven nodal follicular hyperplasias and five FLs.

Statistical Analysis

The main initial and evolutive variables were analyzed for prognostic significance. PD-1–positive cells were analyzed as a continuous variable, except when a threshold was necessary to draw survival curves. Categoric data were compared using χ^2 or Fisher's exact test two-sided P values, whereas for ordinal data, nonparametric tests were used. Bonferroni correction for multiple comparisons was applied when necessary. The multivariate analysis of the variables predicting response was performed by logistic regression. Kaplan-Meier actuarial survival analysis was performed, and the curves were compared using the log-rank test. The independent prognostic value of PD-1, FOXP3-Tregs, and clinical data summarized in the FLIPI was tested by means of stepwise proportional hazards Cox model. The definition of OS and progression-free survival (PFS) were the standard.[18]

RESULTS

Clinical and Histologic Features of the Patients at Diagnosis

The main clinical and histologic features of the 100 patients at diagnosis are detailed in Patients and Methods and summarized in Table 1. The histologic distribution was grade 1 in 20 cases, grade 2 in 59 cases, and grade 3 in 21 cases (20 grade 3a and one 3b). The architectural pattern was follicular (follicular area > 75%) in 93 cases and predominantly diffuse (follicular area < 25%) in seven cases.

PD-1–Positive Cells in FL at Diagnosis

PD-1–positive cells were mainly observed in the follicular areas, whereas in the interfollicular compartment they were infrequent. The

mean proportion of PD-1–positive cells in the 100 diagnostic biopsies was 21.8% (standard deviation [SD] 19.6%; range, 0.12% to 73.6%). Overall, the percentiles 25, 50, and 75 were 5.4%, 14.3%, and 33.5%, respectively (Fig 1). Seven patients had a count less than 1%.

The number of PD-1–positive cells in the follicular compartment was related to the main histopathologic characteristics (Table 2). Patients with grade 3 FL had fewer PD-1–positive cells than patients with low-grade FL. No difference was found between follicular and diffuse patterns. One patient showed a DLBCL component at diagnosis; the number of PD-1–positive cells was lower in the DLBCL than in the low-grade FL area (0.99% *v* 16.3%). Finally, PD-1 was also evaluated in five reactive tonsils, and percentages were similar than in FL (mean ± SD: tonsils, 30.6% ± 17.6%; FL, 21.8% ± 19.7%; $P > .1$).

Characterization of PD-1–Positive Cells and Relationship to FOXP3-Positive Tregs

A significant correlation between PD-1 and FOXP3-positive cells was found by nonparametric tests, both in the total and in the follicular compartment (correlation coefficient = 0.375 and 0.447, respectively; $P < .001$). The relationship between categoric FOXP3-Tregs and quantitative PD-1 is shown in Table 2. Double immunostaining was performed to determine whether PD-1 was expressed in FOXP3-Tregs in five reactive tonsils and 10 FLs. These markers were expressed in different types of cells. Double-positive cells were rare (Fig 2A and 2B). PD-1–positive cells were further characterized by flow cytometry. In tonsils, the mean value (± SD) of PD-1–positive cells was 22.9% ± 10.9%. The majority of them expressed $CD4^+$ (mean ± SD, 70.9% ± 12.3%). This population was enriched in $CD4^+CD57^+$ lymphocytes (mean ± SD, 2.6% ± 1.25% of all tonsil lymphocytes *v* 12.2% ± 2.2% of PD-1–positive tonsil cells; $P = .05$). Besides, more than 90% (mean ± SD, 94.4% ± 5.7%) of the $CD4^+CD57^+$ subpopulation expressed PD-1, constituting a defined population as a result of its high PD-1 expression (Fig 2C). CD25 expression was similar in PD-1–positive and PD-1–negative lymphocytes. In FL samples, the mean value of PD-1–positive lymphocytes (mean ± SD, 21.8% ± 13%) and their relationship to CD4 and CD57 expression was similar to the numbers observed in tonsils.

The ligand PD-L1 was virtually negative in $CD20^+$ cells from reactive or tumor samples (median, 2.4%; range, 0% to 4%), but it was present in a small fraction of $CD3^+$ cells (median, 9%; range, 2.4% to 29%) in both reactive and tumor samples, without differences between $CD4^+$ and $CD8^+$ lymphocytes (Appendix Fig A2, online only).

Table 2. Distribution of PD-1–Positive Cells in 100 Patients With FL at Diagnosis According to Histopathologic Features

Feature	No.	PD-1–Positive Cells, % Mean	SD	*P*
Grade of FL				
1	20	28.3	22	
2	59	22.9	19	
3	21	12.2	17	.003
Architectural pattern				
Follicular	93	21.1	19	
Diffuse	7	30.2	29	> .1
FOXP3-positive Tregs, %				
< 5	16	9.7	14	
5-10	27	16.7	17	
> 10	44	26.9	21	.002
Total	100	21.8	20	

Abbreviations: PD-1, programmed cell death 1; FL, follicular lymphoma; Tregs, regulatory T-cells.

Relationship Between the Number of PD-1–Positive Cells, Clinical Features, and Outcome

PD-1–positive cells were related to the main clinical features of the patients at diagnosis (Table 3). The number of PD-1–positive cells was significantly lower in patients with poor performance status (Eastern Cooperative Oncology Group ≥ 2) and high serum lactate dehydrogenase (LDH).

Patients were treated independently of the number of PD-1–positive cells, and there was no correlation between the type of therapy, overall response or complete response rate, and PD-1–positive cells. Fifty-three of 89 patients treated after diagnosis eventually experienced progression during the follow-up period, with a 5-year PFS rate of 40% (95% CI, 29% to 51%; Appendix Fig A1A). Variables predicting poor PFS were nonambulatory performance status, bone marrow involvement, high serum LDH, and intermediate-risk/high-risk FLIPI. Patients with FOXP3-Tregs less than 10% also had poor PFS. Moreover, PFS was calculated according to PD-1. Five-year PFS rates were 20% (95% CI, 2% to 38%), 46% (95% CI, 30% to 64%), and 48% (95% CI, 26% to 70%) for patients with PD-1 ≤ 5%, 6% to 33%, and more than 33%, respectively ($P = .038$; Fig 3A).

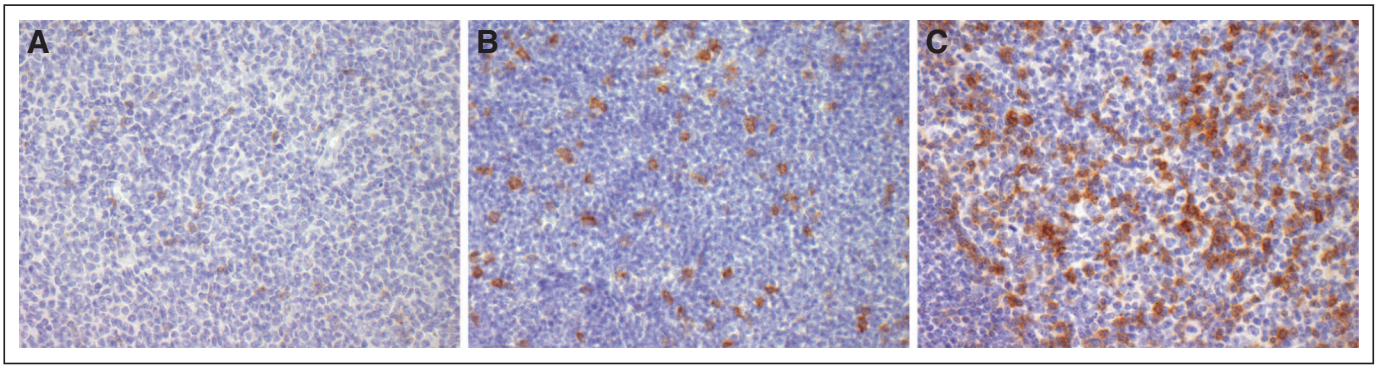

Fig 1. Immunohistochemical staining of programmed cell death 1 in follicular lymphomas. The number of positive cells in the tumors was variable with cases showing (A) ≤ 5%, (B) 6% to 33%, and (C) more than 33% positive cells.

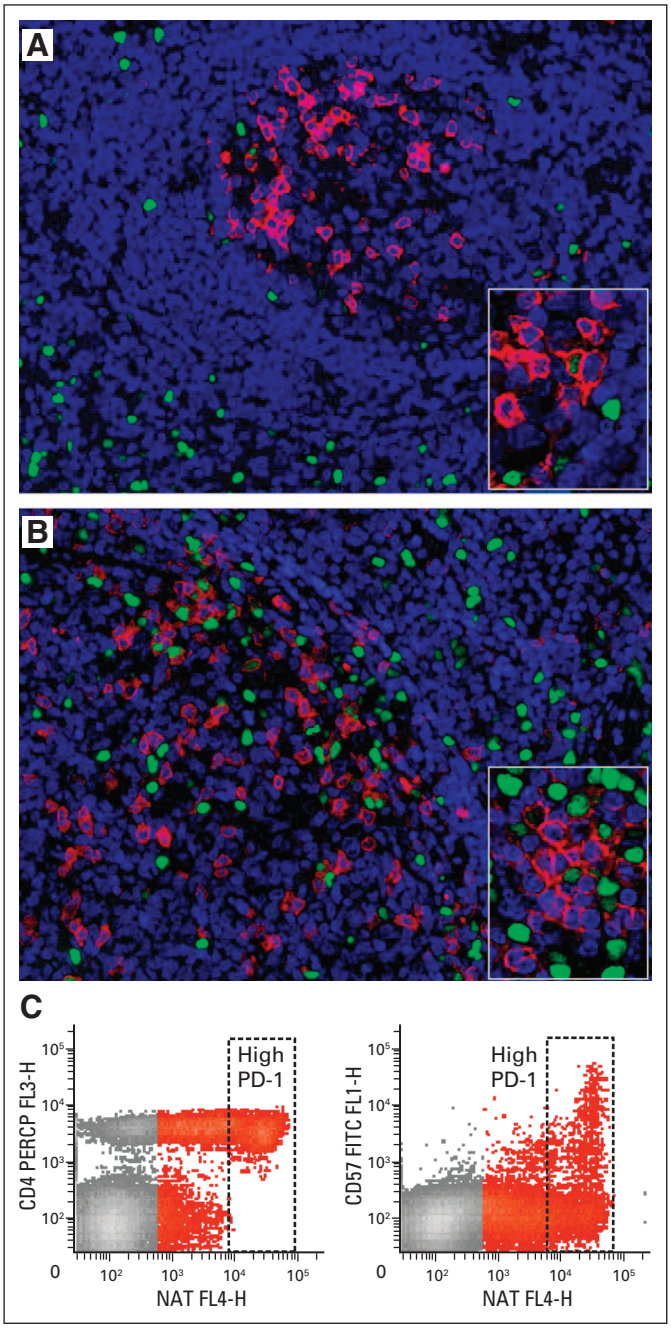

Fig 2. Characterization of programmed cell death 1 (PD-1) –positive lymphocytes. Double immunofluorescence of (A) tonsil and (B) follicular lymphoma (FL; PD-1, red; FOXP3, green). (C) FL flow cytometry showing that most PD-1–positive cells were $CD4^+$ and that virtually all high PD-1–positive cells belonged to the $CD4^+$ subpopulation (right dot plot). In left dot plot, the characteristic high PD-1 in the $CD57^+$ lymphocytes is displayed. PERCP, peridinin-chlorophyll-protein complex; FITC, fluorescein isothiocyanate.

Table 3. Correlation Between Main Clinical Features of Patients With FL and PD-1–Positive Cells in Diagnostic Samples

Feature	No.	PD-1–Positive Cells, % Mean	SD	*P*
Age, years				
< 60	62	22.8	19	
≥ 60	38	20.1	19	> .1
Sex				
Female	47	21.5	20	
Male	53	22.0	19	> .1
ECOG performance status				
< 2	81	23.0	19	
≥ 2	15	12.1	14	.014
Ann Arbor stage				
I to III	37	21.5	19	
IV	60	21.3	20	> .1
Serum LDH				
Normal	72	24.0	19	
High	19	11.9	17	.001
Serum β_2-microglobulin, mg/L				
< 3	60	23.9	19	
≥ 3	21	14.8	18	.06
FLIPI				
Low/intermediate	58	23.9	20	
High	31	17.7	19	.09

Abbreviations: FL, follicular lymphoma; PD-1, programmed cell death 1; SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; FLIPI, Follicular Lymphoma International Prognostic Index.

Thirty-six patients died during the follow-up, with a 5-year OS rate of 76% (95% CI, 66% to 86%). Variables predicting poor OS were age more than 60 years ($P < .001$), presence of "B" symptoms ($P = .002$), nonambulatory performance status (Eastern Cooperative Oncology Group ≥ 2; $P < .001$), hemoglobin less than 12 g/dL ($P = .005$), high serum LDH ($P < .001$), and serum β_2-microglobulin more than 3 mg/L ($P = .001$). Grade 3 FL ($P = .001$) and low FOXP3-Tregs ($P = .009$) also had negative impact on OS. FLIPI score divided the series in three groups with median OS of 4.5 years, 9.0 years, and not reached (Appendix Fig A1B). OS according to PD-1–positive cells is plotted in Figure 3B. The 5-year OS rates of patients with PD-1 ≤ 5%, 6% to 33%, and more than 33% were 50% (95% CI, 30% to 70%), 77% (95% CI, 64% to 90%), and 95% (95% CI, 85% to 100%), respectively ($P = .004$). Particularly, six of seven cases with PD-1 less than 1% eventually died, with a median OS of 2.6 years.

To analyze which of the two variables related to T-cell microenvironment were more important to predict OS, PD-1 and FOXP3 were included in a Cox model, with only PD-1 retaining prognostic interest for OS. Finally, to determine whether the prognostic value of PD-1 was independent of clinical variables, a multivariate Cox analysis was performed, including the number of PD-1–positive cells (≤ 5% *v* 6% to 33% *v* > 33%) and FLIPI score (low *v* intermediate *v* high). In the final model, with 88 assessable cases, PD-1 maintained its predictive value (relative risk, 0.31; $P = .013$) along with FLIPI (relative risk, 3.66; $P < .001$).

The risk of histologic transformation was analyzed according to the number of PD-1–positive cells. In 25 patients with PD-1 ≤ 5%, the transformation risk was significantly higher than in the remainder of patients (5-year risk of transformation, 29% [95% CI, 7% to 51%] *v* 7% [95% CI, 1% to 13%], respectively; $P = .05$).

PD-1–Positive Cells in FL at First Relapse

Thirty-two patients had an available sample at relapse (FL grades 1 to 2, 20 patients; grade 3, seven patients; transformation to DLBCL, five patients). The number of PD-1–positive cells was similar between

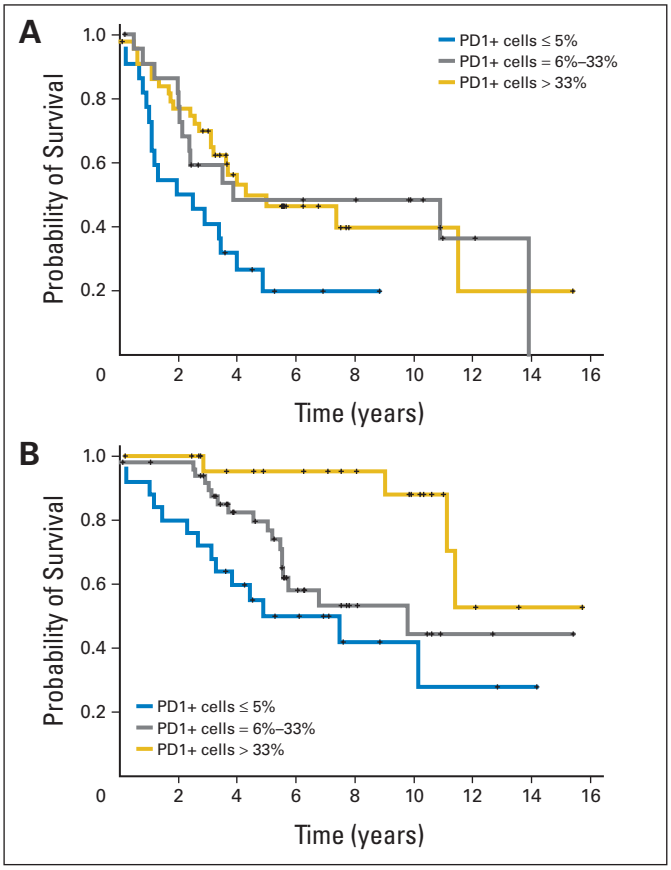

Fig 3. Outcome of the patients with follicular lymphoma (FL) according to the number of programmed cell death 1 (PD-1) –positive cells. (A) Progression-free survival of 89 patients treated for FL. (B) Overall survival of 100 patients with FL.

diagnosis and relapse sample when both corresponded to grade 1 to 2 FL (diagnosis *v* relapse, 24.3% ± 20% *v* 19.8% ± 20%) or grade 3 FL (13.2% ± 17% *v* 20.6% ± 18%).

We studied 10 FLs transformed to DLBCL, one at diagnosis concomitantly with a low-grade FL, five at first relapse, and four at subsequent relapses. The PD-1–positive cells in the DLBCL were significantly lower (mean, 1.04%) than in FL either at diagnosis or relapse ($P < .01$).

Finally, in six transformed cases, a sample was available for PD1 determination both at diagnosis and transformation. PD-1–positive cells in this particular subset was 12.5% ± 13.7% at diagnosis versus 1.4% ± 1.8% at time of transformation to DLBCL ($P = .08$).

DISCUSSION

In this study, we demonstrated that the number of PD-1–positive cells infiltrating the tumor is a predictor of survival in patients with FL, independently of the FLIPI. We also found that PD-1–positive cells decrease when transformation to DLBCL occurs. These results suggest that PD-1 is identifying an important subset of regulatory cells in the microenvironment of FL that may participate in the modulation of the tumor cell behavior and consequently influence clinical evolution.

PD-1 is a member of the CD28 family of membrane receptors that have an important function as attenuating regulators of the T-cell activation and promoters of T-cell tolerance.[13,14,20] PD-1 induces a negative regulation of T-cell receptor–mediated proliferation and cytokine production.[21,22] Its negative immunomodulator role is highlighted by experimental observations indicating that lack of PD-1 is associated with hyperimmune disorders.[14] Interestingly, PD-1 participates also in the regulation of the immune response against cancer cells and may represent an element that facilitates the evasion of tumor cells by the immune system.[23,24] Thus high numbers of PD-1–positive cells in renal cancer is associated with more advanced disease and shorter survival.[25] The negative immune modulation of this T-cell subpopulation in solid tumors seems to be mediated by the expression of its ligand PD-L1 by carcinoma cells, which is also correlated with poor prognostic parameters.[13,14,23,25] In contrast to these observations in solid tumors, we found no expression of PD-L1 in nonneoplastic or tumor FL B cells. Although PD-1 ligands have been detected in Hodgkin's lymphoma cells, our observation is concordant with the lack of expression observed previously in a short series of B-cell lymphomas and B-cell lymphoma cell lines.[26,27]

Our results indicate that a high number of PD-1–positive tumor infiltrating lymphocytes is a protective factor in the evolution of patients with FL. These results parallel the observations on the apparent different role of Tregs in carcinomas and lymphoid neoplasms. Thus high Tregs in solid tumors have been correlated with poor prognosis,[28,29] whereas high Treg content in FL and Hodgkin's lymphoma were associated with improved patient survival.[4,6,30] All these observations suggest that the role of immune-inhibitory pathways mediated by Tregs and PD-1–positive lymphocytes have a different pathogenetic effect in B-cell neoplasms and solid tumors.

The relatively similar results obtained in the present study on PD-1 and in our previous analysis of Tregs in FL prompted us to examine whether PD-1 was expressed in Tregs in human lymphoid tissues. Previous studies have shown that PD-1 is mainly expressed in follicular GC $CD3^+$T-cells, particularly in the $CD4^+CD25^-$ subset, whereas it is negative in B cells, macrophages, and dendritic cells.[16,31] In this study, we have confirmed these observations and showed the mutually exclusive expression of PD-1 and FOXP3 in both reactive tonsils and FL tissues, indicating that Tregs are virtually negative for PD-1. This finding is concordant with the absence of PD-1 in $CD4^+CD25^+$ cells previously observed.[22,27,31,32]

The similar impact on the clinical behavior of FL patients of these two different subsets of T cells involved in negative regulatory pathways of T-cell responses is concordant with the hypothesis that the activated status of the immune microenvironment in FL is an important biologic factor.[10,32,33] Microarray studies have shown that FLs rapidly transforming to DLBCL have an expression profile at diagnosis enriched in genes highly expressed in activated hyperplastic lymphoid tissues.[10] Similarly, Bohen et al[5] found that FL that did not respond well to rituximab therapy had an expression profile signature at diagnosis similar to that of hyperplastic tonsil and contained genes related to an activated immune microenvironment that could facilitate the growth of tumor cells. Immunohistochemical studies have also shown that FL with rapid transformation to DLBCL had a significantly higher number of activated T cells expressing CD69 than tumors without transformation.[10] Taken together, all these observations would be concordant with the idea that the lower numbers of negative immune-regulatory Tregs and PD-1 lymphocytes in aggressive FL may contribute to a more active immune microenvironment that in turn facilitates the growth and progression of tumor cells.

How these subpopulations of T cells may influence the behavior of FL cells is at the present moment unclear. Early studies had shown that FL cells require the contact of stimulatory $CD4^+$ cells and T-cell–derived cytokines to proliferate.[34,35] Tregs interfere with B-cell survival in GCs indirectly by suppressing the function of GC T-helper cells,[36] but also by a direct effect by suppressing B-cell proliferation and inducing B-cell death by a cytotoxic-dependent pathway.[32,37] B-cell lymphoma infiltrating Tregs are functional and able to suppress the proliferation and cytokine production of $CD4^+CD25^-$ T cells needed for T-cell proliferation and survival.[31] This inhibitory effect seems mediated partly by PD-1L expression in Tregs and PD-1 receptor in $CD4^+CD25^-$ T cells.[31] The findings in our study of PD-1 on $CD4^+CD25^-$ cells and PD-L1 in a subset of T cells, including $CD4^+CD25^+$, but not in B cells may suggest that these interactions also occur in vivo in FL. The favorable clinical impact of high numbers of both Tregs and PD-1–positive lymphocytes in FL may be due in part to the inhibitory effect of Tregs on PD-1–positive T cells. Other interactions such a direct effect of Tregs on B cells or negative immune responses mediated by other PD-L1/PD-1 interactions may also influence the behavior of FL cells. It will be interesting to explore the contribution of other elements of these inhibitory pathways in the behavior of FL.

From a clinical standpoint, the study represents an unselected series of patients diagnosed with FL and observed in a single institution. This fact confers the advantage of being representative of a real FL population, but, on the contrary, the therapy given is necessarily somewhat heterogeneous. Nevertheless, the prognostic impact of PD-1 numbers remain similar in both the group of patients who were not treated and those who were treated with rituximab, although this later group was small (data not shown). Given the increasing interest in reliable biomarkers to predict the heterogeneous clinical behavior of follicular lymphoma,[38] our results may justify the inclusion of PD-1 in future studies on larger series of homogenously treated patients.

In conclusion, this study shows that high numbers of PD-1–positive cells predict a better outcome in patients with FL independently of the FLIPI. The common localization of these cells and FOXP3-Tregs in the tumoral follicular compartment and their similar inhibitory role on T-cell activation suggest a contribution to the immune modulation of the microenvironment in FL and a participation in the biologic behavior of the tumor. A better knowledge of these pathways may be of interest to develop therapeutic strategies based on the modulation of the host immune responses in front of the tumor cells.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Elias Campo
Financial support: Elias Campo
Provision of study materials or patients: Armando Lopez-Guillermo, Emili Montserrat
Collection and assembly of data: Joaquim Carreras, Armando Lopez-Guillermo, Emili Montserrat
Data analysis and interpretation: Joaquim Carreras, Armando Lopez-Guillermo, Giovanna Roncador, Neus Villamor, Lluis Colomo, Antonio Martinez, Rifat Hamoudi, William J. Howat, Emili Montserrat, Elias Campo
Manuscript writing: Joaquim Carreras, Armando Lopez-Guillermo, Elias Campo
Final approval of manuscript: Armando Lopez-Guillermo, Elias Campo

REFERENCES

1. Horning SJ: Natural history of and therapy for the indolent non-Hodgkin's lymphomas. Semin Oncol 20:75-88, 1993
2. Montoto S, Lopez-Guillermo A, Altes A, et al: Predictive value of Follicular Lymphoma International Prognostic Index (FLIPI) in patients with follicular lymphoma at first progression. Ann Oncol 15: 1484-1489, 2004
3. Solal-Céligny P, Roy P, Colombat P, et al: Follicular lymphoma international prognostic index. Blood 104:1258-1265, 2004
4. Alvaro T, Lejeune M, Salvado MT, et al: Immunohistochemical patterns of reactive microenvironment are associated with clinicobiologic behavior in follicular lymphoma patients. J Clin Oncol 24: 5350-5357, 2006
5. Bohen SP, Troyanskaya OG, Alter O, et al: Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci U S A 100:1926-1930, 2003
6. Carreras J, Lopez-Guillermo A, Fox BC, et al: High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. Blood 108: 2957-2964, 2006
7. Dave SS, Wright G, Tan B, et al: Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N Engl J Med 351:2159-2169, 2004
8. Farinha P, Masoudi H, Skinnider BF, et al: Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). Blood 106:2169-2174, 2005
9. Glas AM, Kersten MJ, Delahaye LJ, et al: Gene expression profiling in follicular lymphoma to assess clinical aggressiveness and to guide the choice of treatment. Blood 105:301-307, 2005
10. Glas AM, Knoops L, Delahaye L, et al: Gene-expression and immunohistochemical study of specific T-cell subsets and accessory cell types in the transformation and prognosis of follicular lymphoma. J Clin Oncol 25:390-398, 2007
11. Lee AM, Clear AJ, Calaminici M, et al: Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. J Clin Oncol 24:5052-5059, 2006
12. Wahlin BE, Sander B, Christensson B, et al: CD8+ T-cell content in diagnostic lymph nodes measured by flow cytometry is a predictor of survival in follicular lymphoma. Clin Cancer Res 13:388-397, 2007
13. Greenwald RJ, Freeman GJ, Sharpe AH: The B7 family revisited. Annu Rev Immunol 23:515-548, 2005
14. Keir ME, Francisco LM, Sharpe AH: PD-1 and its ligands in T-cell immunity. Curr Opin Immunol 19:309-314, 2007
15. Dorfman DM, Brown JA, Shahsafaei A, et al: Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. Am J Surg Pathol 30:802-810, 2006
16. Roncador G, Garcia Verdes-Montenegro JF, Tedoldi S, et al: Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma. Haematologica 92: 1059-1066, 2007
17. Jaffe ES, Harris NL, Stein H, et al: Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France, IARC Press, 2001
18. Cheson BD, Horning SJ, Coiffier B, et al: Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas: NCI Sponsored International Working Group. J Clin Oncol 17:1244, 1999
19. Mason DY, Micklem K, Jones M: Double immunofluorescence labelling of routinely processed paraffin sections. J Pathol 191:452-461, 2000
20. Keir ME, Butte MJ, Freeman GJ, et al: PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26:677-704, 2008
21. Dong H, Chen L: B7–H1 pathway and its role in the evasion of tumor immunity. J Mol Med 81:281-287, 2003
22. Marinova E, Han S, Zheng B: Germinal center helper T cells are dual functional regulatory cells with suppressive activity to conventional CD4+ T cells. J Immunol 178:5010-5017, 2007
23. Blank C, Kuball J, Voelkl S, et al: Blockade of PD-L1 (B7–H1) augments human tumor-specific T cell responses in vitro. Int J Cancer 119:317-327, 2006

24. Iwai Y, Terawaki S, Honjo T: PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. Int Immunol 17:133-144, 2005

25. Thompson RH, Dong H, Lohse CM, et al: PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. Clin Cancer Res 13:1757-1761, 2007

26. Brown JA, Dorfman DM, Ma FR, et al: Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. J Immunol 170:1257-1266, 2003

27. Yamamoto R, Nishikori M, Kitawaki T, et al: PD-1-PD-1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. Blood 111:3220-3224, 2008

28. Curiel TJ, Coukos G, Zou L, et al: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10:942-949, 2004

29. Liyanage UK, Goedegebuure PS, Moore TT, et al: Increased prevalence of regulatory T cells (Treg) is induced by pancreas adenocarcinoma. J Immunother 29:416-424, 2006

30. Alvaro T, Lejeune M, Salvado MT, et al: Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res 11:1467-1473, 2005

31. Yang ZZ, Novak AJ, Stenson MJ, et al: Intratumoral CD4+CD25+ regulatory T-cell-mediated suppression of infiltrating CD4+ T cells in B-cell non-Hodgkin lymphoma. Blood 107:3639-3646, 2006

32. Yang ZZ, Novak AJ, Ziesmer SC, et al: CD70+ non-Hodgkin lymphoma B cells induce Foxp3 expression and regulatory function in intratumoral CD4+CD25 T cells. Blood 110:2537-2544, 2007

33. de Jong D: Molecular pathogenesis of follicular lymphoma: A cross talk of genetic and immunologic factors. J Clin Oncol 23:6358-6363, 2005

34. Schmitter D, Koss M, Niederer E, et al: T-cell derived cytokines co-stimulate proliferation of CD40-activated germinal centre as well as follicular lymphoma cells. Hematol Oncol 15:197-207, 1997

35. Umetsu DT, Esserman L, Donlon TA, et al: Induction of proliferation of human follicular (B type) lymphoma cells by cognate interaction with CD4+ T cell clones. J Immunol 144:2550-2557, 1990

36. Lim HW, Hillsamer P, Kim CH: Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. J Clin Invest 114:1640-1649, 2004

37. Lim HW, Hillsamer P, Banham, et al: Cutting edge: Direct suppression of B cells by CD4+ CD25+ regulatory T cells. J Immunol 175:4180-4183, 2005

38. Davies AJ: Clinical and molecular prognostic factors in follicular lymphoma. Curr Oncol Rep 8:359-367, 2006

Acknowledgment

We thank Professor Ming Qing Du from the Department of Pathology, Cambridge University, for his support.

