High ratio of interfollicular CD8/FOXP3-positive regulatory T cells is associated with a high FLIPI index and poor overall survival in follicular lymphoma

MAJDA SAIFI[1,2], AURÉLIE MARAN[1], PIERRE RAYNAUD[1,2], MARIE CHRISTINE PICOT[3], PHILIPPE QUITTET[4], GUILLAUME CARTRON[2,4], JEAN FRANÇOIS ROSSI[2,4] and VALÉRIE COSTES[1,2]

[1]Department of Pathology, [2]INSERM U847, [3]Medical Information Department, [4]Onco-Hematology and Biotherapy Department, CHU Montpellier, 34295 Montpellier Cedex 5, France

Abstract. Several studies have highlighted the importance of the microenvironment in the behaviour of follicular lymphoma (FL). We conducted an immunohistochemical analysis to assess the role of different cell subpopulations, i.e. CD8, CD68 and forkhead box protein P3 (FOXP3)-positive regulatory T cells (Treg cells) in 84 lymph nodes of 58 patients with FL (58 at diagnosis, 26 at relapse). Since the overall effect of Tregs is considered to depend on their number in relation to CD8+ effector T cells, we determined the corresponding ratio for each patient and correlated the results with clinical parameters. The interfollicular CD8/FOXP3+ cell ratio was significantly higher in patients with histological grade 3 tumours (2.04 vs. 1.63) and with a high risk FLIPI index (2.99 vs. 1.53) compared to those with grade 1-2 tumours or a low-intermediate FLIPI index. Similar results were obtained for the follicular CD8+/FOXP3+ cell ratio. The interfollicular CD8/FOXP3 ratio was found to have prognostic value [a 5-year overall survival (OS) of 82 vs. 59% for a ratio of ±1.68]. In addition, an interfollicular FOXP3+ cell number of more than 86 cells/mm² was correlated with a more favourable outcome (p=0.03). When patients at diagnosis and relapse were compared, a significant difference (p=0.05) in the localization (interfollicular vs. intrafollicular) of FOXP3+ cells was observed. The CD8/FOXP3 ratio in the interfollicular areas was significantly different (1.66 at diagnosis vs. 2.2 at relapse, p=0.05). The presence of a small number of FOXP3+ cells with a high CD8/FOXP3 ratio is probably the indicator of an active immune response during tumour development, with lymphoma cells acting as targets or bystanders.

Correspondence to: Dr Valérie Costes, Department of Pathology, CHU Montpellier, 80 Av A. Fliche, 34295 Montpellier Cedex 5, France
E-mail: v-costes_martineau@chu-montpellier.fr

Introduction

Follicular lymphoma (FL) is the most common indolent B-cell lymphoma. It is generally associated with translocation t (14; 18) (q32;q21), which participates in tumour cell survival through overexpression of the anti-apoptotic Bcl2 protein (1,2). Numerous treatments have been proposed, from delayed therapy after a watch-and-wait period to autologous transplantation or immune therapy, from allogenic transplantation to vaccination or the use of anti-CD20 monoclonal antibodies, with or without chemotherapy. To date, only clinical prognostic classification, such as the Follicular Lymphoma International Index (FLIPI), is used to guide treatment (3).

Accumulation of genomic alterations and clonal selection account for subsequent FL progression and transformation (4). However, a role of the FL cell microenvironment in determining clinical behaviour and disease prognosis has also recently been substantiated (5-8). In this cancer, tumour cells reside and proliferate in follicular structures in close association with helper T cells and follicular dendritic cells (7,9). Biological analyses have been conducted to determine the exact role of these different cellular subpopulations. Staudt and Dave (10) and Dave *et al* (11) initially used gene expression profiling and observed a major role of T-lymphocytes and macrophages, while defining two prognostic subgroups.

Tumours induce immunologic tolerance by several mechanisms involving tolerogenic antigen-presenting cells, Foxp3+CD4+CD25+ regulatory T cells (Tregs). An increased frequency of Tregs has been noted in the peripheral blood of patients with bronchial carcinoma as compared to healthy individuals (12), and similar findings were also reported in patients with a variety of cancer types. Tregs that are found within the tumour microenvironment are highly suppressive and abrogate the effector function of cytotoxic T cells as well as NK cell-mediated cytotoxicity (13).

In follicular lymphoma, FOXP3+ T cells were first suggested to be associated with poor survival (14). Then conflicting results were published indicating the positive impacts of the CD4+CD25+FOXP3+ phenotype (15-17). In addition, CD8 and CD4 T cells were mentioned as being key cells in the progression and/or transformation process (18,19), and mast cells were

found to be associated with a poor outcome (20). Some studies have outlined the unfavourable outcome of FL associated with high numbers of tumour-associated macrophages, with or without rituximab treatment (21-23)

    We thus conducted immunohistochemical analyses to investigate the presence of CD8+ T cells, FOXP3+ regulatory T cells and macrophages in follicular lymphoma, while focusing particularly on their intra- vs. interfollicular localisation. To obtain a more dynamic picture of the immune response, we correlated the CD8+/Treg ratio in these two compartments with clinical parameters and outcome.

Materials and methods

Patients and samples (Table I). Fifty-eight patients consecutively diagnosed at a single institution between 1990 and 2005 were included in the present study. All cases were reviewed according to criteria of the WHO Classification (24). We observed 39 grade 1 (67%), 10 grade 2 (17%) and 9 grade 3 (16%) tumours. The median age of the patients was 58 years (interquartile range, 48-69 years), and the male/female ratio was 32/26. Advanced stage (Ann Arbor IV) lymphomas were noted in 14 cases (24%). The population distribution according to FLIPI was as follows: 21 low risk cases (36%), 26 intermediate risk cases (45%) and 11 high risk cases (19%).

    All patients were treated with combination chemotherapy without rituximab. Thirty-two patients were in complete remission (CR) and two in partial remission (PR). Twenty-four patients died after a median follow-up of 2 years (range 1-9). The median survival was 8.5 years. Fifteen samples were identified from patients whose survival was less than 5 years, and 14 samples were identified from patients whose survival was more than 10 years after diagnosis. In addition to the diagnostic tissue samples, specimens at relapse were available for 26 patients. Ten reactive lymph nodes were used as controls.

Immunohistochemistry. Tissue samples were formalin-fixed and paraffin-embedded. Sections (4-μm) were dewaxed and blocked in a hydrogen peroxide/methanol solution. Antigen retrieval was performed by heating the slides in EDTA (Sigma) (diluted 1/10, pH 7.0) at 100°C. All immunohistochemical staining was carried out manually using anti-CD8 (clone C8/144B, Dako; dilution 1/25), anti-CD68 (KP1, Dako; dilution 1/500) and anti-FoxP3 (236A/E7, CliniSciences; dilution 1/150). Detection was performed with the Dako Real™ detection system, using peroxidase/DAB+ (ref. 5001).

    CD8, CD68 and FOXP3-positive cells were counted in 20 HPF and expressed as the number of cells/mm². Positive infiltrating cells were counted independently in perifollicular and follicular (germinal centre and mantle zone) compartments. A minimum of six different follicular and interfollicular areas were selected in tumours to minimize the heterogeneous distribution of positive cells.

    Double-labelling immunohistochemistry for CD8 and Foxp3 was performed in selected cases using the Benchmark XT automated stainer. After pretreatment as described above, the sections were exposed to CD8 (clone C8/144B, Dako, at a 1:25 dilution). The ultraView Universal DAB detection system was used with DAB chromogen. The sections were then processed for the Foxp3 antibody at a 1:100 dilution with the

Table I. Patient characteristics at diagnosis.

	No (%)
No. of patients	
Female	26 (45)
Male	32 (55)
Total	58 (100)
Histological grade	
I	39 (37)
II	10 (17)
III	9 (16)
Stage	
I	11 (19)
II	11 (19)
III	17 (29)
IV	19 (32)
Status	
Dead	24 (42)
Living	34 (58)
Clinical reponse	
CR	30 (52)
PR	2 (3)
Not reached	26 (45)
FLIPI	
Low risk	23 (39)
Intermediate risk	23 (39)
High risk	11 (19)
Not reached	1 (2)

alkaline phosphatase-based ultraView Red detection system and Fast Red/Naphthol chromogen.

Statistical analysis. The population characteristics were described with proportions for categorical variables and median and interquartile values for continuous unusually distributed variables (Shapiro-Wilk statistics). The immunochemical results were compared according to the clinical features using the Mann-Whitney U test. The Kaplan-Meier method was used to provide estimates of probability of survival. The following period was defined as time from diagnosis to time of last follow-up or death. The survival and potential prognostic factors were compared with the log rank test after conversion of the immunochemical variables into dichotomous variables using the median value. In all of the statistical analyses, $p < 0.05$ was considered significant. All statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA).

Results

Immunohisochemistry (IHC) results. In FL at diagnosis, CD8, POXP3 and CD68-positive cells were more numerous in interfollicular than in follicular locations: 155.35, 86 and 96.2/mm² compared with 33.5, 39.35 and 39.25/mm², respectively (Table II). An example is illustrated in Fig. 1. The CD8/

Table II. CD8, FOXP3 and CD68 immunostaining in 58 FL patients and 10 reactive lymph nodes.

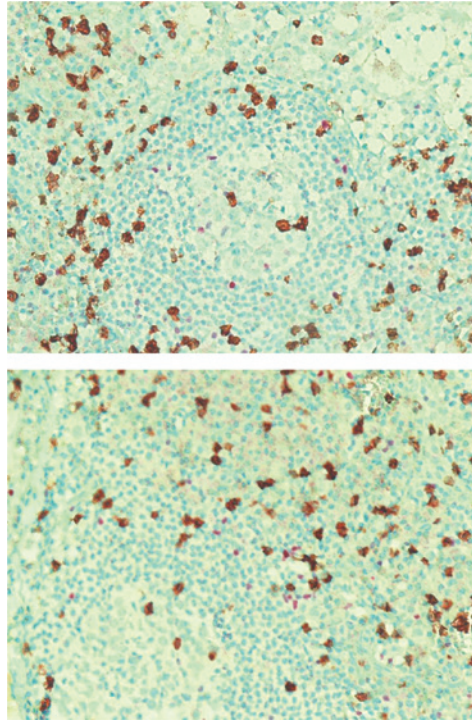|	Location	CD8+ cells/mm² median (IQ 25-75)	FOXP3+ cells/mm² median (IQ 25-75)	CD68+ cells/mm² median (IQ 25-75)	Ratio CD8/FOXP3
FL patients	Follicular	33.50 (20.4-61.0)	39.35 (23.6-55.8)	39.25 (29.4-54.3)	1.04
	Interfollicular	155.35 (118.8-359.2)	86.00 (64.0-164.0)	96.20 (78.2-130.8)	1.66
Reactive lymph nodes	Follicular	25	8	14	2.49
	Interfollicular	411	150	235	4.07

Figure 1. Immunohistochemical double-staining patterns in two FL cases. FoxP3 (regulatory T cells)-positive cells are shown in red and CD8-positive cells are in brown.
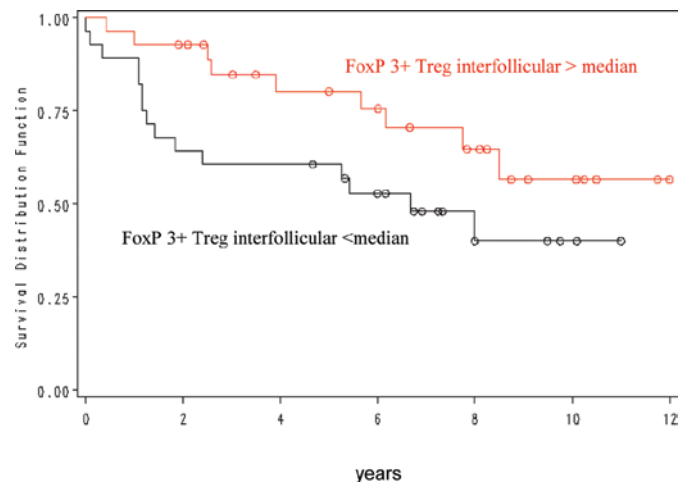

Figure 2. Overall survival in patients with FL in relation to FoxP3-positive interfollicular cells
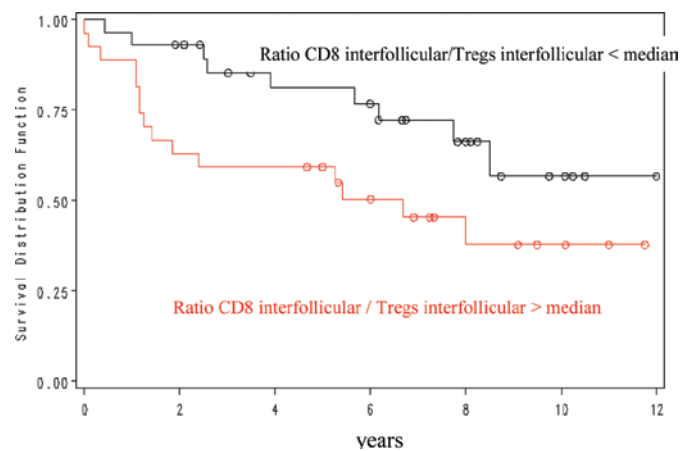
Figure 3. Overall survival in patients with FL in relation to the interfollicular CD8/FoxP3 ratio.

FOXP3 median ratio was 1.66 in interfollicular locations compared with 1.04 in follicular locations.

In reactive lymph nodes, the CD8/FOXP3 median ratio was higher than that in FL, both in interfollicular (4.07 vs. 1.66) and follicular locations (2.49 vs. 1.04). (Table II).

Correlations between IHC results and clinical features. The interfollicular and follicular CD8/Treg ratios were compared with the main clinical features of the patients at diagnosis. The interfollicular CD8/FOXP3+ cell ratio was significantly higher in patients with grade 3 tumours (2.04 vs. 1.63) and with a high risk FLIPI index (2.99 vs. 1.53) compared with those with grade 1-2 tumours or a low-intermediate FLIPI index. The same results were obtained with the follicular CD8+/FOXP3+ cell ratio (Table III). We did not note any significant relationship with stage or patient age.

Correlations between IHC results and outcome. Concerning the interfollicular FOXP3+ cell number, more than 86 cells/

mm² were correlated with a more favourable outcome (p=0.03) (Fig. 2). In contrast, intrafollicular FOXP3, intrafollicular and interfollicular CD8 and CD68 cell numbers were not predictive of patient outcome.

The interfollicular CD8/FOXP3 ratio showed a positive prognostic value for overall survival, with a 5-year OS of 82 vs. 59% for a ratio of less or more than 1.68 (Fig. 3). Other ratios were not significantly correlated with outcome.

Table III. Relationship between CD8/FOXP3⁺ cell ratio and clinical features.

Expressed in median (IQ 25-75)	Grade 1 and 2 (n=49)	Grade 3 (n=9)	P-value
Follicular CD8/FOXP3⁺ cell ratio	0.886 (0.47-1.54)	3.463 (1.66-4.01)	0.002
Interfollicular CD8/FOXP3⁺ cell ratio	1.634 (1.02-2.29)	2.048 (1.65-3.66)	0.050
	FLIPI low and intermediate risk (n= 47)	FLIPI high risk (n=11)	
Follicular CD8/FOXP3⁺ cell ratio	0.916 (0.48-1.57)	2.091 (1.08-8.04)	0.020
Interfollicular CD8/FOXP3⁺ cell ratio	1.536 (1.02-2.01)	2.993 (1.762-8.676)	0.001

Table IV. CD68, CD8 and POXP3-positive cells at first relapse compared with at diagnosis.

Expressed in median (IQ 25-75)	Diagnosis (n=58)	Relapse (n=26)	P-value
Follicular CD68⁺ cells/mm²	39 (29.4-54.3)	32 (20.5-46.8)	0.007
Interfollicular CD68⁺ cells/mm²	96 (86.2-112.8)	62 (54.1-73.2)	0.005
Interfollicular CD8/FOXP3⁺ cell ratio	1.66 (1.07-2.26)	2.2 (1.32-3.87)	0.050

CD8, CD68 and FOXP3-positive cells in FL at first relapse compared with at diagnosis. When comparing these two groups, we observed a significant difference between the number of CD68 cells in intrafollicular (39.23 at diagnosis vs. 32 at relapse, p=0.07) and interfollicular locations (96 at diagnosis vs. 62 at relapse, p=0.06). The CD8/FOXP3 ratio in interfollicular locations was significantly different (1.66 at diagnosis vs. 2.2 at relapse, p=0.05) (Table IV).

Discussion

The present study of 58 diagnostic lymph node samples from FL patients showed that the interfollicular CD8/FOXP3⁺ cell ratio was significantly higher in patients with grade 3 tumours (2.04 vs. 1.63) and with a high risk FLIPI index (2.99 vs. 1.53) compared with those with grade 1-2 tumours or a low-intermediate FLIPI index. We obtained the same results with the follicular CD8⁺/FOXP3⁺ cell ratio. Moreover, the inter-follicular CD8/FOXP3 ratio was found to have a prognostic value for overall survival, with a 5-year OS of 82 vs. 59% for a ratio of less or more than 1.68. An interfollicular FOXP3⁺ cell number of more than 86 cells/mm² was correlated with a more favourable outcome (p=0.03).

These results are in accordance with the findings of two recent studies in which a perifollicular pattern of FOXP3-positive cells was more commonly found in the diagnosis of FL lymph nodes from patients whose subsequent outcome was favourable (15,17). Tzankov *et al* reported a favourable prognostic influence of an increased amount of FOXP3⁺ cells in FL, cHL and in GC-like diffuse large B-cell lymphomas, and defined a cutoff value of 88.6 FOXP3⁺ cells/mm² in a ROC analysis (16). These authors did not take the interfollicular vs. follicular location into account. In our study, an interfollicular but not follicular FOXP3⁺ cell number of more than 86 cells/mm² was correlated with a more favourable outcome (p=0.03).

Recent studies have shown that tumour B cells alone induce conventional T cells to express FOXP3 and acquire a regulatory function (25). The presence of FOXP3-positive cells is also a protective variable in classical Hodgkin's disease in which an inverse relationship between TIA-1 and FOXP3 expression and survival has been demonstrated. Moreover, higher levels of FOXP3 twinned with low TIA-1 were beneficial (16,26,27). In a recent study, De Jong *et al* showed that the presence of CD4/FOXP3⁺ cells is beneficial to the host, irrespective of the treatment used (28).

When comparing the two groups at diagnosis and relapse, we observed a significant difference between the CD8/FOXP3 ratio in interfollicular locations (1.66 at diagnosis vs. 2.2 at relapse, p=0.05). In the Tzankov study, a 2.7-fold (from 94 to 33/mm²) proportional decrease in FOXP3⁺ cells in transformed DLBCL evolving from FL was observed after immunochemical screening, and this was similar to the 4.4-fold decrease observed by Carreras *et al* (9.6-2.2%) (17).

Concerning the presence of CD68⁺ cells, we did not obtain a significant correlation with poor survival. However, a recent study outlined the influence of specific therapeutic regimens on the prognostic impact of CD68-postive macrophages in follicular lymphoma (28). Surprisingly, the numbers of CD68 cells both in intrafollicular and interfollicular locations were significantly lower at relapse as compared to the diagnostic samples (respectively 32 vs. 39.23, p=0.07 and 62 vs. 96, p=0.05).

In inflammatory lymph nodes, we found an interfollicular CD8/FOXP3 ratio (2.74) close to that of the 15 FL patients with less than a 5-year survival (2.02). These findings are in agreement with those of Glas *et al*, who showed that FL with a poor prognosis and a poor response to anti-CD20 therapy had gene expression signatures that were similar to that of normal activated lymphoid tissue (29).

As proposed by De Jong, the clinical behaviour and prognosis of FL seem to be inherited rather than acquired properties according to a dual pathway model (30,31). The poor prognosis pathway may be characterised by genetic alterations inducing highly activated specific T cells. In the 'good prognosis' pathway, different alterations (der18q and +7/+8) and additional anti-apoptotic signals are involved, so protection from apoptosis may therefore be more important than immunologic support. The findings of a recent study support this hypothesis, showing that host genetic variability in immune genes, particularly IL-8, IL-2, IL-12B and IL-1RN, appear to be associated with overall survival in FL (32).

In conclusion, the presence of a high CD8/FOXP3 ratio is the hallmark of an active immune response during tumour development, with lymphoma cells acting as either target or bystander and reflecting a more aggressive disease.

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