VOLUME 24 · NUMBER 31 · NOVEMBER 1 2006

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Number of CD4$^+$ Cells and Location of Forkhead Box Protein P3–Positive Cells in Diagnostic Follicular Lymphoma Tissue Microarrays Correlates With Outcome

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Submitted March 9, 2006; accepted August 28, 2006; published online ahead of print at www.jco.org on October 10, 2006.

Supported in part by Cancer Research UK, the Research Advisory Board of St Bartholomew's Hospital, the Royal London Charitable Foundation (Grant No. RAB03/PJ/07), and Kay Kendall Leukaemia Fund.

Presented at American Society of Hematology, Atlanta, GA, December 10-13, 2005.

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

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0732-183X/06/2431-5052/$20.00

DOI: 10.1200/JCO.2006.06.4642

ABSTRACT

Purpose
To examine the immune microenvironment in diagnostic follicular lymphoma (FL) biopsies and evaluate its prognostic significance.

Patients and Methods
Immunohistochemistry was used to study numbers and location of cells staining positive for immune cell markers CD4, CD7, CD8, CD25, CD68, forkhead box protein P3 (FOXP3), T-cell intracellular antigen-1, and Granzyme B in tissue microarrays of paraffin-embedded, diagnostic lymph node biopsies taken from 59 FL patients who lived less than 5 years (short-survival group; n = 34) and more than 15 years (long-survival group; n = 25).

Results
CD4 and FOXP3 expression were significantly different between the two groups. Samples from the long-survival group were more likely than those from the short-survival group to have CD4$^+$ staining cells and to have FOXP3-positive cells in a perifollicular location.

Conclusion
This study has identified differences in immune cell composition of the diagnostic FL lymph node immune microenvironment and these have the potential for use as prognostic biomarkers in a routine histopathology setting.

J Clin Oncol 24:5052-5059. © 2006 by American Society of Clinical Oncology

INTRODUCTION

Patients with follicular lymphoma (FL) have an indolent clinical course, with a median survival of 10 years, although prognosis is significantly worse for those in whom there is transformation to a more aggressive histology.[1,2] The clinical course of FL is heterogeneous, with many patients experiencing numerous relapses and periods of remission over many years, whereas for others the disease progresses rapidly and survival is short.[3,4]

Although the development of the Follicular Lymphoma International Prognostic Index[5] has helped to stratify patients with this heterogeneous disease, it has limited discriminative power and additional prognostic biomarkers are required. Whole-genome microarray of diagnostic biopsies revealed that FL patients with good and poor outcome were distinguishable by immune microenvironment-associated gene signatures.[6] The gene signature associated with favorable outcome was rich in expression of T-cell genes, whereas the poor outcome gene signature was characteristic of macrophages. In accordance with this finding, immunohistochemical (IHC) analysis of 99 diagnostic FL biopsies from patients who subsequently received uniform treatment has demonstrated a negative prognostic correlation with number of CD68$^+$ macrophages present within the tumor.[7] However, the mechanisms of immune cell–tumor cell influence and interactions have not been elucidated.

The role of the T-regulatory (Treg) cell subset in the tolerance or suppression of malignancy is now being unraveled. Infiltrating Tregs are associated with poor prognosis in ovarian carcinoma,[8] whereas in hematologic malignancy functional Tregs have been identified in B-cell non-Hodgkin's lymphomas.[9,10]

We present a study using IHC on tissue microarray of paraffin-embedded diagnostic FL biopsies from a selected population of 59 patients at the extremes of overall survival (< 5 and > 15 years) that were available in our tissue bank. The relationship between immune microenvironment phenotype and survival in FL is examined. We demonstrate that CD4 expression and a perifollicular location of

forkhead box protein P3 (FOXP3) were significantly more common in the diagnostic biopsy from patients who lived more than 15 years. These findings are in keeping with the hypothesis that in addition to cell number, the location of cells of the immune microenvironment is also important in the host response to FL.

PATIENTS AND METHODS

Patient Samples

From the patients diagnosed with FL at St Bartholomew's Hospital (London, United Kingdom) between 1974 and 1999 for whom initial diagnostic paraffin-embedded lymph node blocks were available were eligible for this study. Thirty-four samples were identified from patients whose survival was less than 5 years and 25 samples were identified from patients whose survival was more than 15 years from diagnosis. Patients were treated according to the current protocol during the 25-year period.[3,11,12] In general, at presentation those with stage I disease were treated with involved-field irradiation; the remainder were managed expectantly, until chemotherapy was instituted when there was an indication for intervention. After remission was achieved, management was again expectant. Ethical approval for this study was obtained from the local regional ethics board.

Tissue Microarray Construction

Tissue microarrays (TMAs) were constructed of 1-mm cores of patient tissue taken from representative areas of lymphoma (> five neoplastic follicles) using premarked sections stained with hematoxylin and eosin and from reactive tonsil and appendix controls, in triplicate using a manual arrayer (Beecher Scientific, Silver Spring, MD) as described previously.[13,14] Approximately 120 cores were applied per slide.

Immunohistochemistry

The test panel of antibodies and dilutions used for immunohistologic analysis are shown in Table 1. TMA blocks were cut to 4 μm and applied to 3-aminopropyltrioxysilane–coated slides, then dewaxed and blocked in hydrogen peroxide/methanol solution. Antigen retrieval was performed by pressure-cooking the sample in citrate, or in the case of FOXP3, EDTA.[15] Sections were then stained using the Vector Elite ABC kit (PK6100; Vector Laboratories, Peterborough, United Kingdom) or for FOXP3, CSA II tyramide amplification system (DAKO, Glostrup, Denmark), followed by diaminobenzidine chromogen (Biostat, Stockport, United Kingdom). Appropriate negative and positive controls were always used.

Table 1. Antibodies Used for the Investigation of the Immune Microenvironment

Test Antibody	Clone	Source	Dilution
CD4	NCL-CD4-368 clone 4B12	Novocastra, Newcastle, United Kingdom	1/100
CD7	NCL-CD7-272 clone 272	Novocastra	1/50
CD8	C8/144B	DAKO, Denmark	1/50
CD25	NCL-CD25-305 clone 4C9	Novocastra	1/100
CD68	KP1	DAKO	1/4,000
Granzyme-B	NCL-GRAN-B clone 11F1	Novocastra	1/40
TIA-1	Ab2712	Abcam, Cambridge, United Kingdom	1/50
FOXP3	Supernatant clone 236A/E7	Gift from Alison Banham, John Radcliffe Hospital, Oxford, United Kingdom[16]	Neat

Abbreviations: TIA-1, T-cell intracellular antigen 1; FOXP3, forkhead box protein P3.

Double Staining for CD4 and FOXP3

After antigen retrieval by pressure-cooking the sample in citrate, staining for CD4 was carried out using Vectastain Elite ABC kit with diaminobenzidine as chromogen. This was followed by staining for FOXP3 using Vectastain Elite ABC-AP kit (alkaline phosphatase) with Fast Red as chromogen.

IHC Analysis

The presence of adequate FL tumor cells in the tissue cores was confirmed by scoring with a diagnostic lymphoma antibody panel comprising CD3, CD5, CD10, CD20, CD21, CD23, BCL-2, BCL-6, Ki-67, TP53, and MUM-1 (data not shown).

Two histopathologists (A.M.L., M.C.) scored the TMAs independently and reached consensus in all cases. The entire 1-mm-diameter core was analyzed at low and high power in each case. Where one of three cores could not be scored due to insufficiency of tissue, consensus of two cores was accepted, although this was necessary in only 10% of the total 1,416 cores analyzed. If two cores of three were insufficient, no scoring was attempted and the result was noted as absent for that sample with the antibody in question, and hence the maximum number of patient cases (34 for the short-survival group and 25 for the long-survival group), was not always assessable for each of the test panel antibodies.

Staining using a test panel of immune-cell–associated antigens CD4, CD7, CD8, CD25, CD68, T-cell intracellular antigen 1 (TIA-1), Granzyme B, and FOXP3 was evaluated for number of positive cells. Scoring was divided into the following categories: less than five cells/high power field (hpf; ×40 magnification), five to 10 cells/hpf, 10 to 15 cells/hpf, 15 to 30 cells/hpf, and more than 30 cells/hpf. Topographic distribution of positive cells was also evaluated. Location relative to the neoplastic follicle (perifollicular, intrafollicular, and not bearing either of those patterns but being evenly distributed) was recorded.

Statistical Analysis

Fisher's exact test was performed to determine whether the incidence of antigen expression was significantly different between samples from patients at the extremes of survival.

RESULTS

Patient Characteristics

Diagnostic FL biopsies from patients whose overall survival lay at the extremes were chosen for study. The histology was reviewed in all cases. The characteristics of these 59 patients are listed in Table 2. Patients who lived less than 5 years from diagnosis, the short-survival group, had a median survival of 2 years and all died as a result of disease. Patients who lived more than 15 years from diagnosis, the long-survival group, had a median survival of 21 years. Although members of the short-survival group were significantly older than those in the long-survival group, with a median age of 61 and 46 years, respectively (Table 2), there was no significant difference in the incidence of transformation, bone marrow involvement, stage, or grade between these two patient groups.

Analysis of the TMAs

For each antibody, two (maximum of 10% patient samples over all the antibodies studied) or three replicate cores were assessable from 31 to 34 patients from the short-survival group and 22 to 25 patients from the long-survival group (Table 3). Scoring was also divided into the following categories: less than five cells/hpf, five to 10 cells/hpf, 10 to 15 cells/hpf, 15 to 30 cells/hpf, and more than 30 cells/hpf. Analysis

Table 2. Demographic and Clinical Characteristics of Patients From the Extremes of Survival

Characteristic	Short-Survival Group		Long-Survival Group		*P*
	No. of Patients	%	No. of Patients	%	
Total No. of patients (N = 59)	34		25		
Sex					
Male	25		16		
Female	9		9		
Survival, years					< .0001
Median	2		21		
Range	0.08-4.8		15.3-30.4		
Age at diagnosis, years					.0001
Median	61		46		
Range	28-80		24-63		
Stage at diagnosis					.29
I	4	12	3	12	
II	3	9	6	24	
III	4	12	4	16	
IV	23	68	12	48	
Histologic pattern					.99
Follicular	33	97	24	96	
Follicular and diffuse	1	3	1	4	
Grade at diagnosis					.85
1	18	53	16	64	
1 and 2	3	9	4	16	
2	8	24	2	8	
2 and 3	1	3	3	12	
3	4	12	0		
3b	0		0		
Proceeded to transformation	9 of 34	26	3 of 25	12	.33
Time to transformation, years					
0-5	9		1		
5-10	0		1		
> 10	0		1		
Bone marrow involvement	17 of 34	50	12 of 25	48	.88
Treatment strategy and No. of lines of treatment*					
Expectant management	1	3	1	4	
1	7	21	6	24	
2	6	18	6	24	
3	4	12	3	12	
4	5	15	3	12	
5	2	6	2	8	
6	6	18	0		
7	3	9	1	4	
8	0		3	12	
Cause of death					
Alive	0		15	60	
Disease	34	100	3	12	
Unrelated	0		7	28	

*Current therapy of the era.

of these categories did not reveal any additional correlation with outcome, and for additional analysis, the categories less than five cells/hpf and more than five cells/hpf were used.

CD4 Incidence Is Significantly Different Between Short- and Long-Survival Groups

CD4 expression was assessable in 56 of 59 (95%) total patients; the three inassessable patients were all from the long-survival group. CD4-expressing cells were observed at levels more than five cells/hpf in 36 of 56 (66%) patients; 18 of 34 (53%) patients from the short-survival group and 18 of 22 (82%) biopsies from the long-survival group ($P \leq .05$; Table 3). Representative CD4-stained TMA cores are shown in Figure 1. The incidence of a high level of expression of CD4 (> 30 cells/hpf) was the same between the two groups (Table 4).

Among all of the patients studied (56 assessable for CD4) those with less than five CD4 cells/hpf in their diagnostic sample had a

Table 3. Expression of Immune Cell Antigens in Diagnostic FL Lymph Nodes

Antigen	No. of Assessable Patient Samples*		< 5 Cells Positive/hpf				> 5 Cells Positive/hpf			
	Short-Survival Group	Long-Survival Group	Short-Survival Group		Long-Survival Group		Short-Survival Group		Long-Survival Group	
			No.	%	No.	%	No.	%	No.	%
CD4	34	22	16 of 34	47	4 of 22	18	18 of 34	53	18 of 22	82
P						< .05				
FOXP3	31	24	11 of 31	35	3 of 24	13	20 of 31	65	21 of 24	87
P						.07				
CD8	33	22	3 of 33	9	0 of 22	0	30 of 33	91	22 of 22	100
P						.27				
CD7	34	23	6 of 34	18	1 of 23	4	28 of 34	82	22 of 23	96
P						.22				
CD25	33	23	11 of 33	33	4 of 23	17	22 of 33	67	19 of 23	83
P						.23				
CD68	33	22	3 of 33	9	2 of 22	9	30 of 33	91	20 of 22	91
P						.99				
TIA-1	32	24	3 of 32	9	1 of 24	4	29 of 32	91	23 of 24	96
P						.63				
Granzyme B	31	25	24 of 31	77	20 of 25	80	7 of 31	23	5 of 25	20
P						.99				

Abbreviations: FL, follicular lymphoma; hpf, high-power field; FOXP3, forkhead box protein P3; TIA-1, T-cell intracellular antigen 1.
*Of a maximum of 34 for short-survival group and 25 for long-survival group, values are number of patient samples that were analyzed. Loss of samples was due to the tissue being used up or damaged.

median age of 62 years (range, 34 to 77 years), whereas the median age of those bearing more than five CD4 cells/hpf was 54 years (range, 24 to 80 years; $P = .08$).

We examined the location of positively staining cells in relation to the neoplastic follicle (intrafollicular, perifollicular, and evenly distributed with no intra- or perifollicular pattern). Absence of CD4 correlated with poor outcome ($P \leq .05$; Table 3), and in samples where $CD4^+$ cells were present, there was a trend toward a significant difference in their location between the two survivor groups (Fisher's exact test, 3×2, $P = .09$; Table 5).

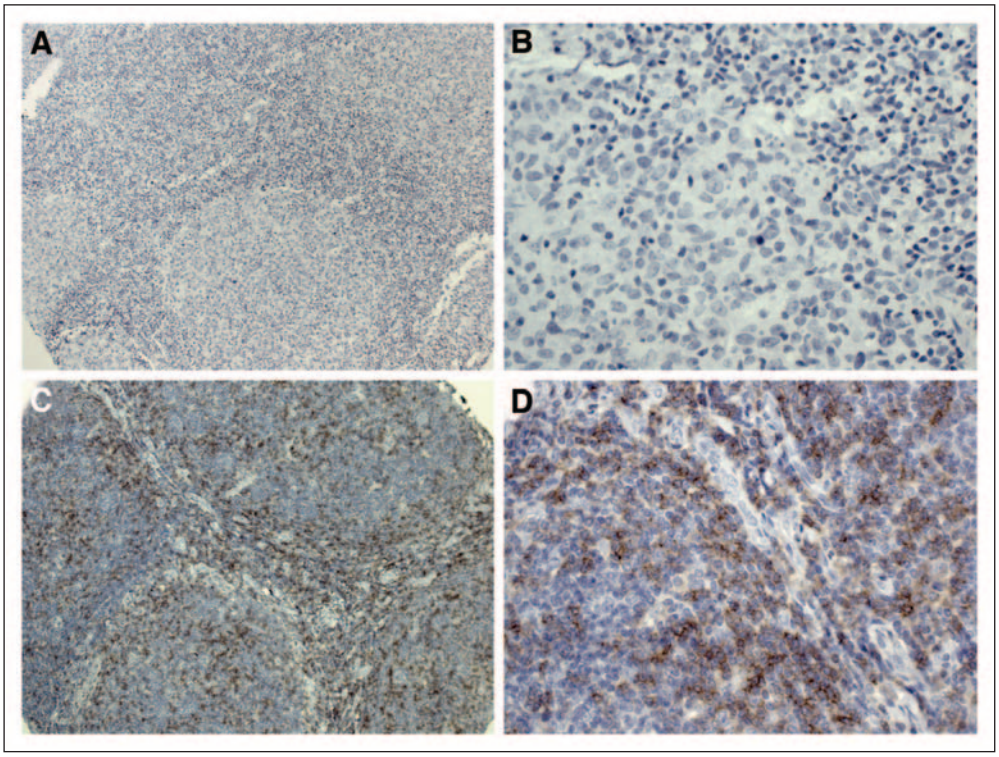

Fig 1. CD4 expression on tissue microarray cores from diagnostic follicular lymphoma biopsies taken at (A, C) low power (×10 magnification) and (B, D) high power (×40 magnification). (A, B) Samples with absence (< five cells/high-power field) and (C, D) presence of CD4 expression (> five cells/high-power field).

Table 4. Incidence of CD4, CD25, and FOXP3 in Same Diagnostic Biopsies of Short- and Long-Survival Groups of FL

	Short-Survival Group (n = 30*)			Long-Survival Group (n = 20*)		
No. of Cells/hpf	CD4	CD25	FOXP3	CD4	CD25	FOXP3
< 5	14	9	10	4	3	3
5-10	2	4	8	4	10	8
10-15	3	7	7	2	6	7
15-30	6	7	4	5	1	1
> 30	5	3	1	5	0	1

Abbreviations: FOXP3, forkhead box protein P3; FL, follicular lymphoma; hpf, high-power field.
*Patient samples that were inassessable for any of the three antibodies to CD4, CD25, and FOXP3 were discounted from this analysis.

Treg Cells and Outcome

There was a trend toward patients with long survival being more likely to have FOXP3-positive cells ($P = .07$), but none of the other immune cell-associated antigens studied showed any significant association between frequency and survival (Table 3). In addition, the pattern of FOXP3 expression was significantly different in the short- and long-survival groups (Fisher's exact test, 3×2, $P = .02$; Table 5). Examples of the different staining patterns observed for FOXP3 expression are shown in Figure 2. Patients from the long-survival group had FOXP3-positive cells at a higher incidence in a perifollicular location than the patients of the short-survival group (collapsed 2×2 Fisher's exact test, $P = .01$; Table 5).

The location of CD25 expression showed a trend toward a significant difference between the two sample sets (Fisher's exact test 3×2, $P = .1$; Table 5) and a trend toward occurrence in a perifollicular location among the samples from patients within the long-survival group (collapsed 2×2 Fisher's exact test, $P = .07$; Table 5).

The findings that CD4 incidence and FOXP3 distribution at diagnosis were important to outcome, and that both CD4 and CD25 distribution showed a trend toward correlation with outcome, suggests involvement of the Treg subset (defined by FOXP3 expression; either $CD4^+$ or $CD8^+$ and variably expressing CD25). The incidence of cells expressing CD4, CD25, or FOXP3 in the same patients from the short-survival group (n = 30) and the long-survival group (n = 20) is shown in Table 4. Double staining with CD4 and FOXP3 revealed cells coexpressing these antigens; an example from the long-survival group is shown in Figures 3A and 3B.

DISCUSSION

This study of 59 diagnostic samples taken from FL patients whose outcome was either good or poor draws attention to the importance of

Table 5. Locations of $CD4^+$, $CD25^+$, or FOXP3-Positive Cells in Short- and Long-Survival Groups With FL, and Correlation With Survival

	CD4				CD25				FOXP3			
	Short-Survival Patients (n = 34)		Long-Survival Patients (n = 22)		Short-Survival Patients (n = 33)		Long-Survival Patients (n = 23)		Short-Survival Patients (n = 31)		Long-Survival Patients (n = 24)	
Parameter	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Location of positive cells												
Absent*	16	47	4	18	11	33	4	17	11	35	3	13
Perifollicular†	10	29	11	50	3	9	7	30	2	6	8	33
Throughout‡	8	24	7	32	19	58	12	52	18	58	13	54
Difference in distribution between the survivor groups (*P*)		.09				.1				.02		
Correlation with survival												
Absent + throughout	24		11		30		16		29		16	
Perifollicular	10		11		3		7		2		8	
Difference in distribution between the survivor groups (*P*)		.16				.07				.02		
Absent + perifollicular	26		25		14		11		13		11	
Throughout	8		7		19		12		18		13	
Difference in distribution between the survivor groups (*P*)		.99				.79				.59		

Abbreviations: FOXP3, forkhead box protein P3; FL, follicular lymphoma; hpf, high-power field.
*< 5 positive cells/hpf (×40).
†Intrafollicular < 5/hpf; perifollicular 5 to > 30/hpf.
‡Positive cells (> 5/hpf) evenly distributed across the cores and no clear perifollicular pattern observed.

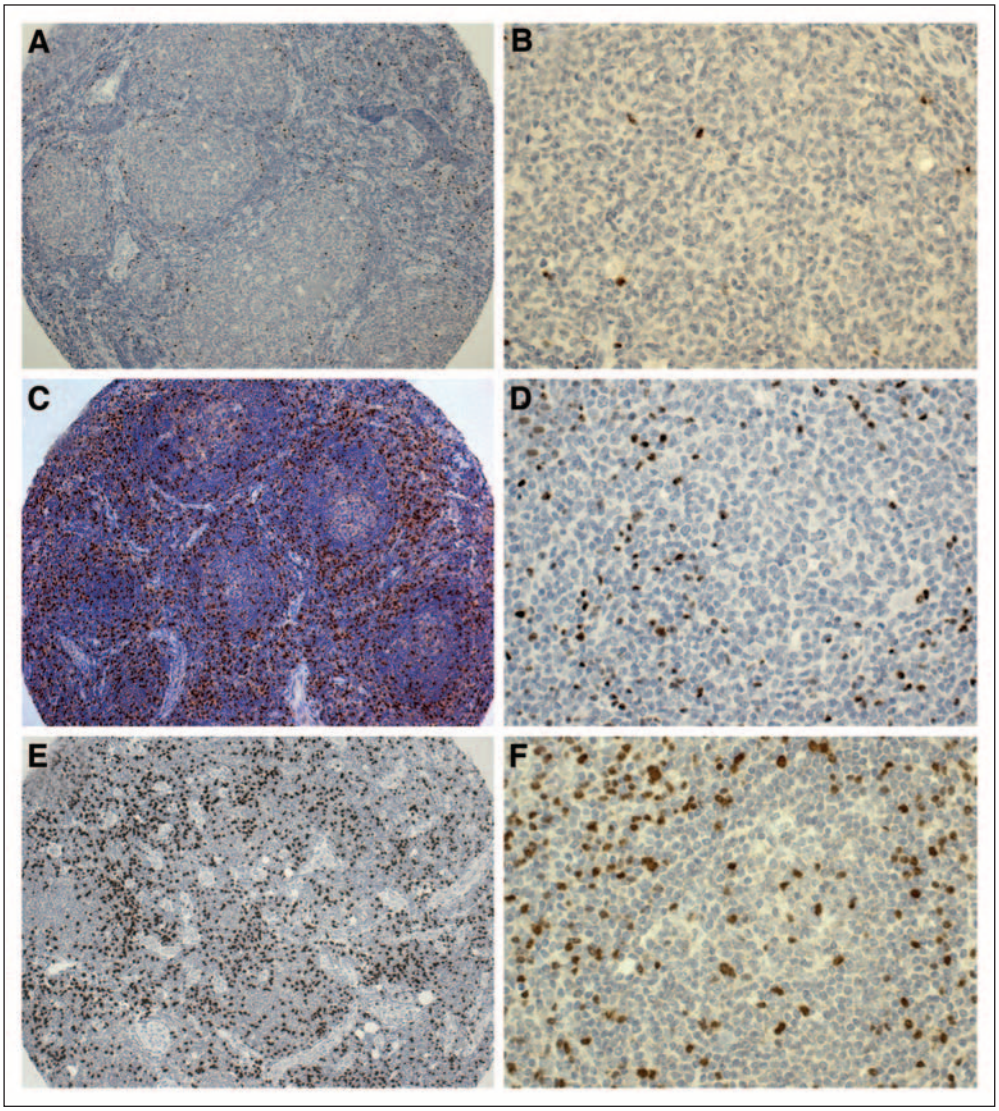

Fig 2. FOXP3 expression on tissue microarray cores from diagnostic follicular lymphoma biopsies taken at (A, C, E) low power (×10 magnification) and (B, D, F) high power (×40 magnification). Samples with (A, B) absence (< five cells/high-power field) and (C, D) presence of FOXP3 expression (> five cells/high-power field) in a perifollicular pattern, or (E, F) evenly distributed throughout the tissue.

the immune system in the setting of hematologic malignancy and provokes speculation about the roles and mechanisms involved. We identified that $CD4^+$ cells and a perifollicular pattern of FOXP3-positive cells are more commonly found in the diagnostic FL lymph node from patients whose subsequent outcome was favorable. These findings are in keeping with the hypothesis that the presence of $CD4^+$ cells are of benefit to the host. Although none of the patients underwent spontaneous regression in this study, significantly more $CD4^+$ cells in the biopsies of untreated FL patients before the tumor regression has been reported.[17]

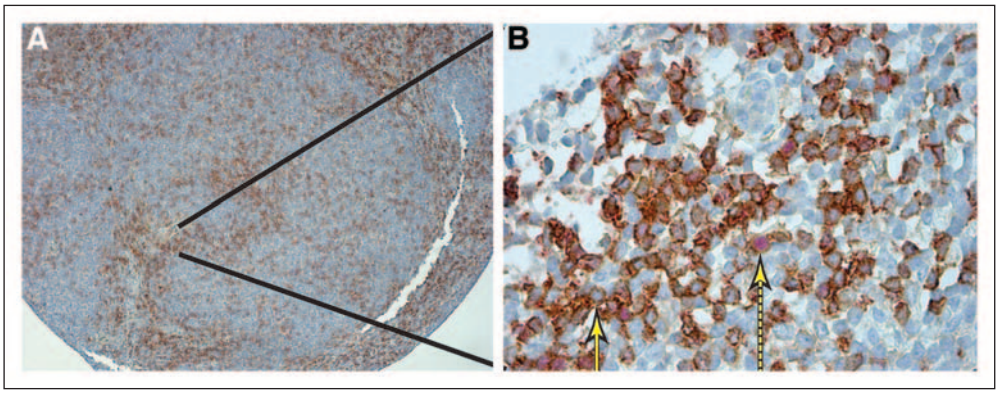

Fig 3. Double staining for CD4 (membranous brown) and FOXP3 (nuclear red). Long-survival group, (A) low power (×10 magnification); (B) high power (×63 magnification). A $CD4^+$ FOXP3-positive cell (dotted-line arrow) and a $CD4^+$ FOXP3-negative cell (solid-line arrow) are highlighted.

Although CD7 appears as a member of the immune-response gene expression signature correlating with better outcome (IR-1) in FL,[6] expression of CD7 protein alone did not correlate with survival in the data presented here. Using CD68 IHC expression as a surrogate for the macrophage-associated IR-2 signature correlating with poor outcome in gene expression studies,[6] Farinha et al[7] reported that more than 15 positively staining cells/hpf in the diagnostic biopsy correlated with short survival. This was not found in the patient groups examined here. *CD7* is only one of 42 genes comprising the *IR-1* gene signature and *CD68* is not one of the 24 genes in the *IR-2* gene signature. We currently are identifying additional antibodies suitable for IHC on paraffin-embedded tissue that detect proteins from genes of the *IR-* signature to validate further the prognostic significance of the *IR-1* and *IR-2* signatures by IHC.

It is known that age-related changes in the immune system occur, and function is impaired as exemplified by reduced vaccination efficacy[18,19] and by higher incidence of certain infections, cancers, and autoimmune disease.[20] Although absolute numbers of lymphocytes are maintained throughout life,[21,22] the T-cell subset composition and distribution may change.[23] To determine whether the distinction in prognosis seen in this study with respect to presence or absence of $CD4^+$ T cells reflects inherent age-related changes (median age of all 59 patients in the study, 56 years) or is the result of tumor-related influences is the focus of ongoing investigation using functional assays of admixed patient and healthy donor immune cell populations. The frequency of $CD4^+$ cells in the FL lymph nodes showed a trend toward correlation with age in our study. Additional studies are ongoing to determine if outcome in FL is associated with age-related changes in the immune cell composition.

CD4 T cells comprise two phenotypic subsets with distinct roles distinguishable by expression of FOXP3. FOXP3-negative $CD4^+$ cells are largely T-helper cells and FOXP3-positive $CD4^+$ cells are Tregs that modulate activated, responding FOXP3-negative T cells.[24] There is variable expression of CD25 on Tregs, which alters in response to the local immune activity.[25] Normally, Tregs restore immune homeostasis by attenuating activated mature CD4 and CD8 T cells after their response to antigen.[26,27] Tregs have an important role in suppression of inappropriate immunoreactivity, which would result in tissue damage. This is demonstrated by patients in whom loss of FOXP3 function results in autoaggressive lymphoproliferation, whereas its overexpression causes severe immunodeficiency.[28] In non-neoplastic lymphoid tissue, distribution of FOXP3-positive cells varies. Within tonsils, FOXP3 cells are in a predominantly perifollicular distribution, whereas within reactive lymph nodes an evenly distributed pattern is seen.[16] The data from this study show FOXP3-positive cells occur in the perifollicular region more commonly in samples from patients with longer survival. FOXP3-positive cells are the rate-limiting subset of the immune response. Their presence surrounding the highly active germinal center in an infected tonsil would support this function and leads to speculation that patients who do well after diagnosis of FL may do so in part because of an active immune response at the time of tumor development, with the lymphoma cells either as target or as bystander.

The Treg-associated markers CD4, CD25, and FOXP3 did not correlate consistently, most likely because these antigens are expressed variably. FOXP3 is also expressed on a subset of CD8 cells[29] and CD25 expression is modulated.[25] Carreras et al[9] reported recently that higher numbers of FOXP3-expressing cells, but not their location, are important to outcome of FL; numbers of $CD4^+$ cells were not important to outcome of FL. Minor differences in their study and the results reported here may be explained by variance in samples and methodology. They used computer analysis of full sections, studied more patients with a mixed and diffuse histology, and defined location differently. Determination of the exact phenotype and function of the cells found to have prognostic significance in our own study and that of Carreras et al[9] will require analysis of cell suspension rather than paraffin-embedded tissue, and such studies are ongoing. Using fixed tissue, we have demonstrated that $CD4^+$ FOXP3-positive cells are indeed present in the perifollicular region in the diagnostic lymph node in the FL patients with long survival.

FOXP3 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; higher levels of FOXP3 twinned with low TIA-1 and were beneficial.[30] No correlation with outcome was observed with this combination in our study of FL. These findings in immune cell malignancies are at odds with specific recruitment of FOXP3-positive Tregs promoting tumor tolerance in epithelial tumors.[8,31] These seemingly contrasting observations lead to the speculation that a critical mass of FOXP3-positive cells in a specific location within the tumor microenvironment may be necessary to maintain a functionally balanced immune response, and which may be pivotal in determining the fate of the cancer patient. However, additional functional and phenotypic investigations are essential to prove this theory definitively.

The prognostic significance of the immune microenvironment in FL at diagnosis may reflect factors involved in determining the inherent aggression of the tumor and its ability to recruit infiltrating cells on the one hand or coexist with naturally occurring cells of the lymph node on the other. To expand the understanding of the mechanisms involved in determining outcome in FL functional assays, examination of the interactions between FL and immune cells is required. In addition, the investigation of immune cell composition during the course of the disease, by analysis of sequential biopsies, will provide additional insight into the natural history of the condition. This study has identified that CD4-expressing cells and perifollicular distribution of FOXP3-expressing cells occur more commonly in the diagnostic tumor microenvironment of FL patients with better outcome compared with those with poor outcome. Elucidating the phenotype and functional capabilities of these cells is likely to provide a better understanding of the impact of the immune microenvironment on tumor progression in FL and may identify novel targets for the potentiation of tumor immunotherapy. The findings in this study can be translated easily to the routine diagnostic histopathology setting and may provide a method to aid identification of patients with potentially aggressive disease at the time of diagnosis.

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Acknowledgment

The assistance of Sharon Love, PhD, Cancer Research UK, Medical Statistics Group, Oxford, and Jean-Baptiste Cazier, PhD, Cancer Research UK, Bioinformatics and BioStatistics, is gratefully acknowledged. We thank the medical and surgical teams of St Bartholomew's Hospital and the patients for donating tissue for research.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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