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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

A Unifying Microenvironment Model in Follicular Lymphoma: Outcome Is Predicted by Programmed Death-1–Positive, Regulatory, Cytotoxic, and Helper T Cells and Macrophages

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Abstract

Purpose: The microenvironment influences outcome in follicular lymphoma. Our hypothesis was that several immune cell subsets are important for disease outcome and their individual prognostic importance should be demonstrable in the same analysis and in competition with clinical factors.

Experimental Design: Seventy follicular lymphoma patients with extreme clinical outcome ("poor" and "good" cases) were selected in a population-based cohort of 197. None of the 37 good-outcome patients died from lymphoma, whereas all the 33 poor-outcome patients succumbed in ≤5 years. Furthermore, the good-outcome patients were followed for a long time and needed no or little treatment. A tissue microarray was constructed from diagnostic, pretreatment biopsies. Cellular subsets were quantified after immunostaining, using computerized image analysis, separating cells inside and outside the follicles (follicular and interfollicular compartments). Flow cytometry data from the same samples were also used.

Results: Independently of the Follicular Lymphoma International Prognostic Index, $CD4^+$ cells were associated with poor outcome and programmed death-1–positive and $CD8^+$ cells were associated with good outcome. The prognostic values of $CD4^+$ and programmed death-1–positive cells were accentuated when they were follicular and that of $CD8^+$ cells were accentuated when they were interfollicular. Follicular $FOXP3^+$ cells were associated with good outcome and interfollicular $CD68^+$ cells were associated with poor outcome. Additionally, high CD4/CD8 and CD4 follicular/interfollicular ratios correlated with poor outcome.

Conclusion: There are many important immune cell subsets in the microenvironment of follicular lymphoma. Each of these is independently associated with outcome. This is the first study showing the effect of the balance of the entire microenvironment, not only of individual subsets. *Clin Cancer Res; 16(2); 637–50.©2010 AACR.*

Follicular lymphoma is the most common nodal indolent B-cell malignancy and is characterized by a variable clinical course. Some patients remain asymptomatic for decades, but the median time until therapy is 3 years (1). Several types of treatment lead to remission, but the disease will eventually relapse. Overall survival times vary greatly, from <1 to >30 years, with a historical average of ~10 years (2). Since the introduction of the monoclonal anti-CD20 antibody rituximab, survival times have increased (3). Still, the disease has no cure, except allogeneic stem cell transplantation. Risk stratification is done with the Follicular Lymphoma International Prognostic Index (FLIPI) based on clinical characteristics (4).

Several recent studies have shown that the immune microenvironment around the follicular lymphoma cells predicts disease outcome (5–18). In these mostly noncorroborating studies, different immune cell subsets have been reported to be important (typically one or two subsets significant and all others insignificant). An overview of earlier findings is given in Table 1. We have shown previously that nodal $CD3^+CD8^+$ cells estimated by flow cytometry are predictive for better survival, independently of the FLIPI (12), and another group had similar results from immunohistochemistry, although not FLIPI independent (10).

In the present study, our hypothesis was that several immune cell subsets are important for disease outcome and

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Translational Relevance

In the study presented here, we worked under the hypothesis that several different immune cell subsets affect outcome in follicular lymphoma. We obtained the original diagnostic specimens from extreme-outcome patients (long-time survivors that needed no or little treatment versus patients who quickly succumbed in spite of anthracycline-containing regimens). Using computerized quantification, we identified five subsets that were associated with disease outcome independently of each other and of the Follicular Lymphoma International Prognostic Index. The significant subsets were programmed death-1–positive, $CD8^+$, $FOXP3^+$, and (especially follicular) $CD4^+$ T cells and macrophages. The $CD4^+$ cells and the macrophages were associated with poor prognosis, but programmed death-1–positive, $CD8^+$, and $FOXP3^+$ cells were associated with good prognosis. This is the first study showing the effect of the balance of the entire microenvironment, not only focusing on individual subsets. We believe our findings could change how we regard the microenvironment in follicular lymphoma and suggest attractive therapeutic targets.

their individual importance should be demonstrable in the same analysis and in competition with clinical factors. Specifically, we hypothesized that (*a*) $CD8^+$ cells are associated with good prognosis (presumably due to tumor cell killing) as are (*b*) cells positive for programmed death-1 (PD-1; CD279) and FOXP3 (due to diminished B-cell stimulation), whereas (*c*) $CD4^+$ cells are associated with poor prognosis (due to B-cell stimulation). We also investigated other subsets of previously reported importance: macrophages, mast and T cells, and NK cells.

Materials and Methods

Identification of extreme-outcome cases. We assumed that patient groups with extreme differences in clinical outcome would also present the largest microenvironmental differences, which thus would be easier to detect than in an unselected cohort. Therefore, we constructed two extreme-outcome groups, focusing on the clinical behavior of follicular lymphoma. In a completely characterized population-based cohort of 197 patients *de novo* consecutively diagnosed with follicular lymphoma between 1994 and 2004 in South Stockholm County (reclassified according to the current WHO criteria; ref. 19), we examined cases that at diagnosis had sufficient material for tissue microarray analysis but not grade 3b or concomitant diffuse large B-cell lymphoma. For the poor-outcome group, we selected all patients who died from a lymphoma-related cause ≤5 years from diagnosis. The causes of death were identified in patient files, amended with data from the National Causes of Death Register in four uncertain cases. For inclusion in the good-outcome group, the patients first had to fulfill two general criteria: no possible lymphoma-related death and no autologous or allogeneic transplantation. Furthermore, for the good-outcome patients, one of the following three statements had to be true: (*a*) never treated against lymphoma and followed for ≥5 years (n = 11), (*b*) never relapsed after first-line antilymphoma treatment and followed for ≥8 years (n = 14), or (*c*) relapsed but never received intensive (transplantation) or frequent (≥3 years between) treatments and followed for ≥10 years (n = 12). The rationale for this approach was our interest in detecting truly disease-specific prognostic microenvironmental factors. These selection criteria rendered 37 and 33 patients in the good- and poor-outcome group, respectively. All 70 patients were identified and grouped before the construction of the tissue microarray. Last follow-up was done in January to April 2009. The clinical characteristics of the 70 patients are given in Table 2, showing striking differences between the two selected groups, as expected: the FLIPI and its constituting factors (except age) differed greatly, but neither follicular lymphoma grade, proportion of diffuse component, nor Ki-67 positivity did. The poor-outcome patients had been treated heavily (91% received anthracyclines and 4 were transplanted) compared with the good-outcome patients who had experienced long survival times with only 30% receiving anthracyclines and none transplanted. The poor-outcome patients had all died from lymphoma-related causes (median, 2.2 years after diagnosis), whereas the good-outcome patients have long follow-up (median, 10.1 years) and no lymphoma-related deaths.

Samples from relapses were also studied. From first relapse, flow cytometry results were available in 24 patients and biopsies for tissue microarray in 13 patients, and from second relapse, flow cytometry results were available in 7 patients and biopsies for tissue microarray in 3 patients.

This study was approved by the Local Ethical Committee of Medical Research, Stockholm.

Tissue microarray. Twin 1.2 mm cores were taken from the diagnostic and relapse tumor biopsies of the patients and put into a paraffin-embedded tissue microarray. One good and two poor diagnostic cases had insufficient material left for successful core production, leaving 67 cases. Sections of the tissue microarray were stained with antibodies (Table 1). The one core of the pairs that had the best quality was selected for each case. The cell populations were quantified using an automated scanning microscope and computerized image analysis system (Ariol SL-50; Genetix) under the supervision of an expert hematologic-pathologic team. The follicular and interfollicular areas within each core were defined because the system allows for precise demarcation by drawing boundaries on the virtual slides (Fig. 1A). The strong staining with most of the nonnuclear antibodies made individual cells difficult to discern by the Ariol system, due to overlapping

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positivity, why these subsets were quantified as the fraction of cellular antibody-positive area divided by the sum of antibody-positive and antibody-negative cellular area. Cells positive for the nuclear marker FOXP3 and CD4 and PD-1 were better quantified as numbers of positive cells divided by sum of total cellular area (Fig. 1B and C). The software algorithm was determined for each marker and applied in all the samples in the same way. For all measurements, validation of the specificity of the staining was done by a hematopathologist. Also, to ensure the robustness of the results, the automated quantification results were verified with flow cytometry.

The follicular and interfollicular areas in each core were separately quantified, to get results in three different compartments (total core, follicular, and interfollicular) for each antibody in each case, except cases with entirely diffuse cores, where only total core values were extracted. The number of entirely diffuse cores were ~11 in each staining.

Flow cytometry. Flow cytometry had routinely been done on all unfixed biopsies as described previously (12). In 64 cases, flow cytometry data were available from the same specimens used in the tissue microarray. All flow cytometry results were re-reviewed.

Statistical analysis. Correlations between continuous and ordinal variables were estimated with linear regression or Spearman's test depending on the nature of the variables. Categorical data were compared using χ^2 or Fisher's exact test. The quantifications of the immune cell subsets were analyzed as continuous variables. Differences in the distribution of subsets between the outcome groups were first estimated with the Mann-Whitney-Wilcoxon rank-sum test in univariate analysis and with logistic regression in bivariate FLIPI-adjusted analysis (Table 3) to reduce the number of competing factors in multivariate analysis. Only the subsets with at least borderline significance [nominal $P < 0.25$ in at least one of the three compartments (total, follicular, and interfollicular) in Table 3] were taken to multivariate analysis, which was done using forward stepwise logistic regression, always in competition with the FLIPI. Because the number of predictors competing in multivariate analysis was a potential concern, forward regression was preferred to backward, because forward regression need not include all competing predictors simultaneously during analysis. The multivariate models were checked for interaction between the factors and none was found. All statistical calculations were done using Stata 9.2 (StataCorp).

Results

Data verification. Using linear regression, the automated subset quantifications from tissue microarray were verified with corresponding flow cytometry results, showing strong correlations between total core tissue microarray and flow cytometry results in all applicable cases: CD3, CD4, CD7, CD8, and FOXP3/CD3$^+$CD25$^+$ (Table 1; Fig. 1D). In the tissue microarray, PD-1 values (being a sub-subset of CD4$^+$ cells) were indirectly verified through its correlations with CD4 ($P < 0.000005$) and TIA-1 and perforin (being mostly expressed in CD8$^+$ cells) through correlations with CD8 ($P = 0.027$ and 0.002, respectively). CD57 was associated with CD3 ($P = 0.007$) and granzyme B was associated with perforin ($P = 0.003$). However, no collinearity was strong enough to complicate multivariate analysis (R^2 always <0.4; data not shown). Investigation of differences in the distribution of subsets between compartments revealed PD-1$^+$ cells to be the only subset more frequent inside than outside the follicles (Table 1).

Differences between good- and poor-outcome groups. The distribution of the subsets in the two outcome groups are shown in Table 3. Cells positive for CD4, FOXP3, PD-1, CD8, granzyme B, TIA-1, perforin, CD57, CD56, and CD68 had a difference between good- and poor-outcome groups with $P < 0.25$ in at least one of the three compartments (total core, follicular, and interfollicular). These subsets were taken to a first multivariate analysis together with the FLIPI, where only total core values were used, because we did not want to lose information from the diffuse cases (Table 4A). The only subsets significantly associated with outcome were CD4$^+$ cells (poor) and CD8$^+$ and PD-1$^+$ cells (good).

To better clarify the role of the cells in the follicular and interfollicular compartments, we did a second multivariate analysis, also FLIPI-adjusted. For CD4, FOXP3, and PD-1, values from the follicular compartment were used. This would adjust the effect of the follicular CD4$^+$ cells to those follicular cells that are positive for FOXP3$^+$ or PD-1$^+$ (and also mostly CD4$^+$). Thus, the contribution of CD4$^+$ population to the model would be from the follicular CD4$^+$PD-1$^-$FOXP3$^-$ cells. Because all other investigated subsets mostly take residence outside the follicles, interfollicular values were used for these. In this model, follicular CD4$^+$ cells had an even stronger association with poor outcome and the inhibitory follicular PD-1$^+$ and FOXP3$^+$ cells with good outcome. Interfollicularly, CD8$^+$ cells increased their correlation with good outcome and CD68$^+$ cells (macrophages) entered as related with poor outcome (Table 4B).

Because of the relative weakness of the CD4 staining, we wanted to ensure that its quantification was not attributable to computerized misinterpretation. To do this, "indirect" follicular CD4 values were calculated from the difference between follicular CD3 and CD8 quantification and inserted in the model. These "indirect" follicular CD4 values also independently correlated with poor prognosis ($P = 0.012$).

Ratios between follicular and interfollicular CD4$^+$ cells and between CD4$^+$ and CD8$^+$ cells. CD4$^+$ cells were the sole subset wherein the ratio between follicular and interfollicular cells was associated with outcome (Table 3). Also, a high CD4/CD8 ratio in all compartments correlated with poor outcome. Note that the low values in the CD4/CD8 ratio are due to different quantification methods for CD4 and CD8. These two ratios were the best dichotomous predictors of outcome (Fig. 2A), but because of their compound nature they were not included in the multivariate models.

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Table 1. Antibody sources, investigated subsets and their distributions in the study population, and associations with flow cytometry results and previous findings

Subset	Antibody source	Best quantification	Compartment	p50
CD3	NCL (L-CD3-565)	Positive area	Total	56.5
			Follicular	38.2
			Interfollicular	66.0
CD7	NCL (L-CD7-272)	Positive area	Total	41.3
			Follicular	27.0
			Interfollicular	50.0
CD4	NCL (L-CD4-368)	Cell count	Total	5.8
			Follicular	3.5
			Interfollicular	6.5
FOXP3	Abcam (ab20034)	Cell count	Total	1.9
			Follicular	1.3
			Interfollicular	2.1
PD-1	Spanish National Cancer Research Centre	Cell count	Total	2.6
			Follicular	3.3
			Interfollicular	2.4
CD8	DAKO (M7103)	Positive area	Total	17.6
			Follicular	7.9
			Interfollicular	22.1
Granzyme B	NCL (L-Gran-B)	Positive area	Total	0.32
			Follicular	0.14
			Interfollicular	0.37
TIA-1	Immunotech (Im2550)	Positive area	Total	1.0
			Follicular	0.56
			Interfollicular	0.94
Perforin	NCL (perforin)	Positive area	Total	0.80
			Follicular	0.44
			Interfollicular	1.0
CD57	DAKO (TB01)	Positive area	Total	9.9
			Follicular	7.1
			Interfollicular	10.9
CD56	NCL (L-CD56-IB6)	Positive area	Total	0.36
			Follicular	0.15
			Interfollicular	0.46
CD68	DAKO (M0876)	Positive area	Total	10.4
			Follicular	6.6
			Interfollicular	11.5
Tryptase	DAKO (AO12)	Positive area	Total	0.86
			Follicular	0.09
			Interfollicular	1.3

NOTE: Each subset's values are given as percentages of area positive for the antibody, except in CD4, FOXP3, and PD-1, where values are numbers of positive cells per 10^{-9} m^2.

Associations of subsets with grade, transformation, and the FLIPI. Only $CD8^+$ cells were associated with histologic grade at diagnosis (negatively; total core $P = 0.0225$). Subsets at diagnosis were also analyzed with respect to subsequent transformation to diffuse large B-cell lymphoma, of which there were 13 pathology-verified cases. Only follicular $CD4^+$ cells had a trend (positive; $P = 0.079$) towards association with transformation. Significant (and positive) associations with the FLIPI could be seen in follicular CD68 ($P = 0.0140$), total core and interfollicular CD57 (total core $P = 0.0127$), and all three compartments of TIA-1 (total core $P = 0.0003$). The study was not designed for these analyses, so these findings were interpreted with caution.

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Table 1. Antibody sources, investigated subsets and their distributions in the study population, and associations with flow cytometry results and previous findings (Cont'd)

p25	p75	Range	Corresponding flow cytometry subset	*P* (association tissue microarray- flow cytometry)	Previous significant prognostic findings (reference)
45.0	70.1	21.2-92.1	$CD3^+$	0.047	Good (15)
29.6	48.4	5.9-88.5			
50.5	78.5	22.2-93.3			
29.2	58.9	2.4-89.4	$CD3^+CD7^+$	0.009	Good (15)
18.0	44.3	1.5-89.4			
33.3	68.7	9.2-89.4			
1.6	8.7	0.05-17.2	$CD3^+CD4^+$	0.004	Good (8)
0.59	5.8	<0.01-17.8			More transformations
2.2	9.1	0.07-15.4			when follicular (11)
1.2	3.0	0.35-9.0	$CD3^+CD25^+$	<0.000005	Good (9, 18)
0.79	2.0	0.10-4.1			
1.3	3.1	0.54-7.7			Good when interfollicular (8)
1.3	4.5	0.03-7.8	—	—	Good (30; published during
1.8	4.4	0.07-10.2			our analysis phase)
1.0	4.3	0.01-8.5			
9.6	26.7	2.0-64.1	$CD3^+CD8^+$	<0.000005	Good (10, 12)
3.9	13.1	0.22-54.7			
14.2	35.3	4.0-66.4			
0.17	0.53	0.05-3.9	—	—	—
0.08	0.33	0.02-1.9			
0.24	0.59	0.06-5.0			
0.50	2.2	0.05-23.2	—	—	—
0.21	1.2	<0.01-21.8			
0.61	2.1	0.05-24.9			
0.45	1.7	0.15-6.5	—	—	—
0.28	1.3	0.07-3.2			
0.51	2.1	0.23-9.0			
5.0	17.7	0.11-71.6	—	—	Associated with high-stage
4.8	14.5	0.10-81.1			disease (10)
5.2	21.2	0.11-63.7			
0.19	0.70	0.02-10.5	—	—	—
0.04	0.32	<0.01-3.3			
0.24	0.72	0.03-12.4			
7.0	14.9	2.8-23.9	—	—	Poor (7, 15). Poor only without
4.7	8.7	1.5-14.1			rituximab (13, 14, 16)
8.3	16.4	3.1-28.4			
0.31	2.2	<0.01-30.6	—	—	Poor with rituximab (17)
0.01	0.23	<0.01-6.0			
0.44	2.6	<0.01-67.2			

Subsequent biopsies. There were no significant differences in subsets or ratios between the diagnostic and the subsequent biopsies when both were follicular lymphoma (n = 13). In four subsequent biopsies, the disease had transformed to diffuse large B-cell lymphoma, with an altered microenvironment: all subsets, except macrophages, were scarcer than at the original follicular lymphoma diagnosis, especially $CD4^+$ cells, and there was, accordingly, a tendency towards lowered CD4/CD8 ratios.

Discussion

Specific immune cell subsets in the microenvironment of follicular lymphoma have been suggested to influence the clinical outcome (5–18). In tissue microarray, using an automatic quantifying method that separates the follicular and interfollicular compartments, we have identified several FLIPI-independent biological differences between patients in two clinical extreme-outcome groups.

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Table 2. Clinical characteristics

Characteristic	Outcome group		*P*
Category	Good, *n* (%)	Poor, *n* (%)	
Grade at diagnosis			
1	14 (38)	7 (21)	
2	14 (38)	14 (42)	
3a	9 (24)	12 (36)	0.281
Proportion of diffuse component at diagnosis			
<25%	30 (81)	26 (79)	
≥25%	7 (19)	7 (21)	0.811
Proportion of Ki-67$^+$ cells at diagnosis			
<25%	10 (36)	11 (44)	
≥25%	18 (64)	14 (56)	0.538
Age at diagnosis (y)			
≤60	19 (51)	10 (30)	
>60	18 (49)	23 (70)	0.072
Lactate dehydrogenase at diagnosis			
Normal	30 (83)	13 (41)	
Elevated	6 (17)	19 (59)	<0.0001
Hemoglobin level at diagnosis (g/dL)			
≥12	35 (94)	21 (66)	
<12	2 (5)	11 (34)	0.002
Involved nodal areas at diagnosis			
≤4	29 (78)	13 (42)	
>4	8 (22)	18 (58)	0.002
Ann Arbor stage at diagnosis			
I-II	16 (43)	3 (9)	
III-IV	21 (57)	29 (91)	0.002
Risk according to the FLIPI at diagnosis			
Low	20 (54)	2 (6)	
Intermediate	10 (27)	9* (27)	
High	7 (19)	22 (67)	<0.0001
B symptoms at diagnosis			
No	33 (89)	19 (59)	
Yes	4 (11)	13 (41)	0.004
Bulky disease at diagnosis			
No	32 (86)	19 (63)	
Yes	5 (14)	11 (37)	0.027
Sex			
Female	24 (65)	14 (42)	
Male	13 (35)	19 (58)	0.060
Rituximab in first line			
No	33 (89)	31 (94)	
Yes	4 (11)	2 (6)	0.479
Ever received rituximab			
No	25 (68)	24 (73)	
Yes	12 (32)	9 (27)	0.638
Anthracyclines in first line			
No	28 (76)	10 (30)	
Yes	9 (24)	23 (70)	<0.0001
Ever received anthracyclines			
No	26 (70)	3 (9)	
Yes	11 (30)	30 (91)	<0.0001

(*Continued on the following page*)

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Table 2. Clinical characteristics (Cont'd)

Characteristic	Outcome group		*P*
Category	Good, *n* (%)	Poor, *n* (%)	
Oral alkylators in first line			
No	32 (86)	26 (79)	
Yes	5 (14)	7 (21)	0.394
Local irradiation in first line			
No	29 (78)	30 (91)	
Yes	8 (22)	3 (9)	0.150
Later transformation to diffuse large B-cell lymphoma			
No	36 (97)	21 (64)	
Yes	1 (3)	12 (36)	<0.0001
Deceased			
No	32 (86)	0 (0)	
Yes	5 (14)	33 (100)	—
Causes of death			
Lymphoma	0 (0)	25 (76)	
Complications to antilymphoma therapy	0 (0)	7 (21)	
Possibly lymphoma	0 (0)	1 (3)	
Dementia	2 (40)	0 (0)	
Cardiac failure	1 (20)	0 (0)	
Cerebral hemorrhage	1 (20)	0 (0)	
Pneumonia 9 y in remission	1 (20)	0 (0)	—
Follow-up time (y)			
Median (range)	10.1 (5.4-15.1)	2.2 (0.4-5.0)	—

*Based on incomplete patient files, one patient was estimated to be in the intermediate-risk group.

To our knowledge, this is the first demonstration of the effect of the balance of the entire microenvironment. Several immune cell subsets were independently associated with outcome (also independently of the FLIPI): CD8, CD4, PD-1, FOXP3, and CD68 (Table 4; Fig. 2B). We believe that prognosis in follicular lymphoma is affected by several subsets simultaneously at work in the microenvironment rather than being dictated by an individual immune cell subset. This multifactorial detection has probably been facilitated by our extreme case selection and with a quantifying method that does not lose information as much as semiquantifying methods do. Although it could be that tissue microarray cores are not representative of the entire lymph node in follicular lymphoma, there was good agreement between tissue microarray and whole-node flow cytometry results. The two groups contain truly clinical extreme-outcome patients, not only with regard to chronologic follow-up time and mortality but also to disease-related clinical characteristics, disease aggressiveness, and need for treatment. Selections based only on survival status and follow-up time would probably have obscured the microenvironmental factors, because such a selection would have been more influenced by differences unrelated to lymphoma biology, such as patients' abilities to tolerate intensive treatment and their age. A weakness of our approach is that it disregards the 60% of patients that do not fit our inclusion criteria. However, our findings should be tested on prospective cohorts.

Our data confirm that $CD8^+$ cells (CTLs) correlate with good prognosis (10, 12), regardless of their distribution patterns in the follicular lymphoma nodes, although the vast majority of CTLs are interfollicular. The influence of CTLs in follicular lymphoma could be through cytokines or from "follicular border patrolling." Markers of activated CTLs had diverging results and did not hold significance in multivariate analysis.

The adverse action of $CD4^+$ cells was especially apparent in the follicular compartment. Based on the findings of worse outcome with (*a*) more $CD4^+$ cells, especially when follicularly located and adjusted to PD-1 and FOXP3, and (*b*) higher follicular/interfollicular CD4 ratios, it is obvious that the adverse subset is follicular and positive for CD4 and negative for FOXP3 and PD-1, which would mean that they are follicularly residing helper T cells. The most frequent T helper subset in lymphatic tissue, especially in follicles, is follicular B helper T cells (T_{FH}), but the $CD4^+FOXP3^-PD\text{-}1^-$ follicular cells also include the Th1 and Th2 subsets (20). No single-staining marker exists to identify T_{FH} with automated image analysis. T_{FH} are

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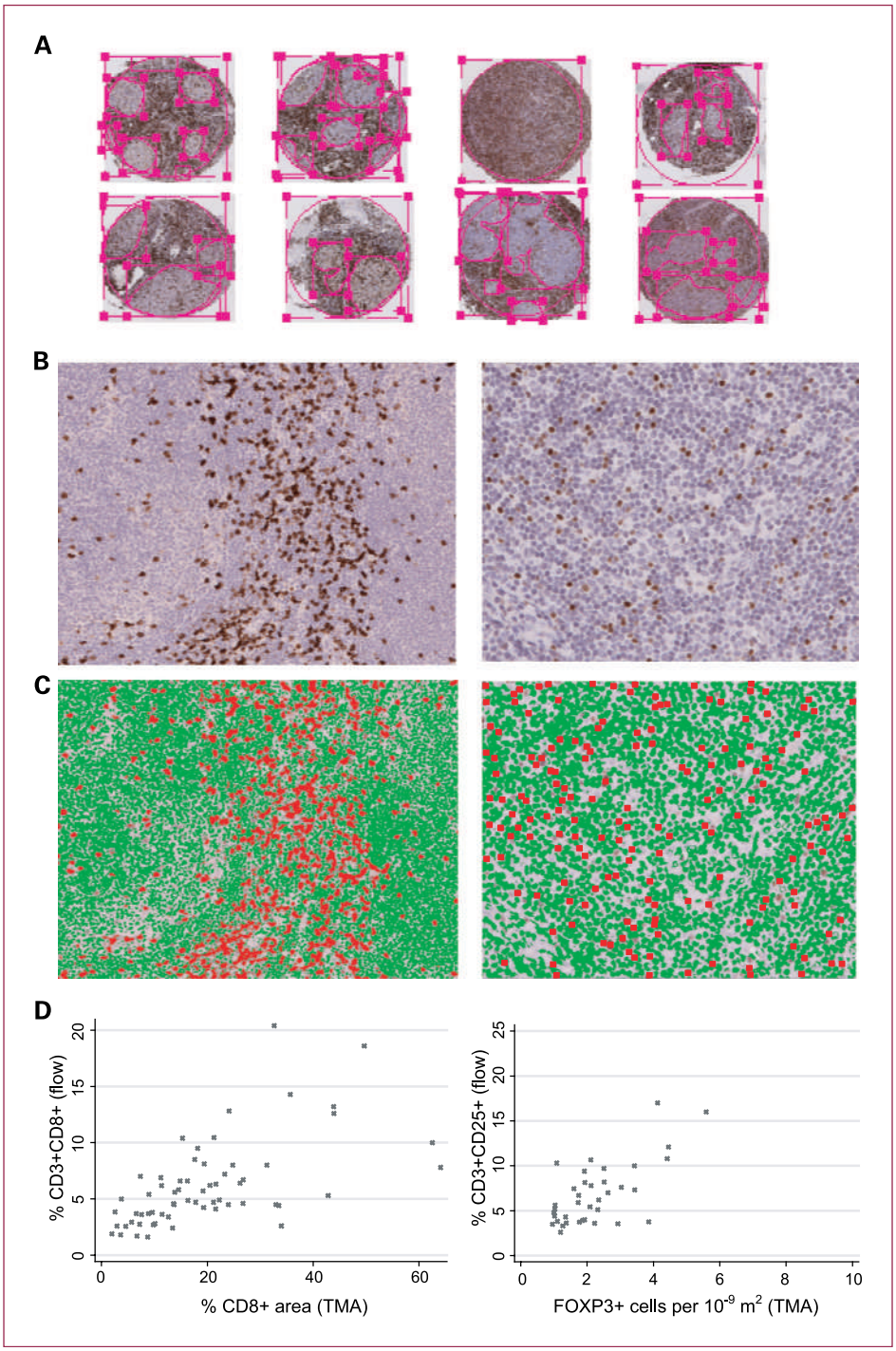

Fig. 1. *A,* examples of follicular-interfollicular demarcation in CD3 staining (one case is diffuse). *B,* nonnuclear CD8 (*left*) and nuclear FOXP3 (*right*) staining. *C,* respective best quantification method: negative green and red positive cellular area in CD8 (*left*) and cell count in FOXP3 (*right*). *D,* values derived with these methods correlated with flow cytometry data.

distinguishable from other helper T cells by several criteria, including chemokine receptor expression (CXCR5), location (B-cell follicles), and function (B-cell help). T_{FH} produce the cytokine interleukin (IL)-21 that potently stimulates the proliferation, isotype switch, and differentiation of B cells through IL-21R (20). Follicular lymphoma cells have been reported to show exceptionally high IL-21R expression compared with other lymphomas (21). It is possible that the T_{FH}-produced IL-21 stimulates follicular lymphoma cells to proliferate, increasing the risk for additional genetic damage and transformation. In the present study, a trend towards increased risk of transformation was seen in patients with more follicular $CD4^+$ cells, in accordance with a previous report (11). IL-21

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greatly enhances the proliferation of $CD4^+CD25^-$ cells, rendering them resistant to regulatory T cell (Treg)–mediated suppression (22). However, to complicate the picture, IL-21 also activates CTLs and NK cells, and antitumoral effects of IL-21 (including apoptosis of follicular lymphoma cell lines) have been seen (23). Helper T cells, and T_{FH} most proficiently, express CD40L, which protects follicular lymphoma cells from apoptosis through CD40 (24), and other signals, such as IL-4, stimulating the follicular lymphoma cells to proliferate (25, 26).

Tregs, defined as $FOXP3^+$ (27), have been associated with good prognosis in follicular lymphoma (8, 9, 18). Tregs negatively regulate T_{FH}- and T_{FH}-dependent B-cell survival (28) and directly suppress B cells (29). In our study, Tregs and the suppressive $PD\text{-}1^+$ subset were both associated with good prognosis. During the data analysis phase of this work, Carreras et al. reported similar results for PD-1, in the same cohort that they had previously studied for Tregs (which now did not retain independent prognostic value; ref. 30). Our independent results confirm the importance of both $PD\text{-}1^+$ cells and Tregs. PD-1 and its ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273), constitute an important inhibitory pathway in T-cell immunity, promoting T-cell tolerance (31). PD-1 is detected in some T-cell lymphomas of T_{FH} origin (32, 33) and is expressed in $CD4^+CD25^-$ (but FOXP3 in $CD4^+CD25^+$) cells. The intrafollicularly concentrated $PD\text{-}1^+$ cells in follicular lymphoma could represent a subset of T_{FH}, different from Tregs and B-cell stimulatory T_{FH} (27, 31). PD-L1 is expressed in several cell types, including Tregs (34), but not follicular lymphoma cells (30, 31). PD-L2 is only expressed in dendritic cells, macrophages, and mast cells (31). PD-1-PD-L interaction leads to negative regulation of the $PD\text{-}1^+$ cell, but the $PD\text{-}1^+$ cell can also affect its surroundings via reverse signaling through PD-L2 and PD-L1. PD-1-PD-L ligation leads to reverse signaling into dendritic cells, inhibiting the induction of the $CD4^+CD25^-$ T-cell response by dendritic cells (31). In summary, $PD\text{-}1^+$ and $FOXP3^+$ cells are $CD4^+$ subsets, the prognostic importance of which in follicular lymphoma probably is due to their inhibition of other immune cells that otherwise assist follicular lymphoma cells.

In our study, $CD57^+$ cells had no association with outcome, but with the FLIPI, similar to a previous report (10). CD57 is expressed in a subset of T_{FH}, but the function of the CD57 molecule and the T cells expressing this NK cell marker is uncertain. $CD4^+CD57^+$ cells have inconclusively been called both an anergic (incapable of producing B-cell helper cytokines) and an energic (highly expressing inducible costimulator) T_{FH} subset (20). PD-1 expression is stronger in $CD57^+$ cells than in other T_{FH} (20), and T cells coexpressing PD-1 and CD57 have been observed in follicular lymphoma (30). $PD\text{-}1^+$ and $CD57^+$ cells seem to be two distinct but sometimes overlapping subsets (Fig. 2B; ref. 33).

Previous studies have shown that tumor-associated macrophages are an adverse prognostic factor in follicular lymphoma (7, 15), circumvented with rituximab-containing regimens (13, 14, 16). The mechanism through which macrophages would be adverse in follicular lymphoma is unclear; one suggestion is that macrophages prevent CTLs from attacking tumor cells (35, 36). The circumvention seen in rituximab-treated patients might depend on macrophagic participation in antibody-dependent cellular cytotoxicity (37, 38). In our study, macrophages were the statistically weakest of the predictive factors. Few patients in this study received rituximab in first line (7%), but more in later lines (cumulatively 30%), which probably dilutes the prognostic effect of the subset. Furthermore, we advocate some caution in interpreting the importance of the macrophages, because, among the subsets significant in multivariate analysis, macrophages were the only one that had not been included in our study hypothesis.

Dichotomizing the individual factors, two compound values, each and together, best predict outcome: the follicular/interfollicular CD4 ratio and the tissue microarray-derived CD4/CD8 (in all compartments but most obvious in the follicles), although the information in these ratios is rather crude, because $PD\text{-}1^+$ cells and Tregs are included in the CD4 value. We could not show any prognostic importance of NK cells, and neither could we confirm that of $CD3^+$, $CD7^+$ (15), or mast cells (albeit the last have only shown effect in rituximab-treated patients; ref. 17).

The standard treatments for problematic cases in this population have been anthracycline-based (CHOP), and our results should be viewed in that context. Rituximab has altered the prognostic value for macrophages and could possibly do that for other subsets as well. For example, a vaccinal effect of rituximab, which would be dependent on CTLs, has been proposed (39) and recently supported with the detection of follicular lymphoma idiotype-specific T cells after rituximab (40). It has also been shown that different chemotherapy regimens could alter the prognostic value of individual immune cell subsets (18). Still, there are follicular lymphoma patients who *never* need treatment, and investigating more of such cases would probably be of great help in understanding the microenvironment of follicular lymphoma. However, in our study, the immune cell numbers in the good-outcome patients that needed *no* and those who needed *some* treatment were similar (data not shown), suggesting that prognostic immune cells are similarly important with or without therapy. Furthermore, the relative stability in the follicular lymphoma microenvironment in sequential biopsies (also after therapy) explains why the diagnostic tissues can predict prognosis far ahead in time.

We conclude that there are different but equally important cells at work in the milieu of follicular lymphoma. From our findings and those of others, we tentatively propose a model in which follicular helper T cells, most probably a B-cell stimulatory T helper subset, promote the survival and proliferation of follicular lymphoma cells, increasing the risk for progressive, non-chemotherapy-responding disease and maybe also for transformation.

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Table 3. Distributions of subsets in outcome groups

Factor	Good outcome			Poor outcome			*P*	*P* (adjusted to the FLIPI)
	p50	p25	p75	p50	p25	p75		
CD3								
Total	55.4	45.0	70.9	59.0	38.6	68.9	0.5939	0.5485
Follicular	35.4	26.6	49.2	39.3	33.0	47.5	0.5988	0.9020
Interfollicular	68.3	51.0	78.2	65.8	49.8	78.7	0.7089	0.8964
CD7								
Total	40.2	28.0	58.9	41.8	30.8	60.1	0.7047	0.5998
Follicular	28.3	12.0	42.5	25.3	18.2	46.7	0.6468	0.9259
Interfollicular	49.8	31.7	70.9	54.6	37.0	68.6	0.8519	0.9796
CD4								
Total	5.0	1.0	8.4	6.4	3.0	10.2	0.0849	0.2668
Follicular	1.8	0.18	5.4	4.2	2.4	6.4	0.0367	0.1827
Interfollicular	6.4	1.2	8.6	7.1	3.3	9.6	0.1506	0.5020
FOXP3								
Total	2.1	1.2	3.4	1.7	1.2	2.7	0.4146	0.4931
Follicular	1.3	0.68	2.8	1.3	0.81	1.7	0.5696	0.0576
Interfollicular	2.3	1.9	3.8	1.5	1.1	2.8	0.0452	0.1429
PD-1								
Total	2.7	1.7	4.3	2.5	1.0	4.5	0.4581	0.1055
Follicular	3.7	2.2	4.9	2.8	1.6	4.1	0.1402	0.0689
Interfollicular	2.2	1.0	4.1	2.5	0.92	4.6	0.9016	0.3031
CD8								
Total	18.5	13.5	32.1	13.7	7.4	23.9	0.1103	0.0662
Follicular	8.6	4.1	16.9	6.2	3.8	10.3	0.2134	0.1228
Interfollicular	22.1	16.7	44.8	18.3	12.0	34.2	0.1447	0.1210
Granzyme B								
Total	0.38	0.26	0.77	0.26	0.13	0.38	0.0178	0.2422
Follicular	0.15	0.11	0.49	0.12	0.05	0.23	0.0818	0.2926
Interfollicular	0.47	0.32	0.83	0.26	0.13	0.43	0.0115	0.2349
TIA-1								
Total	0.68	0.32	1.9	1.1	0.83	2.4	0.0368	0.8611
Follicular	0.25	0.12	1.0	0.91	0.50	1.9	0.0154	0.9464
Interfollicular	0.68	0.35	1.4	1.1	0.90	2.6	0.0146	0.8130
Perforin								
Total	0.75	0.41	1.7	0.92	0.57	1.7	0.4892	0.8037
Follicular	0.40	0.25	0.87	0.52	0.36	1.5	0.1688	0.2511
Interfollicular	0.83	0.44	1.8	1.0	0.64	2.8	0.2953	0.9799
CD57								
Total	7.8	4.3	21.0	10.9	5.5	15.6	0.7573	0.1076
Follicular	7.5	4.5	15.0	6.2	5.0	14.1	0.6504	0.2874
Interfollicular	8.9	3.8	23.2	11.8	5.9	17.8	0.8756	0.1229
CD56								
Total	0.30	0.17	0.70	0.50	0.20	0.78	0.3929	0.5300
Follicular	0.12	0.03	0.25	0.21	0.21	0.08	0.1022	0.9949
Interfollicular	0.46	0.25	0.70	0.45	0.19	1.0	0.7006	0.8451
CD68								
Total	9.0	6.7	14.4	12.4	8.2	15.5	0.2103	0.7405
Follicular	5.7	4.0	9.9	7.9	5.2	8.6	0.3436	0.5153
Interfollicular	10.3	8.3	15.9	14.5	8.2	17.6	0.4010	0.9136

(*Continued on the following page*)

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Table 3. Distributions of subsets in outcome groups (Cont'd)

Factor	Good outcome			Poor outcome			*P*	*P* (adjusted to the FLIPI)
	p50	p25	p75	p50	p25	p75		
Tryptase								
Total	1.0	0.18	2.1	0.83	0.55	2.8	0.4137	0.3688
Follicular	0.10	0.01	0.39	0.09	0.01	0.20	0.7920	0.5773
Interfollicular	1.33	0.38	2.5	0.94	0.74	3.1	0.5257	0.6943
CD4/CD8 ratio								
Total	0.21	0.03	0.37	0.35	0.24	1.1	0.0028	0.0139
Follicular	0.21	0.03	0.47	0.52	0.32	1.0	0.0040	0.0485
Interfollicular	0.14	0.02	0.36	0.28	0.20	0.60	0.0234	0.0293
CD4 follicular/CD4 interfollicular ratio								
	0.44	0.25	0.72	0.67	0.45	0.98	0.0262	0.0381

NOTE: Each factor's values are given as percentages of area positive for the antibody, except in CD4, FOXP3, and PD-1, where values are numbers of positive cells per 10^{-9} m^2 or when specified as ratios.

The helper T cells are inhibited by two subsets, PD-1$^+$ cells and Tregs, maybe through separate mechanisms. Also, Tregs may directly inhibit the follicular lymphoma cells and PD-1$^+$ cells may act via other immune cells, such as dendritic cells. CTLs protect from progressive and aggressive follicular lymphoma, through either cytokines (41) or direct cytotoxicity or both. Macrophages are dual: adverse maybe because they inhibit CTLs but advantageous because they participate in the actions of rituximab. T cells are divided into an ever-increasing number of subsets, and with the identification of more specific T-cell populations, the sketch proposed here will probably improve. Furthermore, there are factors in the microenvironment that we have not addressed, such as dendritic cells, follicular dendritic cells, and angiogenesis, the roles of which remain unclear.

We believe that our focus on extreme clinical behavior has allowed for a unique and novel finding in this study: five immune cell subsets that independently affect disease outcome and also may present some attractive therapeutic targets for specific monoclonal antibodies or cytokines. Our findings are based on long-surviving patients whose lymphomas demand little or no treatment versus patients whose lymphomas have warranted intensive therapy, mostly CHOP-based, yet still been rapidly lethal. Larger studies, preferably on rituximab-treated patients, will be needed for verification of the unifying model we here propose. We cannot say whether these subsets carry the same prognostic information in patients that receive rituximab-containing therapy upfront. Still, many patients will remain asymptomatic and untreated for decades, and for those patients, CTLs, PD-1$^+$ cells,

Table 4. Multivariate models

Model	Factor	Odds ratio (95% confidence interval)	*P*
(A) All patients	CD4 total core	1.26 (1.03-1.54)	0.025
	PD-1 total core	0.58 (0.37-0.92)	0.020
	CD8 total core	0.94 (0.89-0.99)	0.024
(B) Compartmental model	CD4 follicular	2.16 (1.21-3.87)	0.010
	FOXP3 follicular	0.09 (0.01-0.66)	0.018
	PD-1 follicular	0.34 (0.13-0.84)	0.019
	CD8 interfollicular	0.86 (0.76-0.97)	0.014
	CD68 interfollicular	1.36 (1.01-1.82)	0.040

NOTE: The number of observations in analysis A and B was 67 and 53, respectively. All multivariate analyses were done with adjustment to the FLIPI. Competing, nonsignificant factors were total core estimates of FOXP3, granzyme B, TIA-1, perforin, CD57, CD56, and CD68 in analysis A and interfollicular estimates of granzyme B, TIA-1, perforin, CD57, and CD56 in analysis B.

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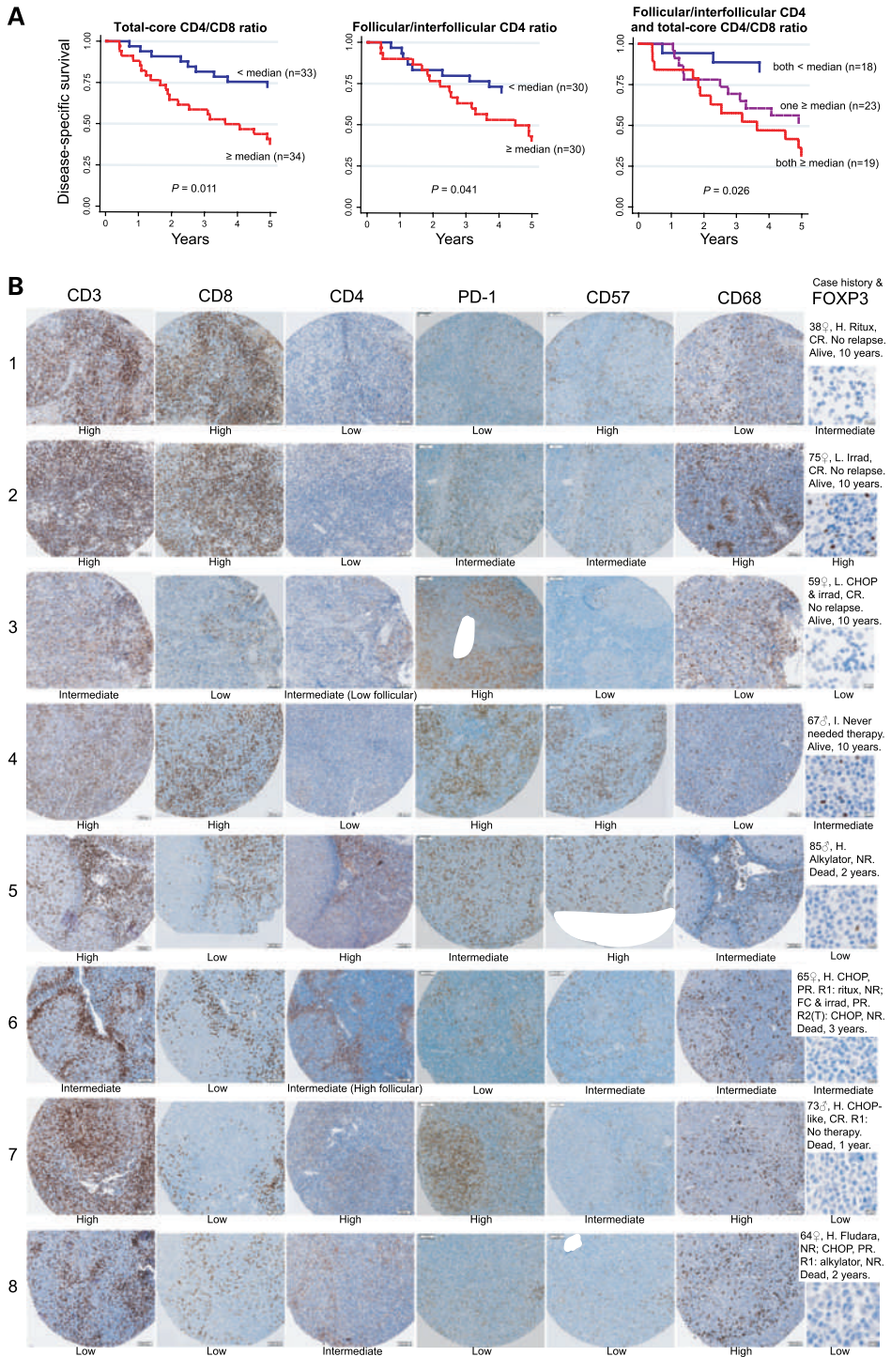

Fig. 2. *A,* Kaplan-Meier curves. The timescale is cut at 5 years because the patients in this study per definition could not die from lymphoma after this time point. *P* values are from logistic regression between outcome groups. *B,* examples of good (1-4) and poor (5-8) outcome. Tertiles from the computerized total core quantification are given below each staining. Case 1 shows a CD8-dominated microenvironment, whereas case 2 is rich in $CD8^+$, $FOXP3^+$, and $CD68^+$ cells. Case 3 is PD-1 dominated (but low in $CD57^+$ cells), whereas case 4 is high in both $CD8^+$ and $PD\text{-}1^+$ (and $CD57^+$) cells. Cases 5 to 7 are examples with few $CD8^+$ and many $CD4^+$ cells; case 7 is also rich in $PD\text{-}1^+$ and $CD68^+$ cells. Case 8 has a CD68-dominated microenvironment. Note: Patients' histories are summarized accordingly: age and sex, FLIPI risk group [low (L), intermediate (I), or high (H)]. First-line treatment, response. Relapse (R) number [and if transformed (T)]: treatment, response. Survival status, follow-up time.

and Tregs are important in halting disease progress, in the years before whatever treatment will be needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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A Unifying Microenvironment Model in Follicular Lymphoma: Outcome Is Predicted by Programmed Death-1–Positive, Regulatory, Cytotoxic, and Helper T Cells and Macrophages

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