Impact of the tumor microenvironment on prognosis in follicular lymphoma is dependent on specific treatment protocols

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ABSTRACT

Background

The clinical behavior of follicular lymphoma is largely determined by properties of the non-malignant tumor microenvironment. The precise nature of the cell populations is still unclear and published data on their prognostic significance are highly conflicting. This may be partly due to heterogeneous composition and treatments.

Design and Methods

Pre-treatment biopsy samples of patients with follicular lymphoma treated in an EORTC/BNLI trial comparing fludarabine to cyclophosphamide, vincristine and prednisone (CVP) chemotherapy could be retrieved for 61 patients in five European countries. Immunohistochemical investigations were performed to evaluate tumor cell characteristics, T-cell subsets, follicular dendritic cells and macrophages and associations with clinical outcome were studied.

Results

Some markers showed a homogeneous prognostic impact, while others had a different and sometimes opposite effect in the treatment arms. CD69 expression on tumor cells was a poor prognostic sign and an interfollicular infiltrate of FoxP3-positive T cells was a good prognostic sign irrespective of the treatment arm. It is suggestive that a dense infiltrate of FoxP3-positive T cells, a dense and interfollicular infiltrate of CD68-positive macrophages and complete follicular dendritic meshworks were associated with a favorable time to progression in CVP-treated patients, while being a poor prognostic sign in fludarabine-treated patients.

Conclusions

Our results suggest that characteristic properties of the microenvironment in follicular lymphoma determines the responses to essentially different chemotherapeutic approaches. These data may provide an explanation for the highly conflicting results on immunohistochemical markers and the prognostic role of the microenvironment in follicular lymphoma reported thus far and lay the basis for the development of predictive assays to tailor treatment in patients with follicular lymphoma.

Key words: follicular lymphoma, microenvironment, prognostic factors, immunohistochemistry.

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Introduction

Follicular lymphoma (FL) is an indolent disease characterized by frequent relapses that generally respond well to various chemotherapeutic approaches. Until the introduction of chemo-immunotherapy, no single treatment regimen had been shown to provide a significantly superior overall survival, despite improvements in progression-free survival.[1] The introduction of rituximab may, however, be changing this situation for the first time.[2,3] The overall survival of FL patients varies greatly, but approximately 20% of the patients die early in the course of the disease. Up to now, clinical prognostic indices, such as the International Prognostic Index and the disease-specific Follicular Lymphoma International Prognostic Index (FLIPI) have been the only practical indicators of the clinical course.[4]

From a biological point of view, the clinical behavior of FL is determined principally by the tumor microenvironment rather than by inherent properties of the tumor cells themselves.[5-7] Specific T-cell and accessory cell populations, including macrophages and follicular dendritic cells, have been reported to have an influence on overall and/or progression-free survival, either as a poor or a good prognostic parameter.[6,8-16] The results of various studies are, however, very contradictory, with specific cell populations being correlated with a poor prognosis in some series, but with a good prognosis or without any significant impact in others. In general, these studies were well-performed and although some of the variability in results might be explained by technical scoring variations,[17] the explanation probably lies more in the diversity of the clinical characteristics of the patients and their treatments (Table 1). Specifically, there is evidence suggesting that very aggressive treatment, as in the series reported by Farinha *et al.*, has a different impact compared to that of the standard, more *indolent* treatment in the UK and the Netherlands, such as the standard CHOP-like treatment commonly used in many other countries.[6,8,9] Rituximab has been suggested to influence the prognostic impact of potential prognostic markers in FL as it does in diffuse large B-cell lymphoma.[13,18-21] Moreover, due to its specific targeting of T cells, fludarabine may have a different impact on the FL microenvironment. The prognostic value of different T-cell populations may, therefore, be different in patients treated with fludarabine. These aspects can only be studied appropriately in the setting of randomized clinical trials.

To gain insight into the possible influence of treatment

Table 1. Comparison of published data and results from this study on clinico-pathological correlations for T-cell populations, macrophages and follicular dendritic cells.

	Lee[9]	Glas[8]	Carreras[11]	Farinha[6]	Alvaro[10]	Klapper[12]	Taskinen[13]		Wahlin[14]	EORTC 20921	
							R-CHOP	CHOP	[d]	FLUDA	CVP
Interfollicular component	nd	-	nd	-	nd	-	nd		nd	-	-
CD3 dense infiltrate	nd	-	-	-	-	-	-	-	nd	Poor	-
CD3 pattern interfollicular	nd	-	-	nd	-	-	-	-	nd	Good	-
CD4 dense infiltrate	Good	-	-	-	Good	nd	nd		-	Good	Good
CD4 sparse infiltrate	Poor	-	-	-	Poor	nd	nd		-	Poor	Poor
CD4 pattern interfollicular	-	Good	-	nd	Good	nd	nd		nd	Good	Good
CD8 dense infiltrate	-	-	nd	-	Good	nd	nd		good	Good	Good
CD8 sparse infiltrate	-	-	nd	-	Poor	nd	nd		poor	Poor	Poor
CD8 pattern interfollicular	-	-	nd	nd	Good	nd	nd		nd	Poor	Good
FoxP3 dense infiltrate	Good	-	Good	Poor[a]	Good	nd	nd		nd	Poor	Good
FoxP3 sparse infiltrate	Poor	-	Poor	Good[a]	Poor	nd	nd		nd	Good	Poor
FoxP3 pattern interfollicular	Good[b]	Good	Good	nd	Good	nd	nd		nd	Good	Good
FoxP3 pattern intrafollicular	nd	-	Good	nd	nd	nd	nd		nd	-	-
CD68 dense infiltrate	-	-	nd	Poor	Good	-	Good[c]	Poor[c]	nd	Poor	Good
CD68 sparse infiltrate	-	-	nd	Good	Poor	-	Poor[c]	Good[c]	nd	Good	Poor
CD68 pattern interfollicular	-	nd	nd	nd	nd	nd	nd	nd	nd	Good	-
MIB1	nd	-	nd	-	nd	-	nd		nd	-	-
CD21/CD23	nd	Poor	nd	-	nd	-	nd		nd	Poor	Good
TIA1	-	-	nd	-	-	nd	nd		nd	nd	
CD57	nd	-	nd	-	Good	nd	nd		nd	nd	

Type of analysis	e-o-s	e-o-s	Continuous	Continuous	Continuous	Continuous	Continuous		Continuous	Continuous	
Number of patients	59	66	97	99	211	158	96	45	139	31	30
End point	OS	transformation	OS	OS, PFS	OS, PFS	OS, PFS	OS,PFS		OS, DSS	PFS	
Treatment	Various indolent regimens	Various indolent regimens, mostly CVP	Various, 58% CHOP, 14% fludarabine	BP-VACOP+RT	Various, 44% CHOP, 15% CVP	MCP/CHOP randomized	R-CHOP	CHOP	Highly various	Fludarabine	CVP
Median age (years)	61/46	52/44	55	44	+/- 56	n.a.	n.a.	n.a.	59.8	56	56
(FL)IPI range (%)	n.a.	51/20/27 versus 91/9/0	37/27/36	59/40/1	61/60/36	22/67/65	61/30/7	51/31/18	32/32/35	26/42/32	27/47/27

Statistically significant association with good, respectively poor prognosis. Borderline significant or trend to good, respectively poor prognosis, in bold: no association with prognosis. n.d.: not done, n.a.: not available, e-o-s: end-of-spectrum analysis, PFS: progression-free survival, OS: overall survival, DSS: disease-specific survival. CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone, R: rituximab, MCP: mitoxantrone, chlorambucil, prednisone, BP-VACOP: bleomycin, cisplatin, etoposide, doxirubicin, cyclophosphamide, vincristine, prednisone, RT: radiotherapy. [a]personal communication, performed on the same series (R.Gascoyne), [b]specifically stated as perifollicular, [c]PFS only, not OS, [d]based on flow data.

on the predictive value of different cell populations in the microenvironment, we evaluated these aspects in patients who were treated in a phase III, prospective, randomized controlled trial comparing fludarabine versus cyclophosphamide, vincristine and prednisone (CVP) in previously untreated patients with FL.[22]

Design and Methods

Patients

Between 1993 and 1997, a phase III, prospective, randomized controlled trial was conducted to compare the safety and efficacy of eight cycles of fludarabine phosphate versus conventional CVP in previously untreated patients with malignant low-grade non Hodgkin's lymphoma. The study was conducted in nine countries and 60 study centers of the European Organization for Research and Treatment of Cancer Lymphoma Group (EORTC LG, n=276 patients) or of the British National Lymphoma Investigation (BNLI, n=105 patients). Patients with various classes of indolent B-cell non-Hodgkin's lymphoma were treated in this study; approximately 60% of the patients had grade 1 or 2 FL.

Representative formalin-fixed paraffin-embedded biopsy samples could be retrieved retrospectively for 61 FL patients from 17 centers in the Netherlands, Belgium, Switzerland, France and Portugal. Of these, 31 samples came from patients treated with fludarabine and 30 from patients in the CVP arm of the study. For local regulatory reasons BNLI centers were not able to participate in the translational research project.

Immunohistochemistry

Tissue micro-arrays were constructed at the National Cancer Institute in Amsterdam from the tumor blocks according to standard method, using triplicate 1 mm cores from pretreatment representative lymph node biopsy samples for each patient. The sections were stained with antibodies to evaluate tumor cell characteristics, T-cell subsets, follicular dendritic cells and macrophages as listed in Table 2. The amount and the patterns of infiltration of tumor cells, T cells, T-cell subsets and macrophages, and the integrity of follicular dendritic cell meshworks were semi-quantitatively assessed by two collaborators in a consensus mode (DJ, SH). The sampling of three cores per case was considered to provide a representative sample of the whole lymph node and results were averaged for a final score.

Scoring was performed as described previously.[6] In brief, for CD20 the pattern of interfollicular tumor cell distribution was scored. MIB1 staining was assessed semi-quantitatively as a proportion of the total number of tumor cells. CD3 was used as a reference for the total number of T cells for subpopulations of CD4, CD8 and FoxP3-positive cells. Staining for these cells was assessed semi-quantitatively into three classes (0-5%, 5-10%, >10%). Moreover, the CD4-positive T-cell distribution pattern was assessed as predominantly intrafollicular, predominantly interfollicular or diffuse. Likewise the pattern of FoxP3-positive cells was recorded as predominantly intrafollicular, predominantly interfollicular or diffuse. CD69, a marker of T-cell activation, was scored relative to the total number of T cells (absent or sporadically positive cells versus uniformly weakly or strongly positive). CD69 was considered positive on tumor cells when more than 75% of the cells were stained. A distinction between T-cell staining and tumor cell staining could be made on the basis of morphology and infiltration patterns. CD21 and CD23 markers of follicular dendritic cell meshworks, were scored in a four-tiered classification (absent, minority of the neoplastic follicles with disrupted meshworks, majority of neoplastic follicles with well-developed meshworks, uniformly well-developed meshworks). CD68-positive macrophages were counted as absolute cell numbers per three representative follicular high power fields (at magnification 600x). Quantification was performed in duplicate. The spatial distribution of CD68-positive macrophages was recorded as predominantly intrafollicular, predominantly interfollicular or diffuse.

The T-cell markers and macrophage marker (CD3, CD4, CD8 and FoxP3) were scored in three categories, follicular dendritic cell patterns (CD21 and CD23) and proliferation (MIB-1) were scored in four classes. Because of the limited sample size, these parameters were analyzed as binary variables and classes were collapsed according to their distribution to result in balanced groups for T cells and macrophages or to biologically meaningful classes for follicular dendritic cell patterns and proliferation. Specifically, proliferation (MIB-1) was assessed with a 10% cut-off level, follicular dendritic cell patterns were assessed as nearly complete/uniform versus absent/mostly incomplete, FoxP3-positive T-cells were analyzed using a cut-off of 5% and CD4 and CD8 using a cut-off of 10%. CD68-positive macrophages were analyzed using a cut-off level matching that described by other researchers (15/high power field).[8,13,15]

Results were analyzed at the EORTC Data Center and correlated with clinical data (MG).

Table 2. Antibodies used in this study.

Antibody	Clone	Source
Ki-67	MIB1	DAKO
CD20	L26	DAKO
CD3	CD3	DAKO
CD4	4B12	Novacastra
CD8	C8/144B	DAKO
FoxP3	236A/E7	AbCam
CD21	1F8	DAKO
CD23	1B12	Novacastra
CD68	KP1	DAKO
CD69	CH11	LabVision

DAKO (Glostrup, Denmark); Novacastra (Newcastle upon Tyne, United Kingdom) Zymed (Invitrogen, Breda, the Netherlands); Becton Dickinson (Franklin Lakes, NJ, USA); Abcam (Cambridge, United Kingdom); LabVision (Fremont, CA, USA).

Statistical analysis

Response to therapy (complete and partial response) and time to progression were chosen as the primary

Table 3. Clinical characteristics of fludarabine- and cyclophosphamide, vincristine and prednisone-treated patients.

	Fludarabine (n=31) N (%)	CVP (n=30) N (%)	Total (n=61) N (%)
FLIPI			
Low	8 (25.8)	8 (26.7)	16 (26.2)
Intermediate	13 (41.9)	14 (46.7)	27 (44.3)
High	10 (32.3)	8 (26.7)	18 (29.5)
Age group			
< 40 years	2 (6.5)	5 (16.7)	7 (11.5)
40-50 years	11 (35.5)	10 (33.3)	21 (34.4)
50-60 years	9 (29.0)	11 (36.7)	20 (32.8)
60-70 years	5 (16.1)	4 (13.3)	9 (14.8)
≥ 70 years	4 (12.9)	0 (0.0)	4 (6.6)
Ann Arbor stage			
III A	8 (25.8)	5 (16.7)	13 (21.3)
IIIB	2 (6.5)	1 (3.3)	3 (4.9)
IIIsB	1 (3.2)	0 (0.0)	1 (1.6)
IV A	15 (48.4)	19 (63.3)	34 (55.7)
IV B	5 (16.1)	5 (16.7)	10 (16.4)
Number of involved nodal areas			
1	0 (0.0)	4 (13.3)	4 (6.6)
2	7 (22.6)	5 (16.7)	12 (19.7)
3	6 (19.4)	2 (6.7)	8 (13.1)
4	10 (32.3)	8 (26.7)	18 (29.5)
5	4 (12.9)	8 (26.7)	12 (19.7)
6 or more	4 (12.9)	3 (10.0)	7 (11.5)
Baseline hemoglobin			
≥120 g/L	25 (80.6)	26 (86.7)	51 (83.6)
<120 g/L	6 (19.4)	4 (13.3)	10 (16.4)
Baseline LDH			
≤250 IU	16 (51.6)	18 (60.0)	34 (55.7)
>250 IU	15 (48.4)	12 (40.0)	27 (44.3)

end-points of this analysis since these data were fully and reliably available in contrast to information on subsequent treatment protocols that were expected to be highly variable and would, therefore, have precluded meaningful interpretations of overall survival.

Time to progression was defined as time from randomization to disease progression; patients who were still progression-free at the last documented follow-up, and patients who died without any sign of progression were censored on the date of death or last follow-up. Because of the limited sample size, all immunohistochemical parameters were analyzed as binary variables. For each end-point, the prognostic value of all parameters was first investigated in univariate prognostic models; multivariate models were subsequently used to test whether the statistical significance was independent of clinical prognostic factors.

Predictive factors (factors predicting the randomized treatment effect) were identified using multivariate models including treatment, the tested covariate, and an interaction factor.

Logistic models and Wald's χ^2 tests were used for all analyses of response, Cox models and Wald χ^2 tests were used for all analyses of time to progression, and the Kaplan-Meier method was used to estimate time to progression.

Results

Patients' characteristics and disease features

The baseline characteristics of the patients included in this study are listed in Table 3. The median follow-up at the time of the current analysis had reached 9.1 years (actuarial estimate). Ten patients were still alive and progression-free at the last documented follow-up, in 2006 (3 patients), 2005 (1 patient), 2004 (1 patient), 2003 (1 patient) or before the final analysis report in 2003 (4 patients). The median overall survival was 10.6 years; the median time to progression, the principle end-point for this study, was 17 months. In a univariate analysis, FLIPI score itself had a borderline prognostic value (p=0.07, HR 1.41, CI 0.97-2.04); lactate dehydrogenase (LDH) concentration, as a single component, had a significant prognostic value (p=0.0086, HR 2.17 CI 1.22-3.86). In a multivariate model, only LDH level retained a prognostic significance for time to progression.

Immunohistochemical analyses and clinical correlations

Immunohistochemical prognostic markers for time to progression for all patients

In a Cox univariate analysis for all patients (Table 4), it was seen that CD69, an activation marker on tumor cells, had a statistically significant (p=0.0195) unfavorable impact. Other tumor cell-related factors, including proliferation as measured with MIB-1, did not have statistically significant effects. The presence of an interfollicular component of FoxP3-positive T cells, irrespective of a concomitant intrafollicular component (interfollicular and diffuse patterns), correlated significantly (p=0.0033) with a better prognosis (in terms of time to progression) (Figure 1A). The amount of FoxP3-positive cells was not relevant in this respect.

In a multivariate Cox model including the statistically significant clinical prognostic factors (FLIPI score and LDH level) and immunohistochemical parameters, FoxP3 spatial distribution retained its significance (p=0.0025 when adjusted for FLIPI score, p=0.0084 when adjusted for LDH level).

Immunohistochemical prognostic markers for time to progression per treatment arm

When we separately analyzed prognostic factors in the randomized treatment arms, we observed that the impact of each factor (measured by its hazard ratio) sometimes differed substantially between the two arms: for some factors, the impact was favorable (HR<1) in one arm and unfavorable (HR>1) in the other arm (Table 4). This suggests that some of the factors, even if they do not show a significant prognostic value in the overall population, may have a predictive value for the treatment effects, i.e. affect the relative efficacy of the investigational treatment (fludarabine) when compared with the control arm (CVP). The predictive value of all factors was formally investigated by using a

multivariate Cox-model and interaction test.

CD23 score was identified as a statistically significant predictive factor (p=0.036, interaction test), while the CD21 score had a borderline predictive value (p=0.062, interaction test) (Figure 1B). For both factors, the presence of meshworks was associated with a longer time to progression in the CVP arm, while absence of this factor was associated with a longer time to progression in the fludarabine arm.

Other such interactions were suggested by the data, but without having a statistically significant predictive value. The presence of a follicular component of CD3 was a relatively poor prognostic sign for patients receiving fludarabine treatment, but of no impact for those treated with CVP. Very interestingly, the density of the FoxP3-positive T-cell infiltrate and CD68-positive macrophage content had opposite effects in the two treatment arms: FoxP3-positive T-cell infiltrates containing more than 5% of T cells were of adverse prognostic impact in the fludarabine treatment arm but of good prognostic impact among patients treated with CVP. The prognostic significance of CD68-positive macrophages was similar but the reverse.

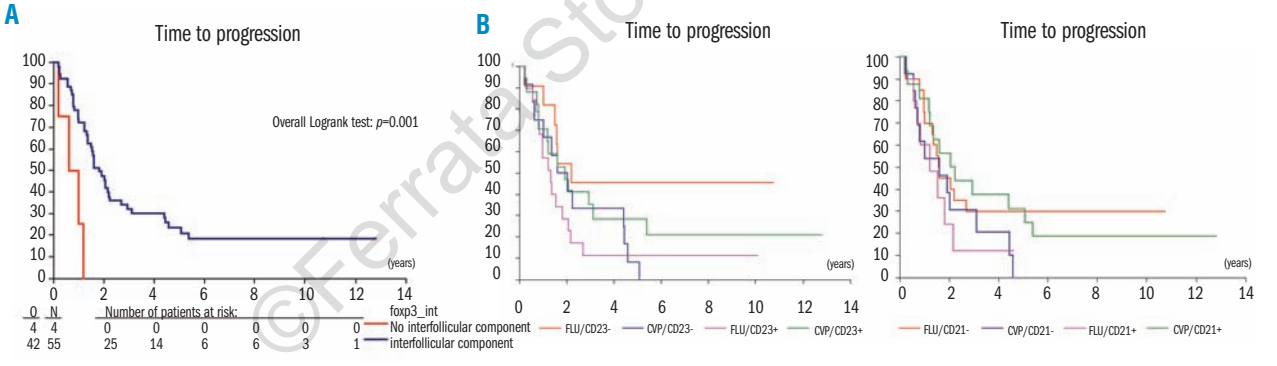
Response to therapy

The overall study results demonstrated an advantage from fludarabine in terms of response to therapy;[18] in the patients included in the current analysis, we observed the same trend. In a univariate analysis studying the response to therapy in all patients defined as reaching complete or partial response, none of the factors showed a statistically significant correlation. Levels of borderline significance were reached for the amount of CD8 (p=0.068) and HLA-DR (p=0.077).

We next investigated the predictive value for the treatment effects, i.e. the relative efficacy of fludarabine treatment as compared to CVP treatment (Table 5). Predictive models were constructed for all immunohistochemical parameters. CD21 score was identified as a predictive factor (p=0.034). In patients with absent or sporadic follicular dendritic cell meshworks, the observed response rate was 95% (18/19) for fludarabine *vs.* 54% (7/13) for CVP (p=0.01, Fisher's exact test). In contrast, among patients with mostly well-formed meshworks, the response rate was 67% (6/9) for fludarabine *vs.* 75% (12/16) for CVP (p=0.82, Fisher's exact test). The data also suggested that an interfollicular component of macrophages, irrespective of the presence of an intrafollicular component (interfollicular only and/or diffuse), was related to a lesser chance of reaching response after fludarabine treatment, but did not influence the response rate after CVP treatment (p=0.08 with an odds ratio of 0.111 for fludarabine, p=1 for CVP), although this was not statistically significant.

Discussion

Various gene-expression and immunohistochemical studies have provided strong evidence of the impor-



CD21	Treatment	N	0	HR	p	Median (CI)	% at 5 years (CI)
Absent/	CVP	13	12	1.00	0.2323	1.59 (0.73, 4.45)	0.00(,)
Sporadic	Fludarabine	21	14	0.63 (0.29, 1.36)		1.56 (1.33, N)	30.00 (12.25, 50.14)
Present/	CVP	16	13	1.00	0.1436	2.14 (1.36, 5.39)	31.25 (11.39, 53.65)
Uniform	Fludarabine	10	8	1.99 (0.78, 5.06)		1.21 (0.68, 2.17)	12.00 (0.66, 40.79)
CD23							
Absent/	CVP	12	12	1.00	0.0943	1.59 (1.02, 4.45)	8.33 (0.51, 31.11)
Sporadic	Fludarabine	12	6	0.44 (0.16, 1.18)		2.18 (1.56, N)	45.45 (16.66, 70.69)
Present/	CVP	17	13	1.00	0.2277	1.91 (1.17, 5.39)	28.24 (9.62, 50.50)
Uniform	Fludarabine	19	16	1.58 (0.75, 3.32)		1.33 (0.96, 2.17)	11.40 (1.92, 30.35)

Figure 1. (A) Survival curves (time to progression) based on the presence of an interfollicular component of FoxP3-positive T cells. (B) Survival curves per treatment arm (time to progression) based on the presence of (near) complete CD23 and CD 21 positive follicular dendritic cell meshworks.

tance of the microenvironment in the clinical behavior of FL.[5-7] The precise nature of the relevant cell populations is still unclear, and published data on the prognostic significance of cell populations, including T-cell subsets, macrophages and follicular dendritic cells, are conflicting.[6,8-16] The analysis of pre-treatment samples from FL patients treated in the EORTC/BNLI 20921 trial enabled us to study the impact of essentially different chemotherapeutic approaches: CVP is principally directed towards the tumor cells, whereas fludarabine is directed towards both tumor cells and the microenvironment, especially the T-cell populations. In this clinical trial overall response rates were significantly better in the fludarabine arm than in the CVP arm, but there were no statistically significant differences in time to progression, time to treatment failure, and overall survival between treatment groups.[18] This was true for the whole cohort of patients as well as for the subgroup

Table 4. Immunohistochemical prognostic factors for time to progression.

		All patients		Fludarabine		Cyclophosphamide, vincristine and prednisone	
	Variable	p	HR	p	HR	p	HR
CD3	Follicular component	0.3830	1.353	0.0922	2.968	0.9470	0.971
	Interfollicular component	0.5081	0.617	0.2158	0.261	0.9202	0.902
	Amount	0.5053	1.297	0.3576	1.598	0.7110	0.792
CD4	Follicular component	0.6725	1.153	0.7827	1.193	0.4630	1395
	Interfollicular component	0.1259	0.392	0.9991	0.000	0.3781	0.507
	Amount	0.3485	0.753	0.8201	0.907	0.2732	0.619
CD8	Follicular component	0.9552	0.976	0.5474	1.400	0.5524	0.643
	Amount	0.4378	0.791	0.8767	0.935	0.4293	0.719
CD21	Score	0.9877	0.995	0.2219	1.731	0.1291	0.527
CD23	Score	0.4142	1.280	0.0469	2.620	0.3164	0.663
CD68	Follicular component	0.6607	1.147	0.6167	1.271	0.9499	1026
	Interfollicular component	0.2516	0.710	0.0794	0.468	0.9058	1052
	Amount	0.4807	1.227	0.1672	1.814	0.5671	0.795
CD69	T-cells	0.1300	0.633	0.7277	0.859	0.0783	0.479
	Tumor cells	0.0195	2.735	0.0797	2.721	0.1381	2640
FOX-P3	Follicular component	0.9368	1.027	0.5855	1.299	0.7655	0.874
	Interfollicular component	0.0033	0.189	0.0315	0.182	0.0580	0.217
	Amount	0.7920	0.924	0.3379	1.512	0.1761	0.554
HLADR	Score	0.1226	0.479	0.0552	0.109	0.2741	0.545
KI67	Score	0.3190	1.340	0.3524	1.488	0.5955	1241

Table 5. Immunohistochemical prognostic factors for response to treatment.

		All patients		Fludarabine		Cyclophosphamide, vincristine and prednisone	
	Variable	p	OR	p	OR	p	OR
CD3	Follicular component	0.3371	2.000	0.3432	3.667	0.9008	1.114
	Interfollicular component	0.9757	>>	0.9800	>>	0.9801	>>
	Amount	0.4571	1.875	0.9612	>>	0.5247	0.500
CD4	Follicular component	0.8544	1.143	0.7373	1.556	0.7527	0.750
	Interfollicular component	0.9757	>>	–	–	0.9734	>>
	Amount	0.5113	1.557	0.4471	2.538	0.9304	1.077
CD8	Follicular component	10.000	1.000	0.6894	1.667	0.9275	0.889
	Interfollicular component	0.9829	>>	0.9800	>>	–	–
	Amount	0.0680	0.309	0.1905	0.200	0.1785	0.333
CD21	Complete	0.5947	1389	0.0781	9000	0.2388	0.389
CD23	Complete	0.8259	0.872	0.5344	2143	0.4958	0.583
CD68	Follicular component	0.9114	0.931	0.7425	1500	0.7889	0.808
	Interfollicular component	0.2768	0.500	0.0781	0.111	1	1000
	Amount	0.6419	1333	0.2421	4199	0.6032	0.667
CD69	T-cells	10000	1000	0.6325	1800	0.7246	0.750
	Tumor cells	0.7700	0.712	0.9717	<0.001	0.8881	0.833
FOX-P3	Follicular component	0.7308	0.775	1	1000	0.6106	0.619
	Interfollicular component	0.6724	0.585	0.9800	>>	0.5829	0.444
	Amount	0.4482	1620	1	1000	0.2316	2708
HLADR	Positive	0.0772	0.179	0.9746	<0.001	0.4885	0.471
KI67	> 5%	0.3221	1852	0.8771	1182	0.2388	2571

with FL.

In the series of samples from FL patients in the EORTC 20921 trial that could be studied using immunohistochemisty, some markers showed an overall prognostic impact, while others had a very different and sometimes opposite effect in the different treatment arms. It should be noted, however, that due to the small size of this series, the effect of most immunohistochemical parameters failed to reach full statistical significance and the results should, therefore, be interpreted with great caution. LDH level was a significant prognostic factor as also reflected in borderline significance for the FLIPI score. CD69 expression on tumor cells was related with a poor outcome in both treatment arms. A dense infiltrate of CD4-positive T cells, especially when located interfollicularly, was a good prognostic sign irrespective of treatment arm. This is in line with previous reports.[9,10]

In view of the highly conflicting results in the literature, the differential effects of FoxP3-positive T cells and CD68-positive macrophages on time to progression found in this series are of most interest. In the patients who were treated with CVP, a dense infiltrate of FoxP3-positive T cells, especially with an interfollicular component, was associated with a better survival. In the patients treated with fludarabine, however, the dense infiltrate was associated with a shorter time to progression. We found a similar treatment-related differential effect of CD68 positive macrophages: a dense infiltrate was a good prognostic sign in CVP-treated patients and a poor prognostic sign in those treated with fludarabine. The presence of complete or nearly complete follicular dendritic cell meshworks expressing CD23 and to a lesser extent CD21 was associated with a longer time to progression in CVP-treated patients, while being a poor prognostic sign in fludarabine-treated patients.

How do these findings relate to results from other published series? In the present study, the poor prognostic impact of a dense infiltrate of CD68-positive macrophages in fludarabine-treated patients was very similar to that of the impact in patients treated with the intensified BP-VACOP, as reported by Farinha *et al.*[8] The prognostic direction in CVP-treated patients was, however, more in line with that of other studies, although most authors did not find a prognostic significance for this marker[9,10] (Table 1). The findings for FoxP3-positive T cells are also highly conflicting with the series of BP-VACOP-treated patients showing opposite results as compared to those of all other series.[8-10] Again, we found that the prognostic trend in fludarabine-treated patients paralleled that in the BP-VACOP-treated patients, while in the CVP-treated patients the impact of both the density of the FoxP3-positive T cells and their spatial distribution reflected that in other series of patients treated less intensively. It should be noted, however, that the treatments in these series were highly varied with mostly *indolent type* regimens in the series reported by Lee and co-workers, but also including CHOP in the two other series. Nevertheless, although these comparisons should all be regarded with caution in view of the highly heterogeneous treatments in most reported series, the different clinical risk distributions, the variations in scoring criteria and the small size of the present EORTC 20921 series, these data do suggest that the microenvironment plays a different role in FL in the context of specific treatment regimens. This notion is further supported by the different prognostic impact that was found for CD68-positive macrophage infiltrates and mast cells in FL patients treated with CHOP with and without rituximab.[13,16,18]

Taken together, based on previous studies by us and others and on the present data, it can be hypothesized that there may be two different classes of FL response: FL that contain a complete, dense microenvironment with many macrophages, FoxP3-positive T cells and CD23-positive follicular dendritic cells have a more favorable outcome after CVP treatment, while FL with these same microenvironmental characteristics are associated with a poor outcome after treatments that are also directed towards the microenvironment (fludarabine and BP-VACOP).[6,7,27] It has been shown that fludarabine has a rather specific effect on FoxP3-positive regulatory T cells in patients with B-cell chronic lymphocytic leukemia, in that both the frequency and the inhibitory functions of these cells are reduced after treatment.[23-25] Very likely, interference with the tumor microenvironment may result in different responses depending on the specific role of the microenvironment in a specific class of FL. Similar effects of cyclophosphamide have been shown on regulatory T cells too.[26]

In conclusion, although this was a relatively small series, the patients were uniformly treated within a clinical trial. The data provide an explanation for the highly conflicting results on immunohistochemical markers and the prognostic role of the microenvironment in FL as reported thus far and lay the basis for the development of predictive assays that may help to tailor treatment in patients with FL. Moreover, this study highlights the importance of translational studies as an integral part of clinical trials in lymphoma. Combined efforts by different trial organizations will be necessary to provide sufficient data to study this issue further.[27]

Authorship and Disclosures

DdJ: design of the study, acquisition of data, data analysis, writing of the manuscript, final approval of the manuscript; AK: acquisition of data, data analysis, final approval of the manuscript; AH: clinical study coördinator of the EORTC 20921 study, data analysis, final approval of the manuscript; JR: design of the study, data analysis, final approval of the manuscript; DV: acquisition of data, final approval of the manuscript; SH: acquisition of data, data analysis, final approval of the manuscript JPdB: data analysis, final approval of the manuscript; MvG: statistical data analysis, writing of the paper, final approval of the manuscript. The authors reported no potential conflicts of interest.

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