Clinical Cancer Research

The Prognostic Impact of CD163-Positive Macrophages in Follicular Lymphoma: A Study from the BC Cancer Agency and the Lymphoma Study Association

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Abstract

Purpose: We aimed to assess the prognostic significance of follicular lymphoma–associated macrophages in the era of rituximab treatment and maintenance.

Experimental Design: We applied immunohistochemistry for CD68 and CD163 to two large tissue microarrays (TMA). The first TMA included samples from 186 patients from the BC Cancer Agency (BCCA) who had been treated with first-line systemic treatment including rituximab, cyclophosphamide, vincristine, and prednisone. The second contained 395 samples from PRIMA trial patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, and randomized to rituximab maintenance or observation. Macrophage infiltration was assessed using Aperio image analysis. Each of the two cohorts was randomly split into training/validation sets.

Results: An increased CD163-positive pixel count was predictive of adverse outcome in the BCCA dataset [5-year progression-free survival (PFS) 38% vs. 72%, respectively, $P = 0.004$ in the training cohort and 5-year PFS 29% vs. 61%, respectively, $P = 0.004$ in the validation cohort]. In the PRIMA trial, an increased CD163 pixel count was associated with favorable outcome (5-year PFS 60% vs. 44%, respectively, $P = 0.011$ in the training cohort and 5-year PFS 55% vs. 37%, respectively, $P = 0.030$ in the validation cohort).

Conclusions: CD163-positive macrophages predict outcome in follicular lymphoma, but their prognostic impact is highly dependent on treatment received. *Clin Cancer Res; 21(15); 3428–35.* ©*2015 AACR.*

Introduction

Follicular lymphoma is the most common indolent lymphoma subtype and is clinically characterized by prolonged survival with median survival times in excess of 10 years (1). Patient outcomes are, however, heterogeneous, and a nonnegligible proportion of patients are at risk of early progression and/or transformation (secondary development of diffuse large B-cell lymphoma), putting them at risk for adverse outcome. Treatment decisions are currently almost exclusively guided by clinical characteristics and range from watchful waiting to anti-CD20 directed immunotherapy alone or in combination with chemotherapy (2).

Despite the presence of highly recurrent genetic alterations such as the t(14;18)(q32;q21) translocation or mutations in epigenetic modifiers, malignant follicular lymphoma cells fail to thrive *in vitro*, suggesting that the tumor microenvironment plays a crucial role in their expansion and survival (3). The landmark study of the Lymphoma/Leukemia Molecular Profiling Project established that gene expression signatures derived from nonmalignant tumor-infiltrating cells influence patients' outcomes (4). The correlation of macrophage and T-cell counts with prognosis is an area of intensive translational investigation. However, for tumor-associated macrophages (TAM) in particular, the results

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Translational Relevance

In follicular lymphoma, the tumor microenvironment has been shown to influence patient outcomes, but the association of tumor-infiltrating macrophages with survival is controversial. Here, we report the correlation of CD163, a marker of alternatively polarized macrophages, with outcome in two large datasets. We show that increased $CD163^+$ macrophages are associated with poor survival in patients treated with R-CVP, and with favorable outcome in patients treated with R-CHOP/R-maintenance. Our findings are of translational relevance as they illustrate that the tumor microenvironment modulates response to therapy and patient outcomes. Furthermore, we demonstrate, for the first time in patient data, the existence of a positive interaction between chemotherapy (doxorubicin) and rituximab, mirroring data from preclinical animal models. In the context of heterogeneous patient outcomes in follicular lymphoma, our findings inform on the rational use of immunochemotherapy in this disease.

are apparently contradictory as their presence correlated with adverse outcome in some studies (5–10) but not in others (11). It is increasingly recognized that the prognostic effect of TAMs is modulated by treatment, and several studies suggest a positive interaction between rituximab and tumor-suppressive functions of macrophages (7, 11, 12).

The monocyte and macrophage lineage is characterized by considerable functional heterogeneity and plasticity, accounting for the implication of these cells in biologic processes as diverse as inflammation, infection, or cancer (13, 14). TAMs adopt a broad spectrum of functional states that may evolve and fluctuate between the extremes of antitumoral M1-type and protumoral M2-type phenotypes (13, 14). In follicular lymphoma, stromal cells recruit monocytes via the secretion of CCL2 and TAMs, in cooperation with stromal cells, increase the proliferation of B-cell cell lines, and prolong the survival of primary follicular lymphoma cells in culture (15). Further, CD36-purified TAMs overexpress IL15, stimulating follicular lymphoma cell survival in combination with CD40 activation (16). CD163, a surface marker that is predominantly found on M2-skewed macrophages (17), has been associated with poor prognosis and increased angiogenic sprouting in follicular lymphoma (10).

We designed this study to ask the question whether the M2-restricted macrophage marker CD163 would provide better prognostic value than CD68 and whether that effect would be consistent across two different patient cohorts. We tried to overcome limitations from prior studies by evaluating outcome correlations in two large datasets with relative treatment uniformity and by using sensitive, state of the art image analysis.

Subjects and Methods

Patients

We constructed a first tissue microarray (TMA) from 1.5 mm duplicate cores using the samples from 186 patients treated between 2004 and 2009 with rituximab, cyclophosphamide, vincristine, and prednisone (R-CVP) at the BC Cancer Agency (BCCA). The median time from date of biopsy (study sample) to initiation of systemic treatment was 1.84 months (interquartile range, 5.57 months). Twelve patients of 186 (7%) had received local therapy (surgery and/or radiotherapy) before R-CVP. From 2006 onwards, rituximab was given as maintenance treatment (once every 12 weeks for 2 years) for patients achieving at least a partial response after R-CVP. The second TMA was built from 1.0 mm triplicate cores using samples from patients who participated in PRIMA, an international randomized phase III trial that assigned patients responding to first-line therapy to rituximab maintenance (every 8 weeks for 2 years) or observation (18). For PRIMA trial patients, we restricted the analysis to those patients receiving rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) as front-line treatment (93% of the population represented on the TMA) in order to ensure treatment homogeneity. The PRIMA trial required the study sample to be taken within 4 months before study registration.

Immunohistochemistry, scoring, and immunofluorescence

Both TMAs were stained in Vancouver with mouse monoclonal antibodies against CD68 (clone KP1; Dako) and CD163 (clone 10D6; Novocastra) on a Ventana BenchMark XT automated slide staining system. Image analysis was performed on an Aperio ScanScope XT using the Positive Pixel Count algorithm with a color saturation threshold (CST) set at 0.1. All slides were reviewed by pathologists (K. Tan and A. Vawda) to exclude nonspecific staining and in rare cases, the CST was increased to 0.15 to reduce background staining. The percentage of positive pixels was determined as described in Tan and colleagues (19) and averaged across all cores from each sample. In addition, visual scoring was performed on the BCCA TMA by estimating percentages of CD68- and CD163-positive cells. Scores were reported in discrete categories (0%, 1%, 5%, 10%, 20%, and 30%).

Double immunofluorescence was performed on a 4-μm formalin-fixed paraffin-embedded section after heat-induced epitope retrieval using an AlexaFluor-647-labeled mouse anti-human CD68 antibody (clone KP1; Santa Cruz) and an unlabeled rabbit anti-human CD163 antibody (clone K20-T; Abnova) in combination with a secondary goat anti-rabbit Cy3 antibody. The immunofluorescent image was acquired using a Coolsnap HQ digital camera on an inverted microscope (IX70 Olympus) in combination with a DeltaVision RT imaging system (Applied Precision). The image was scanned in stacks, deconvoluted, and projected onto a single plane using SoftWoRx (Applied Precision).

Statistical analysis

As, on average, the extent of macrophage infiltration is low in follicular lymphoma and point estimates can be imprecise on TMAs, we required each valid patient sample to have at least two measurements and the relative standard error to be less than 33.33%. Correlation between Aperio and manual scoring was assessed using the Pearson correlation coefficient, whereas the correlation between CD68 and CD163 positivity was determined using Spearman correlation as visual inspection suggested that the underlying assumptions of linear regression were not met. For survival analysis, both the BCCA and the PRIMA cohorts were randomly split into training/validation sets in 1:1 ratios. As the distributions of CD68 and CD163 expression did not reveal natural cutpoints, we used the X-tile software (version 3.6.1; Yale University, New Haven, CT) to bisect these distributions at values that gave the maximum χ^2 values of the log-rank test (20).

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Thresholds of CD68 and CD163 expression were defined using progression-free survival (PFS) in each of the training cohorts. They were then locked and carried forward into the validation cohorts for PFS and the training/validation cohorts for overall survival (OS) analysis. Times to event [relapse or death from any cause (PFS) or death from any cause (OS)] were calculated from date of the first dose of R-CVP for BCCA patients, and from date of registration for PRIMA patients. Associations of clinical characteristics with patient cohorts were evaluated using the χ^2 test, and the distributions of CD163 staining in risk categories were compared using the Mann–Whitney or the Kruskal–Wallis tests. $P < 0.05$ was considered significant.

Results

Patient cohorts

The study overview is shown in Supplementary Fig. S1. After quality assessment, 180 of 186 (97%) and 335 of 436 (77%) patient samples were deemed evaluable for the assessment of either CD68 or CD163 in the BCCA and PRIMA datasets, respectively. The two cohorts differed in several clinical characteristics, including age, stage, performance status, lactate dehydrogenase (LDH), and hemoglobin levels, but after repartition of patients into FLIPI risk categories, the study populations were not significantly different between BCCA and PRIMA patients ($P = 0.329$; Table 1). The median follow-up of living patients was 70.7 and 55.2 months for the BCCA and the PRIMA cohorts, respectively. Rituximab maintenance was intended for 150 of 180 BCCA patients (83%) and randomly administered (according to the study design) to 141 of 335 PRIMA patients (42%).

Table 1. Patient characteristics in each cohort

	BCCA ($n = 180$)		PRIMA ($n = 335$)		
	N	%	*N*	%	*P*
Age at induction					
≤60	87	48.3	226	67.5	<0.001
>60	93	51.7	109	32.5	
Gender					
Female	76	42.2	161	48.1	NS
Male	104	57.8	174	51.9	
Ann Arbor stage					
I–II	31	17.3	23	6.9	<0.001
III–IV	148	82.7	312	93.1	
ECOG[a]					
0	87	48.9	227	67.8	<0.001
1	72	40.5	97	29.0	
2	15	8.4	11	3.3	
3	4	2.3	0	0.0	
LDH[b]					
≤ULN	143	84.1	225	67.4	<0.001
>ULN	27	15.9	109	32.6	
Hemoglobin[c]					
<12 g/dL	17	10.1	69	20.6	0.004
≥12 g/dL	152	89.9	266	79.4	
Nodal areas[d]					
≤4	59	33.9	115	34.3	NS
>4	115	66.1	220	65.7	
FLIPI[e]					
Low risk	40	24.2	62	18.6	NS
Intermediate risk	54	32.7	115	34.4	
High risk	71	43.0	157	47.0	

Abbreviations: ECOG, Eastern Cooperative Oncology Group performance status; FLIPI, Follicular Lymphoma International Prognostic Index; ULN, upper limit of normal.

[a]Data were unavailable for 2 patients from the BCCA cohort.

[b]Data were unavailable for 10 patients from the BCCA cohort and 1 patient from the PRIMA cohort.

[c]Data were unavailable for 11 patients from the BCCA cohort.

[d]Data were unavailable for 6 patients from the BCCA cohort.

[e]Data were unavailable for 15 patients from the BCCA cohort and 1 patient from the PRIMA cohort.

Macrophage staining

Aperio scoring of macrophage infiltration was well correlated with manual assessment of percentage of stained cells ($R = 0.827$ and $P < 0.001$ for CD68; $R = 0.907$ and $P < 0.001$ for CD163; Supplementary Fig. S2). The extent of macrophage infiltration of the tumor was low, as assessed by a median pixel positivity of 3.87% and 2.61% for CD68 and 1.07% and 0.98% for CD163, in the BCCA and PRIMA cohorts, respectively (Supplementary Fig. S3). On average, CD68 stained more pixels than CD163 ($P < 0.001$ in both datasets; Supplementary Fig. S4A). The distribution of CD163 staining was superimposable in the two cohorts ($P = 0.222$), but significantly different for CD68 ($P < 0.001$; Supplementary Fig. S5). CD68 and CD163 staining were not significantly correlated ($R = 0.157$ and $P = 0.057$ for the BCCA cohort, and $R = 0.112$ and $P = 0.068$ for the PRIMA cohort; Supplementary Fig. S6). By immunofluorescence, CD163-positive cells costained positive for CD68 (Supplementary Fig. S4B). These cells were mostly located in inter-follicular areas, whereas scattered CD68-positive/CD163-negative cells tended to be found inside follicles.

Survival analysis in the BCCA cohort

CD68 positivity was weakly associated with PFS in the training cohort, but a significant association could not be found in the validation cohort (Supplementary Fig. S7). High positive pixel counts for CD163 on the other hand were significantly associated with poor PFS and OS in both the training and validation cohorts (Fig. 1). Using the optimal cutoff of 3.97% for CD163, 5-year PFS was 38% versus 72% in the training cohort ($P = 0.004$), and 29% versus 61% in the validation cohort ($P = 0.004$). The CD163-positive pixel count was not significantly associated with clinical risk factors (Table 2). The adverse prognostic impact of $CD163^+$ TAMs remained significant in a Cox multivariate regression model when CD163 was used as a continuous variable and after adjusting for the FLIPI index and use of rituximab maintenance by intention to treat (HR 1.12 for PFS, $P = 0.022$; Table 3).

Survival analysis in the PRIMA cohort

In the PRIMA dataset, even after a new cutpoint optimization using X-tile, CD68 staining was predictive of outcome only in the training but not the validation cohort (Supplementary Fig. S8). For CD163, the 3.97% cutpoint, as defined in the BCCA cohort, failed to validate in the PRIMA training set as it did not separate patients into groups that significantly differed by PFS ($P = 0.313$). This threshold identified 19 of the 144 patients in the training set (13.2%) as having a high CD163-positive pixel count, and the 5-year PFS in these patients was 78%, as opposed to 53% for those 125 of the 144 patients who had a low CD163-positive pixel count (86.8%). Although not significant, these results suggested that the outcome correlation for CD163 might be reversed in the PRIMA data. Importantly, the observed differences in outcome were not explicable by differences in staining between the two TMAs as the percentage of CD163 positivity was equally distributed between the two cohorts (Supplementary Fig. S5). A new cutpoint

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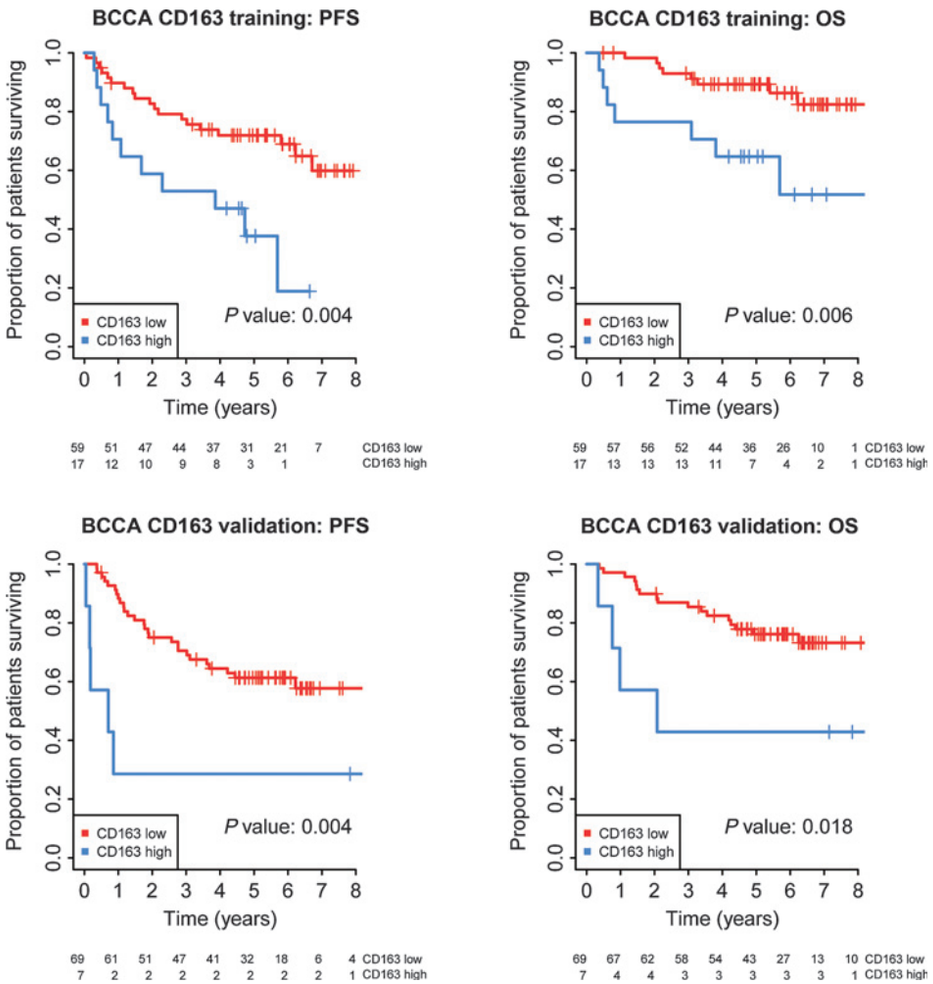

Figure 1.
Outcome correlation for CD163 in the BCCA cohort. The optimal cutoff of 3.97% was defined for PFS in the training cohort and carried forward into the OS analysis and validation cohorts. PFS and OS are calculated from date of initiation of R-CVP. In the training cohort, 17 and 59 patients of 76 were classified as having a high versus low CD163-positive pixel count. In the validation cohort, these numbers were 7 and 69 of 76.

determination using X-tile revealed an optimal cutoff of 0.16% for CD163 in the PRIMA data. Based on this threshold, a high CD163-positive pixel count was indeed associated with favorable PFS in the training cohort (Fig. 2; 5-year PFS 60% vs. 44% and $P = 0.011$), and this association remained true in the validation cohort (5-year PFS 55% vs. 37% and $P = 0.030$). A high CD163-positive pixel count was associated with age > 60 years ($P = 0.018$), female gender ($P = 0.026$), low LDH ($P = 0.022$), and ≤ 4 nodal sites ($P < 0.001$). The CD163-positive pixel count was not associated with the FLIPI index (Table 2).

We then performed a multivariate Cox regression analysis in which CD163 was introduced as a continuous variable in order to avoid overfitting of the model and relying on predefined cutpoints (Table 4). The analysis was stratified by randomization into either the rituximab or observation arms. High CD163 remained significantly associated with favorable outcome when adjusted for the FLIPI in those patients assigned to the rituximab arm (HR 0.73 for PFS, $P = 0.015$), but not in those patients randomized to the observation arm (HR 1.06 for PFS, $P = 0.322$). These latter results confirm the association between a high CD163-positive pixel count and favorable outcome in the rituximab maintenance arm of the PRIMA trial only. They suggest that $CD163^+$ TAMs modulate the efficacy of rituximab, supporting the hypothesis of a favorable interaction between TAMs and response to maintenance rituximab.

Discussion

Here, we investigated the correlation of TAMs with outcome using modern image analysis. We first analyzed a single institution experience with uniform therapy (BCCA cohort, R-CVP) and then compared the findings with those from a prospective, randomized phase III clinical trial (PRIMA, R-CHOP). Computer-assisted scoring has recently been proposed to be a more reliable means for enumeration of microenvironment cell populations than traditional manual scoring (21). We showed that most diagnostic follicular lymphoma samples are infiltrated by few macrophages. Increased staining for CD163 was associated with poor PFS and OS in the BCCA cohort and favorable PFS in the PRIMA cohort. On the other hand, CD68 staining cells did not predict outcome in either cohort.

Of the two markers that were used in the present study, CD163 gave us much cleaner and stronger staining than CD68, as described by our group and by others (19, 22, 23), whereas CD68 has more nonspecific background signal and stains some nonhistiocytic cellular elements (24) These considerations may

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Table 2. Association of CD163 with clinical characteristics

	BCCA		PRIMA	
	CD163-positive pixels count, mean	*P*	% CD163-positive pixels count, mean	*P*
Age at induction				
≤60	1.87	NS	1.52	0.018
>60	2.10		2.32	
Gender				
Female	2.05	NS	1.92	0.026
Male	1.95		1.66	
Ann Arbor stage[a]				
I–II	2.46	NS	2.21	NS
III–IV	1.90		1.75	
ECOG[b]				
0	1.53		1.73	
1	2.39		1.91	
2	3.40		1.78	
3	1.68	NS	NA	NS
LDH[c]				
≤ULN	1.81		1.84	
>ULN	2.93	NS	1.66	0.022
Hemoglobin[d]				
<12 g/dL	2.77		2.04	
≥ 12 g/dL	1.85	NS	1.72	NS
Nodal areas[e]				
≤4	2.26		2.45	
>4	1.82	NS	1.42	< 0.001
FLIPI[f]				
Low risk	2.28		2.19	
Intermediate risk	1.66		1.56	
High risk	2.00	NS	1.78	NS

Abbreviations: ECOG, Eastern Cooperative Oncology Group performance status; FLIPI, Follicular Lymphoma International Prognostic Index; ULN, upper limit of normal.

[a]Data were unavailable for 1 patient in the BCCA cohort.

[b]Data were unavailable for 2 patients in the BCCA cohort.

[c]Data were unavailable for 9 patients in the BCCA cohort and for 1 patient in the PRIMA cohort.

[d]Data were unavailable for 9 patients in the BCCA cohort.

[e]Data were unavailable for 5 patients in the BCCA cohort.

[f]Data were unavailable for 12 patients in the BCCA cohort and for 1 patient in the PRIMA cohort.

contribute to explaining prior conflicting studies and the absence of a correlation between CD68 staining and outcome in our study. CD163 is widely seen as a phenotypic marker of M2 or alternatively polarized macrophages as—at least *in vitro*—its expression is suppressed by proinflammatory mediators such as lipopolysaccharide, IFNγ, or TNFα and stimulated by the Th2 cytokines IL6 and IL10 (17, 25). CD163 is also associated with angiogenesis in both classical Hodgkin lymphoma and follicular lymphoma, supporting its role in identifying M2-type macrophages (10, 26).

Table 3. Cox multivariate regression analysis in the BCCA cohort

	PFS		
Variable	**HR**	**95% CI**	***P***
FLIPI index			
CD163-positive pixel count[a]	1.12	1.02–1.23	0.022
FLIPI risk group	1.46	1.02–2.07	0.037
Rituximab maintenance	0.83	0.44–1.58	0.573
FLIPI individual factors			
CD163-positive pixel count[a]	1.12	1.01–1.24	0.040
Age >60 years	1.60	0.91–2.82	0.100
Stage III/IV	1.74	0.77–3.93	0.182
LDH > ULN	0.78	0.36–1.69	0.527
Hemoglobin <120 g/L	2.09	0.90–4.84	0.086
>4 nodal sites	0.88	0.48–1.59	0.663
Rituximab maintenance	0.80	0.42–1.54	0.505

Abbreviations: CI, confidence interval; FLIPI, Follicular Lymphoma International Prognostic Index; ULN, upper limit of normal.

[a]continuous variable.

The simplistic conclusion that $CD163^+$ TAMs are invariably associated with either an adverse or a favorable outcome is not supported, however, by our study. The differential impact that we observe in the BCCA and the PRIMA groups rather suggests that prognosis is modulated, in addition to $CD163^+$ macrophages, by cohort-specific patient characteristics. In the BCCA data, CD163 staining was not associated with clinical risk factors, whereas in the PRIMA trial, increased CD163 staining was correlated with age > 60 years, the female gender, low levels of LDH, ≤ 4 nodal areas but not the FLIPI index. The Cox multivariate regression analysis shows, however, that the differential prognostic impact of TAMs on PFS is independent of established risk factors and potential confounders. It is noteworthy that one of the main differences in terms of treatment resides in the administration of doxorubicin as part of the R-CHOP regimen that was uniformly given in those PRIMA patients that were evaluated in this study, whereas BCCA patients were invariably managed without an anthracycline as part of their first-line therapy. In an allograft mouse model, depletion of macrophages reduced the efficacy of doxorubicin, but not daunorubicin, and conversely, prior macrophage activation enhanced the efficacy of doxorubicin (25, 27). Recent experimental data have also indicated that anthracyclines are able to modulate the differentiation and function of cells involved in innate immunity toward a tumoricidal phenotype (28). These findings, in combination with our data, suggest that doxorubicin contributes to better outcome in those R-CHOP patients whose samples harbor elevated numbers of macrophages.

Within the PRIMA trial, the randomization also offered an unbiased, controlled comparison of outcome modulation by CD163 and use of maintenance rituximab versus observation. Elevated CD163 staining was significantly associated with favorable PFS only in the rituximab maintenance arm, suggesting that macrophages positively modulate its efficacy. These findings mirror the findings of the FL-2000 trial in which $CD68^+$ macrophages predicted poor event-free survival in the cyclophosphamide, doxorubicin, etoposide, prednisolone and interferon (CHVP-I) arm but not in the rituximab plus CHVP-I arm (7), and the study by Taskinen and colleagues in which elevated numbers of macrophages correlated with favorable PFS in patients treated with R-CHOP but not with CHOP (11). Anti-CD20 antibodies exert their effects through antibody-dependent cell-mediated cytotoxicity, complement-mediated lysis, or direct effects such as antiproliferative signals (29). Macrophages bind therapeutic antibodies on Fc receptors and have been proven to be critical effectors of tumor cell killing in a syngeneic Eμ-*cMyc* mouse model using an anti-CD20 antibody and in a humanized mouse model of chemorefractory B-cell leukemia using alemtuzumab (30, 31). In the latter study, antitumor responses were achieved in the macrophage-rich environment in the spleen but not in the macrophage-poor bone marrow (31). Lastly, M2-skewed macrophages show increased *in vitro* phagocytic capacity of rituximab-opsonized chronic lymphocytic leukemia cells in contrast with M1 macrophages, suggesting that CD163 is a

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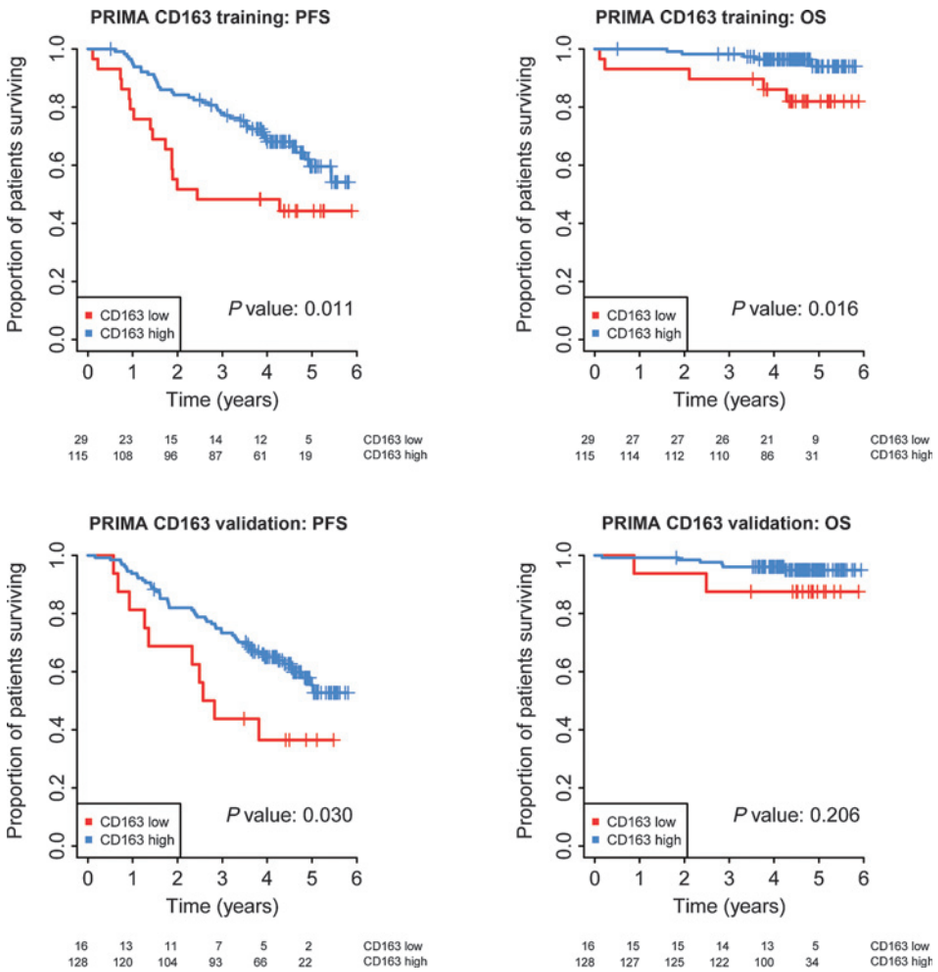

Figure 2.
Outcome correlation for CD163 in the PRIMA cohort. The optimal cutoff of 0.16% was defined for PFS in the training cohort and carried forward into the OS analysis and validation cohorts. PFS and OS are calculated from date of registration in the trial. In the training cohort, 115 and 29 patients of 144 were classified as having a high versus low CD163-positive pixel count. In the validation cohort, these numbers were 128 and 16 of 144.

rational marker to identify those TAMs that participate in rituximab-mediated antitumor responses (32).

In conclusion, our findings lend texture to a rich but highly conflicted literature on tumor microenvironment markers in follicular lymphoma, and the interpretation needs to be nuanced. In the absence of doxorubicin as part of primary immunochemotherapy, increased numbers of $CD163^+$ TAMs correlate with worse outcome, and this negative effect is not compensated by maintenance rituximab. However, when doxorubicin is given, as it was in the R-CHOP–treated PRIMA trial patients, maintenance rituximab emerges as important, and its inclusion reverses the negative influence of $CD163^+$ TAMs. Although the development of a robust biomarker is highly desirable for follicular lymphoma patients in the precision medicine era, the apparently opposite

Table 4. Cox multivariate regression analysis in the PRIMA cohort

	Rituximab arm: PFS[a]			Observation arm: PFS[b]		
Variable	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
FLIPI index						
CD163-positive pixel count[c]	0.73	0.57–0.94	0.017	1.05	0.95–1.17	0.346
FLIPI risk group	2.58	1.45–4.58	0.001	1.35	0.98–1.86	0.068
FLIPI individual factors						
CD163-positive pixel count[c]	0.77	0.58–1.01	0.058	1.02	0.91–1.14	0.715
Age >60 years	1.58	0.75–3.33	0.225	0.79	0.45–1.38	0.402
Stage III/IV	0.85	0.09–8.27	0.889	1.21	0.40–3.67	0.736
LDH > ULN	2.21	1.08–4.52	0.030	1.09	0.64–1.84	0.752
Hemoglobin <120 g/L	0.96	0.43–2.18	0.925	3.19	1.90–5.36	<0.001
>4 nodal sites	3.90	1.15–13.24	0.029	1.52	0.83–2.79	0.174

Abbreviations: CI, confidence interval; FLIPI, Follicular Lymphoma International Prognostic Index; ULN, upper limit of normal.
[a]this analysis was performed using data from all 113 patients who were evaluable for CD163 staining and randomized to the rituximab arm.
[b]this analysis was performed using data from all 147 patients who were evaluable for CD163 staining and randomized to the observation arm.
[c]continuous variable.

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results from the BCCA and the PRIMA studies suggest that prognostication is highly dependent on patient characteristics and/or treatment. Furthermore, as the threshold that most significantly distinguished the outcomes of those BCCA cases with high from those cases with low $CD163^+$ positivity did not validate in the PRIMA cohort, we do not presently recommend staining for CD163 in routine clinical evaluation. Future studies are warranted in well-annotated external patient populations and should also extend to cohorts of patients treated with the nowadays more commonly used bendamustine–rituximab regimen, or new agents such as imids or kinase inhibitors.

Disclosure of Potential Conflicts of Interest

L.H. Sehn reports receiving speakers bureau honoraria from Amgen, Celgene, Gilead, Janssen, Lundbeck, Pfizer, Roche/Genentech, and Seattle Genetics. G. Salles reports receiving a commercial research grant from Roche, and is a consultant/advisory board member for Celgene, Gilead, Janssen, and Roche. No potential conflicts of interest were disclosed by the other authors.

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