Tumor-infiltrating macrophages correlate with adverse prognosis and Epstein-Barr virus status in classical Hodgkin's lymphoma

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Funding: this work was supported by Aarhus University; the Karen Elise Jensen Foundation; the Max and Inger Wørzner Foundation, The Foundation in Commemoration of Eva and Henry Frænkel, The Foundation of Aase and Ejner Danielsen, The Foundation of Poul and Ellen Hertz, The Foundation of Else and Mogens Wedell-Wedellsborg and The A.P. Møller and Chastine Mc-Kinney Møller Foundation for General Purposes. The Center for Stochastic Geometry and Advanced Bioimaging is supported by the Villum Foundation.

Acknowledgments: the authors would like to thank Tom Nordfeld and Mette Hjøllund Christiansen for technical assistance, collaborating pathological departments for retrieval of tissue samples and Jens Overgaard and the Department of Experimental Oncology for scientific inspiration and logistic support.

Manuscript received on August 9, 2010. Revised version arrived on October 27, 2010. Manuscript accepted on October 29, 2010.

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ABSTRACT

Background
Classical Hodgkin's lymphoma is characterized by a minority of neoplastic cells surrounded by a heterogeneous background population of non-neoplastic cells including lymphoma-associated macrophages. High levels of expression of both the monocyte/macrophage lineage-associated antigens CD68 and CD163 have been suggested to have pro-tumor effects. The aim of our study was to correlate expression of CD68 and CD163 with the clinico-pathological features and prognosis of a cohort of patients with previously untreated Hodgkin's lymphoma.

Design and Methods
A tissue microarray was constructed from paraffin-embedded tumor tissues from 288 cases of classical Hodgkin's lymphoma. CD68 and CD163 expression was assessed immunohistochemically and the degree of macrophage infiltration within the tumor was scored using point grid counting. Clinical data were obtained from clinical records.

Results
The patients' median age was 37 years (range, 6-86 years). The male to female ratio was 1.2. In classical Hodgkin's lymphoma (n = 288) high CD68 and CD163 expression correlated, at the univariate level, with poorer overall survival (P=0.002 and P=0.03, respectively) and event-free survival (P=0.03 and P=0.04, respectively). At the multivariate level, high CD68 expression remained significantly predictive of overall survival (P=0.004). In addition, we demonstrated that both high CD68 and CD163 expression were associated with the presence of Epstein-Barr virus in the neoplastic cells (P=0.001 and P=0.0002, respectively).

Conclusions
In classical Hodgkin's lymphoma, high expression of the macrophage/monocyte-related antigens CD68 and CD163 correlates with adverse outcome and with the presence of Epstein-Barr virus in the tumor cell population.

Key words: classical Hodgkin's lymphoma, tumor microenvironment, macrophages, CD68, CD163, Epstein-Barr virus.

Citation: Kamper P, Bendix K, Hamilton-Dutoit S, Honoré B, Nyengaard JR, d'Amore F. Tumor-infiltrating macrophages correlate with adverse prognosis and Epstein-Barr virus status in classical Hodgkin's lymphoma. Haematologica 2011;96(2):269-276.doi:10.3324/haematol.2010.031542

Introduction

Classical Hodgkin's lymphoma (cHL) is a B-cell derived malignant lymphoproliferative disease diagnosed in approximately 0.4 persons per 100,000/year.[1] Although current treatment strategies manage to cure more than 80% of these patients, a substantial proportion still experience relapsing or refractory disease which eventually leads to their death. In order to identify those patients who need novel treatment strategies, several scoring systems have been proposed, including the International Prognostic Score (IPS).[2] The IPS is still considered the "gold standard" for assessing prognosis and has performed consistently well in independent data sets. However, it was originally designed for patients with advanced disease and is, therefore, less suitable for patients with a low-risk profile.[3] Moreover, recent early interim positron emission tomography analysis has been shown to have a prognostic value superior to that of IPS in advanced stage cHL.[4]

The tumor lesion in cHL is characterized by a minority of neoplastic Hodgkin and Reed-Sternberg cells, often accounting for as little as 1% or less of the total cell population. The Hodgkin and Reed-Sternberg cells are embedded in a heterogeneous background of non-neoplastic bystanders, mostly B and T cells, but also macrophages, eosinophils, basophils and plasma cells. There has recently been increasing interest in these bystander cells, both in order to better understand the underlying biology of the disease and in order to identify new biological markers for prognostic and therapeutic purposes. Although, previous studies have suggested that bystander cells such as eosinophils, mast cells[5,6] and T-cell subsets[7] might be of prognostic importance, no specific biological markers have yet been identified as reliable tools for pre-therapeutic risk assessment.

It has been suggested that lymphoma-associated macrophages may have a prognostic role in several different lymphoproliferative entities, including cHL.[8] For example, a number of studies have indicated that an increased content of CD68-positive lymphoma-associated macrophages in follicular lymphoma is associated with an adverse prognostic impact, with regards to both event-free and overall survival.[9,10] In cHL, a considerable variation in the number of intra-tumor macrophages has been observed.[11] Recent gene expression profiling data showed a correlation between up-regulation of genes related to intra-tumor macrophage infiltration (*STAT1*, *ALDH1A1*) and poor response to treatment, suggesting an underlying tumor-promoting role for these cells.[12]

In the current study we used immunohistological expression of two macrophage-associated markers, CD68 and CD163, to quantify the macrophage content in cases of cHL and then correlated the levels of expression of these markers with pre-therapeutic clinico-pathological features and assessed their possible effect on outcome in cHL.

Design and Methods

Patients

We identified a total of 428 patients histopathologically diagnosed with Hodgkin's lymphoma (HL) between 1990 and 2007 at Aarhus University Hospital, and in whom no other concurrent or previous malignancies were found. None of the patients was positive for human immunodeficiency virus. All primary diagnostic tumor biopsies were reviewed and reclassified according to the World Health Organization classification of tumors of the hematopoietic and lymphoid tissues.[13] Eighty-seven patients were excluded because insufficient representative tumor tissue could be retrieved. An additional 22 patients had insufficient material for tissue microarray construction, while data were missing for 17 patients. Fourteen cases were classified as having nodular lymphocyte-predominant HL and were excluded from further analysis. Thus, 288 eligible cases were analyzed. Clinical and follow-up data were obtained from clinical records. Late relapses occurring beyond the routine clinical follow-up period (10 years) were identified through the Danish National Pathology Registry and Pathology Data Bank (Patobank) which contains a nationwide registration of all histopathological diagnoses since January 1st, 2000. For those patient who had died, the precise date of death was obtained from the Danish Civil Registration System.

Clinical and paraclinical information reflecting clinico-pathological features at diagnosis was collected. This information included: histopathological subtype, age, gender, presence or absence of B-symptoms, clinical stage according to the Cotswolds modification of the Ann Arbor classification,[14] presence or absence of bulky disease (maximum diameter ≥ 10 cm), specific anatomical localization of disease, hemoglobin concentration, total leukocyte and lymphocyte counts, and serum albumin concentration. The study was approved by the regional ethical committee and by the Danish Data Protection Agency.

Treatment

All patients were treated at Aarhus University Hospital between 1990 and 2007 according to standard guidelines. Patients with advanced stage disease (stage III or IV) were uniformly treated with ABVD/COPP (adriamycin, bleomycin, vinblastine, dacarbazine/cyclophosphamide, vincristine, procarbazine, prednisone) chemotherapy. Additional radiotherapy was given in cases of pre-therapeutic bulky or localized residual masses. In the period 1990-1997, patients with localized disease (clinical stage I or II) without systemic symptoms or bulky lesions were treated with radiotherapy alone. From 1998 onwards, patients with stage I or II disease were treated according to the recommendations of the Nordic Lymphoma Group. Patients with cHL and no risk factors, i.e. no bulky disease, two or fewer lymph node regions involved and an erythrocyte sedimentation rate of 55 mm or less, were given two cycles of ABVD followed by involved-field radiotherapy. Patients with one or more risk factors were given four cycles of ABVD followed by involved-field radiotherapy. Treatment response was assessed using standardized guidelines.[15]

Tissue samples

A total of 288 formalin-fixed, paraffin-embedded tissue blocks from diagnostic cHL biopsies were retrieved from the archives of ten participating Danish pathology departments.

Immunohistochemistry and in situ hybridization

All immunohistochemical stains were performed on the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA) using 4 μm paraffin tissue sections. The primary antibodies used in this study were: anti-CD68 (clone KP-1, dilution 1:800; Dako, Glostrup, Denmark), anti-CD163 (clone 10D6, dilution 1:50; Novocastra, Newcastle, UK) and anti-latent membrane protein-1 (LMP-1) (clones CS 1-4, dilution 1:100; Dako). All slides were incubated with the primary antibodies for 32 min at 37°C. The Ventana Ultraview™ DAB detection kit was used for detection of the primary antibody. For LMP-1 staining, the signal was enhanced with a Ventana amplification kit. Slides were counterstained with hematoxylin. Non-isotopic *in situ*

hybridization for Epstein-Barr virus (EBV)-encoded RNA-1 and -2 (EBER) was performed and scored as described elsewhere.[16]

Tissue microarray construction

Six tissue microarray blocks containing triplicate 1 mm cores from each patient's sample were constructed with a manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA), according to standard guidelines.[17] Each block also contained samples of normal lymph node, placenta, kidney, and liver tissue as internal controls and for tissue microarray land-marking.

Stereological analysis for the quantification of CD68 and CD163 expression

Immunohistochemical stains were quantified by stereological analysis using a light microscope equipped with a computer assisted stereology system (CAST, Visiopharm, Hoersholm, Denmark). The counting frame covered an area of 146,435 μm^2 and the fields of view was sampled in a systematic, random fashion. A median number of 18 (range, 8-26) and 20 (range, 9-30) counting frames were evaluated for CD68- and CD163-positive signals, respectively. In order to estimate the degree of macrophage infiltration we determined, for each triplet of cores, the area fraction of positive cells for CD68 or CD163. The CD68- or CD163-positive area fraction was determined by point-counting[18] with a x20 lens at a total magnification of x739. Points hitting either necrosis or artifacts were ignored. In order to evaluate the intra-observer variation, Pearson's correlation coefficients were calculated in 10% of the patients (n=30). The inter-observer variation was estimated in ten patients who were chosen in a systematic, random manner. Variability between readings was also assessed by difference-average plots in concordance with the Bland-Altman method.[19] The intra-observer variation was found to be excellent for both CD68 and CD163 with Pearson's correlation coefficients of 0.95 and 0.97, respectively. The two readings of CD68 had a mean deviation of -0.63% (95% CI, -1.4 - 0.13) and a 95% prediction interval from -4.7% to 3.5%. Considering CD163, the mean deviation was -0.18 (95% CI: -1.6 - 1.2) with a 95% prediction interval ranging from -7.7 to 7.4. Pearson's correlation coefficients for inter-observer variation with CD68 and CD163 were 0.99 and 0.97, respectively. The inter-observer variation for CD68 had a mean deviation of 0.06% (95% CI: -0.6 - 0.7) and a 95% prediction interval from -3.5% to 3.7%, while CD163 had a mean deviation of 2.2% (95% CI: -0.5 - 4.9) and a 95% prediction interval from -5.7% to 9.7%.

Validation study

A number of previous studies have validated the use of tissue microarrays in HL.[17,20] Prior to the main cohort analysis, we performed a pilot study in order to validate specifically the use of tissue microarrays for the evaluation of macrophage infiltration. For this purpose, a separate tissue microarray containing three cores from 27 individual patients' samples was created and stained using the anti-CD163 antibody. This experiment showed an overall good concordance in the results when comparing the analysis of whole sections with tissue microarray sections, when evaluating at least two 1 mm cores (Pearson's correlation coefficient 0.98 (95% CI; 0.95-0.99); 95% prediction interval ranging from -6.4 to 2.5). This concordance decreased considerably if only one core was included in the analysis. For this reason only cases with at least two tissue cores present on the final cohort tissue microarray were included in the final analysis. In conclusion, 262 patients were eligible for CD68 evaluation and 279 for CD163.

Statistical analysis

Patients' characteristics were compared using the Wilcoxon rank-sum test and the Kruskal-Wallis equality-of-populations rank test. The follow-up time was defined as the time from diagnosis to either last follow-up or a given event. Event-free survival was measured from the date of diagnosis to either disease progression or discontinuation of treatment for any reason or censoring date. Overall survival was measured from the date of diagnosis to the date of death from any cause or censoring date. For survival analyses related to CD68 and CD163 expression, two groups were considered: the quartile of patients with highest expression values *versus* the lower three quartiles taken as one group. This cut-point was chosen in concordance with previous reports[12] and the resulting absolute cut-off values for CD68 and CD163 were above or equal to 7.8% and above or equal to 21.1%, respectively. Survival was estimated by the Kaplan-Meier method and compared using a log-rank test. A Cox proportional multivariate model was used to assess possible associations of CD68 or CD163 with overall survival and event-free survival, while adjusting for other individual parameters with a significance level below 0.10 at a univariate level. Two-sided *P* values less than 5% were considered statistically significant and *P* values less than 10% were considered of borderline significance. All statistical analyses were done using STATA software version 10.1 (STATA, TX, USA).

Results

Clinico-pathological features

The main clinical and histopathological features at presentation of the study cohort are summarized in Table 1.

Table 1. Demographic and clinico-pathological parameters and their and distribution according to CD68 and CD163 expression.

Characteristic	Total N.	%	CD68 N.	CD68 Mean	CD68 *P*	CD163 N.	CD163 Mean	CD163 *P*
	288		262			279		
Age, years (n=288)								
<45	181	63	165	6.0	ns*	177	13.5	ns*
≥45	107	37	97	7.0		102	14.8	
Sex (n=288)								
Male	156	54	146	6.5	ns*	154	14.8	ns*
Female	132	46	116	6.2		125	13.0	
Ann Arbor stage (n=288)								
I-II	192	67	172	5.7	0.01*	186	11.7	0.0009*
III-IV	96	33	90	7.6		93	18.6	
B symptoms (n=288)								
Yes	138	48	124	6.6	ns*	133	16.2	0.03*
No	150	52	138	6.2		146	12.0	
Bulky disease (>10cm) (n=271)[1]								
Yes	78	29	70	6.6	ns*	76	16.5	ns*
No	193	71	177	6.4		187	13.2	
Histological type (n=288)								
NSI	167	58	149	5.7	0.002**	160	12.2	0.009**
NSII	70	24	64	6.4		70	14.1	
MC	47	16	46	8.7		46	20.4	
cHL, NOS	4	1	3	2.9		3	8.2	
IPS (n=252)[1]								
≤ 2	168	67	153	6.1	ns*	163	11.7	0.001*
> 2	84	33	75	6.9		80	18.3	
EBV status (n=288)								
Positive	95	33	90	7.7	0.001*	93	18.7	0.0002*
Negative	193	67	172	5.7		186	11.6	

*[1]Parameter not available in all 288 patients; *Wilcoxon's rank-sum test, **Kruskal-Wallis equality-of-populations rank test. NSI and NSII, nodular schlerosis type I and II; MC,: mixed cellularity; cHL, NOS: classical Hodgkin's lymphoma, not otherwise specified; IPS: International Prognostic Score; EBV: Epstein-Barr virus.*

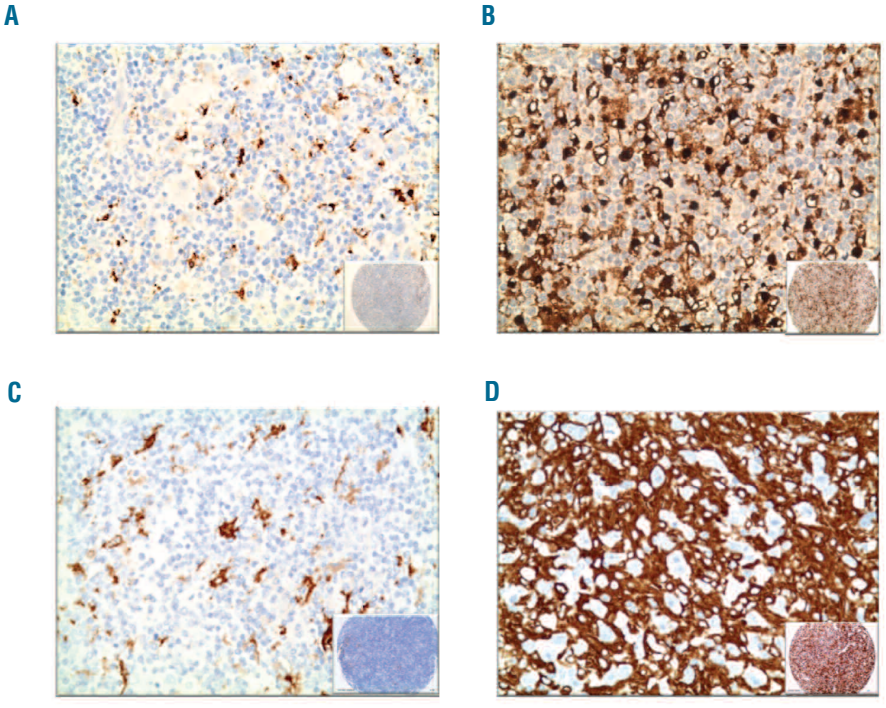

Figure 1. Representative immunohistochemical stains for CD68 and CD163 (x 400). The images show low levels of CD68 and CD163 expression (panels A and C; both nodular sclerosis subtype) and high levels of CD68 and CD163 expression (panels B and D; mixed cellularity and nodular sclerosis subtype, respectively). Insets show low magnification images of the corresponding tissue microarray cores.

The population of patients under study had a median age of 37 years and a male/female ratio of 1.2. The median follow-up of the population was 7 years (range, 0.2-18.6 years). A third of all patients (33%) had disseminated disease (Ann Arbor stage III-IV), almost half of the population (48%) experienced B-symptoms and another third (29%) presented with bulky disease. The median IPS score of the entire cohort was 2 and approximately one third of the patients had EBV infection (positivity for EBER and LMP-1) in Hodgkin and Reed-Sternberg tumor cells. The large majority of the patients (82%) had histological features consistent with the nodular sclerosis subtype (I and II).

CD68 and CD163 expression

The staining patterns for both CD68 and CD163 ranged from cases with almost no expression to cases in which the macrophage signal was predominant (Figure 1). We found a higher area fraction for CD163 positivity than for CD68, with median values of 7.5% (range, 0.1-63.4%) and 4.4% (range, 0.3-30.7%), respectively.

The architectural pattern of macrophage infiltration varied from being diffuse and homogeneous to a more rosetting pattern. This rosetting was seen in both nodular sclerosis and mixed cellularity subtypes and seemed unrelated to the nodules that otherwise characterized nodular sclerosis architecture. As expected, the expression of both macrophage markers was higher in mixed cellularity cases than in nodular sclerosis cases.

CD68 and CD163 expression and clinico-pathological features

Correlations between the results of staining for CD68 and CD163 and clinico-pathological features are listed in Table 1. Higher levels of CD68 and CD163 expression were correlated with the presence of EBV in the tumor cells (P=0.001 and P=0.0002, respectively) and, consequently, also with the mixed cellularity subtype. As expected, the majority of cases of mixed cellularity cHL (68%) were EBV-positive, whereas EBV was found in only 26% of the cases without mixed cellularity histology (P<0.001). However, even excluding the mixed cellularity subtype from the analysis, the significant correlation between EBV and high CD163 expression persisted (P=0.03), whereas the corresponding correlation for CD68 was slightly weakened (P=0.06). Over-expression of both CD68 and CD163 also correlated with disseminated clinical stage and high CD163 expression alone correlated with the presence of B-symptoms and an IPS greater than 2.

Correlation between CD68 and CD163 expression and patients' outcome

At the univariate level, we found that high levels of CD68 and CD163 expression were correlated with an adverse outcome (Table 2). The 10-year overall survival was 82% for cases with low CD68 expression and 61% for cases with high expression. Corresponding values for CD163 were 81% and 64%, respectively. Both high CD68 and high CD163 expression correlated with poorer event-free survival. The 10-year event-free survival for cases with high and low CD68 expression was 61% and 43%, respectively. The corresponding values for patients with high and low CD163 expression were 62% and 42% (Figure 2).

At the multivariate level, over-expression of CD68, but not of CD163, was significantly related to overall survival (Table 3). None of the macrophage stains analyzed correlated significantly with event-free survival in multivariate analysis. When the analysis was conducted only on the cases of nodular sclerosis cHL, high CD68 count was found to have a similar adverse impact on both overall and event-free survival (P=0.001 and P=0.4, respectively). Both at univariate and multivariate levels age 45 years or more was a strong predictor of poorer overall and event-free survival. However, older age was not found to correlate with an increased occurrence of intratumoral macrophages.

Table 2. Univariate analysis of overall survival (OS) and event-free survival (EFS) for patients with cHL.

		Overall Survival				Event-free Survival			
Characteristic	N.	10-Year OS (%)	HR	95 % CI	P	10-Year EFS (%)	HR	95 % CI	P
Age, years									
<45	181	89.3	1	ref	<0.001	64.3	1	ref	0.001
≥45	107	55.9	5.63	3.20-9.87		44.2	1.86	1.27-2.71	
Sex									
Female	132	82.5	1	ref	0.05	59.9	1	ref	ns
Male	156	72.0	1.69	0.99-2.88		54.5	1.15	0.79-1.68	
Ann Arbor Stage									
I-II	192	79.1	1	ref	ns	62.1	1	ref	0.01
III-IV	96	72.7	1.52	0.90-2.54		47.4	1.63	1.12-2.40	
B symptoms									
No	150	83.9	1	ref	0.005	62.9	1	ref	0.04
Yes	138	69.9	2.13	1.25-3.64		51.1	1.49	1.02-2.17	
Internation Prognostic Score									
≤2	168	85.5	1	ref	<0.001	62.5	1	ref	0.01
>2	84	56.0	3.70	2.13-6.43		44.1	1.70	1.13-2.55	
Bulky disease (>10cm)									
No	193	79.0	1	ref	ns	58.1	1	ref	ns
Yes	78	70.7	1.60	0.92-2.78		53.4	1.18	0.77-1.80	
Histological type									
NSI	167	79.2	1	ref	ns	59.6	1	ref	ns
NSII	70	74.5	1.28	0.68-2.40		57.9	1.03	0.64-1.65	
MC	47	75.5	1.39	0.71-2.71		49.6	1.34	0.82-2.19	
cHL, NOS	4	50.0	3.21	0.77-13.4		25.0	2.42	0.76-7.72	
EBV status									
Negative	193	76.5	1	ref	ns	57.7	1	ref	ns
Positive	95	78.0	1.11	0.65-1.89		55.7	0.98	0.66-1.46	
CD68									
Low (<7.8%)	195	81.7	1	ref	0.002	61.0	1	ref	0.03
High (≥7.8%)	67	61.0	2.45	1.40-4.28		41.8	1.62	1.06-2.49	
CD163									
Low (<21.1%)	210	81.3	1	ref	0.03	62.3	1	ref	0.04
High (≥21.1%)	69	64.2	1.82	1.06-3.13		42.1	1.53	1.01-2.31	

NSI and NSII, nodular sclerosis type I and II, respectively; MC: mixed cellularity; cHL NOS: classical Hodgkin's lymphoma not otherwise specified; EBV: Epstein-Barr virus.

Discussion

Macrophages are often found to infiltrate malignant lesions in both solid tumors[21,22] and hematologic cancers. The existence of at least two different subtypes of tumor-infiltrating macrophages has been hypothesized. These two subtypes have different phenotypes and it has been suggested that they exert opposing immunological functions, i.e. pro-inflammatory *versus* immunosuppressive.[23] The pro-inflammatory functions are attributable to the classical (M1) macrophages that are typically induced by interferon-γ or bacterial products, and are capable of antigen presentation through the activation of a type 1 cellular immune response.[24] They are generally considered to be effector cells, capable of killing micro-organisms and tumor cells. On the other hand, the alternatively activated (M2) macrophages have poor antigen-presenting capacity, but possess immunosuppressive, angiogenesis-enhancing and, possibly, tumor-promoting activity.[24]

However, it has been argued that the M1/M2 model is oversimplifying a much more complex macrophage biology and another nomenclature based on these cells' involvement in host defense, wound healing and immune regulation has been proposed.[25] There is increasing experimental evidence that macrophages promote cancer initiation by facilitating an inflammatory environment that is mutagenic and growth-enhancing and that they support tumor progression by stimulating angiogenesis, tumor cell migration, and suppressing antitumor immunity.[26] In contrast to the binary M1/M2 model, a definition of tumor-associated macrophages composed of several distinct populations with greater overall similarity to macrophages involved in developmental processes has now been suggested.[26]

In order to broadly cover the macrophage population present in cHL lesions, we used antibodies directed against CD68 and CD163, both of which are considered to be macrophage-associated antigens. CD68 is widely used as a pan-macrophage marker and has been shown to correlate with an adverse prognosis in a variety of malignancies, e.g. follicular lymphoma.[27] CD163 is a glycoprotein belonging to the cysteine-rich scavenger receptor superfamily[28] and has been suggested to be a narrower macrophage marker than CD68.[29] It is up-regulated in macrophages stimulated by interleukin-10 and dexamethasone.[30] A high number of CD163-positive tumor-infiltrating macrophages has been shown to have an unfavorable prognostic impact in a variety of malignancies, e.g. pancreatic cancer,[31] malignant melanoma[32] and, more recently, also lymphoma.[33] In our material, we found the expression of CD163 was higher than that of CD68. This

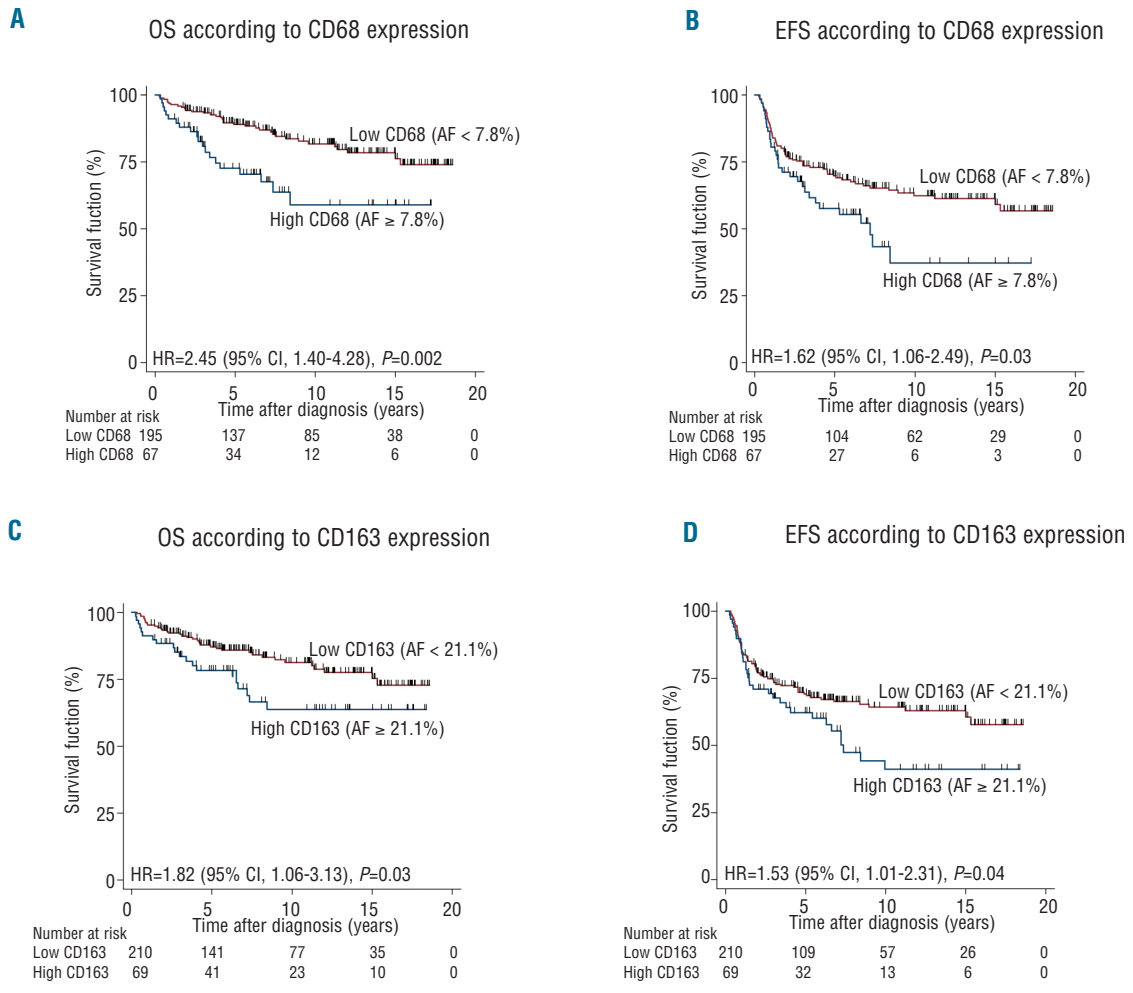

Figure 2. Overall survival (OS) and event-free survival (EFS) according to CD68 and CD163 expression. HR, hazard ratio; AF, area fraction.

finding is in keeping with observations in previous studies[32,34] in which higher counts of tumor-infiltrating CD163 compared with CD68 macrophages were found in other malignancies such as malignant melanoma and leiomyosarcomas.

The results of our study support the prognostic importance of tumor-infiltrating macrophages in cHL. At the univariate level, high expression of both CD68 and CD163 in the tumor microenvironment was significantly associated with adverse prognosis. However, after a multivariate analysis, CD68 retained a significant influence on overall survival, while CD163 did not. A possible explanation could be that CD68 covers a broader range of cells involved in the immune response and thereby better reflects the cellular microenvironment than the more lineage-specific CD163 staining. Furthermore, we observed that the adverse impact of a high CD68 count persisted also when cases with nodular sclerosis histology were analyzed separately. This finding suggests that the predictive value of CD68 is independent of histological subtype. Interestingly, both macrophage-associated markers were found to be strongly correlated with EBV positivity. This held true even after removal of the EBV-associated mixed

Table 3. Multivariate analysis, including CD68 expression.

	Overall Survival			Event-free Survival		
Characteristic	HR	95% CI	P	HR	95% CI	P
High CD68	2.35	1.32-4.19	**0.004**	1.45	0.94-2.25	0.10
Age ≥ 45 yrs	6.03	3.30-10.0	**<0.001**	1.75	1.17-2.62	**0.006**
Male sex	1.43	0.80-2.55	ns	‡		
Stage III-IV	‡			1.43	0.94-2.18	ns
B symptoms	2.31	1.31-4.06	**0.004**	1.35	0.88-2.06	ns

Multivariate analysis including CD163 expression.

	Overall Survival			Event-free Survival		
Characteristic	HR	95% CI	P	HR	95% CI	P
High CD163	1.58	0.92-2.73	0.10	1.30	0.85-1.98	0.23
Age ≥ 45 yrs	6.00	3.36-10.7	**<0.001**	1.84	1.24-2.71	**0.002**
Male sex	1.39	0.80-2.42	ns	‡		
Stage III-IV	‡			1.39	0.92-2.08	0.06
B symptoms	2.10	1.21-3.65	**0.008**	1.45	0.96-2.19	ns

Factors with P<0.1 in univariate analysis were fitted in the multivariate model. Bold font indicates statistical significance. EFS: event-free survival; Ref: reference; HR: hazard ratio; ‡:Not fitted in the model.

cellularity subtype from the analysis. Consistent with this finding, a recent molecular profiling study in cHL showed that the genes for CD68 and CD163 belong to a large group of genes found to be up-regulated in EBV-positive cases.[35] So far, only a few studies have found specific changes in the tumor microenvironment related to the expression of EBV in Hodgkin and Reed-Sternberg cells. IP-10, a chemo-attractant for activated T-cells, has been shown to be up-regulated in the presence of EBV[36] and the recruitment of FoxP3-positive T-regulatory cells has been shown to be facilitated by CCL20 in EBV-positive cases of HL.[37] The biological mechanisms underlying the relationship between a high number of macrophages and EBV positivity in HL remain to be established.

In terms of methodology, our study quantified immunohistochemical stains using a computer assisted stereology system. Whether this method will find a place as a routine diagnostic tool remains to be seen. However, its labor-intensiveness will probably limit its use to the domain of clinical translational research, whereas semi-quantitative methods are more likely to be broadly applied.

Very recently, Steidl *et al.* published a study on cHL which also suggests that lymphoma-associated macrophages are of prognostic significance in this disease.[8] Using microarrays, they identified a gene-expression signature of tumor-associated macrophages that correlated with shortened survival. Against the background of this gene-expression profile, they evaluated the number of intra-tumoral macrophages in an independent cohort of 166 cHL patients using CD68 immunostains. In that study CD68 was not independently associated with progression-free survival, but an association between the CD68-positive macrophage count and disease-specific survival at both univariate and multivariate levels was found.[8] Despite some methodological differences in quantifying CD68-positive macrophages, the findings of Steidl *et al.* are in line with the results of our analysis. Hence, the present study provides an important set of data validating the role of CD68 immunohistochemistry as a biomarker in cHL, whose clinical usefulness should be tested prospectively.

In summary, quantification of CD68 and CD163 expression in the non-neoplastic cellular microenvironment of cHL lesions suggests that the number of tumor-infiltrating macrophages is a novel and important prognostic factor in this lymphoma. We found the prognostic impact of CD68 to be more marked than that of CD163. Both antigens were associated with tumor positivity for EBV. In cHL, as in other lymphoma subtypes, there is now specific evidence that the microenvironment plays an important role in the biology and prognosis of the disease. This observation may contribute to an improved understanding of the pathogenesis of cHL and indicate new biomarkers for outcome prediction and novel therapeutic targeting.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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