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Original Article

Significance of CD68 Tumor Infiltrating Macrophages in Pediatric Classical Hodgkin Lymphoma

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Abstract

Background: Hodgkin Lymphoma (HL) represents a disease of successful outcome due to advances in modern medicine. A significant percentage of patients respond very well to treatment, achieving relapse free survival. However, despite appropriate treatments as many as 20% of these patients die of this disease. Risk stratification allows therapy to be selected based on specific prognostic indicators.

Procedure: A retrospective cohort study containing 25 pediatric classical HL cases were evaluated from the files of Miami Children's Hospital Department of Pathology. The study aimed to analyze tumor-associated macrophages via CD68 immunohistochemistry in tissue obtained at the time of diagnosis. It studied the prognostic value of CD68+ histiocytes against a patient's response to treatment and survival rates, as a possible correlation of this biomarker with outcomes.

Results: Higher levels of CD68+ macrophages was strongly correlated with a significant probability of relapsing from complete response (P=0.005), along with a greater likelihood of death from lymphoma (P=0.024). Furthermore, survival analysis demonstrated a decreased progression-free survival (P=0.001) and disease specific survival (P=0.023) when the microenvironment showed elevation of these macrophages.

Conclusions: The presence of an increased expression of CD68+ macrophages was found to be associated with a worse prognosis in a pediatric patient with HL. This study, establishes a new use for CD68, as a reliable immunohistochemical marker in pediatric patients with equivalent predictor outcomes as those reported in adult cases. This biomarker helps to identify those pediatric patients at higher risk of treatment failure, and thus provide the basis for individualized patient treatment.

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Original Article

Keywords

CD68; Tumor Associate Macrophages; Pediatric Hodgkin Lymphoma; Prognosis

Introduction

Hodgkin Lymphoma (HL) is set apart from other lymphomas as it is consider to be a successful example of contemporary treatment strategies. In fact, the vast majority of patients are able to achieve relapse-free survival with standardized treatment protocols. However, it is still reported that as many as 20% of patients died from relapse or tumor progression [1-3]. Current prognostic models such as the International Prognostic score, nor the individual clinical components, can accurately identify high risk patients and/or predict outcome to standard therapy. It has been suggested that the tumor microenvironment has a significant role in the pathogenesis of the disease [4]. Tissue microarray and gene expression studies have become the latest trend in research, by investigating the microenvironment components of the tumor in classical HL, and have proven to be useful when trying to explain the behavior of the disease [5-12].

A previous study suggested that the presence of CD68+ histiocytes in patients with HL might be associated with a decreased survival [4]; however, there was no clear discrimination between adult and pediatric patients. Devilard *et al.* identified a macrophage gene profile in classical HL that was associated with unfavorable outcomes [10]. Once again, the study included only adult patients. HL is a disease with a significant peak incidence in the pediatric population. It remains uncertain if the presence of CD68+ histiocytes correlates to a similar behavior as that reported with adult patients, or if the pediatric cases follow a different trend. A recent study, analyzed the microenvironment of 100 pediatric classical HL, and reported that CD68+ cells did not display an effect on outcome [13]. However, to date CD68 has not been utilized as marker for treatment stratification. The purpose of this study is to determine if the presence of CD68+ histiocytes in the microenvironment of pediatric HL can further refine post-induction risk stratification.

Methods

Research Design:

Our group searched the pathology database at Miami Children's Hospital for cases of classical HL diagnosed between the years 2000 and 2010. In order to meet criteria, selected cases needed to include: a primary diagnosis of classic Hodgkin Lymphoma after central review, a representative lymph-node tissue, and have received exclusively standard therapy with doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD). Cases were selected based on available blocks and adequate tissue. Suitable diagnostic material was found for 25 patients and most Hodgkin Lymphomas were the nodular sclerosis subtype. We selected formalin fixed, paraffin-embedded lymph-node specimens obtained at the time of diagnosis. Immunohistochemical analysis of tumor-associated macrophages was performed using CD68 stains on representative tissue blocks from each case. Other immunohistochemical stains were used to substantiate a diagnosis of classical HL, which included CD15, CD30, CD45, and CD20.

The immunohistochemical staining process for CD68 was performed as published by others and is summarized as follows: sections were deparaffinized in xylen, rehydrated through graded alcohols and rinsed in distilled water. Heat induced epitope retrieval was performed in a microwave oven using 0.01 M Citrate buffer pH 6.0 for 5 and 20 minutes respectively. Sections were allowed to cool for 30 minutes and then rinsed in distilled water. Antibody incubations and detection were carried out at 37°C on a NEXes instrument (Ventana Medical Systems Tucson, AZ) using Ventana's reagent buffer and detection kits unless otherwise noted. Primary antibody was detected using a biotinylated goat anti-mouse followed by application of streptavidin-horseradish-peroxidase conjugate. The complex was visualized with 3,3 diaminobenzidene and enhanced with copper sulfate. Slides were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted with permanent media. Appropriate positive and negative controls were included with the study sections [14].

Stains were compared with corresponding H&E stain sections. The level of CD68 staining was analyzed by three of the investigators, and graded with respect to the relative percentage of tumor associated macrophages. The few cases with discrepancies were reviewed until agreement was reached. Following the parameters reported by Steidl *et al.* immuno-histochemical scoring for CD68 ranged from Grade I (1) to Grade III (3) with higher scores indicating a greater presence of positives cells [5]. Scoring less than 5% represented Grade I, 5-25% was described as Grade II, and more than 25% correlated with Grade III. A representative sample of CD68+ staining is shown in **Fig-1**.

The association with CD68+ histiocytes and the patient's response to treatment and survival was retrospectively analyzed by reviewing the patient's medical records and further correlating this biomarker with the patient outcome. For comparison analyses, outcome was considered "good" if the patient was disease-free (remission), as opposed to "poor" if the disease recurred (relapse) or patient expired from lymphoma (death).

Clinical variables taken into account in the study included: age, gender, morphology of classical HL and its histologic variations (i.e. Nodular sclerosis, Mixed Cellularity, Lymphocyte Rich, Lymphocyte Depleted), date of diagnosis, and follow-up period. Other variables examined were the presence of systemic symptoms (B symptoms), EBV status (by serology or EBER), clinical stage at the time of diagnosis, treatment (ABVD), and response to treatment: remission (confirmed by PET-CT), relapse, or death from HL.

Statistical Methods:

IBMSPSS program version 19.0 was used for the statistical analyses. Bivariate analysis of grade II (5-25% CD68+) and Grade III (More than 25% CD68+) patients was performed to explore the association between the presence of CD68 and other descriptive statistics as well as its correlation with different outcomes (remission, relapsed, or death from disease). Two sided P-values of less than 0.05 were considered to indicate statistically significance.

Kaplan-Meier method was employed for the survival analyses and the creation of pertinent survival tables. The Time-to-event analyses included two end-points: Disease specific survival (DSS), which we defined as the time (in months) from initial diagnosis to death from HL; and progression free survival (PFS) that is the interval (in months) from initial diagnosis to relapse after complete response.

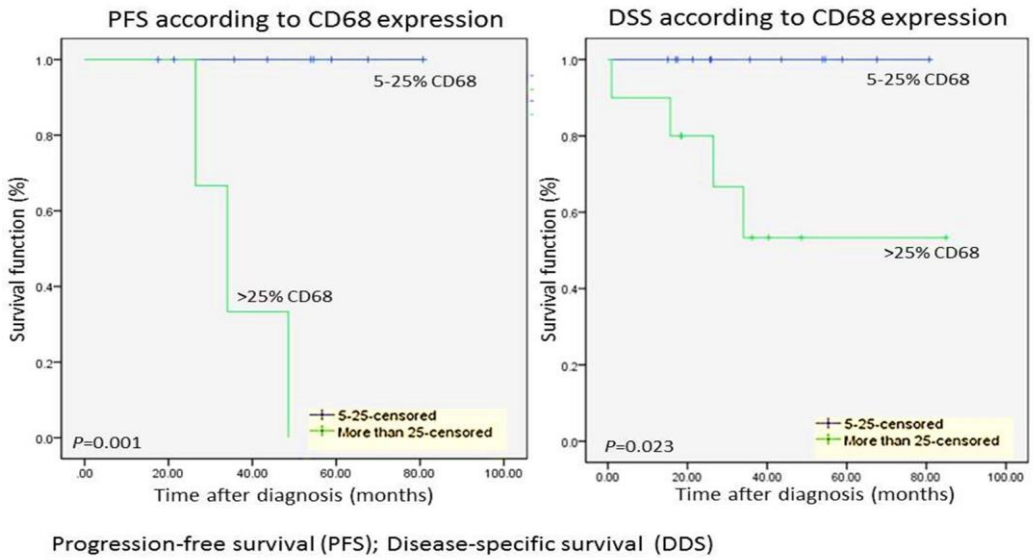

Fig-1:

Survival according to the number of infiltrating CD68+ macrophages in pre-treatment lymph node biopsy specimens

Results

Clinical-Pathological features:

The population's clinical and histo-pathological characteristics, along with the correlation of each variable to the presence of CD68 are summarized in **Table-1**.

The analysis included 25 patients. The initial population of patients had a median age of 16 years with a range of 5–21 years, a mean of 15.6 and a SD of 3.34. Approximately half of the population (52.0%) was male. The most common HL subtype was nodular sclerosis with twenty one patients (84%). Other subtypes included mixed cellularity with three patients (12%), and lymphocyte rich with one case (4.0 %). No patients had lymphocyte depleted morphology.

Almost half of the population (47.8%) had disseminated disease (Ann Arbor stage III-IV). Eighteen patients (72%) experienced B symptoms. The majority (92%) of the patients studied were treated with the initial standard chemotherapy treatment for HL: doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD). There were two patients (8%), who were lost to follow-up: one due to non-lymphoma associated death (car accident) and the other who return to his native country to complete treatment. Approximately half of the patients (52.0%) were positive for Epstein Barr virus based on serology or EBER studies.

Table-1: Demographic, clinical, and pathological parameters according to CD68 expression (N=25)

Characteristics at diagnosis	N (%)	CD 68 Level N (%)		Fisher's Exact 2-tail
		Grade II[a]	Grade III[b]	
Median Age (Range) - yr	16 (5-21)			
Sex				**0.015**
Male	13 (52.0)	4 (30.8)	9 (69.2)	
Histologic Subtype				0.455
Nodular Sclerosis	21 (84.0)	12 (51.7)	9 (48.3)	
Mixed Cellularity	3 (12.0)	2 (66.7)	1 (33.3)	
Lymphocyte Rich	1 (4.0)	0 (0)	1 (100)	
Lymphocyte depleted	0 (0)	0 (0)	0 (0)	
Ann Arbor Stages				**0.012**
I and II	12 (52.2)	10 (83.3)	2 (16.7)	
III and IV	11 (47.8)	3 (27.3)	8 (72.7)	
Unknown	2 (8.0)	0 (0)	0 (0)	
B Symptoms				1
History of B Symptoms	18 (72.0)	10 (55.6)	8 (44.4)	
No history of B symptoms	7 (28.0)	4 (57.1)	3 (42.9)	
Primary Chemotherapeutic Treatment				N/A
ABVD	23 (92.0)	12 (52.2)	11 (47.8)	
Unknown	2 (8.0)	0 (0)	0 (0)	
EBV				0.414
EBV Positive	13 (52.0)	8 (61.5)	5 (38.5)	
EBV negative	10 (40.0)	4 (40.0)	6 (60.0)	
Unknown	2 (8.0)	0 (0)	0	

[a)] Indicates CD 68 level of 5-25% , [b)] Indicates CD68 level of >25%

Association of CD68 Expression and Patient's Outcome:

In order to study the impact of CD68 expression in tumor microenvironment, an immunohistochemical score was implemented emulating the previously reported one by Steidl *et al.* CD68 levels < 5% indicated Grade-I, 5-25% Grade-II, and > 25% Grade-III (**Fig-1**). None of cases were found to have less than 5% of CD68-positive macrophages in the tumor microenvironment. Therefore, all of our 25 cases represented either Grade II or Grade III category. Fourteen (56%) of the cases were classified as Grade-II, while eleven (44%) were recorded as Grade III. Specific outcome and grading score statistics were based in the 23 patients who completed follow up (**Table-2**).

Furthermore, possible confounding variables were also analyzed: An Arbor stage and sex. As for staging there was an association with CD68 level (P=0.012), yet none with outcome (DSS, P=0.056; PFS, P=0.127). A similar finding was noted with respect to sex, where the association with CD68 level was significant (P= 0.015), however no relationship with outcome (DSS, P= 0.590; PFS, P=0.236).

In total, twelve (52.2%) patients achieved complete remission (CR). From this group, nine (75%) patients did not relapse, and belonged to the Grade II CD68 category. Meanwhile, three patients (25%) did relapse after CR, and interestingly 100% of relapses had scores corresponding to Grade III category. The relationship of relapse with higher grading category was found to be highly statistically significant (P= 0.005). Likewise, from our cohort of patients, a total of four (17.4%) patients died of disease, who also belonged to the Grade III category (P= 0.024). From the relative risk analysis, we learned that those patients with lower counts of CD68 (Grade-II) were approximately 17 times less likely to relapse (RR=0.06 (0.004-0.767)) and approximately 11 times less likely to die (RR=0.09 (0.006-1.232)) than those with higher CD68 counts (Grade-III).These results support the hypothesis of a significant association between higher counts of CD68+ macrophages in the tumor microenvironment with poor outcomes.

Table-2: Association of CD68 level and Study Outcome

Outcome	CD68 level		RR	P-value
	Grade II[a] (N= 13)	Grade III[b] (N= 10)	(95% CI)	
Remission (N= 12)	9/13 (69.0)	3/10 (30.0)	2.31 (0.990-5.384)	**0.100**
Relapse* (N= 3)	0/9 (0.0)	3/3 (100.0)	0.06 (0.004-0.767)**	**0.005**
Death from HL (N= 4)	0/13 (0.0)	4/10 (40.0)	0.09 (0.006-1.232)**	**0.024**
Other *** (N= 7)	4/13 (31.0)	3/10 (30.0)		

[a)] Indicates CD68 level of 5-25% [b)] Indicates CD68 level of >25%. * Subgroup of patients: The frequencies for relapse are based on a total of 12 patients that achieved complete remission. ** To calculate relative risk avoiding the issue of empty cells 0.5 was added to all cells. *** Patients who were still alive with active disease, without being able to achieve remission at time of study.

Survival Analysis

Survival comparisons demonstrated that patients with an elevated count of CD68+ cells (Grade-III) had a shorten DSS mean of 36.4 months, whereas a mean of 48.2 months in the Grade-II category. Furthermore, the PFS mean of Grade-III was also decrease at 32.4 months, compared to a mean of 39.8 months of those patients with Grade-II (**Table-3**).

Survival analysis using Kaplan-Meir demonstrated a significant association between the number of CD68+ tumor infiltrating macrophages and shorted PFS (P=0.001). In addition, the expression of these

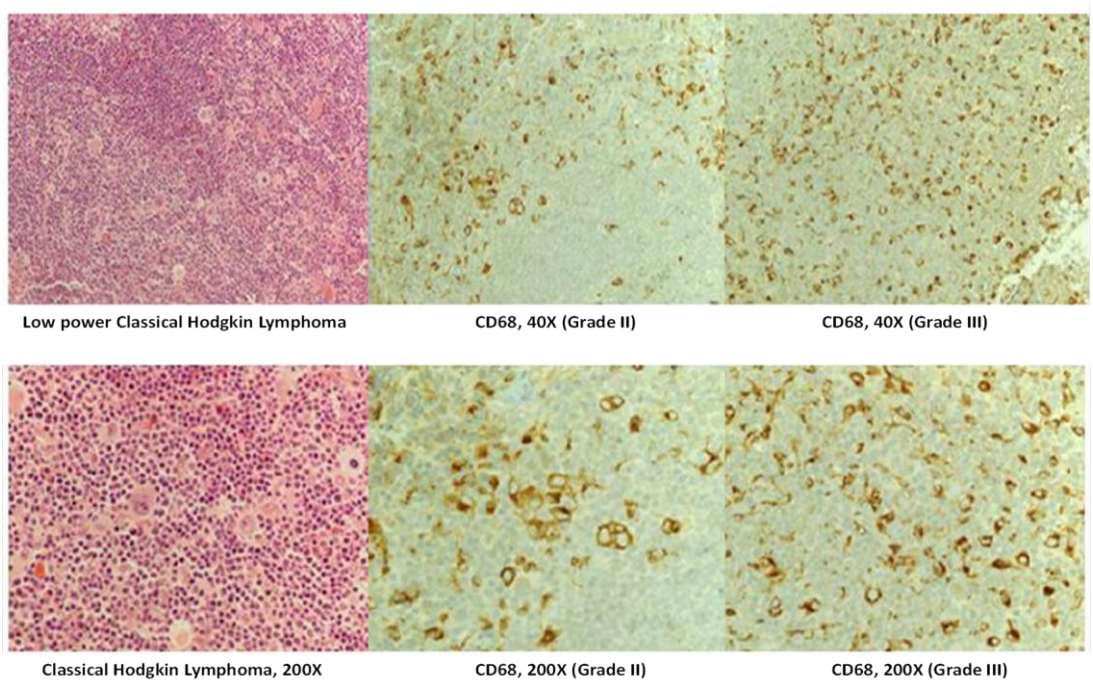

Fig-2: Representative immunohistochemical stains for CD68

Table-3: Survival Indicators of the Population

Population Survival Characteristics	CD68 Level		Total
	Grade II[a]	Grade III[b]	
Disease-Specific Survival (DSS)			
Mean DSS- months	48.2	36.4	45.2
Std. Deviation	20.8	11.2	19.1
Range DDS	17.5-80.7	26.5-48.6	17.5-80.7
Progression-Free Survival (PFS)			
Mean PFS- months	39.8	32.4	36.6
Std. Deviation	21.6	23.1	22.1
Range PFS	15.1-80.7	1.0-85.0	1.0-85.0

[a)] Indicates CD68 level of 5-25% [b)] Indicates CD68 level of 5-25%

macrophages in the microenvironment inversely associated with DSS (P=0.023) (**Fig-2**).

Discussion

It has been proposed that macrophages can stimulate the initiation of a neoplastic process as well as perpetuate it by facilitating inflammation, angiogenesis, tumor cell migration, and immunity suppression [19]. All these processes support and promote mutagenesis, abnormal cell growth, and thus tumor progression. In fact, these cells have been found infiltrating the tumor microenvironments of both solid and hematologic tumors [5-6, 20].

CD68 (Kp-1) is a glycoprotein considered to be a macrophage-associated antigen. It is widely used as a monocyte/macrophage marker. Tumor- associated macrophages have been described to play a key role in the development of Classical HL. The degree of CD68+ macrophages in the tumor microenvironment of classical HL was recently revealed as a potential determinant of risk stratification in these patients. Through bicomputational tools, Steidl *et al.* identified a gene signature of tumor stromal macrophages that

was significantly associated with poor clinical outcomes [5]. An increased number of CD68 was found to be a strong prognosticator for primary treatment failure, leading to decreased PFS and DSS. However, this paramount study was only based on adult patients, and the impact of this biomarker in pediatric cases remains uncertain, which was what prompted our study.

The results of our study support the prognostic importance of tumor-associated macrophages in classical HL. At the bivariate level, high expression of CD68 strongly correlated with a significant probability of relapse after CR (P=0.005), and with a greater likelihood of death from lymphoma (P=0.024). Survival analysis are similar to those reported in adults, by demonstrating a decreased PFS (P=0.001) and DSS (P=0.023) in patients with higher levels of CD68+ macrophages in the tumor microenvironment.

Contradictory results have been reported in the adult literature. Harris *et al.* examined 44 cases of classical HL utilizing the immunohistochemical stains for CD68 and CD163. Here, there was no relationship between these biomarkers and recurrence of disease. Nevertheless, their statistical analysis was performed using probability test as opposed to survival analysis [14]. Their findings support those of another previous study, which also found no link between the percentage of positive cells and survival in 265 cases of uniformly treated classical HL [16]. However, Azambuja, *et at*. does report that higher levels of CD68 and CD163 expression were related with the presence of Epstein–Barr virus positive Hodgkin tumor cells. In our study, similar result were noted as approximately half of the patients (52.0%) were positive for EBV. Of this group, there was a significant association with increased expression of CD68+ cells (P=0.011), along with greater likelihood of death and relapse (P=0.026 and P=0.030 respectively). These findings have been previously suggested by other authors who have correlated tumor-infiltrated macrophages with EBV status and adverse prognosis in classical HL. [11, 13-16].

Based on literature research at the time our study was conducted, there was only one previous investigation that has studied the association of various macrophages with the prognosis in a pediatric population [13]. Surprisingly enough, their results differed from ours as well as the pioneer study on adults by Steild *et al.* where an increased number of tumor-associated CD68+ macrophages was strongly associated with shortened survival rates [5]. Barros *et al.* evaluated the microenvironment of 100 pediatric classical HL, with CD68 and CD163 stains. Interestingly, only elevated percentage of CD163+ cells was associated with worse EFS, while CD68+ cells did not display an effect on outcome. In addition, EBV+ cases exhibited higher numbers of both biomarkers supporting our findings regarding this relationship. Overall, their results suggest that the macrophage composition in pediatric HL is different from that of adults, while ours propose that the composition is indeed very similar.

Our study contributes with evidence that the tumor microenvironment has a definitive role in the pathogenesis and prognosis of classical HL. Our results are supporting for the prognostic value of CD68+ cells in classical HL, and now provide indication of this biomarker in pediatric patients. CD68 is a simple and relatively inexpensive stain that can be easily perform in every case of HL at the time of diagnosis. We present CD68 as single reliable immunohistochemical marker that if implemented can serve as a prognosticator for therapeutic modality. It is an affordable technique, which may help in the stratification of patients that will have a satisfactory response to standard therapies from those at higher risk of treatment failure, and thus establish the basis for individualized patient treatment. Lastly, it would be of interest to study CD68 expression in other lymphomas such as Non-Classical Hodgkin lymphoma and variants of Non Hodgkin lymphoma.

Like any other study ours has several limitations. We are aware our study has a small cohort of cases, and our sample size could be a limiting factor in representing the overall population of pediatric HL. A larger and prospective study is needed to corroborate our findings. We plan on opening a prospective multi-institutional study looking at CD68 expression in

pediatric patients with Hodgkin lymphoma.

Conflict of Interests

The authors declare no conflict of interest.

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