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Flow cytometry for assessment of the tumor microenvironment in pediatric Hodgkin lymphoma

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Abstract

Background: The role of flow cytometry in diagnosis and management of Hodgkin lymphoma (HL) remains limited. As knowledge emerges of the tumor microenvironment in this disease, various methods are being evaluated in its study. This study examines the microenvironment using flow cytometry to assess differences between subtypes and clinicopathologic correlates.

Procedure: A retrospective cross-sectional study was performed analyzing the tumor immunophenotype, by flow cytometry, for 31 children with classical HL. Correlation was made with patient information, including outcome.

Results: The makeup of the tumor microenvironment varies across subtype of HL, with T cells predominating in nodular sclerosis (NS), and similar proportions of B and T cells in mixed cellularity (MC). CD4 cells predominate in NS, whereas CD8 more so in MC subtype. The rate of continuous complete remission is significantly higher in the MC subgroup. Last, the proportion of HLA-DR/CD38 copositive lymphocytes was an independent prognostic factor for relapse/refractoriness.

Conclusions: This study indicates that flow cytometry can be used to examine the tumor microenvironment in HL and that percentage of HLA-DR/CD38 copositive lymphocytes may be a biomarker for relapse and refractoriness in pediatric HL.

Keywords

biomarker; flow cytometry; Hodgkin lymphoma; immunophenotype; tumor microenvironment

1 | INTRODUCTION

With combined modality treatment, Hodgkin lymphoma (HL) in children has an event-free survival at 5 years of about 80%. Despite this success, those with relapsed or refractory disease have poor outcomes. Additionally, currently, at diagnosis, only stage predicts

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

outcome and determines those who receive intensified therapy. Current trials are attempting to use individualized risk-adapted therapy in order to maximize treatment response and minimize long-term sequelae. To this end, biomarkers of disease behavior have become increasingly important.

Few studies have examined HL histologic subtype and its relationship with outcome. These have found an association between mixed cellularity (MC) subtype and superior outcome,^{1,2} and, conversely, linked stage IV disease^{3,4} and nodular sclerosis (NS) 2 subtype⁴ with increased risk of relapse. In developing countries, EBV-associated HL is more common, tends to occur in younger patients, and is more often MC subtype.^{5,6}

Recent studies have linked tumor associated macrophages with outcome.^{7–13} Higher numbers of CD68⁺ macrophages within tumor tissues were associated with shorter progression-free survival.^{7,8,10,11,14} CD163 and CSF1R expression have also been linked to survival.¹³ Emerging data also highlights the potential relationship between the number of regulatory T-cells and outcome in adults.¹⁵ Similar data in children are limited and contradictory^{12,16} and the biologic features shared by patients with relapsed or refractory disease are largely unidentified.

Flow cytometry is useful in diagnosing non-Hodgkin lymphoma, but its use has been limited in HL. Few studies have found patterns in the immunohistochemical profile^{17–25} that may be specific to HL. The immunophenotype of EBV-positive tumors has also been shown to be modulated by the virus.^{12,24,26} However, no single immunophenotype has been associated with either histologic subtypes or prognosis of HL.

Understanding the relationship between the microenvironment and the behavior of HL may allow us to identify biomarkers for disease. Additionally, flow cytometry may help diagnose HL. Here, we describe our analysis of tumor tissue by flow cytometry, in which we found that specific aspects of the microenvironment were associated with clinical characteristics and outcome.

2 | MATERIALS AND METHODS

The study was approved by the Wayne State University Human Investigation Committee. We reviewed records from Children's Hospital of Michigan Pediatric Hematology/Oncology for children initially diagnosed with HL from 1997 to 2009, and for whom flow cytometric analysis of an involved lymph node was available. All diagnoses were made by the Children's Hospital of Michigan Department of Pathology. Patient data including age, sex, HL histologic subtype, laboratory investigations, treatment, and outcome were collected.

3 | FLOW CYTOMETRY

Lymph node biopsy specimens were mechanically dissociated to obtain mononuclear cells and residual red cells were removed by ammonium chloride lysis. The resulting mononuclear suspension cells were resuspended in PBS plus 30% adult bovine serum to block nonspecific antibody reaction, and stained with direct conjugated monoclonal antibodies in three-color flow cytometry using CD45/SS as a primary gate for the malignant

target population. The monoclonal antibodies include isotype controls, CD3, CD4, CD8, CD5, CD7, CD2, HLA-DR, CD19, CD20, CD22, CD10, CD40, CD11b, CD13, CD14, CD34, CD33, CD41, CD61, CD16, CD56, CD57, CD15, CD38, CD1a, CD36 (Beckman-Coulter; Miami, FL), sIg, and Kappa and Lambda light chains (Invitrogen, Carlsbad, CA). Cells were stained in the dark for 30 min at room temperature with agitation, washed with 1 ml cold PBS, and resuspended in 0.5 ml PBS plus fixative (PBS plus 0.5% formaldehyde). Acquisition was performed on a Coulter XL Flow Cytometer (Coulter Corp, Miami, FL) equipped with a 488 nm Argon laser. Results were analyzed using EXPO-32 software.

4 | STATISTICAL METHODS

Data were analyzed with Student's *t*-tests, nonparametric Mann–Whitney U and Kruskal–Wallis tests, and Pearson's correlation coefficient. Logistic regression models were used for both univariate and multivariate analyses. Receiver operating characteristics (ROC) curves were created using outcome of relapsed/refractory or continuous complete remission (CCR) assessed at 2 years from completion of treatment to assess best cut-off values for HLA-DR/CD38 copositive lymphocyte percentage. Cross-validation was performed using the “leave one out” method. Survival was analyzed with the Kaplan–Meier method. Cox proportional hazard regression was used for both univariate and multivariate analysis. Data were analyzed with the SAS 9.3 statistical software program (Cary, NC).

5 | RESULTS

There were 31 qualifying children; one case of nodular lymphocyte predominant subtype was excluded, and the remaining 30 cases of classical HL were analyzed. The histologic subtype was NS in 21, median age 14 years (17 female), mixed cellular (MC) in six, median age 11 years (1 female), and undetermined in three (Table 1). All patients were treated on regimens based on risk classification, regardless of histologic subtype. While T cells predominated in the NS samples, the infiltrate in the MC samples was balanced between T and B cells (Table 2). The mean proportion of CD4-positive T lymphocytes was significantly higher in the NS subtype and, conversely, the mean proportion of CD8-positive cells was significantly higher in the MC subtype. Consistently, the CD4/CD8 ratio was significantly higher in NS. All six MC tumors were in situ EBV-positive, compared to only 24% (5 of 21) of NS tumors ($P = 0.008$). Further analysis of NS cases by in situ EBV status revealed that the CD4/CD8 ratio was intermediate to those of MC group and EBV-negative NS cases (5.5).

The mean white blood cell (WBC) counts and absolute lymphocyte (ALC) counts in the peripheral blood at the time of diagnosis were significantly higher in children with NS than in those with MC (Table 2). There was a positive correlation between WBC and CD4/CD8 ratio and a negative one between WBC and HLA-DR/CD38 copositive lymphocyte proportion ($r = 0.7$, $P < 0.0001$ and $r = -0.51$ and $P = 0.004$, respectively) both in the entire population as well as within the NS subgroup ($r = 0.65$, $P = 0.002$ and $r = -0.49$, $P = 0.015$, respectively). There was no correlation between WBC and CD3 or CD19 proportions in tumor tissue.

The proportion of patients with primary CCR was significantly higher in the MC group ($P = 0.009$). Higher hemoglobin concentrations at diagnosis were associated with primary CCR ($P = 0.07$). Higher HLA-DR/CD38 copositive lymphocyte percentage was also associated with primary CCR status ($P = 0.002$) in the entire study group and when analyzed for the NS group ($P = 0.003$). There was no significant difference in HLA-DR/CD38 copositive lymphocyte proportion by subtype. Furthermore, HLA-DR/CD38 copositive lymphocyte percentage negatively correlated with the CD4/CD8 ratio ($r = -0.59$; $P = 0.004$) in the whole group and in the NS group ($r = -0.62$, $P = 0.01$).

Logistic regression analysis, accounting for age, gender, subtype, and stage, indicated that HLA-DR/CD38 copositive lymphocyte percentage predicted relapse/refractoriness, in a protective manner, in all children (OR, 0.91; 95% CI, 0.85–0.98; $P = 0.01$) and in the NS subgroup (OR, 0.89; 95% CI, 0.81–0.98, $P = 0.013$). The odds ratio of relapse was 0.63 (95% CI, 0.43–0.9) for every 5% increase in HLA-DR/CD38 copositive lymphocytes, that is, a 60% decrease in risk of relapse for every 5% increase in the percentage.

The area under the ROC curve for predicting relapse or refractoriness using HLA-DR/CD38 copositive lymphocyte percentage cut-offs was 0.83 (95% CI, 0.67–1; $P < 0.001$; Figure 1), indicating that this marker could potentially predict patients who will have refractory or relapsed disease. This was supported by cross-validation.

The 5-year event-free survival for all children was 69%. Survival analysis showed that the event-free survival was lower for those with NS subtype, which was expected given that there were no relapses in patients with MC subtype. However, event-free survival differed significantly among four groups based on the percentage of lymphocytes copositive for HLA-DR/38 ($P = 0.017$; Figure 2). Moreover, the hazard ratio for relapse was 0.54 (95% CI, 0.33–0.87; $P = 0.011$) for every 10% increase in HLA-DR/CD38 copositive lymphocytes. In multivariate analysis, accounting for age, sex, stage and subtype, percentage of HLA-DR/CD38 copositive lymphocyte remained a predictive factor ($P = 0.011$).

6 | DISCUSSION

The interactions between Hodgkin Reed-Sternberg cells (HRS) (malignant) cells, which comprise <10% of tumor tissue, and their microenvironment are key to the understanding of the biologic behavior of HL. The microenvironment consists of T cells, most being CD4-positive (including T-helper and T-regulatory cells) and a smaller number CD8-positive, B cells, macrophages, eosinophils, plasma cells, mast cells, and neutrophils. The interactions between the malignant HRS cells and their surroundings support tumor cell proliferation and antiapoptotic features. These interactions and the resulting activation of these cells also contribute to the inflammatory response. The presence of EBV in a significant subgroup of cases also contributes to the inflammatory response, tumor proliferation, and immune escape by HRS cells via similar interactions with the microenvironment.²⁷ T_{reg} and HRS cells also exert inhibitory effects on cytotoxic T lymphocytes (CTLs), as well as lead to decreased CD4 positive T-cell activation. HRS cells overexpress surface molecules that promote peripheral tolerance, including PDL-1, which contributes to T-cell exhaustion.²⁸

Increasing knowledge of the interactions between HRS cells and their microenvironment has led to recent studies evaluating differences in the microenvironment and their correlation with clinical features and outcome. In this study, the predominance of T cells in NS subtype, as opposed to significantly higher numbers of B cells in MC subtype by comparison, is a novel finding and indicates important differences in the tumor microenvironment between the two subgroups. Higher numbers of CD8⁺ T-cells in MC tumor samples as evaluated by immunohistochemistry was also seen in a previous study.²⁴ We observed higher CD4/CD8 ratios in tissue, and higher WBC and ALC counts in the peripheral blood in NS cases, compared to MC cases. We also found a correlation between CD4/CD8 ratio and WBC, indicating a possible link between WBC and tumor biology, although many factors may influence WBC in this setting.

The significantly high incidence of EBV positivity in MC cases and the intermediate CD4/CD8 ratio seen in EBV positive NS cases suggest that EBV is involved in shaping the microenvironment in children with HL, expanding on the existing literature.^{12,24,26} Duffield et al. recently found increased infiltration of CD8⁺ T cells in EBV-positive classical HL samples, which we noted in the patients with MC subtype, all of which were EBV positive.²⁶ We did not note a significant difference, however, when comparing EBV-positive and EBV-negative NS cases. In another study, Barros et al. found that CD8⁺ T cells were increased in MC tumor samples by immunohistochemistry, as well as in EBV-positive cases, similar to our findings.²⁴ Interestingly, they observed a trend toward worse PFS for children with MC subtype in contrast to our observation.

Our findings also indicate a relationship between tumor microenvironment characteristics, as assessed by flow cytometry, and clinical outcomes. A recent study of adults with HL found a correlation between CD8-positive lymphocyte proportions and freedom from treatment failure,²⁹ although previous studies were contrasting.³⁰ We noted that children with MC subtype, whose tumor samples had higher percentages of CD8-positive T cells, had a better CCR rate, although CD8-positive lymphocyte proportion itself was not associated with outcome in this study. However, we did observe that patients whose tumors had a higher percentage of activated HLA-DR/CD38 copositive lymphocytes had higher CCR rates, indicating that this proportion may predict relapse or refractoriness. Additionally, the proportion of HLA-DR/CD38 copositive lymphocytes was negatively correlated with the CD4/CD8 ratio; however, we do not have data as to whether these lymphocytes are CD4 or CD8 positive.

The association between HLA-DR/CD38 copositive lymphocyte proportion and outcome has not previously been reported. The coexpression of HLA-DR and CD38 on lymphocytes is a marker of activation. We postulate that these findings together may be indicators for tumors that are less immune evasive, that is, less successfully inhibitive of cytotoxic T lymphocytes and activated CD4 positive T cells. This is further supported by findings in recent studies evaluating the highly successful checkpoint inhibitors, which have shown that PD-1 blockade acts by promoting T-cell activation.³¹ Biomarkers for response to checkpoint inhibitors in HL remain elusive,³² and this will be evaluated in future studies.

These findings may indicate a better outcome in children with HL who have higher numbers of activated CD8-positive T lymphocytes in the tumor microenvironment and could potentially lead to therapeutic approaches that can exploit these alterations.

7 | CONCLUSIONS

Flow cytometry can characterize the tumor microenvironment in HL, and the percentage of HLA-DR/CD38 copositive lymphocytes may be a biomarker for relapse and refractoriness in children with HL.

Abbreviations:

ALC	absolute lymphocyte
CCR	continuous complete remission
CTL	cytotoxic T lymphocyte
HL	Hodgkin lymphoma
HRS	Hodgkin Reed–Sternberg
MC	mixed cellularity
NS	nodular sclerosis
WBC	white blood cell

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FIGURE 1.
Receiver operating characteristic (ROC) curve for HLA-DR/CD38 co-positive lymphocyte percentage by flow cytometry in involved lymph node at diagnosis

FIGURE 2.

Kaplan–Meier curves of event-free survival according to percentage of HLA-DR/CD38 copositive lymphocytes

TABLE 1

Characteristics of 30 children with Hodgkin lymphoma by histologic subtype

Characteristics	Nodular sclerosis (n = 21)	Mixed cellularity (n = 6)	Undetermined (n = 3)
Sex, n (%)			
Male	6 (29)	5 (83)	2 (67)
Female	15 (71)	1 (17)	1 (33)
Age, median, years (range)	14 (10–17)	11 (3–15)	12 (12–13)
Tumor EBV status, n (%)			
Negative	16 (76)	–	2 (67)
Positive	5 (24)	6 (100)	1 (33)
Stage, n (%)			
I	–	2 (33)	–
II	9 (43)	2 (33)	1 (33)
III	3 (14)	1 (17)	1 (33)
IV	9 (43)	1 (17)	1 (33)
Treatment response, n (%)			
Relapse	10 (48)	–	–
Continuous complete remission	11 (52)	6 (100)	3 (100)

TABLE 2

Tumor lymphocyte makeup, WBC, and ALC of 27 children with Hodgkin lymphoma by histologic subtype

	Nodular sclerosis (n = 21)	Mixed cellularity (n = 6)	P-value
T-lymphocytes (%)	68.4	49.8	0.03
B-lymphocytes (%)	28.2	47.3	0.027
CD4-positive lymphocytes (%)	55	30.5	0.01
CD8-positive lymphocytes (%)	11.3	21.3	0.02
CD4/CD8 ratio	9.6	1.6	0.02
WBC count, mean (n/mm ³)	12	6.1	0.02
ALC, mean (n/mm ³)	3.5	1.5	0.048

WBC, white blood cell; ALC, absolute lymphocyte.