Retrospective Flow Cytometric Data Review of Classic Hodgkin Lymphoma Cases to Explore Disease Outcomes

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A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington

2022

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Program Authorized to Offer Degree:
Laboratory Medicine

University of Washington

Abstract

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Flow cytometry is an emerging method in detecting and diagnosing classic Hodgkin Lymphoma (cHL). There is increasing evidence that the tumor microenvironment (TME) plays a significant role in the prognosis and clinical outcome of the disease. In this study, ten different parameters were assessed and correlated to outcome. A retrospective cross-sectional study was performed by analyzing flow cytometric data collected from 62 patients at presentation who were diagnosed with cHL. Correlation was made from TME cellular composition to clinical outcome. The composition of the TME varied between patients who achieved complete remission compared to those who relapsed or whose disease had progressed. The T-cell rosetted fraction of Hodgkin-Reed Sternberg (HRS) cells showed a significant correlation to outcome. However, the %CD20 B-cells, %CD5 T-cells, %HRS cells, percentage of activated B-cells and T-cells characterized by CD71 expression, ratio of CD4+/CD8+ T-cells, %eosinophils, %neutrophils, and eosinophil/neutrophil ratio did not show significant correlation to outcome. This study shows that the fraction of T-cell rosetted Hodgkin-Reed Sternberg cells at presentation may be a biomarker in predicting outcome in cHL.

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ACKNOWLEDGMENTS

I would like to express my deepest gratitude to Dr. Jonathan Fromm and Dr. Kikkeri Naresh for their invaluable mentorship, patience, and feedback throughout the course of this project. This endeavor would not have been possible without their knowledge and expertise and I am grateful for the opportunity to have learned from them.

I am also thankful to Dr. Steve Polyak and Ms. Heather Eggleston, who have generously provided support, resources, and advice throughout the course of the program. Thanks should also be given to my cohort members and classmates for their moral support and late-night work sessions throughout a uniquely challenging journey.

Lastly, I'd like to recognize my family, especially my parents, who have kept me motivated and inspired with their unconditional support and guidance. Also, to my friends, for their constant encouragement. This process would have been a more challenging feat without them.

Chapter 1
INTRODUCTION

Classic Hodgkin Lymphoma (cHL) is a highly curable disease with current treatment schemes; however, approximately 20% of those diagnosed still succumb to the disease. Current prognostic models predict treatment outcome with imperfect accuracy and there has been minimal advancement in identifying new biomarkers for treatment or prognosis. Treatment is largely dictated by stage at presentation, but this can lead to overtreatment that may have adverse effects later in life, with some patients developing other cancers such lung cancer and thyroid cancer.[1]

A key characteristic and diagnostic criteria of cHL is the presence of binucleated Hodgkin-Reed-Sternberg (HRS) cells, which are frequently accompanied with by T-cells ringing around the HRS cells (T-cell rosetting). Unlike most other malignancies, the malignant HRS cells are outnumbered in the tumor microenvironment (TME) with T-cells being the predominate population.[2] The rosetting T-cells are theorized to act as a protective barrier against an immune response and provide survival signals to the HRS cell.[2, 3, 4, 5, 6] These HRS cells also secrete cytokines and chemokines that attract different T-cells that may also play a role in immune evasion. However, an increased number of regulatory T-cells (Treg) in the TME has been shown to indicate favorable prognosis due to the possible immunosuppressive capabilities on cytotoxic T-cells.[2]

Additionally, the TME of cHL is highly complex and is comprised of many immune cells including T-cells, B-cells, neutrophils, eosinophils, macrophages, and fibroblasts. The HRS cells rarely survive in vitro or in mouse models, indicating that the cells it recruits to the TME is important for its survival.[3, 4, 5] Previous studies have suggested that the composition – including percentage of B cells[6], and percentage activated T-cells[7], eosinophilia[8, 9], the

proportion of CD8 T-cells[10], and macrophages[11] – of the microenvironment is linked to prognosis; however, the data on these factors correlating to outcome is limited.

Flow cytometry (FC) is an emerging method of diagnosing cHL by identifying immunophenotypic signatures for cHL.[12, 13] By utilizing gating strategies of FC data, the cellular composition of the TME can be determined, further analyzed, and correlated to outcome. There has been increasing evidence that the TME can provide insight of prognosis or disease outcome based on the cellular composition. Understanding the interaction and composition of the TME may allow new biomarkers to be identified to predict prognosis or outcome.

In our retrospective study we focused on ten different cell populations at presentation of disease: %CD20+ B-cells, %CD5+ T-cells, %HRS cells, T-cell rosetted fraction of HRS cells, ratio of CD4+ and CD8+ T-cells, %activated T-cells, %activated B-cells and the eosinophil and neutrophil proportions. We were interested in whether these populations were associated to disease outcome. Our results describe that certain populations in the tumor microenvironment are linked to disease outcome, when measured by flow cytometry.

Chapter 2
MATERIALS AND METHODS

2.1 Case Selection

The University of Washington (UW) Hematopathology database was searched using the criteria of patients with FC data, between 2007-2017, with an immunophenotype consistent with cHL. Selection criteria were initial diagnostic specimen analyzed, age, sex, stage and subclassification, treatment, data from follow-up, and outcome. Patients who were lost to follow-up or had no data available in the electronic medical records were excluded from analysis. The diagnostic specimen was confirmed while collecting patient history. There were 326 specimens where cHL was likely detected, but 64 specimens that met our selection criteria. Two cases were excluded from statistical analysis, due to missing portions of FC data. The remaining 62 cases were analyzed.

2.2 Flow Cytometry

All specimens were run at the University of Washington Medical Center (UWMC) on 4-laser, 10-color Becton Dickinson LSRII flow cytometer (Becton Dickinson [BD], San Jose, CA). A 9-color FC tube with the following antibodies: CD95-PB, CD64-FITC, CD30-PE, CD5-ECD, CD40-PE-Cy5.5, CD20-PE-Cy7, CD15-APC, CD71-APC-A700, CD45-APC-Cy7, were used. Cell suspensions were prepared following standard protocols of immunophenotyping at UWMC Hematopathology Laboratory. Involved tumor tissue is finely minced using a scalpel in 3 to 5 mL of RPMI. The cell suspension was filtered through a 40 µm filter, centrifuged at 550g for 10 minutes, washed with phosphate buffered saline (PBS), resuspended in 0.5 mL of RPMI. Cells were incubated at room temperature with titered, fluorescently labeled antibodies in the dark for 15 minutes in approximately 100 µL of RRMI. Cell suspensions were lysed and

fixed with 0.15 mol/L ammonium chloride, pH 4.8 and 0.25% formaldehyde for 15 minutes, washed with 3 mL PBS – bovine serum albumin-azide, and incubated in 0.1 mL of PBS prior to analysis.

2.3 Flow Cytometric Data Analysis

Flow cytometric data from the selected cases were analyzed in Woodlist 3.1.3. Each population from our parameters of interest was gated for each specimen. A particular method was used to define each population.

The HRS cell population as detected by FC is defined as having CD30, CD40, and CD95 expression, lack of bright CD20 expression, and lack of CD64 expression. Unrosetted HRS cells express an immunophenotype of CD5–, CD15+/–, CD20–, CD30+, bright CD40+, low CD45+, CD64–, CD71+, and bright CD95+.[12, 13] T-cell rosetted HRS cells are expected to have the immunophenotype of CD5+, CD15+/–, CD20–, CD30+,bright CD40+, bright CD45+, CD64–, CD71+, and bright CD95+.[12, 13] Thus, the T-cell rosetted HRS fraction was identified by comparing the expression of CD45 and CD5 within the HRS cell population and those expressing CD5 indicate the rosetted HRS population.

B-cells were identified as CD5-, CD20+, and CD45+. T-cells were identified as CD5+, CD20-, and CD45+. Activated B and T-cells were characterized by CD5-, CD20+, CD45+, and CD71+ and CD5+, CD20-, CD45+, and CD71+, respectively. Granulocytes were identified using CD15+, CD45+, and increased side scatter, which were then separated further into the neutrophil and eosinophil populations. Neutrophils were identified as the population expressing higher levels of CD15 compared to eosinophils. CD3+/CD4+ and CD3+/CD8+ populations were obtained from a T-cell specific assay.

2.4 Statistical Analysis

Statistical analysis was performed in RStudio 1.3.1093. Two-sample t-tests were performed to compare parameters among outcome groups (remission and disease progression) and p-values were reported. (Table 3.2)

Chapter 3

RESULTS

3.1 Patient Demographics

There were 64 patients who met all selection criteria, had essential data, and had flow cytometric detection of HRS cells. Two were excluded due to missing clinical flow cytometric evaluation of B-cells and T-cells. The mean age of the cohort was 35.0; 46.8% male and 53.2% female. The clinical outcome included 46 cases (74.2%) reached complete remission, 16 cases (25.8%) had relapse or refractory disease. The histological subtype were 44 nodular sclerosis (NS) (71.0%), 3 mixed cellularity (MC) (4.8%), and 15 (24.2%) subtype not reported. (Table 3.1)

3.2 Correlation to Outcome

Cases that reached complete remission had an increased mean percentage of CD20+ B-cells, increased fraction of T-cell rosetted HRS cells, a higher mean ratio of CD4/CD8 T-cells, higher ratio of eosinophils to neutrophils, and decreased levels of activated T-cells, decreased percentage of eosinophils and neutrophils compared to cases that relapsed or had refractory disease. Patients who relapsed had a significantly lower fraction of T-cell rosetted HRS cells compared to those who achieved complete remission ($p < 0.05$) at presentation (Table 3.2). Other parameters did not indicate significance in relation to clinical outcome.

3.2.1 Percentage CD20 B-cells

The mean percentage of CD20+ B-cells in cases that achieved remission was higher than those that relapsed (27.2% and 20.2% respectively), but it was not statistically significant in our cohort ($p = 0.114$) (Figure. 3.1). Nonetheless, the trend corresponds to other studies

Table 3.1: Cohort demographics; summary of case characteristics

Characteristic	Number (%)
Sex	
Male	29 (46.8%)
Female	33 (53.2%)
Age(yrs), median (IQR)	32.0 (18)
Outcome	
Remission	46 (74.2%)
Relapse/Refractory	16 (25.8%)
Subclass	
Nodular Sclerosis	44 (71.0%)
Mixed Cellularity	3 (4.8%)
Lymphocyte Rich	-
Lymphocyte Depleted	-
Not reported	15 (24.2%)
Stage	
I/II	26 (41.9%)
III/IV	23 (37.1%)
Not reported	13 (21.0%)

that have found the decreased number in B-cells in the TME of cHL to be associated with adverse outcome.

3.2.2 Percentage CD5 T-cells

The mean percentage of T-cells was higher in cases that achieved remission compared to cases that relapsed. In this study, there was no significance between the percentage of T-cells and clinical outcome ($p = 0.700$). (Figure 3.2)

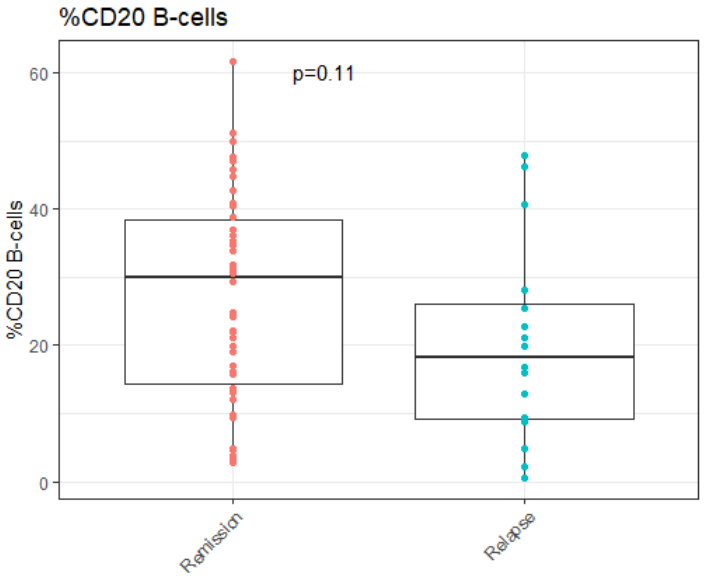


Figure 3.1: %CD20 B-cells by Outcome

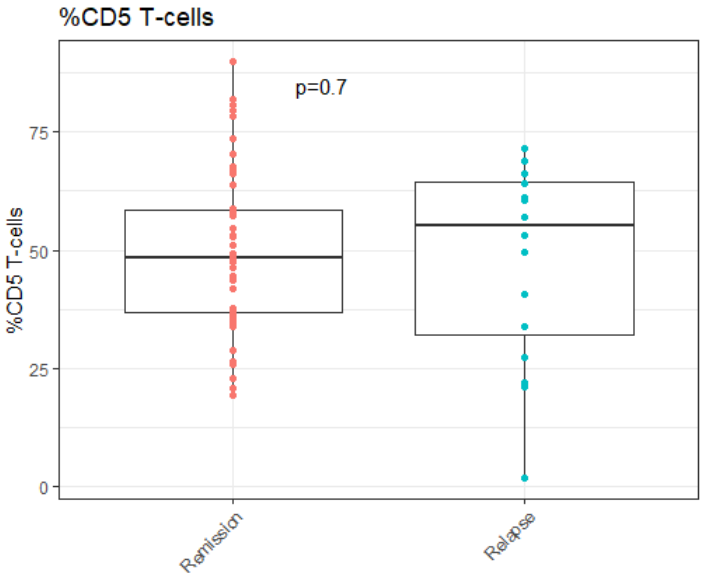


Figure 3.2: %CD5 T-cells by Outcome

3.2.3 Percentage HRS cells

The percentage of HRS cells of all white blood cells (WBC) was lower in cases that achieved remission compared to those that relapsed. There was no significance between the percentage of HRS cells and outcome (p = 0.435). (Figure 3.3)

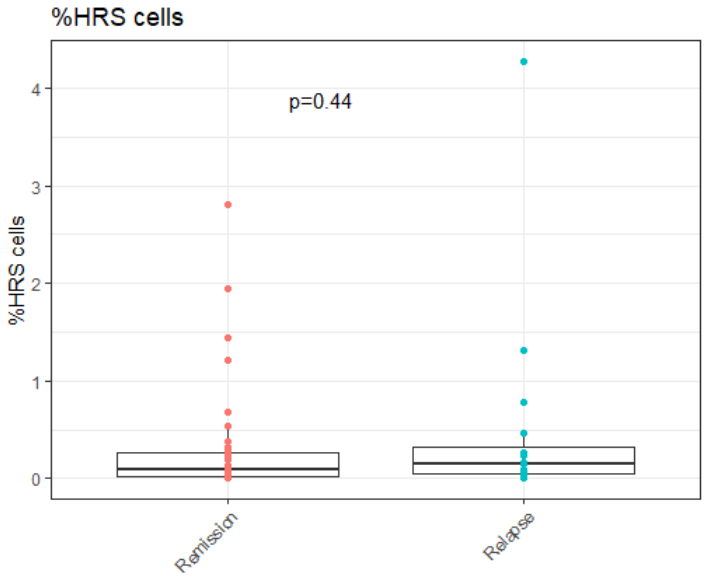


Figure 3.3: %HRS cells by Outcome

3.2.4 T-cell Rosetted HRS cell fraction

The fraction of rosetted HRS cells was significantly higher in cases that achieved remission compared to those where disease relapsed (95% CI, -1.79 – 15.82, $p < 0.05$). (Figure 3.4)

3.2.5 CD4/CD8 ratio

The CD4/CD8 T-cell ratio mean was higher in cases that achieved remission, but showed no significance to outcome (p = 0.503). (Figure 3.5)

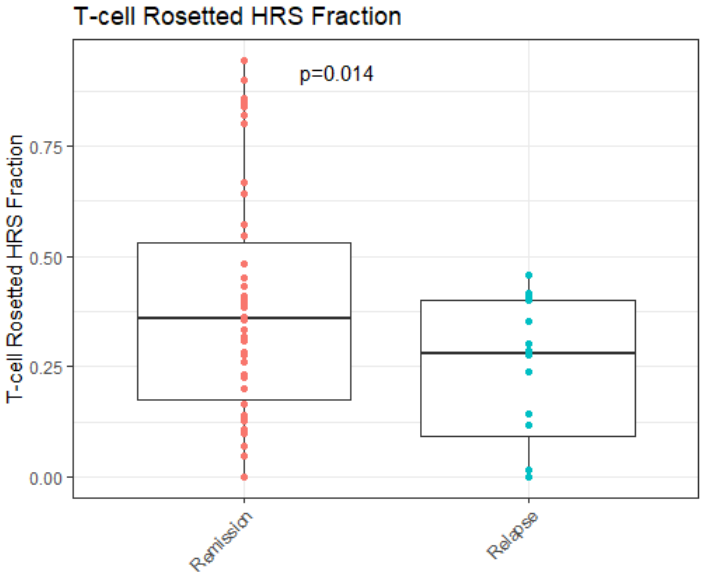


Figure 3.4: T-cell rosetted HRS cell fraction by outcome

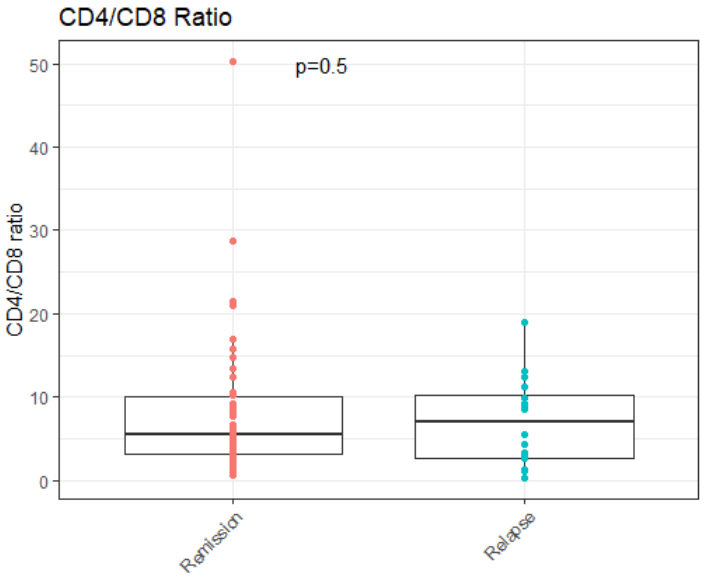
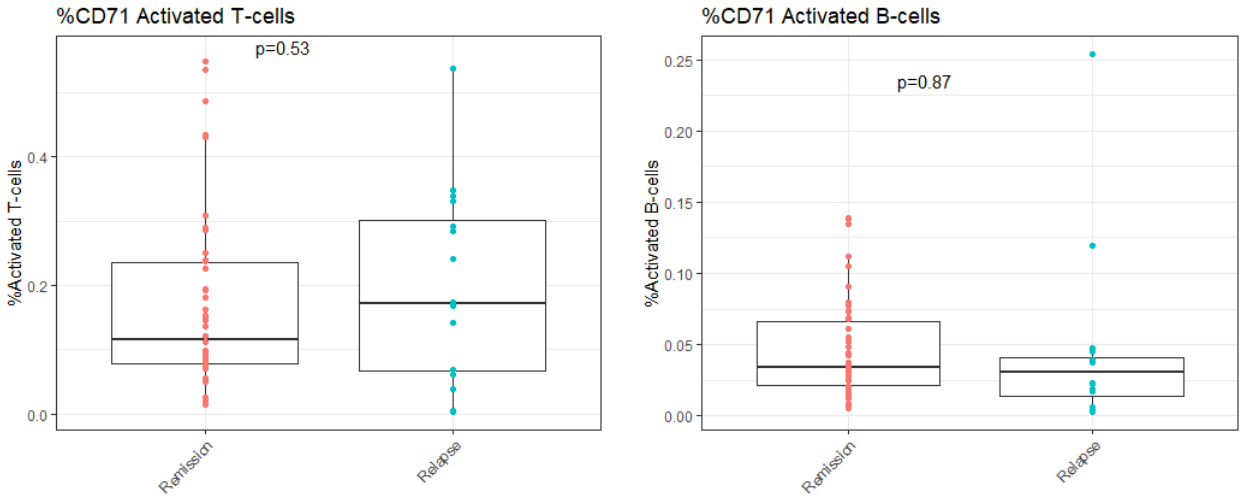


Figure 3.5: CD4/CD8 ratio by Outcome



(a) %CD71 T-cells by Outcome

(b) %CD71 B-cells by Outcome

Figure 3.6: %CD71+ activated B and T-cells by outcome

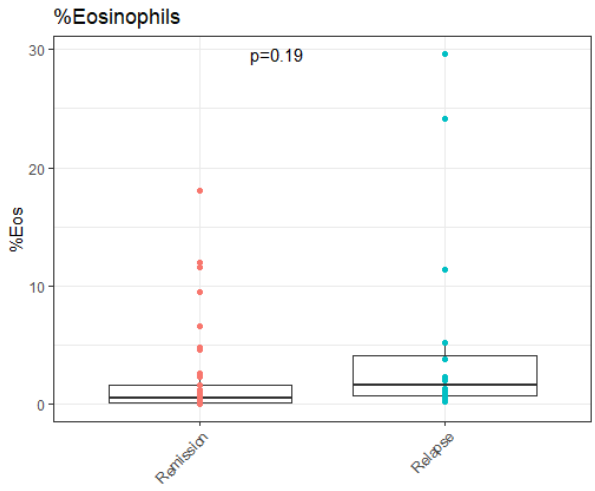
3.2.6 Activated cells characterized by CD71 expression

The mean percentage of activated T-cells characterized by CD71 expression was lower in cases that achieved remission, but showed no significance to outcome ($p = 0.525$) (Figure 3.6a). The percentage of activated B-cells showed almost no difference between the two outcome groups ($p = 0.874$). (Figure 3.6b)

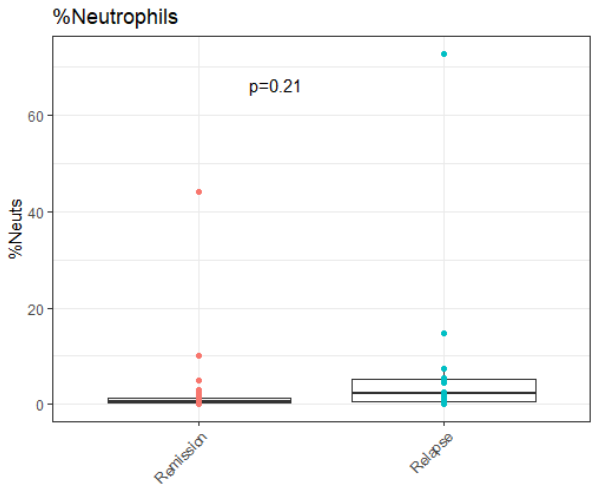
3.2.7 Eosinophils and neutrophils

We tested the eosinophil and neutrophil populations individually to outcome and the ratio of eosinophils/neutrophils to outcome. The percentage of eosinophils and neutrophils, individually, both showed a negative correlation to outcome; a decreased number correlated to a more favorable outcome, but was not statistically significant ($p = 0.191$ and $p = 0.215$ respectively) (Figures 3.7a and 3.7b). Comparing the eosinophil/neutrophil ratio to outcome, a higher ratio was linked to the more favorable outcome, but was not statistically significant

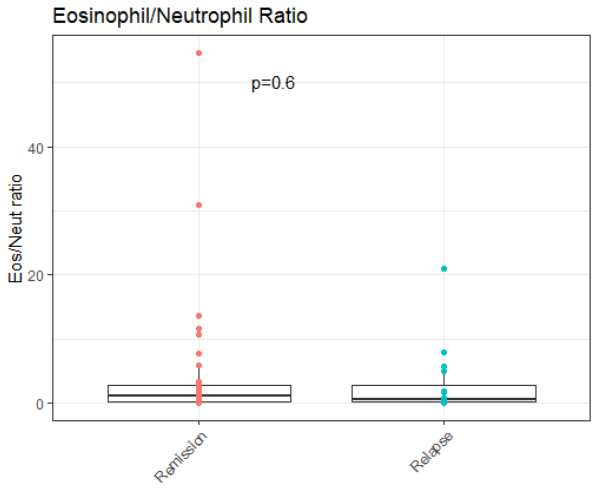
(p = 0.293). (Figure 3.7c)



(a) %Eosinophils by Outcome



(b) %Neutrophils by Outcome



(c) Eosinophils/Neutrophils by Outcome

Figure 3.7: Eosinophil and neutrophil populations by outcome

Table 3.2: Summary of Cell Population Correlation to Outcome

Cell Population - median (IQR)	Remission (n=46)	Relapse (n=16)	p-value
%CD20 B-cells	30.0% (24.0%)	18.3% (16.9%)	0.114
%CD5 T-cells	48.5% (21.6%)	55.1% (32.5%)	0.700
%HRS cells	0.08% (0.24%)	0.15% (0.27%)	0.435
T-cell rosetted HRS fraction	35.8% (35.5%)	28.1% (30.8%)	0.014
CD4/CD8 ratio	5.5 (6.8)	7.0 (7.5)	0.503
%CD71 activated T-cells	11.7% (15.7%)	17.2% (23.5%)	0.525
%CD71 activated B-cells	3.4% (4.4%)	3.0% (2.7%))	0.874
%Eosinophils	0.57% (1.4%)	1.6% (3.5%)	0.191
%Neutrophils	0.54% (0.95%)	2.3% (4.8%)	0.215
Eosinophil/Neutrophil ratio	1.2 (2.5)	0.68 (2.5)	0.293

Chapter 4

DISCUSSION

Understanding the complex interactions that HRS cells have with its microenvironment is an important step in determining the clinical course of cHL. Further, identifying new biomarkers can be the key to improving clinical outcome by allowing clinicians to predict the prognosis more accurately. For example, if a certain immunophenotype is known to be more likely to relapse, a treatment targeting specific immune cell pathways within the microenvironment could be given. With this more targeted approach, it can minimize the possibility of overtreatment for cases with predicted better outcome and conversely, if an immunophenotype is predicted to relapse or progress, an alternative treatment could be given initially with possibly a better response to treatment and later a better clinical outcome. Utilizing FC as a method of diagnosis can allow for the further and more specific analysis of the composition of the TME.

There is increasing evidence that the microenvironment plays a large role in the HRS cell's ability to survive, grow, and proliferate. [3, 14, 15] A unique feature of the cHL microenvironment is that the majority is composed of non-malignant cells (T-cells being predominant population) and less than 1% consisting of malignant HRS cells. The HRS cells secrete cytokines and chemokines that attract a variety of immune and activated cells that allow the HRS cells to evade immune attack.[3, 16, 14] The non-malignant cells appear to play a supportive role to the HRS cells by secreting soluble or produce membrane-bound molecules that contribute to tumor growth and proliferation.[4]

The T-cells that rosette the HRS cells are generally of Th2 and Treg type and express CD40L.[14] While it is hypothesized that the increased number of Th2 and Treg cells helps shield HRS cells from immune attack from cytotoxic T or natural killer (NK) cells, increased

number of Treg cells in the microenvironment has been associated to good prognosis as increased numbers are speculated to cause inhibitory effects on the HRS cells or the cells that support its survival or proliferation.[3] It also appears that the proportions of T-cell subtypes within the microenvironment may also influence the function and a predominant Th2 response is linked to improved prognosis.[15, 17] The relationship between the HRS cells and CD4+ T-cells is speculated to be directly related to immunosuppression.[17]

The growing information of the relationship between HRS cells and the microenvironment has led to more studies of how the differences in the TME relate to clinical features and outcome.[18, 19, 20] However, findings have been inconsistent among studies. In this study, we find that the fraction of T-cell rosetted HRS cells at presentation in the microenvironment vary significantly between cases that achieved complete remission compared to cases that relapsed or progressed. Interestingly, a higher fraction of T-cell rosetted HRS cells at presentation correlated to better outcome (remission). In the other populations we tested, some indicated trends that were consistent in previous cHL microenvironment correlation to outcome studies.

We also observed trends that show cases in which complete remission is achieved have on an increased mean percentage of CD20+ B-cells at presentation. Our cohort also showed that cases which relapsed, had a higher mean percentage of eosinophils. Previous studies have noted correlation between decreased B-cell count and poor prognosis[6], increased activated T-cells and better outcome,[7] tissue eosinophilia and poor prognosis,[8, 9] and the level of CD8 lymphocytes to freedom from treatment failure.[10] This is in contrast to our results where we did not find significance in those populations; however, this may be due to our relatively small cohort as some of our observations match the trend to the results of those studies. Additionally, we did not stratify between histological subclassifications, stage, or age due to the relatively small number of cases in this cohort.

As previously discussed, future studies include stratifying by age, subclassification, and stage. Histological subtype of cHL as each subtype is known to have varying compositions of immune cell types within the microenvironment and can impact the proportions of the

cells types we are interested in. Certain subtypes of cHL have been shown to have better prognosis in children.[7] Other factors such as stage, age, and whether the patient is immunocompromised could also impact the TME composition at presentation.[17] Examining the cases that relapse could provide insight on the differences from presentation and at relapse and additionally, looking at the differences between cases that progress quickly to those that respond to treatment initially then relapse. Factoring the treatment course to analyze the differences between cases that respond positively to treatment to those that treatment fails. Further analysis can be done to correlate these results to progression free survival or overall survival. By exploring other variables to outcome, potentially useful biomarkers may be impactful in determining treatment course or predicting prognosis or outcome in people diagnosed with cHL.

Our findings shows that by analyzing different components of the TME in cHL at presentation, it is possible new biomarkers to predict treatment or disease outcome will be determined. Specifically, the T-cell rosetted fraction of HRS cells is an indicator of prognosis. Further studies that target the specific components of the microenvironment and its relation to outcome can potentially lead to targeted therapies.

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