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RESEARCH ARTICLE

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Flow cytometry assessment of reactive T-cells distinguishes classic Hodgkin lymphoma from benign lymphadenopathy in children

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Abstract

Background: Detection of classic Hodgkin lymphoma (cHL) neoplastic cells using flow cytometric immunophenotyping (FCI) remains limited. We hypothesized that characterization of the reactive infiltrates could assist in diagnosing cHL in children.

Methods: FCI using four-color staining approaches was performed on 156 lymph node specimens with the following histopathologic diagnoses: cHL (25 cases), reactive lymphoid hyperplasia (RLH, 44 cases), and non-Hodgkin lymphoma (87 cases).

Results: The overall concordance of FCI data with the histopathologic results of these cases was 81.4%. A reactive expansion of T-cells with increased expression of CD45RO was present in the reactive infiltrate of cHL (CD45RO/CD3, 67.5%) and Epstein–Barr virus (EBV) infected RLH (62.7%) but not in EBV-negative RLH (28.0%). The mean fluorescence intensity (MFI) of CD7 was higher for cHL and differed significantly from EBV-positive RLH (138.5 vs. 63.8). A proposed diagnostic algorithm markedly elevated the overall concordance rate from 81.4% to 97.4%.

Conclusions: Immunophenotyping the reactive infiltrate of lymphoid tissue using flow cytometry is a reliable supplement to histopathology for the rapid diagnosis of pediatric cHL.

KEYWORDS
classic Hodgkin lymphoma, flow cytometry, reactive T-cell infiltrate

1 | INTRODUCTION

Classic Hodgkin lymphoma (cHL) is an unusual form of lymphoma characterized by a small number of neoplastic Hodgkin and Reed–Sternberg (HRS) cells in an extensive inflammatory background.[1,2] In light of the frequent non-neoplastic causes of lymphadenopathy and involvement of sensitive locations, lymph node fine-needle aspiration (FNA) or core needle biopsy (CNB) as minimally invasive procedures are frequently the first alternatives to obtain lymph node tissue to diagnose lymphoproliferative disorders.[3,4] However, insufficient tissue or the relatively sparse distribution of HRS cells decreases the diagnostic accuracy of cHL using conventional histologic evaluation.[5] Therefore, more sensitive and efficient ancillary tools such as flow cytometry (FCM) can provide unique diagnostic information when the morphologic analysis is unclear. FCI plays a critical role in the accurate diagnosis and classification of non-Hodgkin lymphoma (NHL), but its utility remains limited in diagnosing and managing cHL. Few studies have reported a specific immunohistochemical

profile in the microenvironment of HL-affected lymph nodes.[6–9] Similar data are even rarer in children.[10] Recently, Fromm et al.[11,12] reported that a six- or nine-color flow cytometry assay could be utilized to diagnose cHL with high sensitivity and specificity. However, this method requires more lymph node tissue to ensure that a minimum of 100,000 events were collected for analysis. Also, the fragility of large atypical HRS cells causes them to often escape detection in flow cytometry, producing false-negative results. Therefore, possibly due to technical factors or the lack of quantitative criteria, the utility of flow cytometry for cHL is not widely available.

This study evaluated whether FCI of the reactive infiltrates was diagnostically useful for pediatric cHL. We discovered that the percentage of $CD45RO^+$T-cells could readily distinguish cHL from Epstein–Barr virus (EBV)-negative reactive lymphoid hyperplasia (RLH), but not EBV-positive RLH. Previous research[7] showed that CD7 expression was significantly increased in cHL compared with reactive lymph nodes. Given that EBV can activate T-cells, it remained unclear whether CD7 expression in RLH was affected by EBV. We found that when the mean fluorescence intensity (MFI) of CD7 on T-cells attained a certain threshold, cHL could be distinguished from EBV-positive RLH. Finally, we proposed a diagnostic algorithm to improve the diagnostic accuracy of classic Hodgkin lymphoma.

2 | MATERIALS AND METHODS

2.1 | Sample collection

This work was conducted with the approval of the Medical Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine. We retrospectively searched the database in the division of Pathology for patients who underwent a lymph node biopsy for a suspected lymphoma diagnosis and were confirmed morphologically from January 2006 to December 2020. The histopathologic diagnoses were determined based on morphologic evaluation and immunohistochemistry (IHC). Cases that concurrently underwent flow cytometric analysis in our institution were collected. A list of 156 viable consecutive cases was analyzed to compare the FCI results with the histopathological findings. The study cohort consisted of 125 males and 31 females with a male-to-female ratio of 4:1. The patients' ages ranged from 1 to 16, with a median age of 7.7 years. Within this cohort, 25 cases of cHL and 44 cases of RLH were evaluated for their $CD3^+CD45RO^+$T-cell population and CD7 expression on T-cells. Of the reactive cases, 13 were EBV positive.

2.2 | Pathology and immunohistochemistry analysis

Lymph node biopsy specimens were obtained from the patients, fixed in 10% neutral buffered formalin, and immediately sent to the pathology laboratory. The samples were dehydrated, embedded in paraffin, and sectioned at a thickness of 4 μm in preparation for staining with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), and immunohistochemistry (IHC) for histopathology analysis. Experienced pathologists carried out the macroscopic and microscopic examinations for each specimen. All immunohistochemistry staining was performed using a two-step technique with the DAKO EnVisionTM HRP System. Antibodies to the following CD antigens: CD3, CD20, CD30, CD43, CD79a, CD68, CD15, ALK, epithelial membrane antigen (EMA), and EBV, were obtained from DAKO Denmark. Appropriate positive and negative controls were used.

2.3 | Flow cytometric immunophenotyping

Single-cell suspensions from each lymph node were prepared according to a standard protocol. Briefly, fresh biopsy tissue was finely minced with scissors and then mechanically homogenized. The homogenate was filtered through a mesh (<100 μm), centrifuged, and washed with phosphate-buffered saline (PBS) prior to being resuspended in PBS containing 0.1% sodium azide. The specimens were analyzed for a range of antigens on a flow cytometer (FACSCalibur™; Becton Dickinson) using software (CellQuest, Becton Dickinson) for data acquisition and analysis. Immunophenotyping was performed using four-color immunofluorescent staining with commercially available fluorescent-labeled monoclonal antibodies to the following antigens: Mouse IgG1, Mouse IgG2a, CD1a, CD2, CD3, CD4, CD5, CD7, CD10, CD11c, CD19, CD20, CD23, CD25, CD34, CD45, CD56, CD103, HLA-DR, CD45RA, CD45RO, FMC7, TCRα/β, TCRγ/δ, Cμ, SmIgM, Kappa(κ), and Lambda(λ) (Becton Dickinson, San Jose, CA, USA). CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD25, CD34, CD56, TCRα/β, TCRγ/δ, CD45RA, and CD45RO were used to identify T-cell lymphoproliferative diseases. CD5, CD10, CD11c, CD19, CD20, CD22, CD23, CD34, CD103, FMC7, HLA-DR, Cμ, SmIgM, and surface κ and λ light chains were used to identify B-cell lymphoproliferative diseases. Briefly, 1×10^5 nucleated cells per tube were mixed with four antibodies (20 μl per antibody) and incubated at 4°C for 30 min in the dark. An isotype control was performed at the same time. After incubation, each sample was lysed with a lysing solution and washed twice with PBS. Then, each sample was fixed with 1% paraformaldehyde and analyzed using flow cytometry. At least 10,000 events were acquired and analyzed using the CellQuest (version 3.2) software on a FACSCalibur flow cytometer (Becton Dickinson).

2.4 | Statistical analysis

Continuous data were described as medians and ranges. Categorical data were described as numbers and percentages. The sensitivity and specificity of the flow cytometry analyses were calculated compared with the histopathology findings. Significance between differences in proportions was compared using the $\chi 2$ test or Fisher's exact test. Comparison of antigen expression between normal and neoplastic cells was performed using the unpaired *t* test or Mann–Whitney test,

depending on the normal distribution and homogeneity of variance of the data. Comparisons among three groups were tested using the *Kruskal–Wallis* test. Receiver operating characteristic (ROC) curve analyses were constructed based on the CD45RO frequency and level of CD7 MFI. All analyses were performed using SPSS version 21.0 software. $p < 0.05$ was considered statistically significant for all comparisons. The graphics were plotted using GraphPad Prism version 8.0 software.

$$\text{Sensitivity} = \frac{\text{Number of true postive cases} \times 100}{\text{Number of true postive cases} + \text{False negative cases}}$$

$$\text{Specificity} = \frac{\text{Number of true negative cases} \times 100}{\text{Number of true negative cases} + \text{False postive cases}}$$

3 | RESULTS

3.1 | Histopathology and flow cytometric immunophenotyping

In Table 1, we summarize the histopathology and corresponding flow cytometry data. Of the suspected lymphoma cases, 55.7% (87/156) were diagnosed with non-Hodgkin lymphoma (NHL) and 16.0% (25/156) with Hodgkin lymphoma based on the histopathological features and immunohistochemical results. The NHL group consisted of 27 cases of T-lymphoblastic lymphoma (T-LBL), 10 cases of B-lymphoblastic lymphoma (B-LBL), 20 cases of Burkitt's lymphoma, nine cases of diffuse large B-cell lymphoma (DLBCL), one follicular lymphoma (FL) case, 17 anaplastic large cell lymphoma (ALCL) cases, one NK case, and two cases of NK/T lymphoma.

TABLE 1 Comparation between flow cytometric immunophenotyping and pathological diagnosis

Lymphoma type	Histopathology diagnosis No.	FCI diagnosis No.
T-LBL	27	27
B-LBL	10	10
Mature B lymphoma	30	31
Burkitt lymphoma	20	–
DLBCL	9	–
FL	1	–
ALCL	17	14
NK cell lymphoma	1	1
NK/T lymphoma	2	2
cHL	25	0
RLH	44	71
Total	156	156

Abbreviations: ALCL, Anaplastic large cell lymphoma; B-LBL, B-Lymphoblastic lymphoma; DLBCL, Diffuse large B-cell lymphoma; FL, Follicular lymphoma; T-LBL, T-Lymphoblastic lymphoma.

According to the FCI data obtained, 85 cases were identified as lymphoma from this cohort. The remaining 71 cases were diagnosed as reactive, of which 43 cases were morphologically benign based on histopathology. Also, 28 cases were diagnosed as RLH by FCI, but they were classified as ALCL (three cases) and Hodgkin's lymphoma (25 cases) by histopathology or IHC, respectively.

In order to visually demonstrate the agreement between flow cytometry diagnoses and histological diagnoses, we conducted an exploratory analysis by generating a chord diagram (Figure 1). FCI demonstrated 100% concordance with histopathology in diagnosing all cases of T-LBL and B-LBL. However, there was a difference in mature B lymphoma in that FCI diagnosed 31 cases without further subtype, instead of 30 cases as was indicated by histopathology. One additional case was proven to be RLH based on histopathology. Within the RLH group, the estimated sensitivity was 97.8%, and the specificity was lower (75.0%). The estimated sensitivity and specificity for FCI in diagnosing NHL were 96.5% and 98.6%, respectively, and in the ALCL group, 82.4% and 100%, respectively; Within the cHL group, seven cases exhibited very weak positive expression for CD30, and flow cytometry did not establish a diagnosis for these cases due to the relative scarcity of CD30-positive cells in the specimens. Therefore, flow cytometric analysis alone could not be used to diagnose cHL. All cHL cases in this study were finally diagnosed as false negative by FCI. However, the FCI results were concordant with histopathology in 81.4% (127/156) of lymphoma cases. Excluding cHL, the overall concordance rate was increased to 96.9% (127/131).

3.2 | CD45RO expression on T-cells in lymph nodes from cHL and RLH patients

CD45RO expression on T-cells was examined using FCM in 25 cHL cases. The results were compared with those obtained from the 44 RLH cases. Table 2 contains the characteristics of the patients included in this study, and the majority represented subtypes of cHL. The histologic subtype was nodular sclerosis (NS) in eight patients, mixed cellularity (MC) in 14 patients, and lymphocyte rich in three patients, respectively (Table 2). Ten out of 14 MC tumors were EBER positive, while only 25% (2/10) of the NS tumors were EBER positive. $CD45RO^+$T-cells were calculated as percentages of total T-cells. Representative flow cytometry data and gating strategies for determining the frequency of $CD3^+CD45RO^+$T-cells in cHL and RLH are shown in Figure 2A,B. We found a significantly higher percentage of $CD45RO^+$ T-cells in the cHL group than in the reactive group (67.5%±15.6% vs. 38.2%±18.7%, $p < 0.001$; Figure 3A), suggesting that the percentage of $CD45RO^+$T-cells could have diagnostic value for cHL. To confirm this hypothesis, we performed a ROC test and determined that the percentage of $CD45RO^+$T-cells could distinguish cHL from reactive lymphoid hyperplasia. As shown in Figure 3B, the AUC for CD45RO to predict cHL was 0.871 (95% CI, 0.79–0.95, $p < 0.001$), and the optimal cutoff value was 46%, with a sensitivity and specificity of 92.0% and 72.7%, respectively.

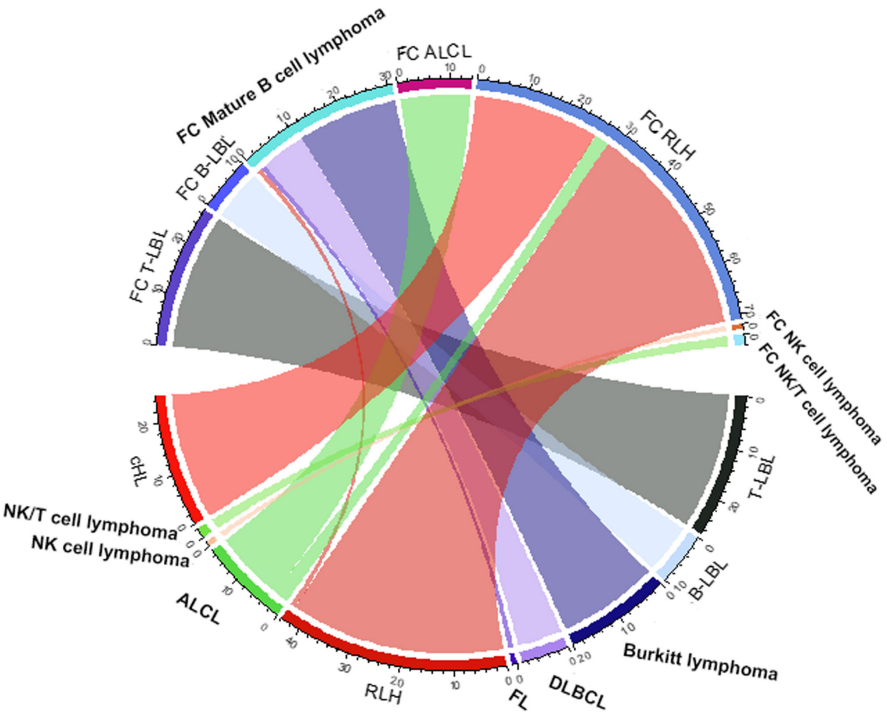

FIGURE 1 Chord diagram representing the agreement between flow cytometry diagnoses (FC is attached to their names) and histological diagnoses.

TABLE 2 Summary of demographic and subtype of classical HL

Characteristic	RLH	Classical HL	EBV-pos RLH
Age, median(range), y	7.7 (1.6–14.6)*	8.4 (2.6–13.0)	7.8 (2.8–14.5)*
Sex, male, No (%)	36 (81.8)*	20 (80.09)	11 (84.6)*
Subtype of cHL			**N (%)**
Nodular sclerosis			8 (32)
Mixed cellularity			14 (56)
Lymphocyte rich			3 (12)
Total			25 (100)

*$p > 0.05$, compared with cHL.

Therefore, CD45RO was a good biomarker to identify cHL from benign lymphadenopathy.

3.3 | Overexpression of CD7 by cHL-infiltrating T-cells

As seen in Figure 3C, CD45RO expression also increased in EBER-positive benign lymphadenopathy (62.7% ± 9.6%). No statistically significant differences were observed between EBV-positive RLH and cHL ($p = 0.49$). Therefore, it was not possible to distinguish malignant lymphoma from all benign lymphadenopathies only based on CD45RO expression. Previous research showed[7] that CD7 expression in $CD4^+$ and $CD8^+$T-cells was increased in cHL compared with reactive lymph nodes. Thus, we determined whether differences existed in the expression of CD7 or fluorescence intensity (MFI) between cHL-infiltrating T-cells and EBV-infected RLH-infiltrating T-cells. All cHL cases and thirteen cases of EBV-positive RLH were retrospectively analyzed, whose FCI data were acquired during the same time frame with a consistent fluorescence intensity on the instrument. Sample FCM CD7 histograms are shown in Figure 3D. The results revealed that the MFI of CD7 was significantly higher in cHL than in EBV-positive RLH ($p < 0.001$, Figure 3E). ROC curve analysis suggested that CD7 expression in T-cells could efficiently distinguish cHL from EBV-positive RLH, with an AUC of 0.908 (95% CI, 0.805–1.0, $p < 0.001$, Figure 3F). To determine the optimal cutoff value to distinguish cHL and EBV-positive RLH, the sensitivity and specificity were calculated at different levels of CD7 in T-cells. The sum of these values was maximized at a CD7 MFI of 89, which provided a sensitivity and specificity of 88% and 85%, respectively.

Furthermore, we analyzed the reactive lymphocyte makeup in children with cHL by the histologic subtype (NS and MC). We found that T-cells predominated in the two subtypes (Table S1). However, there was no significant shift in the percent of $CD45RO^+$T and CD7 MFI between the two subtypes. The major difference between the reactive infiltrates of NS and MC was the proportion of $CD8^+$T-cells, with an increased number of MC, resulting in an elevated CD4/CD8

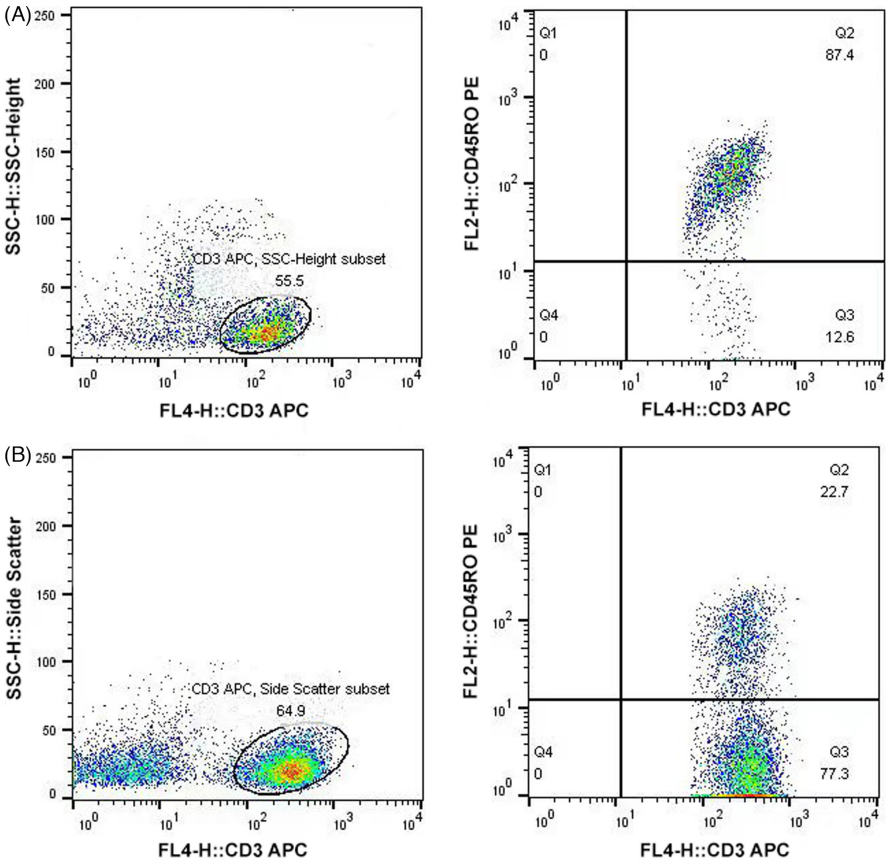

FIGURE 2 Flow cytometry plots for $CD3^+$ $CD45RO^+$T-cell population in two representative cases of (A) cHL and (B) RLH

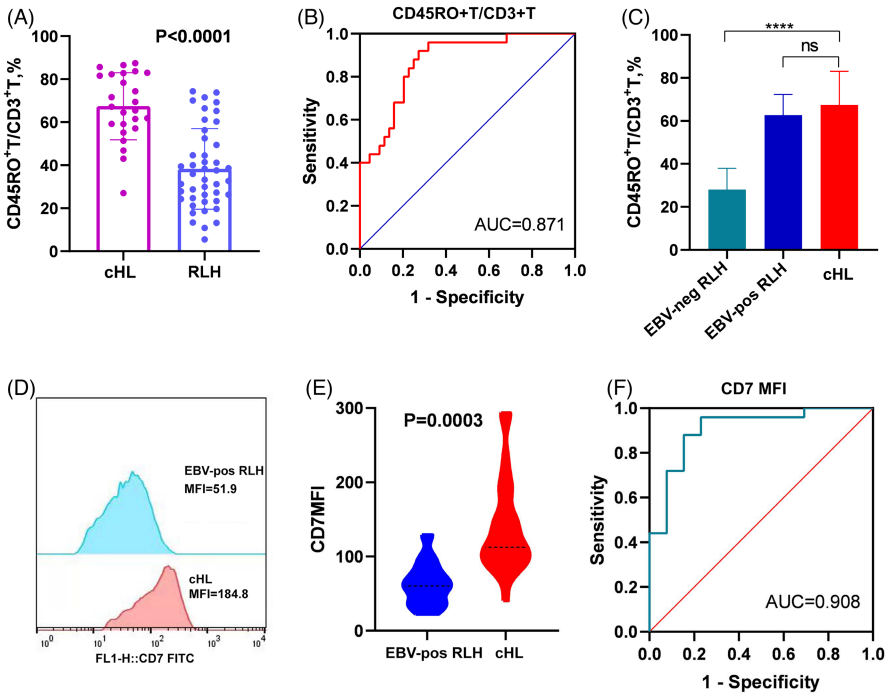

FIGURE 3 Discriminative performance of biomarkers to identify cHL and RLH. (A) The comparison of the percentage of $CD45RO^+$T-cells between cHL and RLH. (B) ROC curves illustrating the performance of CD45RO to predict cHL. (C) Percentage of $CD45RO^+$T-cells. (D) Sample CD7 histograms of FC for a case of EBV-pos RLH and a case of Hodgkin lymphoma. (E) Comparison of CD7 expression on T-cells between cHL and EBV-positive RLH. (F) ROC curves for distinguishing two groups according to CD7 MFI on T-cells. ****$p < 0.0001$; ns, no significance

ratio in NS. To explore whether EBV contributed to the induction of the CD45RO- and CD7-positive T-cell population, we divided the cases with cHL into two groups according to the EBV infection status. No differences were observed between the EBV-positive cHL and EBV-negative cHL groups in terms of their CD45RO- and CD7-positive T-cell population (Table S2).

3.4 | A diagnostic algorithm was useful in the differential diagnosis of lymphomas

Based on these findings, we proposed a new algorithm that could suggest the appropriate diagnosis and provide support when the FCI findings were not entirely conclusive (Figure 4). If the flow cytometry

analysis did not suggest T- or B-NHL, a T-cell population with co-expression of CD45RO and CD3 represented more than 46% of the T-cells, and CD7 MFI was more than 89, cHL should be a diagnostic consideration. However, it should be noted that MFI values are instrument and fluorochrome specific, and it was necessary to establish a laboratory-specific cutoff value. Therefore, we presented an algorithm applying the CD45RO+T-cell percentage and the CD7 MFI to assist with diagnosing cHL using FCM (Figure 4). According to the diagnostic algorithm, the overall concordance rate was elevated markedly from 81.4% to 97.4% [(127 + 25)/156%] ($P < 0.001$).

4 | DISCUSSION

The diagnosis of lymphoma using lymph node biopsies depends on morphology and immunohistochemistry features provided by a pathologist and flow cytometry. Concerning FCI, objective and quantitative evidence for diagnosis are acquired by assessing light chain clonality and simultaneous evaluation of lymphoid B- and T- cell surface antigens.[13]

In this study, we investigated the ability of FCM to diagnose lymphoma compared with the histopathology results accurately. Our results demonstrated that the overall concordance of the FCI data with the histopathologic results was 81.4%, which appeared to be slightly lower than the majority of published reports with an overall diagnostic success rate of approximately 88%.[14,15] An even higher percentage of concordance was reported by Dunphy et al., who demonstrated that 351 out of 373 suspected lymphoma cases (94.1%) were successfully diagnosed using FCI.[16] The sensitivity and specificity of FCI in diagnosing NHL were 96.5% and 98.6%, respectively. Comparable data have been published previously.[14] A recent meta-analysis by Cozzolino et al.[13] evaluating lymph node FNC combined with FCI for the diagnosis and classification of NHL in various sites reported the ranges of sensitivity (75%–99%) and specificity (87%–100%).

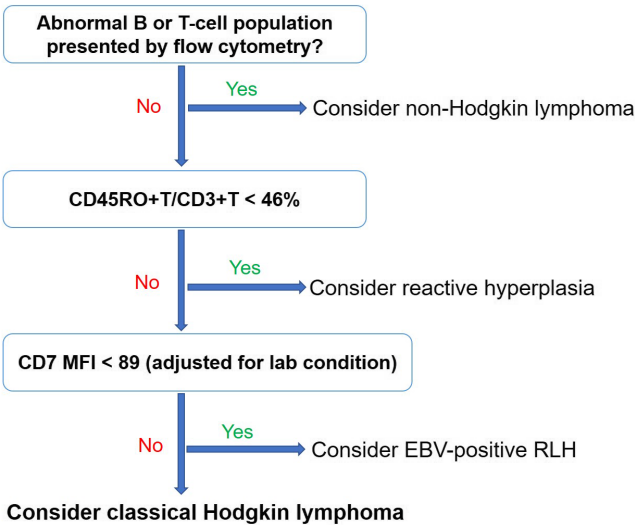

FIGURE 4 Proposed algorithm for using flow cytometry finding of T-cell infiltrates to facilitate histologic diagnosis of cHL.

All cases of T-LBL and B-LBL in our study diagnosed using four-color FCM perfectly matched the histological diagnosis. We also have shown that FCI could correctly diagnose NK cell and NK/T-cell lymphomas, but there were only three patients that underwent FCI and histology. Although the NK cell or NK/T-cell lymphomas were rare, they could be diagnosed unambiguously through FCI for their specific immunophenotyping.

Our results also indicated that FCI could easily diagnose mature B-cell lymphomas by detecting specific surface antigens and light chain ratios. Nevertheless, it was not useful in differentiating subtypes. Based on the expression of CD10/CD5, typical and aberrant phenotypes might simultaneously occur in DLBCL as well as BL, BL-like, and FL.[17,18] For these cases, relying on flow cytometry alone may be impossible, and additional techniques like ICC and FISH are needed for accurate classification.

As reported by others, FCI performed poorly in the identification of cHL. In the current study, no Hodgkin lymphomas were diagnosed by this technology but were classified as normal or reactive conditions. Flow cytometry was traditionally considered unreliable in diagnosing cHL because the large and fragile neoplastic cells represent less than 1% of the total number of cells in the extensive inflammatory background.[2] Recently, researchers have paid increasing attention to the phenotypic characteristics of HRS cells as well as alterations in background lymphocytes.[19,20] Efforts have been made to employ these cells to identify and diagnose cHL through FCM. Fromm et al.[11] reported a highly sensitive and specific method to identify HRS cells using six-color flow cytometry, with a panel of antibodies reactive to antigens on HRS cells, including CD64, CD30, CD40 CD20, CD95, and CD3. The HRS populations showed positive expression of CD30, CD40, and CD95, with increased forward and side scatter. They were negative for CD64 and negative or low for CD20, while CD3 showed variable expression probably caused by T-cell binding rosettes. Nonetheless, this method is not available in most clinical laboratories as they are not routinely equipped with a six-color flow cytometer. Moreover, this method presents some limitations. On the one hand, there is a requirement for enough lymph node tissue to ensure that a minimum of 100,000 events are collected for analysis. On the other hand, detecting HRS cells with flow cytometry may lead to false-negative results due to the fragility of large atypical neoplastic cells that can be easily disrupted during sample processing.

Current research in the field is focused on alterations in the immunophenotype of the background T-cells found in HL. For instance, an elevated CD4/CD8 ratio, overexpression of CD7 and CD45 on $CD4^+$T-cells, and increased expression of $CD25^+$Treg cells were demonstrated in cHL compared with reactive lymph nodes.[6,7,21] In this study, we demonstrated that the presence of reactive T-cells with strong expression of CD45RO was suggestive of cHL or EBV-positive RLH, and CD7 could be used to distinguish them. It is known that the CD45RO isoform represents memory T-cells that respond to antigenic recall by proliferation and provide assisted activation for antigen-specific antibody synthesis.[22] The presence of the CD45RO-positive T-cell population may be induced by an immune response against viral antigens (especially EBV).[23] On the other

hand, the increased CD7-positive T-cell population in cHL might not be explained by the modulation effect of EBV infection but rather the physiological changes in the immune system. Our data, as described and summarized in Tables S1,S2, support the above interpretation that CD7-positive T-cell population is not related to EBV infection. However, additional work is needed to gain more insights into the pathobiological significance of the CD45RO-positive T-cell population in pediatric Hodgkin lymphoma.

Based on the reactive T lymphocytes found in cHL, RLH, and EBV-associated RLH, we presented an algorithm that could suggest the correct diagnosis and facilitate the histologic diagnosis of cHL. If an abnormal B- or T-cell population is present, a diagnosis of non-Hodgkin lymphoma should be considered. If reactive $CD45RO^{+}$T-cells represent >46% of the infiltrated $CD3^{+}$T-cells, RLH without EBV-infected should be excluded. If CD7 MFI < 89, EBV-positive RLH is the suggested diagnosis; otherwise, cHL should be a diagnostic consideration. However, it needs to be mentioned that MFI values are instrument and fluorochrome specific, and it was necessary to establish a laboratory-specific cut-off value. We presented an algorithm analysis that can be easily transferred into clinical application using multi-color flow cytometry, as most clinical laboratories can routinely obtain these data. In conclusion, immunophenotyping the reactive infiltrate of the lymphoid tissue by flow cytometry is a reliable auxiliary to histopathology for rapid diagnosis of pediatric cHL.

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CONFLICT OF INTEREST

No potential conflicts of interest relevant to this article are reported.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL

The study was approved by the Medical Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine (Hangzhou, China). The ethics committee number of our study is 2022-IRB-022.

CONSENT FOR PUBLICATION

All authors approved the final version of the article and agreed with the order of the authors before submission and publication.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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