Tumor-associated Macrophages in Pediatric Classical Hodgkin Lymphoma:
Association with Epstein-Barr virus, Lymphocyte Subsets and Prognostic Impact

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Tumor-infiltrating macrophages are potential independent indicators of poor outcome in classical Hodgkin lymphoma (cHL) in adults and represent possible therapeutic targets. In this study, we investigated macrophages and dendritic cells in pediatric cHL taking into account Epstein-Barr virus (EBV) status and lymphocyte composition of the tumor microenvironment. Our results show that the prognostic role of macrophages in pediatric cHL is affected by EBV status of the Hodgkin and Reed-Sternberg cells. Furthermore, we suggest that use of microenvironment variable as prognostic indicators in cHL may require the combined analysis of cell populations from the innate and adaptive immune systems. Our results emphasize differences in the microenvironment between adult and pediatric cHL and suggest that it may be necessary to re-evaluate studies of adult patients taking EBV status into account.
ABSTRACT

Purpose: Tumor-infiltrating macrophages are associated with adverse outcome in adult classical Hodgkin lymphoma (cHL). We have previously demonstrated age-related changes in the lymphocyte composition of pediatric cHL. We therefore hypothesized that the number, function and prognostic impact of macrophages in pediatric cHL would be different from adult cases. Experimental Design: We analyzed the number of macrophages and dendritic cells (DCs) in the tumor microenvironment of pediatric cHL by immunohistochemistry (IHC). Results were analyzed in context of age, histological characteristics, EBV-status, clinical follow-up and our previous study of T-cell populations in these cases. Results: 100 cHL cases were studied, including 69% nodular sclerosis (NS) and 23% mixed cellularity (MC) cases. 44.8% of cases were EBV-positive. Patients ≤10 years displayed more CD14+ cells (P= 0.025). In comparison to NS, MC was characterized by higher numbers of CD14+, (P= 0.003) and CD163+ cells (P= 0.027). EBV+ cases exhibited higher numbers of CD14+ (P< 0.0005), CD68+ (P= 0.005) and CD163+ cells (P= 0.02). CD68-positive cells did not display an effect on outcome. Worse overall survival was observed in cases with CD163/CD8 ratio >2 (P= 0.007). High numbers of CD163+ cells were associated with worse progression-free survival (PFS) (P= 0.015). Furthermore, high numbers of CD163+ and Granzyme B+ cells were associated with worse PFS in EBV-negative (P= 0.005) but not in EBV-positive cases. Conclusion: Our results suggest that macrophage composition in pediatric cHL is distinct from adults. Functional status of macrophages and their value as prognostic indicators in pediatric cHL may depend on EBV status.
INTRODUCTION

Classical Hodgkin lymphoma (cHL) microenvironment is modulated by cytokines and chemokines produced by HRS cells and is thought to contribute to tumour cell growth and escape from immunosurveillance (1). We have shown recently that in EBV+ pediatric cHL the tumor microenvironment is characterized by a cytotoxic/Th1 profile (2). Furthermore, cases with a small number FOXP3+ regulatory T-cells and a FOXP3/CD8 ratio <1 in the tumor microenvironment were associated with an improved outcome. We, therefore, hypothesized that in pediatric EBV+ cHL an effective cytotoxic immune response directed against viral or tumor antigens may be triggered in the tumor microenvironment (2-3).

Recent studies have consistently reported that high numbers of tumor-associated macrophages (TAM) are associated with adverse outcome in adult cHL (4-6). As yet, the specific role of TAM in pediatric cHL has not been studied.

Macrophages can be polarized toward a pro-inflammatory phenotype (M1 macrophages) with tumoricidal activity or toward a regulatory function (M2 macrophages) which are characterized by functions in tissue repair, remodeling and promotion of T helper 2 (Th2) immune response (7-9). It is generally thought, that M2 macrophages are predominantly found in the microenvironment of malignant tumours. In agreement with this notion, macrophages expressing CD163 (considered to be a marker of M2 macrophages) were associated with adverse outcome in a study of adult cHL (5).

Dendritic cells (DCs) are critically important in the initiation and regulation of immune responses, driving Th1 or Th2 type responses, or promoting antigen-specific tolerance (10-12). Additionally, plasmacytoid DC (pDC) are able to induce regulatory T (Treg) cells (13-14).

As immune reactions are shaped by cross-talk between innate and adaptive immune system (15-16), it is likely that the number, type and functional status of
lymphocytes present in the tumor microenvironment of cHL are engaged in the functional status of TAM and DCs.

Based on the hypothesis that the lymphocytes from the tumor microenvironment modulate and in turn are modulated by macrophages and DCs, we have examined TAMs and DCs in pediatric cHL in the context of our previous analysis of lymphocyte subsets (2-3) and we have investigated their impact on prognosis.

**MATERIAL AND METHODS**

**Patients**

One hundred HIV-negative children and adolescents (up to 18 year old) diagnosed with cHL at the Instituto Nacional do Câncer (INCA, Brazil) between 1999 and 2006 were included in this study. The clinical features of these cases have been previously described (2). All patients were treated according to standard pediatric protocols as previously described (2). For five cases there was not sufficient clinical data and these were excluded from the survival analysis. Children were classified in two age groups (≤ 10 years vs. >10 years) to investigate the characteristics of macrophages and DCs in younger vs older children (17-21). This study was approved by the INCA Ethics Committee.

**Histology**

Diagnosis of cHL was established by morphologic criteria according to the WHO classification (22). All cases were independently reviewed by two pathologists (MHMB and GN) as previously described (2).

**Tissue Microarray Design and Immunohistochemistry**

Construction of tissue microarray (TMA) blocks has been described previously (2). From each case, two 1-mm-diameter cores, selected from two different tumor areas
rich in neoplastic cells, were included. TMA blocks were sectioned at a thickness of 3 μm. Antigen retrieval was performed by heat treatment in a pressure-cooker for 1 minute. Buffers used for antigen retrieval and primary antibodies are listed in the Supplemental Material (Table S1). Briefly, the antibodies used were CD14 (clone 7, Novocastra), CD68 (clone PGM1, Dako), CD163 (clone 10D6, Novocastra), CD83 (clone 1H4b, Novocastra) and CD207 (LANGERIN) (clone 12D6, Abcam). After incubation with primary antibody (30 minutes), immunodetection was performed using ZytoChem Plus HRP polymer kit (Zytomed Systems, Berlin, Germany) (Supplemental Material, Table S1), employing diaminobenzidine (DAB) chromogen as substrate. Sections were counterstained with hematoxylin. External and internal controls included in the TMA were taken into consideration to interpret staining results.

**EBV Detection**

Latent EBV infection was determined in all cases by in situ hybridization (ISH) with fluorescein-conjugated probes for EBV-encoded RNAs (EBER-ISH) and by IHC against latent membrane protein 1 (LMP1) as described (23).

**Computer Assisted Microscopical Analysis**

For cell subset quantitative evaluation, each core was photographed using AxioCam MRc camera (Zeiss, Germany) at a 200x magnification. The numbers of labeled macrophages and DC were determined per 1mm² using the image analysis software HISTO (Biomas, Elangen, Germany).

**Thresholds**
For each cell subset, the 25th and 50th percentiles were used to categorize the intensity of the cell infiltration. Cases displaying no labeled cell in both cores were considered not evaluable for technical reasons.

**Statistical Analysis**

Pearson’s chi-square and Fisher’s exact test were used to test association between dichotomous variables. Mann-Whitney test was used to compare medians, while Spearman’s rank correlation was used to test association between continuous variables. First-order partial correlations were used to correlate two or more variables while keeping constant one or more additional variables and were computed as rank correlations (continuous variables) or point biserial rank correlations (categorical vs. continuous variables). Differences were considered significant at $p < 0.05$ in 2-tailed tests. Hierarchical cluster analysis using average linkage and binary simple matching measure allowed to explore the structure of association among variables of the tumor microenvironment and EBV-status. Primary treatment was defined as a failure if the lymphoma had progressed at any time after the initiation of therapy; treatment success was defined as the absence of progression or relapse. Progression-free survival (PFS) was the interval (in months) from diagnosis to progression at any time, relapse from complete response, or initiation of new, previously unplanned treatment or to the last follow-up in the patients with treatment success. Overall survival (OS) refers to the interval (in months) from the diagnosis to death or last follow-up. Survival distributions were estimated by the Kaplan-Meier method and differences were compared using log-rank test. Multivariate Cox proportional hazard regression method was used to determine the independent prognostic value of statistically significant variables in univariate analyses. Data were analyzed using Statistical Package for the Social Sciences 13.0 (SPSS).

**RESULTS**
Clinical-pathological data of this pediatric group have been described previously (2). In brief, the age at diagnosis ranged from 3 to 18 years (median 14 years) and 27% of children were ≤10 years. The majority of patients had stage I/II disease (59/95, 62.1%) and favorable clinical presentation (50/95, 52.6%). Extranodal disease was observed in 11 children (11/95, 11.6%). NS was the predominant subtype (69/100, 69%), followed by mixed cellularity (MC) (23/100, 23%). Granulomas were observed in 37% of cases (37/100 cases) (2). As described previously, EBV-positive HRS cells were detected in 44.8% of cases and no association with age-groups was observed (3). A detailed description of EBV-association in relation to histological features has been published previously (2-3). Distribution of lymphocyte subsets in the tumor microenvironment in relation to age group, histology, EBV-status and their prognostic impact has been reported previously (2-3) and are summarized in table S3. The main clinical and histological characteristics of the patients are described in the Supplemental Material (Table S2).

**Macrophages and Dendritic Cells in Pediatric cHL**

CD14+ monocyte/precursor cells, as well as CD163+ macrophages, CD83+ and CD207+ dendritic cells were distributed uniformly in the tumor microenvironment, without specific distribution pattern (Figure 1). CD68+ cells also showed uniform distribution in the majority of cases, but in 5 cases a rosette-like arrangement around HRS cells was observed.

The results of the quantitative analysis of CD68+, CD163+, CD14+, CD207+ and CD83+ cells are summarized in Table 1. In general, the number of CD68+ cells was higher than that of CD163+ cells and of CD14+ cells. As expected, a strong correlation between CD68+ and CD163+ cells was observed (p<0.001), as well as between CD68+ and CD14+ cells (p<0.001).
The numbers of CD83+ cells and CD14+ cells were similar. By contrast, LANGERIN+ cells were infrequently detected in the tumor microenvironment (Supplemental Material, Figure S1). No significant bivariate correlation was observed between CD83+, CD207+ and CD14+ cells.

**TAM are Associated with Clinical and Histological Features**

The young age group (<10 years) was characterized by a higher number of CD14+ cells (median 70 cells/mm² vs. 44 cells/mm² for the old-age group, \( P=0.025 \), Mann-Whitney test). No other age-related differences were observed.

The numbers of CD14+, CD68+, CD163+, CD83+ and CD207+ cells were not associated with clinical stage, favorable or unfavorable clinical presentation, B-symptoms, number of involved anatomic sites, lymphopenia or anemia.

In comparison to NS subtype, MC subtype was characterized by higher numbers of CD14+ (median 105 cells/mm² vs. 49 cells/mm² to NS, \( P=0.003 \), Mann-Whitney test) and CD163+ cells (median 191 cells/mm² vs. 117 cells/mm² to NS, \( P=0.027 \), Mann-Whitney test) (Supplemental Material, Table S3).

Cases with granulomas exhibited a higher number of CD14+ (median 64 cells/mm² vs. 49 cells/mm² without granulomas, \( P=0.04 \), Mann-Whitney test) and CD207+ cells (median 3 cells/mm² vs. 1 cell/mm² without granulomas, \( P=0.03 \), Mann-Whitney test). Although not statistically significant, the cases with granulomas also exhibited higher numbers of CD68+ cells (median 310 cells/mm² vs. 269 cells/mm² without granulomas, \( P=0.1 \)) and CD163+ cells (median 167 cells/mm² vs. 126 cells/mm², \( P=0.2 \)).

**TAM and Dendritic Cells are Associated with Lymphocyte Subsets in Tumor Microenvironment**
The analysis of lymphocyte subset distribution in this pediatric cHL series has been published previously, and all data regarding the quantitative assessment of lymphocytes have been taken from those studies (2-3) (Supplemental Material, Table S3).

Numbers of macrophages and DCs were lower than the total number of CD3+ T cells. However, the number of CD68+ cells was higher than that of CD20+ B cells and the number of CD163+ cells was similar to that of CD8+ cells (Supplemental Material, Figure S1).

As previously shown, the size of these lymphocyte subpopulations differed according to age, with a more cytotoxic composition observed in the ≤10 years group compared to a more suppressive profile in children older than 10 years. (2) (Supplemental Material, Table S3). To investigate if macrophages and DCs are associated with lymphocyte subpopulations a partial rank correlation analysis was carried out, controlled by age.

The details of this analysis are provided in the supplemental material (Table S4). In general, the numbers of CD14+ cells showed a direct correlation with the numbers of TBET+ (P< 0.0005), CD8+ (P= 0.019), TIA1+ (P= 0.024) and Granzyme B+ (P= 0.009) cells.

The numbers of CD68+ cells were directly correlated with the numbers of FOXP3+ (P< 0.0005), TIA1+ (P= 0.001) and Granzyme B+ (P = 0.001) cells (Supplemental Material, Table S4).

Numbers of CD163+ cells were directly correlated with those of TBET+ (P= 0.04), CD8+ (P= 0.025), TIA1+ (P= 0.001) and Granzyme B+ (P< 0.0005) cells (Supplemental Material, Table S4).

Finally, numbers of CD83+ cells were directly correlated with the numbers of CD4+ cells (P= 0.01), FOXP3+ cells (P= 0.003) and CD20+ lymphocytes (P= 0.018) (Supplemental Material, Table S4).
EBV-Status of HRS Cells is Associated with the Number of TAM and Dendritic Cells in Pediatric cHL

Since EBV has been shown to modulate the tumor microenvironment composition in adult and childhood cHL (2, 24), a preliminary data classification strategy using hierarchical cluster analysis was performed to identify underlying patterns of tumor microenvironment cell subsets according to EBV status (Figure 2). In this analysis, 2 distinct clusters emerged: cluster I included the EBV-associated cases and cases with high numbers of CD8+, TIA1+, TBET+, CD14+, CD68+, CD163+ and CD207+ cells; cluster II was mainly composed of EBV-negative cases and those with high numbers of FOXP3+, CMAF+ and CD83+ cells.

Statistical associations between the numbers of macrophages/DCs and EBV status of HRS cells were investigated with the Mann-Whitney test. EBV-associated cases displayed higher numbers of CD14+ (median 83 vs. 38 cells/mm² in EBV-negative cases, P< 0.0005), CD68+ (median 331 vs. 234 cells/mm² in EBV-negative cases, P= 0.005) and CD163+ cells (median 164 vs. 111 cells/mm² in EBV-negative cases, P= 0.02). No association between EBV status of HRS cells and numbers of DCs was observed. Specifically, we did not observe any association of EBV status of HRS cell with the number of tumor infiltrating CD207+ cells.

To assess the correlations between the numbers of macrophages, DCs and lymphocytes subpopulations, independently of the EBV status in HRS cells, point biserial rank correlations were performed, using EBV status as controlling variable (Table 2). CD14+ cells were directly correlated with the number of TBET+ cells independently of EBV-status. The same was observed for CD68+ and Granzyme B+ cells, CD163+ and Granzyme B+ cells and CD83+ and FOXP3+ cells.
On the other hand, a positive correlation was observed between CD68+ and FOXP3+ cell numbers only in EBV-negative cases, indicating that this is the source of the overall association observed between these two subpopulations in the microenvironment (see above). These analyses in EBV-negative cases also disclosed a relationship of CD163+ cells with both TBET+ cells and TIA1+ cells (Table 2).

**TAM and Dendritic Cells are Associated with Outcome**

The OS at 60 months was 89.4%; stage and type of clinical presentation did not have impact on the OS (2). No association was observed between single macrophage or DC populations and OS. A worse 5-year OS rate was observed in cases with CD163/CD8 ratio >2 (64.7% vs. 91.4% for ratio <2, \( P = 0.009 \), Log-rank test) and with CD83/CD20 ratio >2 (50% vs. 89.7% for ratio <2, \( P = 0.001 \), Log-rank test) (Figure 3). The other ratios between macrophages, DCs and lymphocyte populations were not associated with survival. In the multivariate Cox regression, CD83/CD20 ratio >2 maintained statistical significance (\( P = 0.008 \); HR 7.3; 95%CI 1.6 to 31.5), while CD163/CD8 ratio showed a borderline significance (\( P = 0.06 \); HR 3.3; 95%CI 0.9 to 11.6).

PFS at 60 months was 78.6% (2). High numbers of CD163+ cells (> 50th percentile) were associated with worse 5-year PFS rate (\( P = 0.015 \), Log-rank), while CD68+ cells showed no influence on survival (Figure 4). Extranodal disease (54.5% vs. 81% for absence of extranodal disease, \( P = 0.028 \), Log-rank), leukopenia (50% vs. 80% for absence of leukopenia, \( P = 0.034 \), Log-rank) and presence of granulomas (63.6% vs. 84.5% for absence of granulomas, \( P = 0.026 \), Log-rank) were also associated with worse 5-year PFS rate (2). Stage and type of clinical presentation did not impact on PFS (2). Considering the variables statistically significant in the Log-rank test, only extranodal disease maintained statistical significance in the Cox regression (\( P = 0.025 \); HR 3.4; 95%CI 1.1 to 10.2).
We have reported previously that high numbers of Granzyme B+ cells were also associated with worse 5-year PFS rate (69.6% vs. 90.9%, \( P = 0.045 \), Log-rank) (2). Next, we combined both microenvironment variables significantly associated with PFS: CD163+ and Granzyme B+ cells. This allowed the distinction of 3 patient groups: Group 1) high numbers of Granzyme B+ cells and CD163+ cells; Group 2) high numbers of Granzyme B+ cells or high number of CD163+ cells and, Group 3) low numbers of Granzyme B+ cells and CD163+ cells. Based in this approach, the worst PFS was observed in cases with high numbers of both, CD163+ and Granzyme B+ cells (\( P = 0.031 \), Log-rank) (Figure 4). In the Cox regression, extranodal disease (\( P = 0.01 \); HR 4.1; 95%CI 1.4 to 12.1) and the combination of CD163+ and Granzyme B+ cells (\( P = 0.038 \); HR 2.2; 95%CI 1.0 to 4.9) maintained the statistical significance.

**EBV Infection of HRS Cells May Influence the Prognostic Significance of CD163+ Cells in the Tumor Microenvironment**

Given that EBV appears to be able to modulate the tumor microenvironment composition and local EBV-specific immunity (2, 24-25), the impact of the numbers of CD14+, CD68+, CD163+, CD207+ and CD83+ cells on PFS was investigated, according to EBV-status. In the EBV-negative group, a worse PFS rate was observed for cases with high numbers of CD163+ cells (>50<sup>th</sup> percentile) (44.4%, \( P = 0.008 \), Log-rank), while no influence of a high number of CD163+ macrophages was observed in the EBV-positive group, although this was the group which generally exhibited higher numbers of CD163+ macrophages. The other cell populations showed no influence on PFS when cases were stratified by EBV-status.

Concerning the combined effect of Granzyme B+ and CD163+ cells, again, the impact on PFS was observed only in the EBV-negative group, with the worst PFS rate
occurring in cases with high numbers of both CD163+ and Granzyme B+ cells (35.7%, \( P = 0.005 \), Log-rank) (Figure 4).

The effects of microenvironment variable on 5-year OS rate were also affected by EBV status. CD163/CD8 ratio >2 and CD83/CD20 >2 were associated with worse prognosis only in EBV-negative cases (50%, \( P = 0.005 \) and 40%, \( P = 0.002 \), respectively. Log-rank) (Figure 3).

**DISCUSSION**

In comparison to adults, children display distinct physiologic immunological features (17-21). In pediatric cHL, these distinct features may contribute to a different immune response against HRS cells reflected by qualitative and quantitative differences in lymphocyte subset distribution in the tumor microenvironment (2). Adaptive and innate immune systems are integrated and cross-talk between these systems contributes to a robust and balanced immune response (15-16). Therefore, the joint analysis of TAM, DCs and lymphocytes in the tumor microenvironment is essential to understand the nature of the local immune responses against in cHL.

We have demonstrated previously, that pediatric cHL cases in the \(<10\) years age group are characterized by a cytotoxic immune profile as demonstrated by a prevalence of CD3+, CD8+, TIA1+ and TBET+ T-cells (2). Here we show in the same cohort, that CD14+ cells are also more prevalent in cHL cases of the younger age group. It is unknown if age related differences exist in the frequencies of CD14+ cells in peripheral blood or tissues; therefore at present it is not possible to say if our result is secondary to a particular immune response against HRS cells in the young age-group or merely reflects an age-related physiological phenomenon. CD14 is expressed on cells of the myelomonocytic lineage, including monocytes, a proportion of macrophages and Langerhans cells (26). Since we did not observe age-related changes in the numbers of
macrophages or Langerhans cells, it is possible that the majority of the CD14+ cells represents monocytes (macrophages precursors). However, this issue requires further analysis.

As described previously in adult cHL (5, 27), we observed higher numbers of TAM in EBV-positive cases, independently of their maturation/activation status, as evidenced by CD14, CD68 and CD163 immunohistochemistry. It is well recognized that the immune axis shaped by Th1 cells, i.e., CD8+ cells, macrophages and DCs, is essential for the control of virus infection (16, 28). In conjunction with our previous observations (2), these results raise the possibility that in EBV-associated pediatric cHL, an antiviral immune response may be triggered against viral proteins present on HRS cells, resulting in an increased recruitment of macrophages and cytotoxic/Th1 cells to the tumor microenvironment (2). In addition, a putative cross-talk of Th1 and CD8+ cells with macrophages (15), might explain the direct correlation observed between the number of macrophages and the number of TBET+, CD8+, TIA1+ and Granzyme B+ cells seen in this study. At present, it is not understood how monocyte/macrophages are recruited to the microenvironment of HRS cells and how these and other non-tumoral cells contribute to the final phenotype.

In contrast to in vitro studies, animal studies or investigations of solid tumors (7, 9), we did not observe a direct correlation between numbers of putative M2 macrophages (CD163+ cells) and the Treg numbers cells. Moreover, unexpectedly we noticed a direct correlation between the numbers of CD163+ cells and Granzyme B+ cells, independently of EBV status. These results suggest that a high number of CD163+ macrophages per se does not reflect in an immunosuppressive tumor microenvironment composition. It is possible that CD163+ macrophages in a cytotoxic environment may either represent part of a “negative-feedback loop” to avoid an excessive tissue destruction by cytotoxic T cells or alternatively, may have different differentiation/activation status, as it has been
described in non-small cell lung tumor (29) and in cutaneous squamous cell carcinoma (30).

Moreover, it is of note that in our pediatric series, therapy response was not negatively affected by the presence of TAM in EBV+ cases, despite the high number of these cell subset that characterized the EBV+ cases. This reinforces the idea that, at least in the EBV+ cases, most TAMs are not of the M2 pro-tumoral subset and that CD163 probably is not a definitive marker of M2 polarization of macrophages.

In addition, we found a correlation between the numbers of CD68+ macrophages and Tregs in the EBV-negative cases, which again points to differences in the macrophage differentiation in the EBV+ and EBV- groups.

TAM are potentially one of the most powerful independent markers for clinical prediction in cHL and represent possible therapeutic targets (4-5, 27, 31-32). However, a number of technical issues, such as a consensus about the best quantification strategies, as well as marker selection, including monoclonal antibodies and molecular markers, are still matters of debate (27).

We did not observe an independent prognostic impact of CD68+ cells on OS, contrary to what has been reported for adult cHL (5). We used the macrophage-specific PG-M1 clone, contrary to many studies of adult cHL which employed CD68 KP1 clone to identify macrophages (4-5, 33). As KP1 is known to cross-react with different cell populations, including large lymphocytes (34-35), it is possible other cell populations stained with the KP1 antibody contribute to the adverse prognostic effect observed in previous studies. The absence of impact on the OS was also observed for CD163+ cells. It remains unclear in our specific pediatric population, if the absence of impact of macrophages and DC subpopulations (when analyzed separately) on OS was due to the success of rescue therapy or secondary to any other factor. Nevertheless, we observed that CD163/CD8 ratio ≥2 and CD83/CD20 ≥2 were associated with worse OS, suggesting
that the immune system may contribute to treatment response; and that tumor microenvironment variables can be informative about this.

High numbers of CD163+ cells were associated with worse PFS only at the univariate level, in line with previously published results in adult cases (5). However, when this cell population was analyzed together with Granzyme B+ cells, we observed a worse progression free survival in cases with high numbers of both Granzyme B+ cells and CD163+ cells in both univariate and multivariate analysis. Because of the complexity of microenvironment cell interactions, prognostic prediction in cHL may require combining microenvironment variables in an index or score, as shown here for the combination of the numbers of CD163+ cells and Granzyme B+ cells. A similar strategy was also proposed in a previous study of adult cHL (6) and may be fruitful for future clinical studies focused on the modulation of tumor microenvironment.

CD83 (clone 1H4b) has been shown to be a specific marker for mature and activated DCs and does not cross-react with macrophages (26, 36). In support of this notion, in our study, numbers of CD83+ cells did not correlate with those of CD14+, CD68+ or CD163+ cells. Also, we observed that the numbers of CD83+ DCs were directly correlated with the numbers of FOXP3+ cells. As it has been described that pDCs are able to induce primary Treg cell differentiation (13-14), it is possible that part of these CD83+ cells were pDC and that they were engaged in Treg cell differentiation. However, the CD83 antibody is not able to differentiate myeloid DCs from pDCs (36-37) and this issue requires further investigation. The prognostic impact of tumor-associated DCs on survival of cancer patients is controversial; in some studies a higher number of these cells was associated with a better outcome (38-39), while in others, the specific pDC subset has been associated with worse outcome (40-42). Here, an excess of DCs was associated with worse OS, when analyzed in relation to B cells (CD83/CD20 ratio >2). This highlights
the necessity to understand better the contribution of DCs to the immune response against HRS cells.

CD207 (Langerin) is a type II membrane-associated C-type lectin exclusively expressed by Langerhans cells (LCs), a subtype of DC (43). Recently, it has been suggested that the presence of CD207+ LCs in the tissue microenvironment may be an exclusive feature of EBV-associated disease, including cHL (44). Our results are different in two respects. Firstly, we found CD207+ cells in comparable numbers in both, EBV-positive and EBV-negative pediatric cHL cases. Moreover, in our study, these represented only a very minor cell population. The reasons for these discrepancies are currently unclear but likely are due to technical differences, e.g., related to the choice of CD207 antibodies. The Langerin (CD207) antibody (clone 12D6, Abcam) used in our study produced reproducible staining of epidermal Langerhans cells in control experiments (not shown). Thus, our results suggest that CD207+ cells are not specifically recruited to the microenvironment of pediatric cHL and, at least in this setting, the presence of CD207+ cells is not a specific feature of EBV+ cases.

We are aware that the number of cases in this study imposes limitation in relation to the analysis of prognostic impact and prospective studies are mandatory to confirm our results. However, our series of sequential cases with similar distribution in relation to EBV-status is appropriate for the immunological evaluation performed here.

In summary, our results suggest that in pediatric cHL the macrophage composition is numerically and functionally distinct from adults, and that the functional status of macrophages and their value as prognostic indicators in pediatric cHL may depend on EBV status of HRS cells. Finally, our results emphasize the complexity of the cHL microenvironment and suggest that for an assessment of the impact on outcome, multiple cell populations of the innate and adaptive immune systems may have to be considered together.
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AUTHOR CONTRIBUTIONS

Contribution: M.H.M.B. designed and performed the experiments, collected and analyzed data and wrote the manuscript; R.H. analyzed data and co-wrote the manuscript. G.N. reviewed the cases and co-wrote the manuscript.
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LEGEND OF FIGURES

Figure 1: Examples of the diversity of immunostains for APCs (antigen-presenting cells) markers. Some cases displayed high numbers of CD68+ cells (A), while in others, only few CD68+ cells were seen (D) (original magnification: 10x). Similarly, there were cases with high numbers of CD163+ cells (B), while others exhibited few number of these cells (E) (original magnification: 10x). Panel C shows a typical example of CD83 expression by dendritic cells (original magnification: 40x). CD207+ cells were infrequent in the tumor microenvironment of pediatric classical Hodgkin lymphoma (F) (original magnification: 40x).

Figure 2: Dendrogram using average linkage obtained by hierarchical cluster analysis. Two main identified clusters (I and II) are indentified by brackets. Num: order of variable input. CD14 high: high number of CD14+ cells/mm² (>50th percentile). CD163 high: high number of CD163+ cells/mm² (>50th percentile). TBET high: high number of TBET+ cells/mm² (>50th percentile). CD68 high: high number of CD68+ cells/mm² (>50th percentile). EBV positive: Epstein-Barr virus associated classical Hodgkin lymphoma. CD8 high: high number of CD8+ cells/mm² (>50th percentile). CD20 high: high number of CD20+ cells/mm² (>50th percentile). CD4 high: high number of CD4+ cells/mm² (>50th percentile). TIA1 high: high number of TIA1+ cells/mm² (>50th percentile). LANGERIN high: high number of LANGERIN (CD207)+ cells/mm² (>50th percentile). EBV negative: absence of Epstein-Barr virus association in classical Hodgkin lymphoma. CMAF high: high number of CMAF+ cells/mm² (>50th percentile). Granzyme B high: high number of Granzyme B+ cells/mm² (>50th percentile). FOXP3 high: high number of FOXP3+ cells/mm² (>50th percentile). CD83 high: high number of CD83+ cells/mm² (>50th percentile).

Figure 3: Kaplan-Meier curves of variables associated with overall survival in pediatric classical Hodgkin lymphoma. A) Overall survival according to CD163/CD8 ratio. B) Overall survival according to CD83/CD20 ratio. C) Overall survival according to CD163/CD8 ratio in Epstein-Barr virus (EBV) negative patients.

Figure 4: Kaplan-Meier curves of variables associated with progression-free survival in pediatric classical Hodgkin lymphoma. A) Progression-free survival according to number of CD163+ cells. B) Progression-free survival according to combined numbers of CD163+
cells and Granzyme B+ cells. C) Progression-free survival according to combined numbers of CD163+ cells and Granzyme B+ cells in Epstein-Barr virus (EBV) negative patients.
Figure 1. Example of the diversity of the immunostains to APCs markers.
Figure 2: Dendrogram using average linkage obtained by hierarchical cluster analysis.
Figure 3. Kaplan-Meier curves of the variables associated with overall survival in pediatric classical Hodgkin lymphoma.
Figure 4: Kaplan-Meier curves of progression-free survival (PFS) in pediatric classical Hodgkin lymphoma.
Table 1. Description of the macrophage and dendritic cell subsets in the tumor microenvironment of pediatric classical Hodgkin lymphoma and their association with progression free survival and overall survival.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Labeled Cells (cells/mm$^2$)</th>
<th>Cases Analyzed (%)</th>
<th>5-Year PFS Rate (%)</th>
<th>Univariate Analysis (p) for PFS</th>
<th>5-Year OS Rate (%)</th>
<th>Univariate Analysis (p) for OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14+ (cells/mm$^2$)</td>
<td>Range (Mean / Median) 0 to 524 (83.1 / 53)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 23 (25th percentile)</td>
<td>-</td>
<td>20/78 (25.6)</td>
<td>78.9</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 23 (25th percentile)</td>
<td>-</td>
<td>58/78 (74.4)</td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>≤ 53 (50th percentile)</td>
<td>-</td>
<td>39/78 (50)</td>
<td>80.6</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 53 (50th percentile)</td>
<td>-</td>
<td>39/78 (50)</td>
<td>71.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD68+ (cells/mm$^2$)</td>
<td>Range (Mean / Median) 44 to 601 (290.81 / 284)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 194 (25th percentile)</td>
<td>-</td>
<td>20/81 (24.7)</td>
<td>78.9</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 194 (25th percentile)</td>
<td>-</td>
<td>61/81 (75.3)</td>
<td>73.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>≤ 284 (50th percentile)</td>
<td>-</td>
<td>43/81 (53.1)</td>
<td>67.5</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 284 (50th percentile)</td>
<td>-</td>
<td>38/81 (46.9)</td>
<td>82.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD163+ (cells/mm$^2$)</td>
<td>Range (Mean / Median) 0 to 560 (164.1 / 143)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>≤ 76 (25th percentile)</td>
<td>-</td>
<td>20/79 (25.3)</td>
<td>84.2</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 76 (25th percentile)</td>
<td>-</td>
<td>59/79 (74.7)</td>
<td>71.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>≤ 143 (50th percentile)</td>
<td>-</td>
<td>40/79 (50.6)</td>
<td>86.8</td>
<td>-</td>
<td>0.015</td>
<td>-</td>
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<tr>
<td>&gt; 143 (50th percentile)</td>
<td>-</td>
<td>39/79 (49.4)</td>
<td>61.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD207+ (cells/mm$^2$)</td>
<td>Range (Mean / Median) 0 to 32 (4.32 / 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 2 (50th percentile)</td>
<td>-</td>
<td>49/76 (64.5)</td>
<td>74.4</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 2 (50th percentile)</td>
<td>-</td>
<td>27/76 (35.5)</td>
<td>69.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD83+ (cells/mm$^2$)</td>
<td>Range (Mean / Median) 11 to 410 (66.9 / 55)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 38 (25th percentile)</td>
<td>-</td>
<td>21/79 (26.6)</td>
<td>80</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 38 (25th percentile)</td>
<td>-</td>
<td>58/79 (73.4)</td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>≤ 55 (50th percentile)</td>
<td>-</td>
<td>40/79 (50.6)</td>
<td>77.8</td>
<td>-</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 55 (50th percentile)</td>
<td>-</td>
<td>39/79 (49.4)</td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PFS: progression free survival. OS: overall survival.
Table 2: Correlation between macrophages/dendritic cells and lymphocytes from the tumor microenvironment, according to Epstein-Barr virus status.

<table>
<thead>
<tr>
<th>LYMPHOCYTES</th>
<th>CD14+ CELLS</th>
<th>CD68+ CELLS</th>
<th>CD163+ CELLS</th>
<th>CD83+ CELLS</th>
<th>CD207+ CELLS</th>
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<tbody>
<tr>
<td></td>
<td>EBV+ C</td>
<td>EBV- C</td>
<td>P</td>
<td>EBV+ C</td>
<td>EBV- C</td>
</tr>
<tr>
<td>CD3+</td>
<td>↓ 0.03</td>
<td>-</td>
<td>-</td>
<td>↓ 0.04</td>
<td>-</td>
</tr>
<tr>
<td>CD4+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOXP3+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TBE+</td>
<td>↑ 0.009</td>
<td>↑ 0.014</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD8+</td>
<td>-</td>
<td>-</td>
<td>↑ 0.028</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TIA1+</td>
<td>-</td>
<td>-</td>
<td>↑ 0.04</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Granzyme B+</td>
<td>↑ 0.003</td>
<td>-</td>
<td>-</td>
<td>↑ 0.002</td>
<td>↑ 0.034</td>
</tr>
<tr>
<td>CD20+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Number of cells /mm². C.: correlation. "-" no statistical correlation. ↑ direct correlation. ↓ inverse correlation. EBV+: Epstein-Barr virus association. EBV-: Absence of Epstein-Barr virus association. The P values are from point biserial Spearman’s correlations.
Tumor-associated Macrophages in Pediatric Classical Hodgkin Lymphoma: Association with Epstein-Barr virus, Lymphocyte Subsets and Prognostic Impact

Mário Henrique Magalhães Barros, Rocio Hassan and Gerald Niedobitek

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