Human Cancer Biology

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

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Abstract

Purpose: Tumor-infiltrating macrophages are associated with adverse outcome in adult classical Hodgkin lymphoma (cHL). We have previously shown 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 (DC) in the tumor microenvironment of pediatric cHL by immunohistochemistry. Results were analyzed in context of age, histologic characteristics, Epstein-Barr virus (EBV) status, clinical follow-up, and our previous study of T-cell populations in these cases.

Results: One hundred cHL cases were studied, including 69% nodular sclerosis and 23% mixed cellularity cases. A total of 44.8% of cases were EBV-positive. Patients ≤ 10 years displayed more $CD14^+$ cells ($P = 0.025$). In comparison with nodular sclerosis, mixed cellularity 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. *Clin Cancer Res; 18(14); 3762–71. ©2012 AACR.*

Introduction

Classical Hodgkin lymphoma (cHL) microenvironment is modulated by cytokines and chemokines produced by Hodgkin/Reed–Sternberg (HRS) cells and is thought to contribute to tumor cell growth and escape from immunosurveillance (1). We have shown recently that in Epstein-Barr virus $(EBV)^+$ pediatric cHL the tumor microenvironment is characterized by a cytotoxic/T-helper cell 1 (T_H1) profile (2). Furthermore, cases with a small number $FOXP3^+$ regulatory T cells (Treg) and a FOXP3/CD8 ratio

less than 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 proinflammatory 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_H2 immune response (7–9). It is generally thought, that M2 macrophages are predominantly found in the microenvironment of malignant tumors. 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 (DC) are critically important in the initiation and regulation of immune responses, driving T_H1 or

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Translational Relevance

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 reevalute studies of adult patients taking EBV status into account.

T_H2 type responses, or promoting antigen-specific tolerance (10–12). In addition, plasmacytoid DC (pDC) are able to induce Tregs (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.

On the basis of 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.

Materials 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 5 cases, there was not sufficient clinical data and these were excluded from the survival analysis. Children were classified in 2 age groups ($\leq$10 years vs. >10 years) to investigate the characteristics of macrophages and DCs in younger versus 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 World Health Organization classification (22). All cases were independently reviewed by 2 patholo-

gists (M.H.M. Barros and G. Niedobitek) 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 2 different tumor areas rich in neoplastic cells, were included. TMA blocks were sectioned at a thickness of 3 μm. Antigen retrieval was carried out by heat treatment in a pressure cooker for 1 minute. Buffers used for antigen retrieval and primary antibodies are listed in the Supplementary Material (Supplementary 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 conducted using ZytoChem Plus HRP Polymer Kit (Zytomed Systems; Supplementary Material, Supplementary Table S1), using 3,3'-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 immunohistochemistry against latent membrane protein 1 (LMP1) as described (23).

Computer-assisted microscopic analysis

For cell subset quantitative evaluation, each core was photographed using AxioCam MRc camera (Zeiss) at a ×200 magnification. The numbers of labeled macrophages and DCs were determined per 1 mm^2 using the image analysis software HISTO (Biomas).

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 χ^2 and Fisher exact test were used to test association between dichotomous variables. Mann–Whitney U test was used to compare medians, whereas Spearman rank correlation was used to test association between continuous variables. First-order partial correlations were used to correlate 2 or more variables while keeping constant 1 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

Clinicopathologic 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 of 95, 62.1%) and favorable clinical presentation (50 of 95, 52.6%). Extranodal disease was observed in 11 children (11 of 95, 11.6%). Nodular sclerosis was the predominant subtype (69 of 100, 69%), followed by mixed cellularity (23 of 100, 23%). Granulomas were observed in 37% of cases (37 of 100 cases; ref. 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 histologic 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 Supple-

mentary Table S3. The main clinical and histologic characteristics of the patients are described in the Supplementary Material (Supplementary Table S2).

Macrophages and DCs in pediatric cHL

$CD14^+$ monocyte/precursor cells, as well as $CD163^+$ macrophages, $CD83^+$ and $CD207^+$ DCs were distributed uniformly in the tumor microenvironment, without specific distribution pattern (Fig. 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. In contrast, Langerin$^+$ cells were infrequently detected in the tumor microenvironment (Supplementary Material, Supplementary Fig. S1). No significant bivariate correlation was observed between $CD83^+$, $CD207^+$, and $CD14^+$ cells.

TAMs are associated with clinical and histologic features

The young age group (≤ 10 years) was characterized by a higher number of $CD14^+$ cells (median, 70 vs. 44 cells/mm^2 for the old age group, $P = 0.025$, Mann–Whitney U 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 with nodular sclerosis subtype, mixed cellularity subtype was characterized by higher numbers of $CD14^+$ (median, 105 vs. 49 cells/mm^2 to nodular sclerosis,
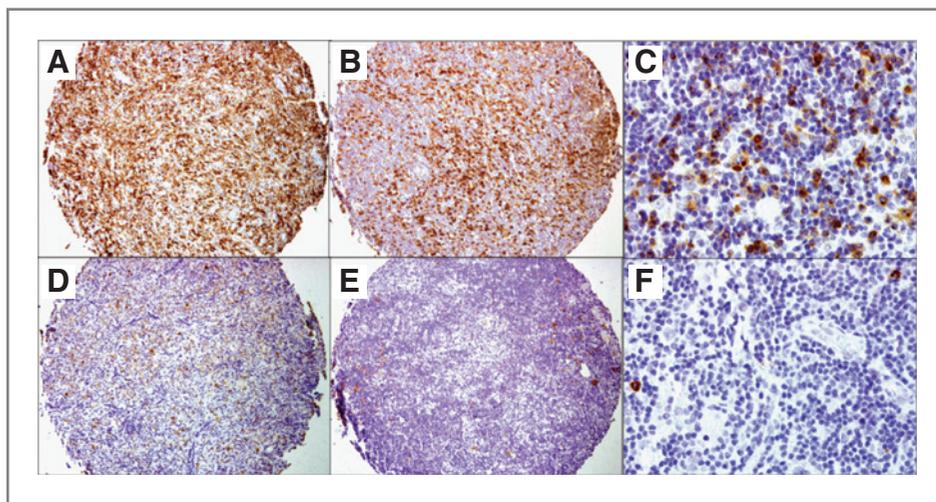

Figure 1. Examples of the diversity of immunostains for antigen-presenting cells markers. Some cases displayed high numbers of $CD68^+$ cells (A), whereas in others, only few $CD68^+$ cells were seen (D; original magnification, ×10). Similarly, there were cases with high numbers of $CD163^+$ cells (B), whereas others exhibited few number of these cells (E; original magnification, ×10). C, a typical example of CD83 expression by DCs (original magnification, ×40). $CD207^+$ cells were infrequent in the tumor microenvironment of pediatric cHL (F; original magnification, ×40).

Table 1. Description of the macrophage and DC subsets in the tumor microenvironment of pediatric cHL and their association with PFS and OS

Variable	Labeled cells/mm^2	Cases analyzed (%)	5-Year PFS rate (%)	Univariate analysis (P) for PFS	5-Year OS rate (%)	Univariate analysis (P) for OS
CD14$^+$ (cells/mm^2)						
Range	0 to 524					
(Mean/median)	(83.1/53)					
≤23 (25th percentile)		20/78 (25.6)	78.9	0.8	85	0.6
>23 (25th percentile)		58/78 (74.4)	75		87	
≤53 (50th percentile)		39/78 (50)	80.6	0.3	84.6	0.6
>53 (50th percentile)		39/78 (50)	71.4		88.6	
CD68$^+$ (cells/mm^2)						
Range	44 to 601					
(Mean/median)	(290.81/284)					
≤194 (25th percentile)		20/81 (24.7)	78.9	0.6	84.2	0.8
>194 (25th percentile)		61/81 (75.3)	73.2		86.4	
≤284 (50th percentile)		43/81 (53.1)	67.5	0.1	85.4	0.9
>284 (50th percentile)		38/81 (46.9)	82.9		86.5	
CD163$^+$ (cells/mm^2)						
Range	0 to 560					
(Mean/median)	(164.1/143)					
≤76 (25th percentile)		20/79 (25.3)	84.2	0.2	85	0.8
>76 (25th percentile)		59/79 (74.7)	71.7		85.5	
≤143 (50th percentile)		40/79 (50.6)	86.8	0.015	89.7	0.2
>143 (50th percentile)		39/79 (49.4)	61.8		80.6	
CD207$^+$ (cells/mm^2)						
Range	0 to 32					
(Mean/median)	(4.32/2)					
=0 (25th percentile)		49/76 (64.5)	74.4	0.6	86.7	0.9
>0 (25th percentile)		27/76 (35.5)	69.2		85.2	
≤2 (50th percentile)		39/76 (51.3)	70.3	0.6	82.1	0.2
>2 (50th percentile)		37/76 (48.7)	75		90.9	
CD83$^+$ (cells/mm^2)						
Range	11 to 410					
(Mean/median)	(66.9/55)					
≤38 (25th percentile)		21/79 (26.6)	80	0.6	90	0.6
>38 (25th percentile)		58/79 (73.4)	75		85.5	
≤55 (50th percenstile)		40/79 (50.6)	77.8	0.7	83.8	0.6
>55 (50th percentile)		39/79 (49.4)	75		89.5	

$P = 0.003$, Mann–Whitney U test) and CD163$^+$ cells (median, 191 vs. 117 cells/mm^2 to nodular sclerosis, $P = 0.027$, Mann–Whitney U test; Supplementary Material, Supplementary Table S3).

Cases with granulomas exhibited a higher number of CD14$^+$ (median, 64 vs. 49 cells/mm^2 without granulomas, $P = 0.04$, Mann–Whitney U test) and CD207$^+$ cells (median 3 vs. 1 cell/mm^2 without granulomas, $P = 0.03$, Mann–Whitney U test). Although not statistically significant, the cases with granulomas also exhibited higher numbers of CD68$^+$ cells (median, 310 vs. 269 cells/mm^2 without granulomas, $P = 0.1$) and CD163$^+$ cells (median, 167 vs. 126 cells/mm^2, $P = 0.2$).

TAM and DCs 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 about the quantitative assessment of lymphocytes have been taken from those studies (refs. 2, 3; Supplementary Material, Supplementary 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 (Supplementary Material, Supplementary Fig. 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 with a more suppressive profile in children older than 10 years (ref. 2; Supplementary Material, Supplementary Table S3). To investigate whether 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 Supplementary Material (Supplementary 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 (Supplementary Material, Supplementary 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 (Supplementary Material, Supplementary 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 ($P = 0.018$; Supplementary Material, Supplementary Table S4).

EBV status of HRS cells is associated with the number of TAM and DCs in pediatric cHL

Because 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 conducted to identify underlying patterns of tumor microenvironment cell subsets according to EBV status (Fig. 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 U 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.
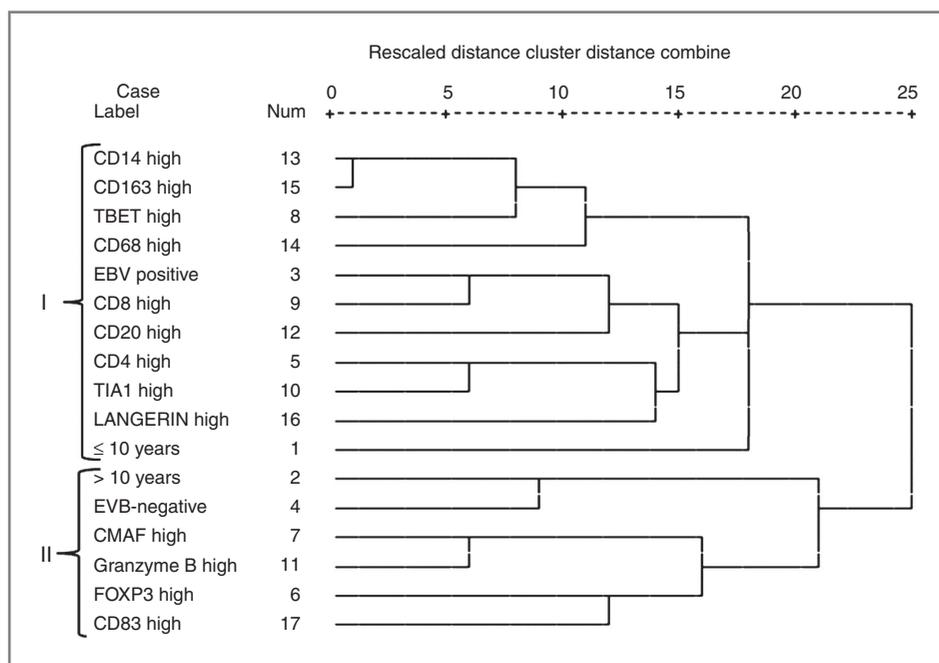

Figure 2. Dendrogram using average linkage obtained by hierarchical cluster analysis. Two main identified clusters (I and II) are indentified by brackets. Num, order of variable input. CD14 high, high number of $CD14^+$ cells/mm² (>50th percentile). CD163 high, high number of $CD163^+$ cells/mm² (>50th percentile). TBET high, high number of $TBET^+$ cells/mm² (>50th percentile). CD68 high, high number of $CD68^+$ cells/mm² (>50th percentile). EBV-positive, EBV-associated cHL. 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 EBV association in cHL. 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).

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 carried out, 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 2 subpopulations in the microenvironment (see earlier). These analyses in EBV-negative cases also disclosed a relationship of CD163+ cells with both TBET+ cells and TIA1+ cells (Table 2).

TAM and DCs 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; Fig. 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% confidence interval (CI), 1.6–31.5], whereas CD163/CD8 ratio showed a borderline significance ($P = 0.06$; HR, 3.3; 95% CI, 0.9–11.6).

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 test), whereas CD68+ cells showed no influence on survival (Fig. 4). Extranodal disease (54.5% vs. 81% for absence of extranodal disease, $P = 0.028$, log-rank test), leukopenia (50% vs. 80% for absence of leukopenia, $P = 0.034$, log-rank test), and presence of granulomas (63.6% vs. 84.5% for absence of granulomas, $P = 0.026$, log-rank test) 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, 1.1–10.2).

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 test; ref. 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. On the basis of this approach, the worst PFS was observed in cases with high numbers of both, CD163+ and granzyme B+ cells ($P = 0.031$, log-rank

Table 2. Correlation between macrophages/DCs and lymphocytes from the tumor microenvironment, according to EBV status

Lymphocytes[a]	CD14+ cells[a]				CD68+ cells[a]				CD163+ cells[a]				CD83+ cells[a]				CD207+ cells[a]			
	EBV+		EBV−		EBV+		EBV−		EBV+		EBV−		EBV+		EBV−		EBV+		EBV−	
	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
CD3+	↓	0.03	—	—	↓	0.04	—	—	—	—	—	—	—	—	—	—	—	—	↑	0.038
CD4+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
FOXP3+	—	—	—	—	—	—	↑	0.001	—	—	—	—	↑	0.047	↑	0.05	—	—	—	—
TBET+	↑	0.009	↑	0.014	—	—	—	—	—	—	↑	0.022	—	—	↑	0.045	—	—	—	—
CD8+	—	—	↑	0.028	—	—	—	—	—	—	↑	0.003	↑	0.002	—	—	—	—	—	—
TIA1+	—	—	↑	0.04	—	—	↑	0.01	—	—	↑	0.01	—	—	—	—	—	—	—	—
Granzyme B+	↑	0.003	—	—	↑	0.002	↑	0.034	—	—	↑	0.028	—	—	—	—	—	—	—	—
CD20+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	↑	0.001	—	—	—	—

NOTE: The P values are from point biserial Spearman correlations.

Abbreviations: C, correlation; —, no statistical correlation; EBV+, EBV association; EBV−, absence of EBV association; ↑, direct correlation; ↓, inverse correlation.
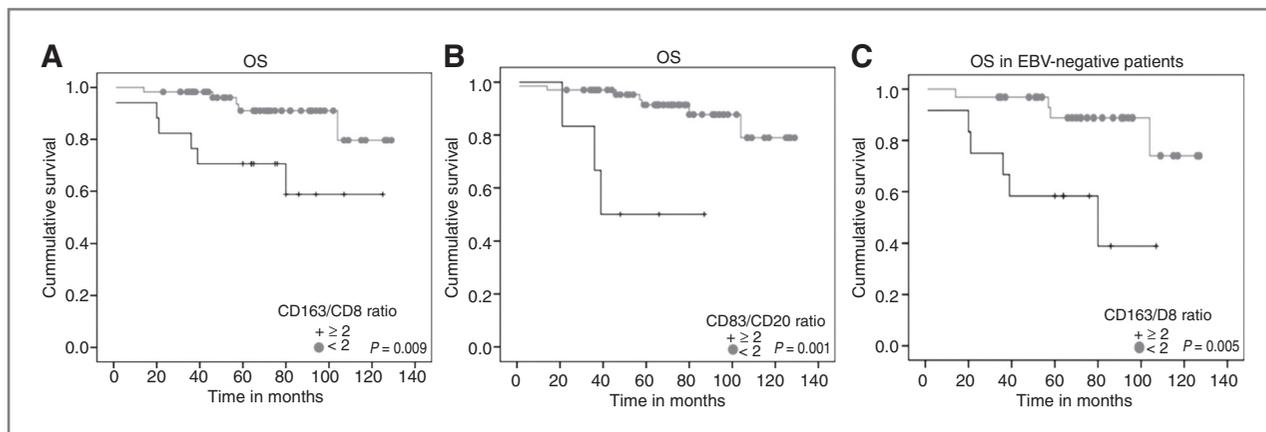
[a] Number of cells/mm2.

Figure 3. Kaplan–Meier curves of variables associated with OS in pediatric cHL. A, OS according to CD163/CD8 ratio. B, OS according to CD83/CD20 ratio. C, OS according to CD163/CD8 ratio in EBV-negative patients.

test; Fig. 4). In the Cox regression, extranodal disease ($P = 0.01$; HR, 4.1; 95% CI, 1.4–12.1) and the combination of CD163$^+$ and granzyme B$^+$ cells ($P = 0.038$; HR, 2.2; 95% CI, 1.0–4.9) 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 (>50th percentile; 44.4%, $P = 0.008$, log-rank test), whereas no influence of a high number of CD163$^+$ macrophages was observed in the EBV-positive group, although this was the group that 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 test; Fig. 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 test; Fig. 3).

Discussion

In comparison with adults, children display distinct physiologic immunologic 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
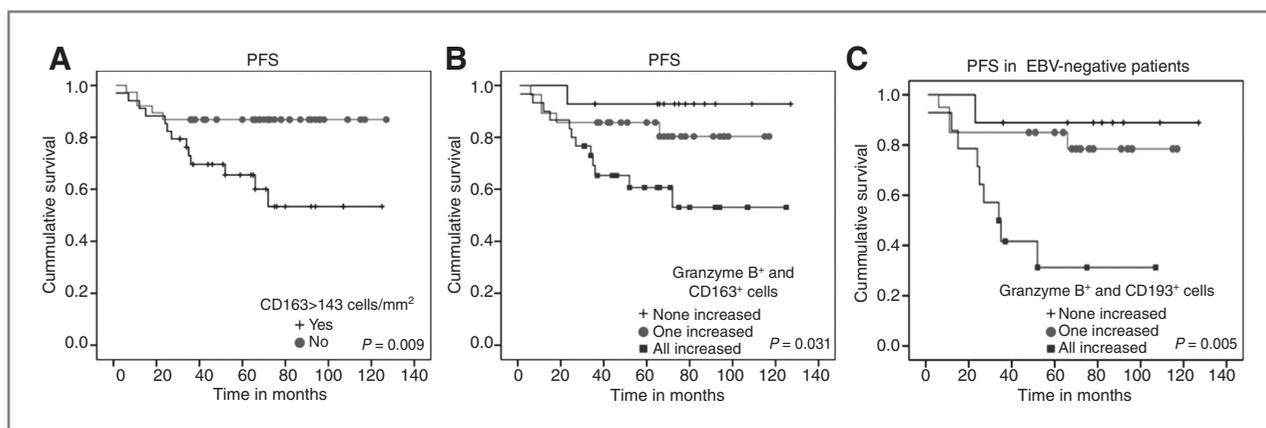

Figure 4. Kaplan–Meier curves of variables associated with PFS in pediatric cHL. A, PFS according to number of CD163$^+$ cells. B, PFS according to combined numbers of CD163$^+$ cells and granzyme B$^+$ cells. C, PFS according to combined numbers of CD163$^+$ cells and granzyme B$^+$ cells in EBV-negative patients.

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 shown previously, that pediatric cHL cases in the ≤10 years age group are characterized by a cytotoxic immune profile as showed 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 physiologic phenomenon. CD14 is expressed on cells of the myelomonocytic lineage, including monocytes, a proportion of macrophages and Langerhans cells (26). Because 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 represent 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 T$_H$1 cells, that is, CD8$^+$ 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/T$_H$1 cells to the tumor microenvironment (2). In addition, a putative cross-talk of T$_H$1 and CD8$^+$ 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 nontumoral 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 numbers of Tregs. 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 protumoral 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.

TAMs 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 used 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 PFS 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 patients with cancer is controversial; in some studies a higher number of these cells was associated with a better outcome (38, 39), whereas 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 $\geq$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, a subtype of DC (43). Recently, it has been suggested that the presence of CD207^{+} Langerhans cells in the tissue microenvironment may be an exclusive feature of EBV-associated disease, including cHL (44). Our results are different in 2 respects. First, 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, for example, 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 immunologic evaluation conducted 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: M.H.M. Barros, G. Niedobitek
Development of methodology: M.H.M. Barros
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.H.M. Barros, G. Niedobitek
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.H.M. Barros, R. Hassan
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.H.M. Barros
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Tumor-Associated Macrophages in Pediatric Classical Hodgkin Lymphoma: Association with Epstein-Barr Virus, Lymphocyte Subsets, and Prognostic Impact

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