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ORIGINAL INVESTIGATION

The interplay between local immune response and Epstein–Barr virus-infected tonsillar cells could lead to viral infection control

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Abstract

Epstein Barr virus (EBV) gains access to the host through tonsillar crypts. Our aim was to characterize microenvironment composition around EBV+ cells in tonsils from pediatric carriers, to disclose its role on viral pathogenesis. LMP1 expression, assessed by immunohistochemistry (IHC), was used to discriminate EBV + and – zones in 41 tonsil biopsies. Three regions were defined: Subepithelial (SE), interfollicular (IF) and germinal center (GC). CD8, GrB, CD68, IL10, Foxp3, PD1, CD56 and CD4 markers were evaluated by IHC; positive cells/100 total cells were counted. CD8+, GrB+, CD68+ and IL10+ cells were prevalent in EBV+ zones at the SE region ($p < 0.0001$, $p = 0.03$, $p = 0.002$ and $p = 0.002$ respectively, Wilcoxon test). CD4+ and CD68+ cell count were higher in EBV + GC ($p = 0.01$ and $p = 0.0002$ respectively, Wilcoxon test). Increment of CD8, GrB and CD68 at the SE region could indicate a specific response that may be due to local homing at viral entry, which could be counterbalanced by IL10, an immunosuppressive cytokine. Additionally, it could be hypothesized that CD4 augment at the GC may be involved in the EBV-induced B-cell growth control at this region, in which macrophages could also participate.

Keywords Epstein Barr virus · Tonsil · Children · Immune response

Introduction

More than 90% of the worldwide population carries Epstein–Barr virus (EBV), which has sophisticatedly evolved to persist life-long in memory B lymphocytes of infected individuals [1]. Disruption of this tightly regulated B cell infection could result in EBV-associated B cell lymphomas [2]. EBV is transmitted through saliva and gets access to submucosal B cells by transmigration across oral epithelial cells or through areas of the tonsillar crypt epithelium in which tight junctions are disrupted [3].

Classical B cell growth transformation is achieved through the action of nine latent antigens: EBV nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C and LP; latent membrane proteins (LMPs) 1 and 2 [4]; and non-coding RNAs (EBERs). Their expression defines three patterns: latency III expresses all viral antigens; in latency II non-coding RNAs, EBNA1, LMP1 and -2 are expressed; whereas latency I only displays the non-coding RNAs as well as EBNA1 [5]. Many of these viral proteins elicit both antibody and cell-mediated immune responses, but the latter appear to be crucial controlling primary infection as well as limiting reactivation of

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the persistent infection from its latent reservoir. The main effectors of these cellular responses are natural killer (NK), CD4+ and CD8+ T cells [4].

In developing countries and lower socioeconomic groups in developed populations, primary EBV infection occurs in early childhood, and results in a subclinical infection with few, if any symptoms. However, if infection is delayed until adolescence, around 25–75% of infected individuals will develop infectious mononucleosis (IM) [6]. EBV infection in children below 10 years old are often overlooked, either because they are entirely asymptomatic or because they do not present with a typical IM syndrome [7]. In Argentina, EBV infection is mostly subclinical and 90% of patients are seropositive by 3 years, resembling an underdeveloped population. Furthermore, EBV is statistically associated to B cell lymphoma in patients younger than 10 years, which may suggest a close relationship between low age of EBV seroconversion and higher risk of B cell lymphoma development [8].

In adult patients undergoing primary infection, an immunological disturbance, mainly driven by the expansion of activated CD8+ T cells, was described once the symptoms appeared. In fact, homogenized tonsillar preparations taken from IM patients showed a substantial number of EBV-specific CD8+ T cells, along with high viral load. In contrast, there is little evidence of antigen-driven monoclonal or oligoclonal expansion of CD4+ T cells [6]. The scenario in children is quite different, since they exhibited elevated amounts of activated EBV-specific CD8+ T cells without a significant global expansion of this compartment [9]. NK cells also have a key role in early response against EBV infection in children, preventing the development of IM [10, 11]. Once the infection has been established, an enrichment of EBV-specific CD8+ T cells is seen in the tonsil, whereas the memory CD4+ T cell response to individual epitopes is less significant [6].

It is recognized that T cells, B cells and macrophages in the microenvironment plays an active role in the multiple steps of lymphomagenesis [12]. EBV influence on microenvironment composition has been largely characterized in Hodgkin lymphoma (HL), in which a coexistence of functional Th1 cell infiltrate with T regs cells was described, and a marked number of activated CD8+ T cells and natural killer (NK) cells were observed [13]. Furthermore, a pronounced infiltration of macrophages associated with EBV presence was also illustrated [14]. Specifically in pediatric HL, EBV+ cases younger than 10 years old displayed an intense cell infiltrate, exhibiting a cytotoxic/Th1 profile in addition with M1 polarization [15–17].

Given that immune cell composition surrounding infected zones could be involved in viral control, our aim was to characterize it around EBV+ compared to EBV− zones at the tonsil, to disclose possible differences that reveal the interplay between EBV presence and immune cells and their potential role in the pathogenesis of EBV infection in our pediatric population.

Materials and methods

Patients and samples

This study was conducted in a cohort of 48 children aged between 2 and 14 years (median 7 years) attending Otorhinolaryngology Service at the Ricardo Gutierrez Children's Hospital, Buenos Aires, Argentina. Samples were surgically removed during routine tonsillectomy. Tonsillar hyperplasia was diagnosed according to international routine protocols for recurrent chronic inflammation. Tonsils were not acutely swollen at the time of removal [18]. Formalin-fixed paraffin-embedded (FFPE) samples were retrospectively selected for the present study. EBV presence was assessed by immunohistochemistry (IHC) as mentioned below, and all patients were defined as carriers, based on previous epidemiological findings in Argentina, and lack of reported IM symptoms on medical records [8, 19]. Serological information for other viruses from the studied sample patients was not available on medical records.

Institutional guidelines regarding human experimentation were followed, according to the Helsinki Declaration of 1975. The protocol was approved by the Ethical Committee of our hospital, and written informed assent and consent was obtained from all patients or patient's parents depending on age.

Given that EBV epidemiological characteristics resemble those of an underdeveloped or developing population, it is very difficult to find EBV seronegative patients. Therefore, it was very difficult to include more EBV-negative patients in our series. The Otorhinolaryngology Service in our institution only performs tonsillectomy due to recurrent chronic inflammation, for that reason, we were unable to compare tonsil from EBV carriers with tonsils neither from EBV-associated malignancies nor autoimmune disease.

EBV detection and infected zone delimitation

IHC for LMP1 (mAb CS1-4, Dako) and EBNA2 (clones 1E6 and R3, kindly provided by Dr. Kremmer, Institut fur Immulogie, Munchen, Germany) was used to detect and localize EBV latent protein expression. A FFPE EBV-positive cell line was used as positive control, whereas an isotype control was used as negative control, according to each immune staining in specific tissue. We defined latency profiles based on LMP1 and EBNA2 expression, as has been previously described [19].

LMP1 expression was employed to discriminate EBV+ and EBV− cases and positive and negative zones within them, given the fact that we have previously observed LMP1+ expression in all EBERs+ cases (18), even in a few EBERs- ones. To validate the results made in the EBV− zones from EBV+ cases that were used in the present work; we contrasted immune cell counts with a group of EBV− cases ($n=7$). As this analysis displayed no statistical differences ($p>0.05$, Mann–Whitney test), we considered valid to compare microenvironment composition around infected and non-infected zones within EBV-positive cases.

Immune cell detection and cell counting

IHC was performed on 4 µm serial FFPE tonsil sections to characterize cell populations with the following antibodies: CD8 for cytotoxic T lymphocyte (CTL) (clone SP57, Ventana Roche, Tucson, USA), Granzyme B (GrB) for activated cytotoxic cells (clone GB7, AbD Serotec, Oxford, UK), CD68 for macrophages (clone KP-1, Ventana Roche), IL10 for anti-inflammatory cytokine-productive cells (Abcam), Foxp3 for regulatory T lymphocytes (Treg) (Abcam, Cambridge, UK), PD1 (CD279) for T cell-negative regulator (AbD Serotec), CD56 for NK cells (Leica, Buffalo, IL, USA) and CD4 for T helper (Th) (Ventana Roche). Lymph node reactive hyperplasia tissue was used as positive control. Negative controls for each case consisted in substituting the primary antibody with antibody dilution buffer and an isotype control. The stains were developed using diaminobenzidine (DAB).

Given the fact that we previously characterized viral antigen expression in lymphocytes at germinal center (GC), interfollicular (IF) and subepithelial (SubEp) regions, the same approach was used for microenvironment characterization at those three histological regions [19, 20].

All cell markers were observed and counted by two pathologists in serial slides on the basis of the best-preserved areas around LMP1+ and LMP1− zones. The results were expressed as immunopositive cells *n*°/100 total cell *n*°.

Statistical analysis

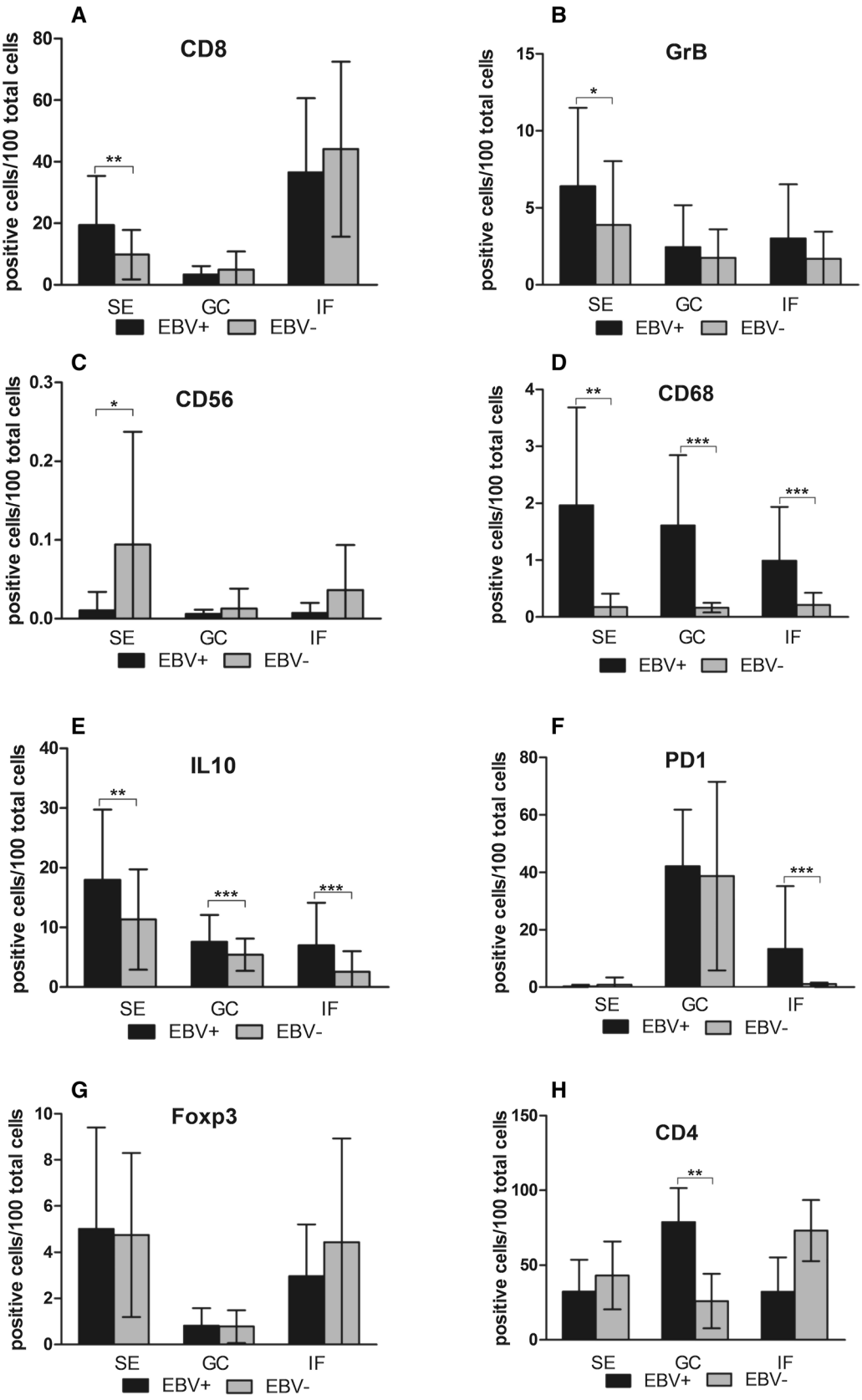
Statistical analysis was performed using GraphPad Prism 5 and InStat 3 software (GraphPad Software Inc., San Diego, California USA). Kolmogorov–Smirnov test was used to test the normal distribution of the data. Wilcoxon matched pairs test was used to compare means by ranking of cellular markers between LMP1+ and LMP1− zones. Correlations between data were determined using Spearman's rank correlation index. All tests were two-tailed, $p<0.05$ was considered statistically significant.

Results

Viral presence was assessed by LMP1 expression, given that we have previously observed that all cases with EBERs also showed LMP1 positivity [19]. Based on this observation, EBV+ and EBV− zones were defined within the tonsil sample of each EBV+ case to disclose cellular immune composition specifically in the vicinity of EBV-infected cells. LMP1-positive staining was observed in 41/48 cases analyzed. The seven EBV− cases were used to validate the results made in the EBV− zones defined by LMP1 expression. As this analysis displayed no statistical differences ($p>0.05$, Mann–Whitney test), we considered valid to compare microenvironment composition around infected and non-infected zones within EBV-positive cases. Once those zones were defined, serial slides were stained for CD8, GrB, CD68, IL10, Foxp3, PD1, CD56 and CD4 for microenvironment characterization. In addition, viral latent antigens were assessed by LMP1 and EBNA2 IHC expression, indicating EBV latency II and III patterns, respectively, while latency I was not detected [19].

LMP1 expression was observed in three main tonsillar histological locations: Subepithelial (SE), interfollicular (IF) and germinal center (GC) regions. Positive cells for each immune cell marker were counted in each histological region, and their quantity was compared between EBV+ and EBV− zones (Fig. 1). Median and *p* values are summarized in Table 1. All cases displayed LMP1 expression at these three locations, whereas EBNA2+ staining was observed exclusively in macrophage cytoplasm, probably due to phagocytosis of infected cells (Electronic Supplementary Material Fig. 1). Since we did not find EBNA2+ lymphocytes, we assume these 41 cases as EBNA2−, and categorized them showing Latency II.

When cell count were compared according to EBV presence and considering histological regions individually, only the SE region displayed a statistical higher mean value of CD8+ cells around the LMP1+ milieu ($p<0.0001$, Wilcoxon test) (Figs. 1a, 2). Furthermore, the ratio CD4/CD8 cells comparing EBV+ SE region versus EBV− one was statistically significant ($p=0.0173$, Wilcoxon test). In line with this, GrB+ cells were also statistically increased exclusively around EBV+ zone at SE region ($p=0.03$, Wilcoxon test). Conversely, NK+ cells counts were smaller in EBV+ SE zones ($p=0.0177$, Wilcoxon test) (Fig. 1b, c). Macrophage presence at the EBV+ microenvironment, evaluated by CD68 expression, was considerably increased in each of the three histological locations studied, namely SE ($p=0.002$, Wilcoxon test), GC ($p=0.0002$, Wilcoxon test) and IF ($p=0.001$, Wilcoxon test) regions (Fig. 1d). IL10 was also statistically prevalent


A
CD8
positive cells/100 total cells
80
60
40
20
0
**
SE
GC
IF
EBV+
EBV-
B
GrB
positive cells/100 total cells
15
10
5
0
*
SE
GC
IF
EBV+
EBV-
C
CD56
positive cells/100 total cells
0.3
0.2
0.1
0.0
*
SE
GC
IF
EBV+
EBV-
D
CD68
positive cells/100 total cells
4
3
2
1
0
**
***
***
SE
GC
IF
EBV+
EBV-
E
IL10
positive cells/100 total cells
40
30
20
10
0
**
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***
SE
GC
IF
EBV+
EBV-
F
PD1
positive cells/100 total cells
80
60
40
20
0
***
SE
GC
IF
EBV+
EBV-
G
Foxp3
positive cells/100 total cells
10
8
6
4
2
0
SE
GC
IF
EBV+
EBV-
H
CD4
positive cells/100 total cells
150
100
50
0
**
SE
GC
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EBV+
EBV-

◂**Fig. 1** Comparison of immune cells markers (**a** CD8; **b** GrB; **c** CD56; **d** CD68; **e** IL10, **f** PD1, **g** Foxp3 and **h** CD4) analyzed according to EBV status, determined by LMP1 expression, in tonsillar hyperplasia. Positive cells for each immune cell marker were counted in three histological regions: Subepithelial (SE), germinal center (GC) and interfollicular (IF). Bar-plot graph shows the mean ± SD distribution of each cell subset studied in the tonsil microenvironment. Light-gray bars indicate EBV− region and dark-gray bars indicate EBV+ region. The p value is from Wilcoxon test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

surrounding LMP1+ histological regions separately (SE $p = 0.002$, GC $p = 0.03$, IF $p < 0.0001$, Wilcoxon test) (Fig. 1e). In contrast, PD1+ cells were increased exclusively at the LMP1+ IF region ($p = 0.004$, Wilcoxon test) (Fig. 1f). Foxp3 expression, marker of regulatory T cells, displayed no statistical differences regarding EBV status in any histological region analyzed ($p > 0.05$, Wilcoxon test) (Fig. 1g). Finally, CD4+ cell counts were statistically higher at the LMP1+ GC region ($p = 0.01$, Wilcoxon test) (Table 1; Fig. 1h). Moreover, this observation was reinforced by the statistical difference in the CD4/CD8 cells ratio between EBV+ GC region versus EBV-one ($p = 0.0004$, Wilcoxon test).

We further continued the study of the microenvironment characteristics in the vicinity of infected and non-infected cells by means of a correlation analysis between immune cell populations according to LMP1 status of the region. Regarding EBV+ zones, a statistically significant direct correlation was observed between the number of CD68+ cells either with IL10+ cells ($r = 0.77$; $p = 0.003$, Spearman's correlation test) or with CD4+ cells ($r = 0.70$; $p = 0.008$, Spearman's correlation test) in GC. Remarkably, this analysis in LMP1− zones showed no statistically significant correlation.

It is well known that early age of acquisition impacts the host's ability to control primary EBV infection, mainly due to immune system maturation [21]. Therefore, to assess this observation, each cell population according to LMP1 status was correlated with patients' age. Remarkably, positive correlation between CD68+ cells and age was demonstrated in nearly all the LMP1+ histological regions (SE: $r = 0.71$, $p = 0.006$; IF: $r = 0.66$, $p = 0.01$; Spearman's correlation test), while at the LMP1− zones this correlation was not proved. Furthermore, this correlation was reinforced in patients younger than 7 years (SE: r = 0.72, $p = 0.013$; IF: $r = 0.71$, $p = 0.0002$; Spearman correlation test), In line with this, IL10+ and PD1+ cells in the presence of EBV displayed a statistical positive correlation with age at the GC and SE regions, respectively ($r = 0.551$, $p = 0.002$; $r = 0.526$, $p = 0.0002$; respectively, Spearman's correlation test), whereas this finding was absent around all EBV− zones.

Discussion

In Argentina, most children are infected by EBV during early childhood [8], in contrast to developed populations, where adolescents are primarily infected and often develop IM. Infections during childhood tend to have minor self-limiting illnesses that often go undetected, and still it is not fully understood why this asymptomatic EBV infections during childhood happens. There are only few studies about EBV-immune pathogenesis performed in the context of asymptomatic infections [9, 22]. Even though EBV is associated with several malignancies, the immune system is usually successful in prevent disease despite persistent infection [3]. To understand this delicate balance, our aim was to characterize the immune cellular composition of the microenvironment in the vicinity of EBV-infected cells from a cohort of asymptomatic EBV pediatric carriers.

Microenvironment composition in EBV-associated tumors has been described in pediatric as well as in adult HL patients, in which EBV exhibits the ability to generate an immunosuppressive milleu [15–17, 23, 24]. However, the interplay between virus and immune environment in a benign condition such as pediatric virus carriers is still unexplored. In adults with IM, EBV-specific CD8+ T cell numbers were far lower in tonsils than periphery, despite very high virus genome loads found in these tonsillar preparations [6]. In contrast, once the infection was solved, tonsils from adult carriers were enriched with EBV-specific CD8+ T cells against latent viral antigens, probably destined for appropriate homing to oropharyngeal sites, where growth transformation of B cells requires being controlled [25]. Asymptomatic EBV infection in children from Africa elicits a virus-specific CD8+ T cell response in peripheral blood that controls the infection without general over-expansion [9]. In our pediatric series, CD8+ cells were predominantly around EBV-infected cells at the SE region. It has been demonstrated a key role of NK cells in EBV infection early response in children [10, 11]. In our series, NK cell response around EBV-infected cells is not recruited at the SE region to restrict viral spread. In addition, we previously described that in younger children, the presence of viral antigens was preferentially restricted to subepithelial B lymphocytes [19]. Therefore, specific CD8+ cell presence may be due to local recruitment at the site of viral entry, to control EBV infection. Furthermore, GrB+ cells at the LMP1+ SE region were also increased, suggesting that cytotoxic activity could play a key role involving control at the site of viral infection and reactivation. This increment of a cytotoxic marker at the SE region could be related to CD8+ CTL cells. An increment in GrB expression was also described in EBV-associated malignancies such as pediatric HL [15] and pediatric diffuse

Table 1 Comparisons of median and *p* values from counted immune cells markers (A. CD8; B. GrB; C. CD68; D. IL10; E. PD1, F. Foxp3, G. CD56 and H. CD4) analyzed according to LMP1 expression, in tonsils from children. Each immune cell marker was counted in three histological regions: Subepithelial (SE), germinal center (GC) and interfollicular (IF)

	SE		GC		IF	
	EBV+	EBV−	EBV+	EBV−	EBV+	EBV−
CD8						
p	< 0.0001***		> 0.05		> 0.05	
Median	17	8	3	4	33	42
GrB						
p	0.03*		> 0.05		> 0.05	
Median	6	3	1.25	1.00	2	1
CD56						
p	0.02*		> 0.05		> 0.05	
Median	0.003	0.004	0.006	0.000	0.003	0.001
CD68						
p	0.002**		0.0002***		0.001**	
Median	2	0	2	0.1	0.7	0.2
IL10						
p	0.002**		0.03*		< 0.0001***	
Median	15	10	6	5	5	2
PD1						
p	> 0.05		> 0.05		0.004**	
Median	0.00	0.00	37	30	6	1
Foxp3						
p	> 0.05		> 0.05		> 0.05	
Median	3	4	0.6	0.5	3	4
CD4						
p	> 0.05		0.01*		> 0.05	
Median	27	22	39	29	86	72

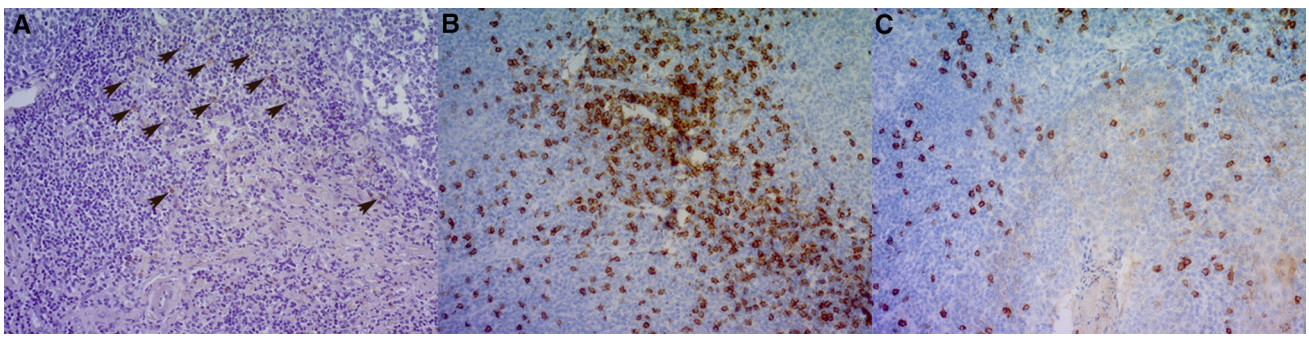

Fig. 2 Expression of latent membrane protein 1 (LMP1) and CD8 in tonsils from pediatric EBV carriers by IHC. **a** Membranous localization of LMP1 in lymphocyte around crypt region. **b** Membranous localization of CD8+ cells on serial FFPE slides located in the vicinity of LMP1+ lymphocytes at the crypt. **c** Membranous localization of CD8+ cells on serial FFPE slides located in the vicinity of LMP1- lymphocytes at the same case but other crypt. The stains were developed using diaminobenzidine (DAB). Digital images were obtained with an AxioCamErc 5 s (Zeiss) camera and acquired using Digital Axio Vision Rel. 4.8 image acquisition software. Original magnification ×200

large B cell lymphoma [26]; but, as far as we know, this is the first report to describe this feature in pediatric carriers.

Moreover, an expansion in CD68+ cell numbers at the LMP1+ SE region was also established. This macrophage proliferation could occur perhaps in response to EBV presence in this region to limit the spread of the infection in the host and to control LMP1 expression. Furthermore, EBNA2+ staining was only observed within macrophage cytoplasm, in line with EBNA3A-specific staining exclusively found in SE macrophages that we have previously reported [19]. These observations could probably reflect a phagocytosis of cells expressing latency antigens, therefore,

reducing the amount of infected cells, perhaps collaborating with CD8+ cell expansion at the same region. Macrophages are thought to be relevant for EBV recognition and certain innate immune receptors, as TLR2, TLR3 and TLR9, have been implicated in this process in vitro [27, 28]. However, TLR2 interaction was described to be involved in the secretion of IL10 in response to EBV infection, among other mechanisms activated by the virus that could contribute to the release of this cytokine [28]. Actually, IL10 expression was significantly higher around LMP1+ cells located at the SE region in our carriers, probably triggered by viral presence, to impair effective immune response.

In EBV + HL, markers of suppression such as IL10 are also raised, along with an increase of the IL10 secreting CD4+ T cells in the surroundings of EBV+ tumor cells. This suggests that the expression of viral proteins in these cells could enable their escape from the virus-specific CTL response [29]. It seems plausible that at the SE region, a delicate balance between phagocytic–cytotoxic immune response (CD68+, CD8+ and GrB+ cells) is counterbalanced by immunosuppressive features, such as IL10 presence. We may think that immune response surrounding subepithelial EBV-infected cells could lead to an effective control of the infection, dropping the number of infected cells but allowing a small percentage of circulating infected memory B lymphocytes to remain for life in young children. Given positive correlation of CD68+ cells with age, we can speculate that recruitment of macrophages begins at young ages and increases at older ages, leading to an efficient immune response against EBV infection.

GCs are the histological structures dedicated to B cell maturation. Distinct types of GC-derived lymphomas originate from cells that are blocked at different stages of this process [30, 31]. Therefore, the GC is one of the most important regions to study EBV biology in normal carriers to disclose the lymphomagenesis process. The GC structure is comprised of a dark zone, which contains highly proliferating B cells, and a light zone in which B cells are mixed together with follicular dendritic cells, T cells and macrophages [30]. In our series, CD4+ cell count at GC was higher in EBV+ than in EBV− zones. CD4+ T cell responses are induced against a variety of epitopes within latent cycle antigens. Even though they are at least tenfold smaller than the corresponding CD8+ T cell responses [32], it seems that, when EBV+ zones are discriminated, T helper response could be involved at the GC region. Since EBV infects and persists in B cells, which constitutively express MHC class II, CD4+ T cells may be able to act as direct effectors, or at least provide support to the overall immune response [6]. Moreover, the positive correlation between CD4+ and CD68+ cells at the GC could suggest the local cooperation between both subpopulations to response against EBV infection. CD4+ T cells can recognize and kill EBV-transformed B cells [33], while they may reduce proliferative capacity by releasing soluble factors [34]. As LMP1 is the most important oncogenic viral protein, we may hypothesize that the increase in both CD4+ and CD68+ cells around LMP1+ GC region observed in our series could be related to an efficient recruitment destined to control EBV-induced B cell growth, in contrast to previous suggestion studied in IM patients [9].

B cells within GC-expressing high-affinity antibodies develop and differentiate into antibody-secreting plasma cells and memory B cells [35], whereas GC B cells with low-antigen affinity and autoreactivity are eliminated via apoptosis and cleared by macrophages [34]. At the EBV+ GC region, we observed a considerable augment in CD68+ expression, suggesting that, as previously proposed here for the EBV+ SE region, macrophage activity could play an important role in EBV infection control at this LMP1+ zone. Besides the phagocytosis activity, the production of IL10 by phagocytes during clearance of apoptotic cells is critical to prevent inflammatory and autoimmune reactions in a physiological state, through its immunosuppressive function [36]. We found that IL10+ cells were significantly prevalent at the EBV+ GC, whereas its presence also correlates with CD68+ expression exclusively at this region, either to prevent inflammatory development or as an immune evasion mechanism in the context of an EBV+ environment.

In summary, to the best of our knowledge, this is the first report deeply characterizing microenvironment composition around EBV-infected cells in pediatric carriers from a developing population. This study reveals that local response at the site of viral entry, in particular at the SE and GC regions of the tonsils, is well mounted. Additionally, that the immune cell populations, described in this study, could play a specific role in each analyzed region; either participating directly as effector cells, or by helping to the overall immune response. Ultimately, this joint between innate-and-acquired immunity may control the infection at the site of viral entry. Our results shed some light on the interplay between EBV infection and local microenvironment in normal pediatric carriers from a developing country.

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Compliance with ethical standards

Institutional guidelines regarding human experimentation were followed, according to the Helsinki Declaration of 1975. The protocol was approved by the Ethical Committee of our hospital, and written informed assent and consent was obtained from all patients or patient´s parents depending on age.

Conflict of interest The authors declare no conflict of interest.

References

1. Ok CY, Li L, Young KH (2015) EBV-driven B-cell lymphoproliferative disorders: from biology, classification and differential diagnosis to clinical management. Exp Mol Med 47:e132. https://doi.org/10.1038/emm.2014.82
2. Young LS, Yap LF, Murray PG (2016) Epstein–Barr virus: more than 50 years old and still providing surprises. Nat Rev Cancer 16(12):789–802. https://doi.org/10.1038/nrc.2016.92
3. Münz C (2016) Epstein Barr virus—a tumor virus that needs cytotoxic lymphocytes to persist asymptomatically. Curr Opin Virol 20:34–39. https://doi.org/10.1016/j.coviro.2016.08.010
4. Rickinson AB, Long HM, Palendira U, Munz C, Hislop AD (2014) Cellular immune controls over Epstein–Barr virus infection: new lessons from the clinic and the laboratory. Trends Immunol 35(4):159–169. https://doi.org/10.1016/j.it.2014.01.003
5. Mesri EA, Feitelson MA, Munger K (2014) Human viral oncogenesis: a cancer hallmarks analysis. Cell Host Microbe 15(3):266–282. https://doi.org/10.1016/j.chom.2014.02.011
6. Hislop AD, Taylor GS (2015) T-cell responses to EBV. Curr Top Microbiol Immunol 391:325–353. https://doi.org/10.1007/978-3-319-22834-1_11
7. Odumade OA, Hogquist KA, Balfour HH Jr (2011) Progress and problems in understanding and managing primary Epstein–Barr virus infections. Clin Microbiol Rev 24(1):193–209. https://doi.org/10.1128/CMR.00044-10
8. Chabay PA, Preciado MV (2013) EBV primary infection in childhood and its relation to B-cell lymphoma development: a mini-review from a developing region. Int J Cancer 133(6):1286–1292. https://doi.org/10.1002/ijc.27858
9. Jayasooriya S, de Silva TI, Njie-Jobe J, Sanyang C, Leese AM, Bell AI, McAulay KA, Yanchun P, Long HM, Dong T, Whittle HC, Rickinson AB, Rowland-Jones SL, Hislop AD, Flanagan KL (2015) Early virological and immunological events in asymptomatic Epstein–Barr virus infection in african children. PLoS Pathog 11(3):e1004746. https://doi.org/10.1371/journal.ppat.1004746
10. Chijioke O, Muller A, Feederle R, Barros MH, Krieg C, Emmel V, Marcenaro E, Leung CS, Antsiferova O, Landtwing V, Bossart W, Moretta A, Hassan R, Boyman O, Niedobitek G, Delecluse HJ, Capaul R, Munz C (2013) Human natural killer cells prevent infectious mononucleosis features by targeting lytic Epstein–Barr virus infection. Cell Rep 5(6):1489–1498. https://doi.org/10.1016/j.celrep.2013.11.041
11. Azzi T, Lunemann A, Murer A, Ueda S, Beziat V, Malmberg KJ, Staubli G, Gysin C, Berger C, Munz C, Chijioke O, Nadal D (2014) Role for early-differentiated natural killer cells in infectious mononucleosis. Blood 124(16):2533–2543. https://doi.org/10.1182/blood-2014-01-553024
12. Dolcetti R (2015) Cross-talk between Epstein–Barr virus and microenvironment in the pathogenesis of lymphomas. Semin Cancer Biol 34:58–69. https://doi.org/10.1016/j.semcancer.2015.04.006
13. Wu R, Sattarzadeh A, Rutgers B, Diepstra A, van den Berg A, Visser L (2016) The microenvironment of classical Hodgkin lymphoma: heterogeneity by Epstein–Barr virus presence and location within the tumor. Blood Cancer J 6:e417. https://doi.org/10.1038/bcj.2016.26
14. Chetaille B, Bertucci F, Finetti P, Esterni B, Stamatoullas A, Picquenot JM, Copin MC, Morschhauser F, Casasnovas O, Petrella T, Molina T, Vekhoff A, Feugier P, Bouabdallah R, Birnbaum D, Olive D, Xerri L (2009) Molecular profiling of classical Hodgkin lymphoma tissues uncovers variations in the tumor microenvironment and correlations with EBV infection and outcome. Blood 113(12):2765–3775. https://doi.org/10.1182/blood-2008-07-168096
15. Barros MH, Vera-Lozada G, Soares FA, Niedobitek G, Hassan R (2012) Tumor microenvironment composition in pediatric classical Hodgkin lymphoma is modulated by age and Epstein–Barr virus infection. Int J Cancer 131(5):1142–1152. https://doi.org/10.1002/ijc.27314
16. Barros MH, Hassan R, Niedobitek G (2012) Tumor-associated macrophages in pediatric classical Hodgkin lymphoma: association with Epstein–Barr virus, lymphocyte subsets, and prognostic impact. Clin Cancer Res 18(14):3762–3771. https://doi.org/10.1158/1078-0432.CCR-12-0129
17. Barros MH, Segges P, Vera-Lozada G, Hassan R, Niedobitek G (2015) Macrophage polarization reflects T cell composition of tumor microenvironment in pediatric classical Hodgkin lymphoma and has impact on survival. PloS One 10(5):e0124531. https://doi.org/10.1371/journal.pone.0124531
18. Strowig T, Brilot F, Arrey F, Bougras G, Thomas D, Muller WA, Munz C (2008) Tonsilar NK cells restrict B cell transformation by the Epstein–Barr virus via IFN-gamma. PLoS Pathog 4(2):e27. https://doi.org/10.1371/journal.ppat.0040027
19. Vistarop AG, Cohen M, De Matteo E, Preciado MV, Chabay PA (2016) Analysis of Epstein–Barr virus infection models in a series of pediatric carriers from a developing country. Sci Rep 6:23303. https://doi.org/10.1038/srep23303
20. Fossum CC, Chintakuntlawar AV, Price DL, Garcia JJ (2016) Characterization of the oropharynx: anatomy, histology, immunology, squamous cell carcinoma and surgical resection. Histopathology. https://doi.org/10.1111/his.13140
21. Balfour JHH, Verghese P (2013) Primary Epstein–Barr virus infection: impact of age at acquisition, coinfection, and viral load. J Infect Dis 207(12):1787–1789. https://doi.org/10.1093/infdis/jit096
22. Silins SL, Sherritt MA, Silleri JM, Cross SM, Elliott SL, Bharadwaj M, Le TTT, Morrison LE, Khanna R, Moss DJ, Suhrbier A, Misko IS (2001) Asymptomatic primary Epstein–Barr virus infection occurs in the absence of blood T-cell repertoire perturbations despite high levels of systemic viral load. Blood 98(13):3739
23. Scott DW, Gascoyne RD (2014) The tumour microenvironment in B cell lymphomas. Nat Rev Cancer 14(8):517–534. https://doi.org/10.1038/nrc3774
24. Dolcetti R, Dal Col J, Martorelli D, Carbone A, Klein E (2013) Interplay among viral antigens, cellular pathways and tumor microenvironment in the pathogenesis of EBV-driven lymphomas. Semin Cancer Biol 23(6, Part A):441–456. https://doi.org/10.1016/j.semcancer.2013.07.005
25. Hislop AD, Kuo M, Drake-Lee AB, Akbar AN, Bergler W, Hammerschmitt N, Khan N, Palendira U, Leese AM, Timms JM, Bell AI, Buckley CD, Rickinson AB (2005) Tonsillar homing of Epstein–Barr virus-specific CD8+ T cells and the virus–host balance. J Clin Investig 115(9):2546–2555. https://doi.org/10.1172/JCI24810
26. Cohen M, Vistarop AG, Huaman F, Narbaitz M, Metrebian F, De Matteo E, Preciado MV, Chabay PA (2017) Cytotoxic response against Epstein Barr virus coexists with diffuse large B-cell

lymphoma tolerogenic microenvironment: clinical features and survival impact. Sci Rep 7(1):10813. https://doi.org/10.1038/s41598-017-11052-z
27. Gaudreault E, Fiola S, Olivier M, Gosselin J (2007) Epstein–Barr virus induces MCP-1 secretion by human monocytes via TLR2. J Virol 81(15):8016–8024. https://doi.org/10.1128/JVI.00403-07
28. Fiola S, Gosselin D, Takada K, Gosselin J (2010) TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells. J Immunol 185(6):3620
29. Morales O, Mrizak D, Francois V, Mustapha R, Miroux C, Depil S, Decouvelaere AV, Lionne-Huyghe P, Auriault C, de Launoit Y, Pancre V, Delhem N (2014) Epstein–Barr virus infection induces an increase of T regulatory type 1 cells in Hodgkin lymphoma patients. Br J Haematol 166(6):875–890. https://doi.org/10.1111/bjh.12980
30. Basso K, Dalla-Favera R (2015) Germinal centres and B cell lymphomagenesis. Nat Rev Immunol 15(3):172–184. https://doi.org/10.1038/nri3814
31. Vockerodt M, Yap L-F, Shannon-Lowe C, Curley H, Wei W, Vrzalikova K, Murray PG (2015) The Epstein–Barr virus and the pathogenesis of lymphoma. J Pathol 235(2):312–322. https://doi.org/10.1002/path.4459
32. Hislop AD, Taylor GS, Sauce D, Rickinson AB (2007) Cellular responses to viral infection in humans: lessons from Epstein–Barr virus. Annu Rev Immunol 25:587–617. https://doi.org/10.1146/annurev.immunol.25.022106.141553
33. Savoldo B, Cubbage ML, Durett AG, Goss J, Huls MH, Liu Z, Teresita L, Gee AP, Ling PD, Brenner MK, Heslop HE, Rooney CM (2002) Generation of EBV-specific CD4+ cytotoxic T cells from virus naive individuals. J Immunol 168(2):909–918
34. Rahman ZS (2011) Impaired clearance of apoptotic cells in germinal centers: implications for loss of B cell tolerance and induction of autoimmunity. Immunol Res 51(2–3):125–133. https://doi.org/10.1007/s12026-011-8248-4
35. De Silva NS, Klein U (2015) Dynamics of B cells in germinal centres. Nat Rev Immunol 15(3):137–148. https://doi.org/10.1038/nri3804
36. Zhang Y, Kim HJ, Yamamoto S, Kang X, Ma X (2010) Regulation of interleukin-10 gene expression in macrophages engulfing apoptotic cells. J Interferon Cytokine Res 30(3):113–122. https://doi.org/10.1089/jir.2010.0004