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PATHOGENESIS AND IMMUNITY

M1 Macrophage Polarization Prevails in Epstein-Barr Virus-Infected Children in an Immunoregulatory Environment

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ABSTRACT Macrophages can be polarized toward a proinflammatory phenotype (M1) ($CD68^+$) or to an anti-inflammatory one (M2) ($CD163^+$). Polarization can be triggered by cytokines such as IFN-γ for M1, or IL-10 and TGF-β, for M2. In the context of pediatric Epstein Barr virus (EBV) infection, little is known about macrophage polarization in EBV primary or persistent infection. When studying tonsils of patients undergoing primary infection (PI), healthy carrier (HC), reactivation (R), and not infected (NI), M1 profile prevailed in all infection status. However, an increase in M2 cells was observed in those patients with broader expression of latency antigens, in particular EBNA2. Tonsils from primary infected patients showed an increased IL-10 expression, whereas, unexpectedly, TGF-β expression correlated with M1 marker. Furthermore, an inverse correlation was demonstrated between CD68 and IFN-γ. Therefore, in the context of asymptomatic infection in children, M1 macrophage polarization prevails, even in the presence of IL-10 and TGF-β immunomodulatory cytokines, and it might be independent from lymphomagenesis process. Our finding indicates that macrophages may have a significant plasticity in response to different types of extrinsic stimuli, and further studies are required to investigate M1 polarization under anti-inflammatory stimuli.

IMPORTANCE Most studies on Epstein Barr virus (EBV) primary infection have been performed in adolescents and young adult populations with Infectious Mononucleosis (IM) in developed countries. Furthermore, studies related to macrophage polarization were assessed in EBV-associated lymphomas, but little is known about macrophage polarization in the context of primary infection at the site of viral entry and replication, the tonsils. Therefore, the aim of this study was to characterize macrophage response in children undergoing EBV primary or persistent infection, in order to enlighten the role of macrophages in viral pathogenesis, in a population with a high incidence of EBV-associated lymphomas in children younger than 10 years old. This study may contribute to explain, at least in part, the asymptomatic viral infection in children from an underdeveloped region, given that M1 polarization pattern prevails, but in a regulatory environment.

KEYWORDS Epstein Barr virus, macrophages, polarization, children

The Epstein Barr Virus (EBV) is a gamma herpesvirus that infects more than 90% of the human population. It spreads among individuals through saliva, reaching the tonsil, where it finds its target cell, the B cells. As all herpesviruses, it can establish two infectious cycles, lytic and latent cycles, resulting in a lifelong persistence in the infected host (1). This persistence is achieved through a complex mechanism through which EBV progressively downregulates its latency antigens expression, allowing it to mimic B cell biology. As suggested by the "germinal center" model of infection, once the virus infects naive B Cells, by mean of the growth transcription program (Latency III), which includes the expression of all EBNAs, LMPs proteins, as well as the EBERs and BARTs transcripts, EBV

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drives the infected cell to become a proliferating blast that migrates to the germinal center, where it switches to the default program (Latency II). The viral proteins (LMP-1 and LMP-2) expressed in this program have the ability to act as a surrogate antigen and provide T cells survival signals, that enables the infected cell to leave the follicle as a resting memory B cell. At this point the virus displays either the Latency I (EBNA-1) program, which drives its replication along with the cellular genome in dividing B cells, or Latency 0 (EBERs) where the virus persists silently as a circular genome in the resting memory B cell (2). Those profiles were defined in tonsils from adults with EBV primary infection as well as in healthy carriers persistently infected by EBV (3, 4). In children undergoing tonsillectomies, Latency II and III viral antigens expression were observed mostly in healthy carriers, whereas in the context of primary infection, Latency 0 and I antigens were prevalent (5, 6). During the transition through latency profiles, the virus becomes invisible to the immune surveillance, due to the downregulation of viral proteins that act as targets for the immune system (2). The EBV and the immune system seem to coexist in a delicate balance. However, under some circumstances, this balance could be disturbed, allowing the virus to express its oncogenic proteins, leading to the development of some EBV associated lymphomas (7, 8).

It is clear that EBV infection remains as unnoticed as possible to the immune system for life; nevertheless, there are two very different scenarios concerning immune response in primary infected individuals. When it comes to adults, there is a substantial expansion of $CD8^+$ T cells, but little expansion of $CD4^+$ compartment. $CD8^+$ response results in infectious mononucleosis (IM). In contrast, in children the infection is mostly asymptomatic, there is no disruption within the T-cell compartment, and it has been demonstrated that natural killer cells play a key role in preventing the development of IM symptoms (9). However, little is known about the implicance of the other cells that comprise the innate immune system. Dendritic cells (DCs) can sense EBV, in order to activate and recruit granulocytes as well as NK cells. In addition, macrophages can sense EBV, specifically the Epstein Barr encoded RNAs (EBERs) via toll-like receptor 3 (TLR3), whereas monocytes sense unmethylated EBV DNA via TLR9. Moreover, EBV-encoded secreted BARF1 (sBARF1) was found to inhibit M-CSF, thereby inhibiting monocyte differentiation and macrophage function, but their role still remains enigmatic (10). Most macrophages originate from differentiated monocytes once they migrate into tissue, though it has also been demonstrated the existence of embryonic tissue associated macrophages. They contribute to immune homeostasis through their phagocytic and tissue repair ability, as well as linking intrinsic and adaptive immune systems by secreting a plethora of molecules (11, 12).

Macrophages are vastly heterogeneous, and their differentiation depends on reciprocal interactions with the microenvironment (13). The presence of different factors can drive macrophages toward one or another polarization profile. Macrophages can be polarized toward a proinflammatory phenotype (M1) ($CD68^+$) with tumoricidal activity or, instead, toward a regulatory function (M2) ($CD163^+$), which promote tissue repair and Th2 immune response generating a tumorigenic propitious environment. Stimuli such as IFN-γ and its receptor, TLR and IL1R signaling, and tumor necrosis factor (TNF) result in a M1 classical activation. In contrast, glucocorticoid hormones, or cytokines such as IL-4, IL-13, IL-10, or TGF-β, induce the M2 "alternative activation," which can be further subdivided. In spite of this classification, macrophages have a notable plastic gene expression, which can be shaped not only by different types of extrinsic stimuli, but also by the concentration, longevity of exposure, and even by cytokines secreted by macrophages themself. Moreover, macrophages placed in different microenvironments display different functions when exposed to the same stimulus, validating microenvironments' key role in macrophages behavior (14, 15). Tumor environments are another peculiar scenario, given that, in this context, macrophages are known as TAM (tumor associated macrophages), and display typically M2 phenotype and are associated with a worse prognosis (16).

Regarding tumor environments, our group studied several pediatric lymphomas associated with EBV, revealing a higher prevalence of pediatric lymphomas (Hodgkin, Burkitt and Diffuse large B cell lymphoma) associated with the virus in children younger than 10 years old (17). In addition, a characteristic recruitment of macrophages surrounding

TABLE 1 Latent antigens expression

Latency antigens	Patients	Infection status[a]
Latency 0 (EBERs-/LMP1-/EBNA2-)	10	PI: 2 HC: 6 R: 2
Latency I (EBERs+/LMP1-/EBNA2-)	18	PI: 7 HC: 8 R: 3
Latency II (EBERs+/LMP1+/EBNA2-)	18	PI: 8 HC: 8 R: 2
Latency III (EBERs+/LMP1+/EBNA2+)	23	PI: 3 HC: 16 R: 4

[a]PI, primary infected; HC, healthy carriers; R, reactivation.

EBV+ cells were observed in tonsils (18), as well as a prevalence of cytotoxic M1 polarized environment in pediatric Hodgkin lymphoma (HL) (19). Macrophage polarization patterns in the context of viral infections are still poorly explored. In addition, it is unknown if macrophages are involved in the disruption of the balance between EBV and the immune system to develop viral associated lymphomas. Therefore, our aim was to characterize macrophage response in children undergoing EBV primary or persistent infection, in order to enlighten the role of macrophages in viral pathogenesis.

RESULTS

Serological profile. According to the presence of EBV sera antibodies, we classified our cohort into four groups (Table S1), as described in a previous study (5). From the 74 analyzed patients, 38 (52.0%) were classified as healthy carriers (HC), 20 (27.0%) were primary infected (PI), 11 (14.9%) were undergoing reactivation (R), and 5 (6.8%) were not infected (NI).

Viral characterization. The expression of viral latent antigens was studied by EBERs ISH and IHC (Table 1, Table S2 and Fig. S1). Eighteen patients expressed Latency I antigens (EBERs+/LMP1-/EBNA2-), 18 Latency II (EBERs+/LMP1+/EBNA2-), 23 Latency III (EBERs+/LMP1+/EBNA2+), 10 Latency 0 (EBERs-/LMP1-/EBNA2-), while 19 cases also exhibited cells expressing lytic cycle BMRF1 antigen. Viral type was detectable in 40 patients. Twenty-five were infected with EBV-1, 12 with EBV-2, and 3 were co-infected with both types. On the other hand, mean viral load was of 190 copies/μg. LMP1, EBNA2, and BMRF1 positive cells in the whole tonsil section was assessed, and mean cell count for each protein was 14.98, 6.64, and 2.52 cells/cm^2, respectively. When the count of cells expressing each latency or lytic marker were correlated with age or viral load, no statistical differences were demonstrated ($P > 0.05$, Spearman). In addition, no differences in the mean cell count of each viral marker was observed when EBV-1, EBV-2, and co-infected patients were compared ($P > 0.05$, ANOVA).

Macrophages' polarization profile. Given that the median age in our cohort was 5 years, the mean cell count of macrophages' polarization markers was compared in younger versus older than 5 years, and there was no significant difference regarding age ($P > 0.05$, Mann-Whitney [M-W]). It was previously demonstrated in pediatric Hodgkin lymphomas that M1 profile was prevalent (13, 19). When polarization markers were defined by the CD68/CD163 ratio in our cohort, 89% of patients displayed a M1 profile. In line with this, CD68$^+$ cell count was higher than CD163$^+$ and CD169$^+$ in the whole series as well as within groups ($P < 0.05$, one-way ANOVA) (Fig. 1A, B, and C). CD68$^+$ and CD163$^+$ cells were statistically higher in interfollicular (IF) region in the whole series as well as in the different in subgroups analyzed ($P < 0.05$ *t* test), whereas CD169$^+$ cells were statistically increased at the germinal center (GC) in the cohort, specifically in HC ($P < 0.05$ *t* test) (Fig. 2A and B and C).

Regarding viral load, no correlation was demonstrated for CD68, CD163, or CD169 cell counts in the entire cohort and within PI, HC, or R subgroups ($P > 0.05$, Spearman). Patients were clustered into cases expressing Latent 0–I and II–III antigens to evaluate

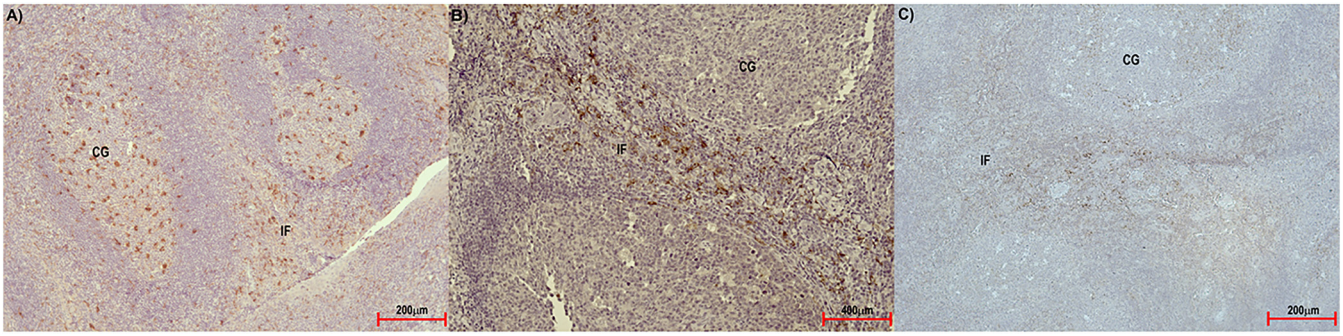

FIG 1 Macrophages' polarization profile markers expression in pediatric patients' tonsil by IHC, membranous positive staining in germinal centers (GC), and interfollicular region (IF) of (A) CD68, (B) CD163, (C) CD169.

the influence of latent EBV proteins expression in macrophages' polarization profile markers. Only CD163$^+$ cell count was statistically higher in cases expressing Latent II–III antigens ($P = 0.002$, M-W), whereas neither CD68$^+$ nor CD169$^+$ mean cell counts displayed differences between latency groups ($P > 0.05$, M-W). Furthermore, CD163$^+$ cells showed a statistical positive correlation with EBNA2+ cell count ($r = 0.303$, $P = 0.0113$, Spearman). No differences in the mean cell counts of CD68, CD163, or CD169 macrophages' polarization markers were demonstrated either in the entire cohort nor within subgroups (PI, HC, and R) when EBV-1 and EBV-2 were compared ($P > 0.05$, one-way ANOVA).

Cytokine gene expression. In order to evaluate cytokines expression in each group of patients, IL-10, IFN-γ, TGF-β, TNF-α, and IL-1β expression was assessed by IHC and qPCR (Fig. 3A, B, and C) (Table S3). Expression of IL-10 was significantly higher between PI and HC ($P = 0.01$, M-W). Furthermore, when PI and HC patients were compared individually with R patients, both expressed significantly more TNF-α than R patients ($P < 0.05$, M-W). In contrast, no significant differences were demonstrated among infection status (PI, HC, and R) for IFN-γ, TGF-β, or IL-1β expression, ($P > 0.05$, one-way ANOVA and M-W) (Fig. 4). Then, the correlation between cytokines and macrophages polarization profiles were analyzed. IFN-γ+ cells negatively correlated with CD163$^+$ cells ($r = -0.232$; $P = 0.047$, Spearman) in the whole cohort, and, unexpectedly, in PI patients with both CD68$^+$ ($r = -0.52$; $P = 0.019$, Spearman) and CD169$^+$ cells (r = –0.465; $P = 0.039$, Spearman). TGF-β + cells showed a statistical positive correlation with CD68$^+$ ($r = 0.25$; $P = 0.033$, Spearman), particularly PI ($r = 0.57$; $P = 0.009$, Spearman), whereas in R patients TGF-β + cells positively correlated with CD169$^+$ ones ($r = 0.741$; $P = 0.012$, Spearman). Finally, when assessed by qPCR TNF-α expression negatively correlated with CD169$^+$ cells ($r = –0.697$, $P = 0.025$, Pearson) only in PI cases. In contrast, no correlation between CD68, CD163, or CD169 cell counts with IL-10+ cell count by IHC or IL-1β expression assessed by qPCR were demonstrated ($P > 0.05$).

With the purpose of evaluating if age is involved in differences in the cytokines analyzed, patients were divided into two groups, younger and older than 5 years, based on the median age of 5 years in our cohort. Patients less than 5 years old expressed significantly more IL-1β ($P = 0.046$; M-W), whereas no significant differences regarding age were shown for the other cytokines ($P > 0.05$, M-W).

When the expression of Latent 0-I versus II-III antigens were analyzed, TNF-α gene expression was statistically increased in the 0-I latency cases ($P = 0.018$, t test). In line with this, TNF-α gene expression quantification by qPCR displayed a negative correlation with LMP-1+ cells ($P = 0.031$, $r = -0.401$, Spearman). Specifically in HC patients, TNF-α negatively correlated with BMRF-1+ cells ($P = 0.02$ $r = –0.744$, Spearman), while in R patients BMRF-1+ cells positively correlated with IL-1β ($P = 0.048$, $r = 0.802$, Spearman).

Finally, no correlation between viral load with any of the evaluated cytokines (IL-10, IFN-γ, TGF-β, TNF-α, and IL-1β) were proved ($P > 0.05$, Spearman). Moreover, when cytokine expression was compared between EBV-1 and EBV-2, no differences in mean cell count of IL-10, IFN-γ or TGF-β, or mean quantitative expression of TNF-α or IL-1β

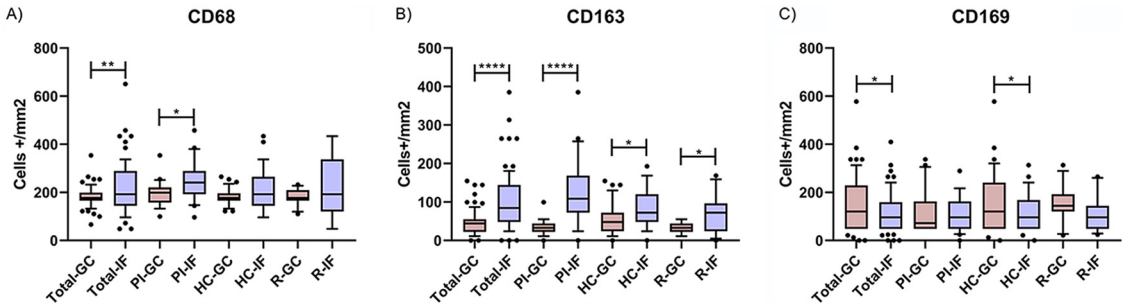

FIG 2 Histological distribution of macrophages markers. (A) CD68$^+$ cells in germinal center (GC) and interfollicular region (IF) in the entire cohort (Total), in primary infected patients (P), healthy carriers (HC), and patients undergoing reactivation (R); (B) CD163$^+$ cells in germinal center (GC) and interfollicular region (IF) in the entire cohort (Total), in primary infected patients (P), healthy carriers (HC), and patients undergoing reactivation (R); (C) CD163$^+$ cells in germinal center (GC) and interfollicular region (IF) in the entire cohort (Total), in primary infected patients (P), healthy carriers (HC), and patients undergoing reactivation (R). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

cytokines were demonstrated in the entire cohort or within subgroups (PI, HC, and R) ($P > 0.05$, M-W).

DISCUSSION

EBV infection has been widely described in adult patients, yet little is known about virus behavior, immune response and its involvement in lymphomagenesis in children. As far as we know, this is the first work that characterizes macrophage's role and the tonsil microenvironment, the site of viral entrance, in a large cohort of pediatric patients.

In Argentina, EBV primary infection in children occurs in the first few years of life, and seroconversion is often observed by ages 3–4 years, while infection in developed countries is often delayed until adolescence (17). Therefore, most patients included in this study were healthy carriers, or children undergoing primary infection or viral reactivation. Viral latent and lytic antigen expression was studied by EBERs ISH and IHC, as previously defined for Diffuse Large B cell lymphomas in Argentina (22). Remarkably, as previously observed in our pediatric population, Latent II and III proteins expression were prevalent in HC, whereas Latent 0 and I prevailed in PI children (5). In line with this, LMP1 prevailed over EBNA1 and EBNA2 in tonsillar B cells in 20 EBV infected adult patients, also detected by IHC (23). In adult patients with IM, double IHC proved that tonsillar B cells displayed Latency IIa, IIb, and III, at the GC and IF regions (24). In patients younger than 18 years old, Latency II pattern by RT-PCR was described in tonsillar GC B-cells (3, 25), whereas Latency III pattern was restricted to naive B-cells (26).

Macrophages can play different, even opposite functions depending on the surrounding environment. Macrophages are able to modulate their metabolic functions from a killing/inhibitory capacity (M1 macrophages) to a heal/growth promoting setting (M2 macrophages) (27), and the latter were described in several types of tumors, known as tumor

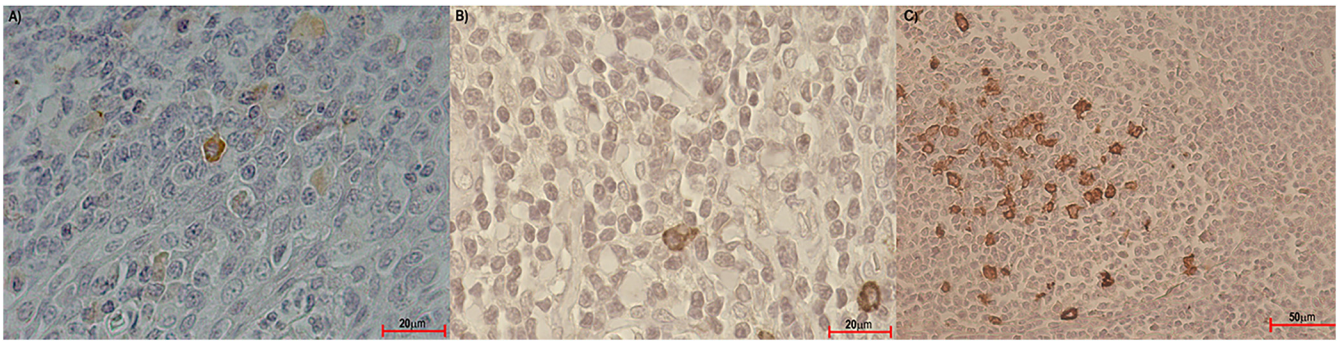

FIG 3 Cytokine expression in pediatric patients' tonsil. Membranous positive staining of (A) TGF-b; (B) IL-10; (C) IFN-γ.

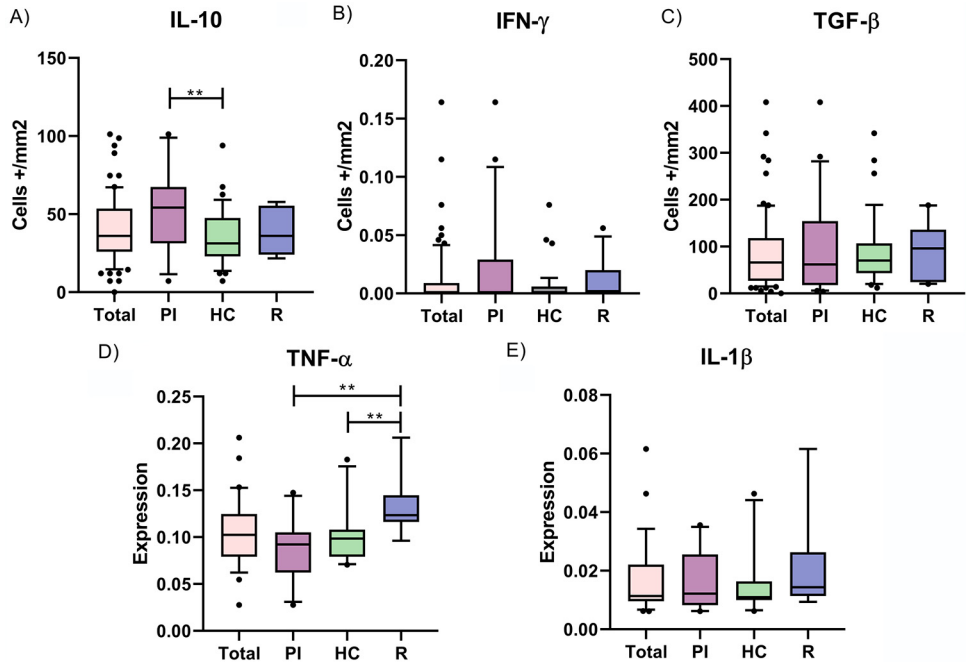

FIG 4 Cytokine expression in the entire cohort (Total), in primary infected patients (P), healthy carriers (HC), and patients undergoing reactivation (R). (A) IL-10 by IHC, (B) TGFα by qPCR, (C) IL1β by qPCR, (D) TGFβ by IHC, (E) IFN-γ by IHC. *$p < 0.05$ by ANOVA when the 3 infection groups were compared, or M-W when comparison were made between 2 groups.

associated macrophages (TAMs) (28). In the context of EBV infection, M2 polarization was described in nasopharyngeal carcinoma (29), as well as in Burkitt lymphoma (30). In contrast, M1 polarization was described in EBV-associated pediatric Hodgkin lymphoma (13, 19), and in tonsils from adults with infectious mononucleosis (24). In line with this, in our analyzed cohort, M1 macrophages (CD68$^+$), characterized for proinflammatory and tumoricidal functions prevails. In fact, the prevalence of CD68$^+$ over CD163$^+$ was observed not only in primary infected patients, but also in patients undergoing reactivation and even in healthy carriers, given that no significant differences in CD68$^+$ cell count between the status of EBV infection. These results may indicate that M1 polarization profile is characteristic of pediatric patients, regardless of EBV infection status, suggesting that the status of viral infection does not play a key role in polarization toward M1 or M2. Nevertheless, when latent viral protein expression was analyzed, an increase in CD163$^+$ cells in the context of viral Latent II and III antigens was evidenced. Furthermore, the increase in CD163$^+$ cell count and its correlation with EBNA2+ cells, suggest that Latency III antigens might be able to recruit M2 macrophages. M2 polarization markers were previously described in Burkitt lymphoma expressing LMP2A Latency II viral protein (30), as well as in posttransplant lymphoproliferative disorders, in which Latency III pattern prevails (31). Our group previously described a prevalence of Latency 0 and I antigens in the context of EBV primary infection (5), while in this study, 23 out of 74 patients displayed Latent III antigens; therefore, the prevalence of M1 pattern could be related to low expression of Latency III viral proteins.

The CD169 is expressed by macrophages found within the subcapsular sinus (SCS) and the medulla of lymph nodes and marginal zone in spleen. So far, there is not sufficient evidence to include CD169$^+$ macrophages within M1 or M2 pattern. CD169$^+$ macrophages are mainly involved in the regulation of the immune system, to maintain immunological tolerance (28). They could contribute to antiviral immune surveillance (32). Furthermore, the lack of CD169$^+$ cells led to impaired adaptive immunity against vesicular stomatitis virus (VSV), suggesting its essential for an efficient adaptive immune response (33). Therefore, the increase in CD169$^+$ cells at the germinal center in HC could be related to immune control of viral reactivations at this specific histological region, from which most B cell EBV-associated lymphomas are originated (34).

M1 macrophages polarization occurs in an inflammatory environment dominated by IFN-γ signaling, and they are able to produce proinflammatory cytokines such as IL-1β, IL-6, IL-12, and TNF-α. In contrast, an IL-4, IL-10, IL-13, or TGFβ productive environment is able to polarize macrophages toward the M2 phenotype (35). Because all the studied cytokines could be produced by other immune cells besides macrophages, correlation analysis was assessed as indirect markers. In order to characterize cytokines production at the microenvironment, IFN-γ, TNF-α, and IL-1β, as indirect markers of M1 polarization, as well as TGF-β and IL-10 expression, as indicators of M2, were studied. In a primary infection scenario, we expected to find a proinflammatory environment and predominance of M1 polarization macrophages as an immune response against the virus. Even though proinflammatory IFN-γ production by CD56bright NK and CD4+T cells was described in tonsils from adults undergoing EBV primary infection (36), unexpectedly, no difference was observed in the expression of both proinflammatory IFN-γ and IL-1β cytokines in the studied PI children. To add more, in this specific group, IFN-γ negatively correlated with both $CD68^+$ and $CD169^+$ cells, along with a negative correlation of TNF-α $CD169^+$ cells. Therefore, a proinflammatory environment, in particular in relation to IFN-γ expression, may not be as determinant as expected in the polarization of macrophages, since M1 still prevails in the context of a non-inflammatory environment.

The expression of an unrestricted Latency II (EBERs+, EBNA1+, LMPs+) or III (EBERs+, EBNA1+, LMPs+, and EBNAs) patterns, is expected to induce an inflammatory environment. In fact, the adult infectious mononucleosis syndrome, characterized by EBV-infected cells that express Latency III proteins, is induced by cytokine release from activated cells (36). In children, whose infection are mostly asymptomatic, it seems that, conversely, proinflammatory TNF-α expression is restricted when a wider Latency II or III patterns are expressed. In fact, the quantification of TNF-α displayed a negative correlation with LMP1 expression. Surprisingly, no differences in TNF-α quantification were demonstrated between PI and HC patients when compared, indicating perhaps that, in our series, these proinflammatory cytokines are not induced at the tonsils in primary infection. In addition, when BMRF1 lytic antigen is expressed, as observed in HC (5), a decrease in TNF-α was proven. Only IL-1β, a proinflammatory cytokine, is increased with lytic antigen expression in R patients. Thus, it could be hypothesized that the lack of symptoms in infected children could be related to decrease expression of pro-inflammatory cytokines at the site of viral infection and reactivation, that may be counterbalanced by an immune-regulatory environment.

The presence of a regulatory environment, particularly in PI children with asymptomatic infection, may be related to a higher IL-10 expression in this specific subgroup. In our series, IL-10 expression was not associated with M2 polarization profile, as could be expected, given that no correlation for CD68 or CD163 markers was observed. These results suggest the prevalence of an M1 profile even in an IL-10 regulatory milieu, triggered by EBV primary infection. The recruitment of IL-10 productive cells around EBV+ cells was described in tonsils, as well as in EBV-associated lymphomas (21, 37, 38), in which also its expression in serum was associated with shorter survival (39). Alternatively, IL-10 expression could be upregulated in EBV infected cells by viral latent antigens such as LMP2A, EBERs, as well as lytic BZLF1, as it was demonstrated *in vitro* (40–42). EBV and its lytic gene product BZLF1 have been shown to induce TGF-β production and secretion in Burkitt lymphoma cells (43), which may contribute to the shift from M1 to a M2 polarization at the microenvironment. In contrast, in our series, even though the immunomodulatory cytokine TGF-β was not increased in PI patients, its expression was positively correlated with $CD68^+$ cells in the whole cohort, and in particular in PI. Therefore, in the context of EBV primary infection, the TGF-β regulatory environment does not induce M2 polarization but is also related to M1 polarized macrophages.

In summary, our group previously described a cytotoxic M1 polarization profile in children with EBV-associated lymphomas (19), that also was observed in different EBV infection status, suggesting that M1 prevalence is independent of lymphomagenesis process. In the context of asymptomatic infection in children, a regulatory environment

is triggered by EBV infection, which could be related to the lack of symptoms observed in pediatric cases. In this immune regulatory environment, M1 macrophage polarization prevails, even in the presence of IL-10 and TGF-β regulatory cytokines. This finding indicates that macrophages may have a significant plasticity in response to different types of extrinsic stimuli, and further studies are required to investigate M1 polarization under anti-inflammatory stimuli.

MATERIALS AND METHODS

Ethical statement. All samples were collected after written consent (for patients older than 12 years and legal guardians of children younger than 12 years), and assent (7- to 12-year-old patients and legal guardians of children older than 12 years) was obtained, following the national and international ethics standards and under the supervision of the Ethical Committee of the Ricardo Gutiérrez Children's Hospital, in accordance with the Helsinki Declaration of 1975.

Patients and samples. We studied 74 patients aged between 1 and 15 years (median age of 5 years) undergoing tonsillectomy due to nonreactive hyperplasia at the Otorhinolaryngology Division, Ricardo Gutierrez Children's Hospital (Buenos Aires, Argentina). Part of the tissue was preserved at −70°C for the later extraction of ADN and ARN. The remaining biopsy tissue was formalin fixed paraffin embedded (FFPE) at the Pathology Division of Ricardo Gutierrez Children's Hospital (Buenos Aires, Argentina). A concomitant blood sample was obtained to assess the serological profile for EBV.

Serological profile. Five mL of blood samples obtained from each patient were centrifuged for 10 min at 2,500rpm, and the serum was separated and stored at −20°C for indirect immunofluorescence assay, as previously reported (5). Primary infected (PI) patients were defined by the presence of IgM and IgG antibodies to VCA (Viral Capsid Antigen), the healthy carrier (HC) state was established by the presence of IgG antibody to VCA and EBNA1, whereas viral reactivation (R) was identified by the developed of rising titers of IgG anti-VCA, anti-EBNA1 and anti-EA (Early Antigen) antibodies. A semiquantitative IgG anti-VCA (diluted 1/10, 1/40, and 1/320) was performed in order to further characterize serological analysis.

Viral antigen expression and latency profile classification. In order to explore the expression of EBV latent and lytic antigens, immunohistochemistry for LMP-1 (CS1-4 pool of clones, Dako), EBNA2 (1E6 y R3 clones, Abcam), both latent antigens, and BMRF1 (G3-E31 clone, Abcam) early lytic antigen, were detected in formalin fixed paraffin embedded (FFPE) tonsil samples, as described (5). Positive cell count in the whole tonsil section was assessed for each protein and results were expressed as positive cells by area (cells/cm^2). FFPE EBV+ Raji cell lines were used as positive controls for EBNA2, P3HR1 stimulated with TPA (12-O-tetradecanoylphorbol-13-acetate, Sigma) for lytic infection (BMRF1), and EBV+ Hodgkin Lymphoma for LMP1. For negative controls, the same methodology without primary antibody was performed. In addition, in-situ hybridization with ViewRNA ISH Tissue 1-Plex Assay and specific probes (Affymetrix) was performed to detect the presence of EBERs according to manufacturer protocol.

Viral load and typification. After mechanical tissue disruption, commercial columns (Qiagen) were used for DNA isolation according to manufacture instructions. The purified DNA was quantified using NanoDrop One (Thermo Fisher) and its integrity checked by human ß-globin PCR assay. Viral load was assessed by quantitative PCR- Real Time, using a plasmid EBNA1 standard curve and TaqMan specific probe, as previously reported (5, 20). Results were analyzed with Step One Software v2.3. Typification of EBV1 or EBV2 was defined by PCR, as described (5).

Macrophages' polarization profile. To assess macrophagés polarization profile, immunohistochemistry (IHC) with specific antibodies was performed, CD68 as M1 marker (clone KP-1, Roche Ventana), CD163 as M2 marker (clone MRQ-26, Roche Ventana) and CD169 as specific lymphoid tissue resident macrophage (Abcam). Primary antibody signal amplification was performed with a commercial kit (ScyTek) for detection of rat, mouse, and goat primary antibodies, followed by detection with DAB (Vector) as chromogen. Slices were observed at optical microscope (A1 AxioScope, Carl Zeiss) and positive cells were count discerning between the germinal center and the interfollicular regions, while results were expressed as cells/mm^2. M1 profile was defined when CD68/CD163 ratio was greater or equal to 1.5, and M2 when the ratio CD163/CD68 was higher or equal to 1.5.

Cytokine expression analysis. Cytokine expression was evaluated by RT-PCR and IHC. For RT-PCR, total tissue RNA was obtained using RNeasy minikit (Qiagen) following the manufacturer's protocol. Subsequently, a retrotranscription with SuperScript Reverse Transcriptase (Invitrogen) and random hexamers was performed to obtain cDNA. RNA was obtained for 32 specifically selected cases, representative of each group (10 PI, 10 HC, 9 R, and 3 NI). The specific primers for target genes IL-1β and TNF-α (involved in M1 polarization) and the endogenous HPRT gene were obtained from http://primerdepot.nci.nih.gov/. qPCR was performed and validated as previously described (21) using a StepOne real-time detection system (Applied Biosystems), results were analyzed with StepOne Software v2.3. The normalized transcription values were calculated by the Pfaffl Method. The mean value of the CT of an equivalent quantity of RNA input from stimulated PBMC was used as a calibrator.

For cytokines detection by IHC, specific primary antibodies against IFN-γ (polyclonal, Abcam) (involved in M1 polarization), IL-10 (polyclonal, Abcam), and TGF-β (polyclonal, Abcam) (involved in M2) were used. Detection was performed with a commercial detection kit (Vector), according to manufacturer's instructions. Slices were observed at optical microscope and positive cells count was performed discerning between the germinal center and the interfollicular regions and results were expressed as cell/mm^2.

Statistical analysis. The data was analyzed using GraphPad Prism 8 software. Normality test was performed using Shapiro-Wilks test. Comparison between groups was assessed by one-way ANOVA or

Kruskal-Wallis tests according to normality test results. Correlations were performed using Spearman or Pearson tests. Categorical variables were analyzed with Fisher exact test or Chi square test. Outliers were defined using the Robust test to compare data median absolute deviation (Mad) in Excel. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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