BURKITT LYMPHOMA WITH GRANULOMATOUS REACTION: A M1/TH1-POLARIZED MICROENVIRONMENT ASSOCIATES WITH CONTROLLED GROWTH AND SPONTANEOUS REGRESSION.


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Aims

Burkitt lymphoma (BL) is an aggressive B-cell lymphoma, which in some instances, may show a granulomatous reaction associated with a favourable prognosis and occasional spontaneous regression. In the present study, we aimed to define the tumour microenvironment (TME) in four of such cases, two of which regressed spontaneously.

Methods and Results

All cases showed aggregates of tumour cells with the typical morphology, molecular cytogenetics and immunophenotype of BL surrounded by a florid epithelioid granulomatous reaction. All four cases were Epstein-Barr virus (EBV) positive with type I latency. The investigation of the tumour microenvironment (TME) showed similar features in all four cases. The analysis revealed a pro-
inflammatory response triggered by Th1 lymphocytes and M1 polarized macrophages encircling the neoplastic cells with a peculiar topographic distribution.

Conclusions

Our data provide an in vivo picture of the role that specific immune cell subsets might play during the early phase of BL, which may be capable of maintaining the tumour in a self-limited state or inducing its regression. These novel results may provide insights to explore new potential therapeutic avenues in EBV-positive BL patients in the era of cellular immunotherapy.

KEYWORDS: Burkitt lymphoma; granulomatous reaction; Th1 T cells; M1 polarized macrophages; Microenvironment; EBV; In Situ lymphoid neoplasia.
INTRODUCTION

Burkitt lymphoma (BL) is an aggressive B cell lymphoma characterized by a diffuse proliferation of medium sized monomorphic lymphoid cells with basophilic cytoplasm and cohesive growth. A so-called starry sky pattern is usually present, which is due to the presence of numerous macrophages with tangible apoptotic bodies. However, some cases show a florid granulomatous reaction and are characterized by a favorable outcome and in rare instances, spontaneous regression. More recently, this issue was discussed in the Lymphoma Workshop of the 18th meeting of the European Association for Haematopathology (EAHP) in 2016 in Basel. Three cases of BL with granulomatous reaction were presented, two of which showed spontaneous remission without further therapy after needle core biopsy or lymph node excision. The almost identical morphological features of these cases may provide a link between the excellent prognosis of these patients and the peculiar immune reaction of the host highlighting the necessity of further investigations with a comprehensive characterization of the tumour microenvironment (TME). In fact, none of the previous studies have characterized the nature of the inflammatory infiltrate and its polarization towards an activated (pro-inflammatory) or tolerant (pro-tumoral) state.

The cellular background in which lymphoma cells thrive has only in the last decade become an important target of inquiry. The functions of what used to be considered passive bystanders are quickly becoming elucidated in order to provide potential targets for immunotherapy. In the last years a model has been developed to describe the complex mechanism of macrophage activation as a polarization towards two opposite states, namely M1 and M2, with pro-inflammatory and pro-tumoral properties respectively. The M1/M2 nomenclature has been inspired by the Th1 vs. Th2 concept. Th1 lymphocytes and M1 macrophages are the primary sources of pro-inflammatory cytokines that also promote cancer immunosurveillance and cytotoxicity. On the other hand, these effects are counterbalanced by M2 macrophages, Th2 lymphocytes and regulatory T-cells (T Regs) exhibiting anti-inflammatory and pro-tumoral effects. In particular, Th1 cells drive the type-1 pathway (“cellular immunity”) to fight viruses and eliminate cancerous cells, while Th2 cells drive the type-2 pathway (“humoral immunity”), up-regulating antibody production. Differentiation of CD4+ T cells into Th1 and Th2 effector cells is controlled by the transcription factors T-bet (T-box protein expressed in T cells, also called as TBX21) and GATA3, respectively. T-bet overexpression causes differentiation into the Th1 lineage whereas loss of T-
bet induces default commitment to Th2 and Th17 lineages. T-Bet directly activates IFN-γ gene transcription and enhances development of Th1 cells. Therefore, CD4+/T-Bet+ cells are considered as Th1 cells, while CD4+/GATA3+ cells are assumed as Th2 cells.

Th1 lymphocytes are well known IFN-γ secretors, involved in the regulation of macrophage polarization, promoting and maintaining the formation of granulomas. IFN-γ activates the IRF/STAT (via STAT1) pathway, switching the macrophage function towards the M1 phenotype.

However, the immunophenotype features that distinguish M1 and M2 macrophages are still not standardized. Given the fact that the scavenger receptor CD163 alone in recent papers is not considered a reliable marker of M2 polarization, the combination of C-Maf, an essential transcription factor for interleukin (IL)-10 gene expression in macrophages, and CD68 as generic macrophage marker may be used to identify M2 macrophages. On the other hand, an antibody against pSTAT1, the phosphorylated form of STAT1 may be used to identify M1-macrophages and IFN-γ primed dendritic cells.

EBV-specific T-cell responses in BL have not been fully elucidated. Indeed, EBNA1, which is usually the only latent protein expressed in EBV+ BL, possesses the ability to inhibit proteasomal processing through its gly-ala repeat domain. In addition, studies have shown defects in MHC Class I antigen processing in BL. These features are well known aspects of BL cells potentially explaining its escape from immune surveillance. On the other hand, other authors have shown that EBNA1 specific CD4+ Th1 cells can be isolated from EBV-seropositive healthy adults and that these cells are cytolytic for constitutively HLA class II expressing BL cell lines.

Therefore, the aim of this study was to investigate the immune landscape in four cases of EBV+ BL with granulomatous reaction, focusing on the quantitative and topographic distribution of the innate immunity (M1/M2 macrophages, plasmacytoid dendritic cells and natural killer cells) and adaptive immunity (Th1/Th2 lymphocytes, regulatory T cells) cell subsets.

MATERIALS AND METHODS

A formalin-fixed paraffin-embedded (FFPE) BL case was retrieved from the Departments of Medical Biotechnologies, University of Siena, Siena (Italy) (case 1), and two cases (case 2 and
case 3) were retrieved from the Lymphoma Workshop (LYWS) of the 18th EAHP meeting, in Basel, Switzerland, 2016 and were included in the workshop report. The third case submitted to the LYWS was not included in this study because of lack of available sections. The fourth case (case 4) was retrieved from the 1st Workshop to support the NIHR-RIGHT Aggressive Infection Related-East African Lymphoma (AI-REAL) Study in Dar-es-Salaam (Tanzania), in February 2020.

The diagnosis of BL was issued by expert haematopathologists following the criteria described in the 2017 WHO classification of tumours of Haematopoietic and Lymphoid Tissue. Diagnostic immunohistochemistry was carried out on the Bond III Autostainer (Leica Microsystems, Newcastle upon Tyne, UK) following the manufacturer’s instructions. In situ hybridization for EBERs was performed on deparaffinized 4 mm-thick, FFPE tissue sections using a Dako Detection Kit for EBV-encoded small RNA (EBER, DakoCytomation, Denmark). The slides were counterstained with hematoxylin and fixed in Faramount (DakoCytomation).

Fluorescence in situ hybridization (FISH) evaluation for MYC gene rearrangement was performed for each case, using break-apart probe (BAP, Vysis MYC Dual Colour Break Apart Rearrangement Probe, Abbott, Germany) and dual fusion probe (ZytoVisionGmbh) following the manufacturer’s instructions. For each specimen, a 4 µm thick tissue embedded in paraffin was cut. Briefly, the slides were incubated in heat pre-treatment solution, washed, digested, dehydrated, hybridized with probes. At least 100 intact non-overlapping nuclei were analysed manually on a Leica DM 600B (Leica Microsystems, Switzerland) fluorescence microscope equipped with DAPI, Spectrum Green, and Spectrum Orange filters.

The antibody panels used for multiplex immunohistochemistry (mIHC) consist of 8 different triple and double staining: a) CD68, CD163 and C-Maf; b) PD-L1, CD-163 and C-Maf; c) CD4, CD25 and FOXP3; d) CD8, PD1 and LAG3; e) p-STAT1, CD68 and CD123; f) CD4 and T-bet; g) CD4 and Gata3; h) CD20 and T-bet (Supplementary Table 1 for antibody characteristics). CD56 and CD57 were included as single stainings. The double and triple stainings were performed using an automated staining system (DISCOVERY ULTRA IHC/ISH research platform, Roche Diagnostics) for open procedures according to manufacturer’s protocols. The detailed protocols for each stain are reported in the supplement. The CD68+/p-STAT1+, CD123+/p-STAT1+, CD163+/CD68+/C-Maf+, CD163+/C-Maf+, CD4+/T-Bet+, CD4+/GATA3+, CD4+/CD25+/Foxp3+, CD8+/PD-1+/LAG3+ cells were evaluated manually and independently by three experienced
pathologists (MG, VM and TM) by counting the individual cell types in 10 HPF using a 40x objective 172 (NIKON Eclipse E400), taking into account either nuclear and surface stains, reported as percentages for each cell type in scores rounded up to the nearest 5%.\textsuperscript{23-24} Only CD163+/CD68+/C-Maf+, CD163+/CD68-/C-Maf+ were considered as M2-polarized macrophages, while we designated CD163-/CD68+/C-Maf- and CD68+/pSTAT1+cells as M1 macrophages and CD123+/pSTAT1+ cells as IFN-γ primed plasmcytoid dendritic cells.\textsuperscript{25}The inter-observer reproducibility of each cell count was assessed by the coefficient of variation in percent, CV\%, which is the ratio of the standard deviation to the mean of the three percent observations. For each variable, percent agreement across the 4 cases analyzed was then expressed as the mean of 100-CV\% and its 95% confidence interval (CI\%), estimated using the bias-corrected and accelerated bootstrap technique (BCa).The percentages of each cell type for each observer and the level of inter-observer reproducibility for each variable are shown in the supplementary table (Supplementary Table 2).

Tissue sections from the same set of cases and without antibody/chromogens were used as negative control. Three tonsils, three reactive lymph nodes and tree cases of conventional BL with typical starry sky pattern were used as positive controls.

RESULTS

Clinical History

Case 1 refers to a 65-year-old woman with an isolated right axillary lymphadenopathy and no signs of systemic infection. The patient underwent excision of the enlarged lymph node, and a diagnosis of focal involvement by EBV-positive B cells with features of BL was made, associated with a reactive lymphadenitis, and granulomatous reaction. The CT-PET scan showed no evidence of other hypermetabolic lymph nodes. Bone marrow biopsy displayed normal haematopoiesis and flow cytometry analysis showed a normal phenotype. Therefore, the patient was followed closely and underwent a "watch and wait" strategy without any chemotherapy. The patient is well and in good conditions 30 months after the initial diagnosis.

Case 2 is related to a 16-year-old patient, who presented with recurring swelling on the left side of the neck. First, he was treated with antibiotic therapy for upper respiratory tract infection. After
seven months, a diagnosis of viral infection was rendered without specific treatment. Because of persistent lymph node enlargement, an excisional lymph node biopsy was performed. The lymph node was 6 cm in diameter and a diagnosis of BL with granulomatous reaction was made. The patient had no signs of other lymphadenopathies, splenomegaly or hepatomegaly. The levels of LDH and β2-microglobulin were normal. A CT-PET scan showed no evidence of disease and the bone marrow biopsy was also negative. The patient refused multi-agent chemotherapy. After four years, he was in complete remission without any treatment.

Case 3 a 47-year-old female, who presented with cervical swelling for one month without B symptoms. CT scan showed a 33x24 mm lymphadenopathy next to the left parotid. Peripheral blood and biochemical parameters were normal, except for LDH: 512 (range 225-450). The left submandibular lymph node was excised for further investigation and a diagnosis of BL with granulomatous reaction was proposed. Multi-agent chemotherapy was initiated. After three cycles of chemotherapy the patient was in complete remission and after two years of follow-up, the patient showed no evidence of disease.

Case 4 is related to a 12-year-old male patient from Kigoma, Tanzania. The patient was admitted to the local hospital for a swelling in the right lower jaw region persisting for more than two months. Prior to the swelling, there was a history of trauma followed by mobility of the lower jaw and regional pain. After tooth extraction there was no reduction of the symptoms and there was further increase of the swelling. The patient was sent to a reference centre for further management. The CT scan of the head showed features suggestive of BL in the right lower jaw region, mild right maxillary sinusitis, no evidence of cervical and mediastinal lymphadenopathy and no evidence of brain involvement. Surgical enucleation and curettage of the tumour was performed. After 6 cycles of chemotherapy with Rituximab, Cyclophosphamide, Oncovin (Vincristine), Doxorubicin, Methotrexate (R-CODOX), the patient showed no residual disease and was discharged. After 16 months of follow-up the patient is asymptomatic.

Microscopic and immunohistochemical findings The morphological and immunophenotypical features of all 4 cases are summarized in table 1. The common features of all the cases in the study were the presence of a focal or diffuse infiltrate by BL cells and a prominent granulomatous reaction. All cases were EBV positive with latency 1.
In particular, in case 1, morphological examination of the surgical specimens revealed an overall preserved lymph node architecture characterized by follicular hyperplasia with reactive germinal centres and prominent perisinusoidal and perifollicular clear areas composed of monocytoid B lymphoid cells. Exclusively within the monocytoid B lymphoid cell areas, nests of dark appearing, atypical cells were occasionally present (Figure 1A). Some nests appeared to coalesce focally forming wider ill-defined aggregates composed of medium sized lymphoid cells with round nuclei, finely clumped chromatin, multiple small peripheral nucleoli, and small to moderate amounts of basophilic cytoplasm, morphologically consistent with BL cells (Figure 1B). Mitotic figures and apoptotic bodies were seen within the nests and focal ill-defined aggregates. In addition, a florid granulomatous reaction, sometimes encircling the neoplastic clusters of cells was present (Figure 1B, HE). The immunophenotype (CD20, CD10, BCL-6, IgM positivity, BCL-2 negativity, high proliferative index by Ki-67 >95%) (Figures 2A, 2B, 2C, 2D, Table 1), MYC protein expression (Figure 2E), and cytogenetics (MYC-translocation by both dual fusion and BAP; Figure 2E, inset) were consistent with BL (Table 1). The tumour cells were also EBV positive by EBER in situ hybridization (Figures 2F). IHC revealed only the expression of EBNA-1 among EBV latency genes, while LMP1, LMP2, EBNA2 and BZLF1 were negative (Table 1). Therefore, the diagnosis of a focal involvement by EBV+ BL in the background of a reactive lymphadenitis was made.

Cases 2, 3 and 4 (Figure 3) showed a clearly effaced lymph node architecture with the presence of an overt BL with a florid granulomatous reaction, in contrast to the limited and interfollicular pattern found in Case 1.

Moreover, case 2 was characterized by the presence of patchy BL cells intermingled histiocytes, fibroblast and abundant collagen deposition. On the other hand, case 3 showed extensive necrotic areas with conspicuous foci of apoptotic debris.

**Microenvironment features**

The analysis of TME in all the four cases showed comparable characteristics Overall, in all four cases the analysis of the macrophages encircling the tumor areas by multiplex IHC showed a
prevalence of M1 macrophages, defined as CD68+/CD163−/c-Maf− cells, accounting for 80-95% of the total macrophages whereas M2 macrophages (CD68+/CD163+/c-Maf+) were scarce (5-20%). (Figures 4A, 4B, Table 2). In the reactive lymph nodes the plasticity of M1 and M2 may be more active in controlling the immune response to different antigens. Most of the macrophages in germinal centers are M2, and this is possibly the reason for the high number of M2 macrophages that we found in reactive lymph nodes. (Personal communication of Prof. Claudio Tripodo, Human Pathology Section, Department of Health Sciences, University of Palermo; Supplementary figure 1).

The pSTAT1/CD68/CD123 triple staining highlighted that macrophages (CD68+) express nuclear pSTAT1 indicating IFNγ priming and confirming a M1 phenotype of the macrophages (Figures 4D, 4E; Table 2). The number of IFNγ primed macrophages (CD68+/pSTAT1+) ranged from 35% to 75% of the total CD68 positive cells, compared with the considerably lower value detected in the controls (< 5%). In addition, pSTAT1 nuclear staining was also found in scattered and small clusters of CD123+ cells, in the proximity of granulomas, possibly representing activated pDCs, ranging from 15% to 35% (Figures 4A, 4B, Table 2). On the other hand, only few CD123+/pSTAT1+ were found in the controls (< 5%). In addition, PD-L1/CD163/c-Maf panel showed that epithelioid granulomas were significantly positive for PD-L1, as secondary effect of IFN-γ activation (Supplementary figure 2). The dominant reactive lymphoid infiltrate was mostly constituted by CD4+ T cells ranging from 75% to 90%. In particular, Th1 cells (CD4+/T-bet+ cells) were consistently represented ranging from 20% to 35% of the total CD4+ T cells (Figure 5A; Table 2). Interestingly, Th1 lymphocytes were found in the immediate proximity of the granulomas further underlining the cooperation between innate and adaptive immunity. By contrast, the tumour-free areas and the reactive lymph node control showed a scant or absent Th1 infiltrate (5-10%) (Supplementary figure 3A; Table 2). However, CD4/T-bet double staining disclosed a subpopulation of CD4 negative T-bet expressing cells. Double staining with CD20 showed the B cell origin of such cells, more likely representing a subset of monocytoid B cells, as previously reported by Jöhrens et al. This finding was particularly evident in case 1, where BL cells were nested in areas of florid monocytoid B-cell hyperplasia (Supplementary Figure 4), but were also detected in the other cases, maintaining the topographic correlation with the granulomas. Th2 cells (CD4+/GATA3+ cells) were also represented in the tumour areas in a range of 10% to 25%. However, these values were lower as compared to the ones in the reactive tissue.
DISCUSSION

In this study, we report four cases of localized (Stage I) EBV positive BL with prominent granulomatous reaction and a similar TME consisting of Th1 lymphocytes and M1 macrophages. The TME described above is quite different from conventional BL with typical starry sky pattern where the most prominent component of TME is represented by M2 macrophages. Such cases usually present with bulky disease, multiple localization and intensive chemotherapy is required. On the other hand, cases of BL with florid granulomatous reaction typically present at an early stage of disease and have a particularly good prognosis with some cases showing spontaneous regression. Although a pro-inflammatory immune reaction may be responsible for the favourable outcome, a thorough characterization of the reactive infiltrate in these cases was lacking. Using mIHC, we now show that the TME exhibits features of an activated cellular immune response characterized by the prevalence of Th1 lymphocytes and M1 polarized macrophages forming granulomas enclosing neoplastic cells. Th1 lymphocytes are well known IFNγ secreting cells that activate inflammatory pathways mainly via macrophage polarization towards a M1 functional status. In addition, they promote granuloma formation and inhibit Th2 lymphocyte proliferation (Figure 6). Accordingly, the vast majority of macrophages were primed by IFNγ, as demonstrated by p-STAT1 nuclear positivity. In addition, due to the dichotomous role of IFNγ, which activates from one side the immune innate response and on the other the checkpoint inhibitors, the positivity for PD-L1 in the granulomas’ epithelioid macrophages may indicate in our context a strong activation of the innate immune response.

Another important observation in this study was the peculiar topographic distribution of the reactive inflammatory cells. In fact, Th1 lymphocytes, pDCs and M1 macrophages were detected in the immediate proximity of the BL cells. Intriguingly, we also observed numerous positive CD20+/T-bet+ cells, more likely representing monocytoid B cells, around the clusters of BL cells. There are recent data suggesting that T-bet is a central regulator of antiviral immunity in all lymphocytes lineages and that T-bet positive B cells may be an important component of ongoing
immune responses during chronic viral infections. Furthermore, our results are in accordance with previous observations, which underline the role of EBNA1-specific CD4-positive T cells in the regulation of the immune response in healthy carriers of EBV. Our data may well represent an in vivo picture of a T-bet mediated immune response fostered by IFNγ and subsequent granuloma formation comprising M1 polarized macrophages with an activated STAT1 signalling pathway.

We believe that the fractions of cells that we score in our IHC experiments are reflecting specific Th states according to the expression of selected Th proteins. However, we need to consider a variety of states and transitions which may be part of the highly dynamical tumor-infiltrating T-cell landscape that we are not taking into account. Besides Th1 and Th2, in addition Th0, Th17, Th9, and Th22 cells and other potentially relevant Th phenotypes could contribute to the overall Th infiltration of BL, such as BCL6 and/or c-Maf+ Tfh cells, which are variably represented among CD4+ T cells in lymphomas of the GC phenotype, or variants in regulatory subsets that may be aberrantly expanded in tumors, such as Eomes+ GzmK+ Tr1-like CD4+ effectors.

Therefore our finding need to be confirmed in further studies using different methods that allow a more comprehensive characterization of the tumor microenvironment (i.e. gene expression analysis)

The interplay with TME may be a crucial event in the outcome of EBV-related diseases and the viral interaction with host immune surveillance requires further investigation in correlation with the EBV latency program. It has been shown that the pattern of viral protein expression determines the immunogenicity of the infected cells. The virus encodes eight antigenically distinct latent-cycle proteins which display a marked hierarchy of immunodominance for the CD8/CD4+-T-cell response. Epitopes derived from the EBNA3A, -3B, and -3C family of proteins tend to induce the strongest responses across a range of different HLA class I alleles. LMP1-specific responses are extremely rare, while LMP2 is more frequently immunogenic but almost always induces low-frequency subdominant responses. Although initially EBNA1 was supposed to mount an immunologically weak response, more recent studies demonstrated that healthy EBV carriers exhibited an efficient CD4+ T cell response to this antigen and were found to be mainly T helper type 1 in nature. On these grounds, conventional BLs with EBV latency I may show a different immune signature from those exhibiting a heterogeneity of latency program characterized by the expression of LMP1, LMP2 and/or lytic genes (non-canonical latency program).
Indeed, all the cases reported here showed a latency I program with the sole expression of EBNA1.

A further observation of this study was the distinctive morphological features of case 1. In fact, in this case the nests of BL cells were found exclusively within prominent areas of clear cells identified as monocytoid B-cells which might represent a possible niche from which BL cells originate and spread as it has been previously reported for HL.\,\,\,40,\,\,41 Although there is consensus that BL is related to germinal centre (GC) B-lymphocytes, it has been hypothesized that EBV-positive BL cases may derive from a later developmental stage of B-cells, i.e. post-germinal centre/memory B cells.\,\,\,42,\,\,43 This is in line with the fact that in healthy carriers, EBV resides in memory B-cells that re-enter the GC reaction following antigenic stimulation.\,\,\,44-46 Only one case with similar features has been recently reported in the literature in a human immunodeficiency virus (HIV) positive female,\,\,\,47 and reported as “Burkitt-microlymphoma”. We have also observed a similar case also in an HIV positive patient (Supplementary Figure 5). Both cases developed an overt BL with unfavourable outcome shortly after the diagnosis, while the case reported here occurred in an immunocompetent host and was characterized by granulomatous reaction and spontaneous regression, possibly representing an early phase of BL.

BL in the setting of granulomatous reaction and conspicuous monocytoid B cell hyperplasia might be easily overlooked and making the correct diagnosis may be challenging.\,\,\,6 Awareness of these features is important to avoid misdiagnosis. Our study provides for the first time an in vivo picture of the immune surveillance of BL with granulomatous reaction that might explain why an aggressive lymphoma is kept in check and behaves in such a self-limiting way. In contrast to conventional BL, the important role of Th1 and M1 macrophages in the TME is highlighted. In addition, the recognition of TME features of BL may be helpful to identify a subset of BL cases with a better prognosis, thus deserving a less burdensome therapy. Further studies are warranted to investigate the reason why similar tumor cells trigger different immune responses. In addition, our data may provide the rationale for new potential therapeutic avenues to explore in EBV-positive BL patients in the era of immunotherapy and in particular of adoptive T-cell therapy (ACT) with EBNA1-specific Th1 cells.\,\,\,48,\,\,49
AUTHORS’ CONTRIBUTIONS

SR, FV, DSG, PB, SE, AC, BN, TT, DAS, ML, DS: provided tumour samples and clinical data;
AA, ES, RG, ML: performed immunohistochemistry and cytogenetic analysis;
GM, LS, MV, SR, VF, GC, CT, DSG, PM, SE, AC, DS, MT, QML, FF, LL: analysed and interpreted the data;
GM, LS, MT, QML, FF, LL: designed and coordinate the study;
GM, LS, QML, FF, LL: interpreted the data and wrote the manuscript;

COMPLIANCE WITH ETHICAL STANDARDS

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

This was a non-interventional study on archived tissue samples.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.
REFERENCES


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FIGURE LEGENDS

Figure 1. Morphology of case 1: Nests of dark appearing atypical cells were occasionally present in the marginal zone of the otherwise reactive lymph node (1A) Small to medium-sized lymphoid cells with round nuclei, finely clumped chromatin, multiple small peripheral nucleoli, surrounded by granulomatous epithelioid reaction (1B). Original magnification (O.M.): A: 10x; B: 20x.

Figure 2. Immunophenotype of case 1: The immunophenotype of the atypical cells is CD10 positive (A), BCL-6 positive (B), BCL-2 negative (C), high proliferation index by Ki-67 >95% (D), MYC protein expression (E) and cytogenetics (Myc rearrangement by break apart probes; E inset) was consistent with BL. These cells were also EBV positive by EBER in situ hybridization (F). O.M.: A-I: 10x.

Figure 3. Morphological features of case 2, case 3 and case 4. A prominent granulomatous reaction, comprising of epithelioid histiocytes and fibroblasts surrounding Burkitt lymphoma cells in case 2 (A), case 3 (B), case 4 (C). O.M.: A, B, C: 20x.

Figure 4. Immunohistochemical characterization of macrophages: Triple staining using c-Maf (brown), CD163 (red), and CD68 (blue) and p-STAT1 (blue), CD68 (brown), CD123 (red). High number of M1 (CD68+/CD163-/c-Maf') macrophages (blue arrows) in the granulomas surrounding the clusters of Burkitt cells in case 1 (A, a), case 2 (B); by contrast in the conventional BL case control (C) the predominant macrophages population is represented by M2 (CD68+/CD163+/c-Maf') macrophages (red arrows). IFN-γ primed macrophages and plasmacytoid dendritic cells expressing nuclear p-STAT1: case 1 (D), case 2 (E). On the other hand, only few CD123+/pSTAT1+ were found in the conventional BL case control (F) O.M.: A-F: 40x.

Figure 5. Characterization of Th1 infiltrate with CD4/T-bet double staining (CD4 red; T-bet brown): A reactive lymphoid infiltrate mostly composed by CD4+ T cells, in particular Th1 cells (CD4+/T-bet+) is
depicted and found in the immediate proximity of the granulomas in case 1 (A, A inset: higher magnification double positive stains); on the other hand only few scattered Th1 cells were detected in the conventional BL case control (B, B inset: ) O.M.:A-B: 10x; A-B insets: 63x.

Figure 6. The role of Th1 lymphocytes: Th1 lymphocytes are well known IFNγ secreting cells that activate inflammatory pathways mainly via macrophage polarization towards a M1 functional status. In addition, they promote granuloma formation and inhibit Th2 lymphocyte proliferation.

SUPPLEMENTARY FIGURES

Figure 1. High number of M2 macrophages in reactive lymph node: Triple staining using c-Maf (brown), CD163 (red), and CD68 (blue). Most of the macrophages in germinal centers are M2, and this is possibly the reason for the high number of M2 macrophages that we found in reactive lymph nodes OM: A 5x, B 30x.

Figure 2. Dichotomous role of IFNγ: PD-L1(brown), CD163 (red) c-Maf (blue) triple staining of case 1 showed that granulomas were significantly positive for PD-L1 as secondary effect of IFN-γ activation. O.M.: 20x.

Figure 3. Th1 and Th2 expression in the controls: only few scattered Th1 cells (CD4+ red, T-bet+ brown) were present in the reactive lymphoid tissue of controls. (A). Th2 cells (CD4+ red, GATA3+ brown) in the reactive tissue ranged from to 10 to 25% (B) O.M. A, B 10x.

Figure 4. Expression of T-bet in B cells: CD20 positive B cells (red) expressing nuclear T-bet (brown) were particularly evident in case 1 where the BL cells were found in the perifollicular areas. (O.M. 20x).

Figure 5 Partial lymph node involvement by BL in a HIV positive patient: Small to medium-sized nests of atypical medium-sized cells with round nuclei, finely clumped chromatin, multiple small peripheral nucleoli, and small to moderate amounts of basophilic cytoplasm morphologically consistent with Burkitt cells were occasionally present in the immediate proximity of the monocytoid B cells hyperplasia. Mitotic figures and apoptotic bodies were seen within the nests and focal ill-defined aggregates (A, B: HE C: Giemsa). No granulomatous reaction was seen.
The immunophenotype CD20 positive (not shown), CD10 positive (D), BCL-6 (E), IgM positivity (not shown), BCL-2 negativity (not shown), high proliferative index by Ki-67 >95% (F), MYC protein expression (G) and cytogenetics t(8;14) translocation by break apart probes; (inset) of the atypical cells was consistent with BL. These cells were also EBV positive by EBER in situ hybridization (H). CD4/CD8 ratio was altered with a higher number of CD8 (Figure L) than CD4 lymphocytes (I). Original magnification (O.M.): A-H: 10x; I-L: 20x.

SUPPLEMENTARY TABLE

Table 1: antibody characteristics

Table 2: The individual scores of the observers and the agreement for all separate cell types: approximate percentages of 80-90% inter-observer agreement (or reproducibility) were calculated by considering all cases and all variables together. The inter-observer reproducibility of each measure was assessed by the coefficient of variation in percent, CV%, which is the ratio of the standard deviation to the mean of the three percent observations. A CV%=0 value indicates perfect agreement; a value greater than 100% indicates virtually no agreement. For each variable, percent agreement across the 4 cases analyzed was then expressed as the mean of 100-CV% and its 95% confidence interval (CI%), estimated using the bias-corrected and accelerated bootstrap technique (BCa).
# TABLE 1. CLINICAL+IMMUNOPHENOTYPICAL FEATURES

<table>
<thead>
<tr>
<th>Case</th>
<th>Age and sex</th>
<th>Location</th>
<th>Stage</th>
<th>Treatment</th>
<th>Prognosis</th>
<th>EBV</th>
<th>MYC protein expression</th>
<th>MYC translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>65 yo, female</td>
<td>Right axillary lymph node</td>
<td>I</td>
<td>None</td>
<td>Healthy after 30 months follow-up</td>
<td>IHC: EBNA-1+ (LMP1-, LMP2-, EBNA-2-, BZLF1-)</td>
<td>Yes (100%)</td>
<td>t(8;14) break apart and fusion probes</td>
</tr>
<tr>
<td>Case 2 LYWS-353</td>
<td>26 yo, male</td>
<td>Mandibular lymph node</td>
<td>I</td>
<td>None</td>
<td>Healthy after 48 months follow-up</td>
<td>IHC: EBNA-1+ BZLF1-</td>
<td>Yes (100%)</td>
<td>t(8;14) break apart and fusion probes</td>
</tr>
<tr>
<td>Case 3 LYWS-360</td>
<td>47 yo, female</td>
<td>Cervical lymph node</td>
<td>I</td>
<td>Three cycles of chemotherapy</td>
<td>Healthy after 24 months of follow-up</td>
<td>IHC: EBNA-1+ BZLF1-</td>
<td>Yes (100%)</td>
<td>t(8;14) break apart and fusion probes</td>
</tr>
<tr>
<td>Case 4</td>
<td>12 yo, male</td>
<td>Mandibular mass</td>
<td>I</td>
<td>Six cycles of chemotherapy</td>
<td>Healthy after 16 months of follow-up</td>
<td>IHC: EBNA-1+ (LMP1-, LMP2-, EBNA-2-, BZLF1-)</td>
<td>Yes (100%)</td>
<td>t(8;14) break apart and fusion probes</td>
</tr>
</tbody>
</table>

*N/A stands for not available

** rituximab plus hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone followed by methotrexate and cytarabine
<table>
<thead>
<tr>
<th>Cell populations</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macrophages</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>M1 (CD68+/CD163-/cmaf-)</td>
<td>95%</td>
<td>85%</td>
<td>90%</td>
<td>80%</td>
<td>25-35%</td>
</tr>
<tr>
<td>M2 (CD68+/CD163+/cmaf+)</td>
<td>5%</td>
<td>15%</td>
<td>10%</td>
<td>20%</td>
<td>55-70%</td>
</tr>
<tr>
<td>CD68+/pSTAT1+/CD123-</td>
<td>65%</td>
<td>75%</td>
<td>40%</td>
<td>35%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>CD68-/pSTAT1+/CD123+</td>
<td>35%</td>
<td>20%</td>
<td>15%</td>
<td>15%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td><strong>pSTAT1+DCs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>90%</td>
<td>80%</td>
<td>85%</td>
<td>75%</td>
<td>50-70%</td>
</tr>
<tr>
<td>Th1 (TBX21+/CD4+)</td>
<td>35%</td>
<td>20%</td>
<td>30%</td>
<td>25%</td>
<td>5-10%</td>
</tr>
<tr>
<td>Th2 (GATA3+/CD4+)</td>
<td>10%</td>
<td>15%</td>
<td>25%</td>
<td>15%</td>
<td>25-40%</td>
</tr>
<tr>
<td>T Reg (CD25+/CD4+/Foxp3+)</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
<td>5-15%</td>
</tr>
<tr>
<td>Cytotoxic (CD8+)</td>
<td>15%</td>
<td>20%</td>
<td>15%</td>
<td>25%</td>
<td>30-40%</td>
</tr>
<tr>
<td><strong>NK</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CD56+,CD57+</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

Table 2. TME results