UNIVERSITÀ
DI SIENA
1240

DEPARTMENT OF MEDICAL BIOTECHNOLOGIES

DOCTORATE RESEARCH IN MEDICAL BIOTECHNOLOGIES

Ciclo XXXV

Coordinator: Professor Lorenzo Leoncini

" Burkitt Lymphoma: from B-Cell Receptor activation to Tumor Microenvironment"

Supervisor: Professor Lorenzo Leoncini

Co-Supervisor: Professor Cristiana Bellan

Candidate:

Maria Chiara Siciliano

Maria Chiara Siciliano

Firmato digitalmente da Maria Chiara Siciliano
Data: 2023.04.11 11:13:11 +02'00'

Academic year: 2021-2022

INDEX

1. B cell receptor (BCR)

1.1 STRUCTURE AND ORGANIZATION OF THE BCR

The human immune system has the responsibility of recognize and dispose foreign organism and their antigens invaders. There are two different mechanism, namely natural and adaptative immunity, by which these tasks are accomplished. Natural immunity is the first line of defense against pathogens and is an immediate, general, and non-specific complement system. Conversely, adaptive immunity is built up as we are exposed to disease and is based on the ability of B and T cells to respond specifically and selectively to challenges by different antigens [1]. In this scenario, B cells play a central role in the adaptive immune response and provide durable protection against pathogens by differentiating into plasma cells that secrete pathogen-specific antibodies. Discovered in the early 1960s together with T cells, B cell are small (6-10 μm) with a dense nucleus and little cytoplasm and can be distinguished phenotypically only by analysis of cell surface markers. B-cell activation is triggered by antigen recognition by the B-cell receptor (BCR), resulting in B-cell proliferation and affinity maturation within germinal centers. Notably, B cell activation is critical for vaccine development and molecular understanding of the role of B cells in autoimmune diseases [2].

The BCR is a complex composed of a membrane immunoglobulin (mIg) molecule and an associated Igα/Igβ (CD79α-β) heterodimer (Figure 1). Specifically, the BCR is a structure of four (in the case of IgD) or five (IgM) immunoglobulin domains in the heavy chain linked by a hinge and a short intracellular domain consisting of just three amino acids: lysine, valine, and lysine (KVK) [3]. The cytoplasm portion of Igα/Igβ heterodimer contains immunoreceptor tyrosine-based activation motifs (ITAM), whereas mIg itself does not contain any signaling motifs [4]. The BCR is a highly specialized recognition protein that functions as a receptor for antigens secreted by bacteria, viruses, or other pathogens in the acquired immune system. Structurally, it is composed of two heavy (H) and two light (L) chains held together by bisulfite bonds. Each chain contained a variable (V) and a constant (C) region. The variable regions consist of two different isotypes of immunoglobulins: mature naïve B cells that present IgM or IgD and memory B cells and plasma cells that express IgG, IgA, or IgE [5]. The V regions react with a specific antigen and allow B lymphocytes to act as effective antigen-presenting cells in their native form. The membrane bound BCR of B lymphocytes has no immune activation effector function as its constant region remains embedded in the B cell membrane. The function of BCR is to recognize and bind antigens through the variable region exposed on the

cell surface and transmit a signal that, by activating B lymphocytes, leads to clonal expansion and the production of specific antibodies.

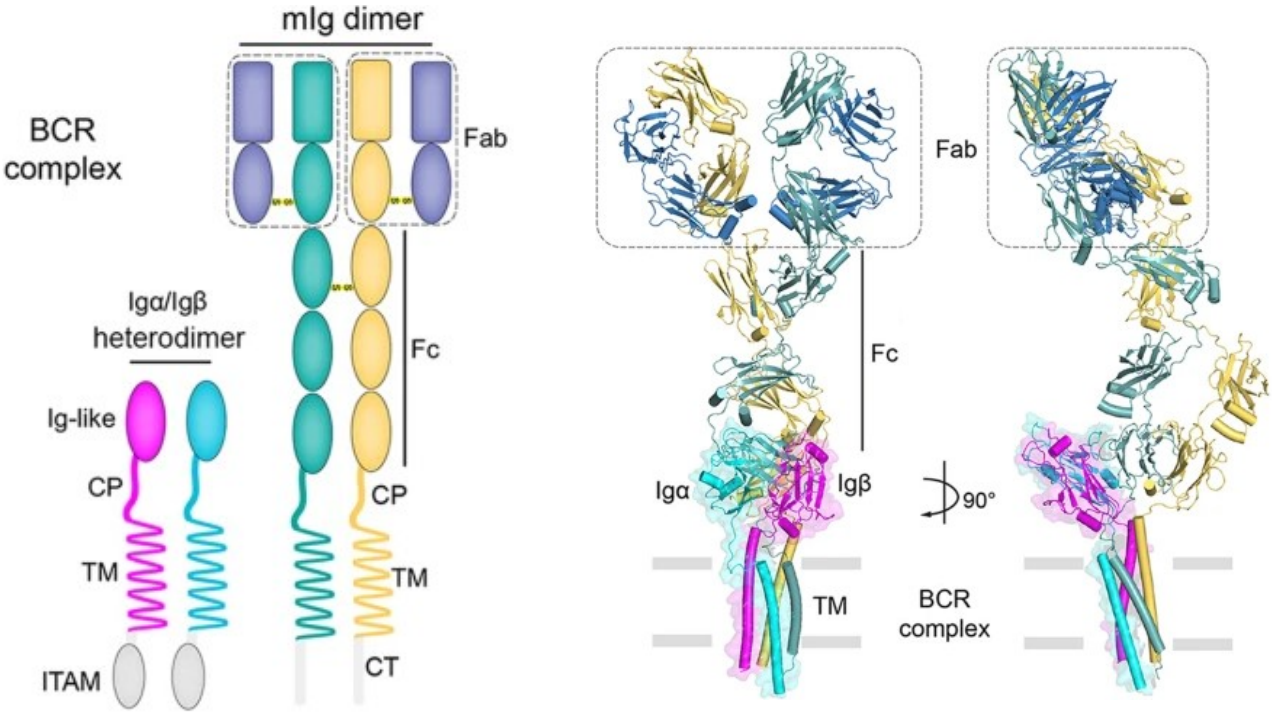


Figure 1. ***Structure of BCR***

In fact, BCR is associated with a set of intracellular signaling proteins responsible for the signal transmission of the B lymphocyte receptor complex. The antigen-binding immunoglobulin on the cell surface is associated with accessory protein chains, Ig-α and Ig-β, through hydrophilic, uncharged interactions between their transmembrane regions. This complex is necessary for both the transport of the receptor to the surface and signal transmission function of the BCR. Ig-α and Ig-β are single-chain proteins composed of an extracellular immunoglobulin-like domain connected to the cytoplasmic tail via a transmembrane domain. Ig-α and Ig-β are held together by a sulfide bond and form a heterodimer called CD79, which is associated with immunoglobulin heavy chains and makes it possible for them to be transported to the cell surface, thus ensuring that fully assembled B cell receptors are present on the cell. CD79α and CD79β possess an immunoreceptor activation motif based on tyrosine residues (ITAMs) in their cytoplasmic tails, which is essential for signal transmission capacity [6].

1.2 SIGNATURE OF BCR DIVERSITY IN B LYMHOCYTES

The BCR signaling pathway is activated when the membrane immunoglobulin (mIg) subunits bind a specific antigen, resulting in receptor aggregation, while the α/β subunits transduce signals to the cell interior. In this context, the complex formed by the CD79α/CD79β heterodimer is called the pre-B-

cell receptor (pre-BCR). Pre-BCR is capable of transducing a signal into the cell that is essential for further B cell development and increase the number of B lymphocytes [7]. Activation of this receptor by the appropriate signals induces multiple binding (cross-linking) of surface immunoglobulins and the recruitment and activation of Src family protein tyrosine kinases: Lyn, Blk, and Fyn as well as the Syk and Btk tyrosine kinases. The BCR-associated kinases phosphorylate ITAM residues of the receptor complex. In the phosphorylated form, ITAMs recruit other molecules that are essential for signal transduction, including Syk [8,9] Once activated, Syk protein kinase phosphorylates other targets, including the BLNK bridging protein, which has multiple sites for phosphorylation and recruits various proteins containing Src homology 2 (SH2) domains, including enzymes and adaptor proteins, to form several different multi-protein signal transduction complexes [10]. Two phosphotyrosine-binding SH2 domains of the Syk tyrosine kinase bind to ITAMs and trigger an activation cascade that further activates other tyrosine kinases such as Btk, serine/threonine kinase such as protein kinase C, the Ras pathway, and calcium signaling pathways. Different adaptor proteins, like CD19 and BLNK, and signaling enzyme such and PLCγ2 and PI3K, are important mediators of the signaling cascade. Recruitment and activation of tyrosine kinases result in the activation of multiple downstream signaling pathways, which generates diacylglycerol and inositol phosphate second messengers, leading to calcium entry and activation of the NFAT factor, which is responsible for the induction of transcription of specific genes, resulting in cell proliferation and differentiation [11]. In addition, the outcome of the activation response is determined by the maturation state of the cell, the nature of the antigen, the extent and duration of BCR signaling and signals from other coreceptors of B cells, such as CD40, the IL-21 receptor and BAFF-R [12]. Antigen binding leads to the formation of BCR microclusters containing 50-500 BCR molecules. The formation of BCR microclusters requires BCR-induced cytoskeletal rearrangement, because actin polymerization inhibitors prevent microcluster formation. However, another study showed that the BCR signalling pathway leads to the disassembly of actin filaments at the membrane, allowing BCR to flow freely in these regions to form microclusters [13].

The complexity of BCR signaling permits many distinct outcomes, including survival, tolerance or apoptosis, proliferation, and differentiation into antibody-producing cells or memory B cells.

1.3 THE BCR IN CONTROL OF TUMOR B-CELL FITNESS

In both normal and malignant B cells, the signals emanating from surface Ig activate a complex set of downstream pathways, including the PI3K\AKT, the NF-kB, the me mTOR and the RAS\MAPK pathways [14]. Feedback mechanism, including the activation of phosphatases targeting BCR signaling components, and the internalization of the BCR prevent continuous signaling from the

antigen receptor. In addition, nanocluster formation is another mechanism that limits the continuous activation of the BCR. In this case, the spatial organization within the plasma membrane causes IgM to assemble with the nanoclusters, keeping them physically separated from other similar ones containing critical BCR positive signaling effectors such as CD19.

During the differentiation process of mature B-cells, any alteration, such as a genetic alteration, copy number variation or chromosomal aberration, can negatively interfere with terminal death and differentiation, leading to uncontrolled proliferation and, ultimately, the emergence of B-cell non-Hodgkin lymphomas (B-NHL). In fact, antigen-experienced B-cells, such as GC cells and memory B-cells, are believed to represent the progenitor cells of several types of B-NHL, notably follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma (BL) (Figure 2) [15].Despite the diversity in the mechanism driving malignant B cell transformation, genetic evidence supports the concept that surface expression of a functional BCR is needed for both the initiation and the maintenance of all form of mature B-cell neoplasm (except classical Hodgkin Lymphoma), keeping the expression of surface BCR in the form of IgM (eg, eBL), or as isotype-switched antibody (eg, DLCBL and FL). Among the different types of B-cell malignancies, several different tactics are applied for activation of the BCR signaling, including *antigen-dependent* and *antigen-indipendent* activation [16]. “Chronic” exposure to microbial antigens is important in promoting clonal expansion of B cells expressing distinctive BCRs and growth of the neoplastic clone. Chronic activation has been implicated in the pathogenesis of different lymphomas, such as Helicobacter pylori in mucosa associated lymphoid tissue lymphoma, Chlamidya Psitaci in ocular adnexa lymphoma, Hepatits C virus in splenic marginal zone lymphomas [17-19].

On the other hand, in diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL), next-generation sequencing (NGS) studies have revealed recurrent mutations of genes belonging to the BCR pathway, converging in the two different types of intrinsically deregulated downstream BCR signals—namely, chronic active signaling in DLBCL and tonic signaling in BL [20,21].

In DLBCL, mutations affecting the BCR pathway are particularly enriched in cases showing the activated B-cell–like (ABC) molecular phenotype. In particular, mutations of CD79B and CARD11 have been identified in about 10% of ABC DLBCL cases. These mutations are sufficient to intrinsically activate survival signaling in the malignant B cells and obviate the need for upstream BCR signaling. Most of sporadic Burkitt lymphomas (sBLs) (~70%) harbor gain-of-function mutations affecting the *TCF3* gene or mutations disrupting the *TCF3*-negative regulator *ID3*. The progressive acquisition of mutations in the TCF3/ID3 genes resulting in intrinsic activation of BCR signaling, thus neoplastic clone may grow independently of EBV. Conversely, in endemic Burkitt Lymphoma (eBL), due to chronic Plasmodium falciparum malaria stimulation continues to reactivate

latently EBV-infected memory B cells, the intrinsic activation of BCR is not the more relevant pathogenetic mechanism [22-24].

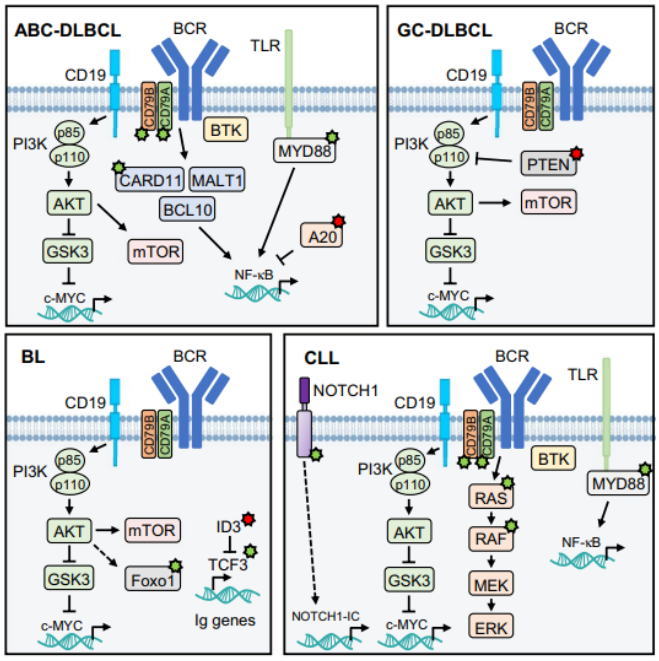


Figure 2. ***BCR activation in B-cell lymphomas***

1.3.1 BCR activation in Burkitt Lymphoma (BL)

In BL, different data suggested that a strong antigenic pressure could potentially be related to prolonged microenvironment interactions, by which virus such as Epstein Barr Virus (EBV) may directly trigger malignant B-cell transformation by "chronically" activating B-cell clonotypes expressing BCRs reacting against selected viral epitopes and thus promoting neoplastic clone growth. In EBV+ BL cases, viral LMP2A protein mediates viral latency by mimicking a constitutively activated BCR. In vivo, LMP2A provides developmental and survival signals to BCR-negative B cells, allowing them to survive in peripheral lymphoid organs, cooperating in reprogramming the function of normal B-lymphocytes and enhancing MYC-driven lymphomagenesis through the activation of the PI3K pathway [26, 25]. "Tonic" BCR signalling, which provides an antigen-independent constitutive baseline signal, induces cell survival in the majority of BL. An important component of the tonic BCR signalling pathway is Transcription factor 3 (TCF3) which normally increases the expression of BCR, leading to increased PI3K signalling; BL largely depends on TCF3 because it enhances pro-survival PI3K signalling. In some instances, TCF3 is mutated which is usually a gain of function. These mutations can promote the cells to become independent of EBV. Another potential cellular compensation through the gain of function of TCF3 mutations is the loss of expression of LMP2A, which is known to activate the PI3K pathway to mediate B cell survival

thus, a mutation that removes the need to stimulate the PI3K pathway might allow for the loss of expression of LMP2A. Through hierarchical clustering of both endemic and sporadic cases on TCF3 target genes, Abate et al; found that the TCF3 pathway was more activated in EBV-negative cases, as indicated by the significant negative enrichment of TCF3 target genes in EBV-positive samples. It is possible that these mutations are found in EBV-negative BL because they lose their dependence on EBV and, therefore, no longer maintain EBV DNAs following the acquisition of these mutations in TCF3, along with other compensatory cellular mutations (Figure 3) [27-29].

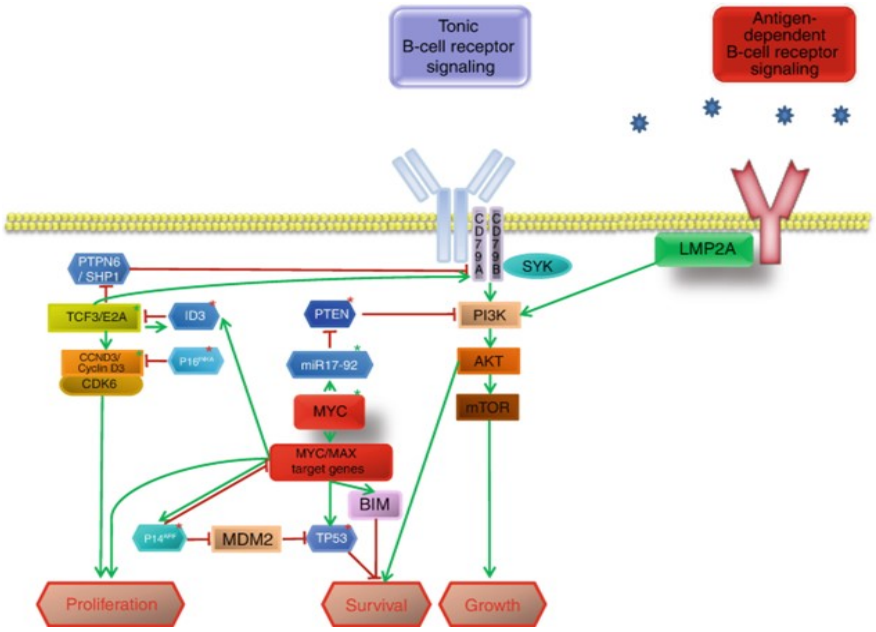


Figure 3. ***"Tonic" and "chronic" activation of BCR***

1.4 CAN MICROENVIRONMENT INTERACTIONS REPLACE BCR FUNCTION IN PROMOTING LYMPHOMA GROWTH?

The relationship of B cells to their microenvironment is crucial for the establishment of altered proliferative/apoptotic pathways, which are hallmarks of lymphoma pathogenesis. The generation of Ig-negative B cells during tumor progression should result in these cells lacking essential survival and proliferation signals provided through BCR signaling [30]. This selective pressure for BCR-negative cells means that only those that receive sufficient compensatory signals can survive and possibly persist. Following genetic alterations accumulated by tumor cells before the loss of the BCR, compensatory signals are activated, which lead these cells to resistance to apoptotic stimuli and to better proliferation [31]. In a scenario like that of the tumor microenvironment, BCR-negative malignant B cells could also remodel the interaction with surrounding immune and stromal cells, possibly receiving extrinsic cell survival and proliferation signals through the activation of alternative signaling pathways. Clues to the nature of microenvironment-related stimuli that could be positively

influencing. It is still unclear how the microenvironment might positively influence the expansion of BCR-negative lymphoma cells [32]. Stromal adhesion and microenvironment-derived signals that underpin BCR-deficient malignant B cells could be related to a disruption of some chemokines. For example, in GC B cell-derived tumors, interference with BCR expression/function could increase CXCR4 expression, and thus, possibly, its signaling following binding to CXCL12 released from neighboring stromal cells [33,34].

2. Tumor microenvironment (TME) in B cell lymphomas

2.1 TME: A PHYSICAL BARRIER AROUND TUMOR CELLS

The tumour microenvironment is a complex and continuously evolving network of various interactions between immune cells and non-cellular components such as immune cells, stromal cells, cytokines, blood vessels, and extracellular matrix components, including sclerosis, whose composition is guided by the neoplastic cells and which in turn, influence tumour initiation, progression, and drug resistance. The role of TME is a key characteristic of lymphomagenesis and progression, in addition to somatic mutations and inflammation, thus sustaining tumour proliferative signalling, resisting cell death, and evasion of growth suppressors [35,36]. In addition, tumour cells also use these interactions to generate immunosuppressive mechanisms that promote tumour escape from immune surveillance, leading to disease progression. and immune escape mechanism. Therefore, TME is a dynamic and interactive entity that forms a physical barrier around tumour cells. A full understanding of TME biology and the interaction between lymphoma cells and TME, as well as the host immune system and the TME, is necessary to provide distinctive insights into therapeutic potential [37,38]

2.2 TME OF B CELL LYMPHOMAS

The TME of B-cell lymphomas is highly variable in terms of both the spatial configuration and composition of cells, playing an important role in the origination, invasion, and metastasis of B cell lymphomas. Scott and Gascoyne suggested three models that divide the wide range of TME described in B-cell lymphomas with a range of tumour cell content, ranging from ~1% in Hodgkin's lymphoma to typically more than 90% in Burkitt's lymphoma (figure).

The determinants of composition and spatial arrangement of the TME include the genetic aberrations that are harboured by the malignant cells, the degree to which these cells remain dependent on external stimuli for survival, proliferation and immune evasion, and the inflammatory response of the host. The interplay between these elements produces three main pattern of tumour microenvironments observed within the various lymphoma subtypes.

FL best represents the *'re-education'* model. In this case, during the course of the disease, malignant cells depend on cells in the microenvironment for survival and proliferation signals. In many cases, the composition and spatial arrangement of cells within the tumour resemble those of normal lymphoid tissue, with neoplastic nodules resembling reactive germinal centres with prominent follicular dendritic cells (FDC) and follicular T helper cells (TFH). The second patter is the '*recruitment'* model, recognizable in classical Hodgkin's lymphoma (cHL). The tumour microenvironment comprises an extensive supportive environment of non-malignant cells that are distinct from the composition of normal lymphoid tissue, surrounding the infrequent malignant Hodgkin Reed-Sternberg cells (HRS cells). Last, the *'effacement'* model comprehends Burkitt lymphoma (BL), where genetic aberrations, such as MYC translocation and cooperating mutations, cause the microenvironment to grow and survive independently. In these lymphomas, the composition of microenvironment is poor, with extensive infiltration by malignant cells. The only microenvironment is the occasional Tingible-body macrophages. In fact, these tumors are characterized by a scarcity of reactive T cells (Figure 4) [39].

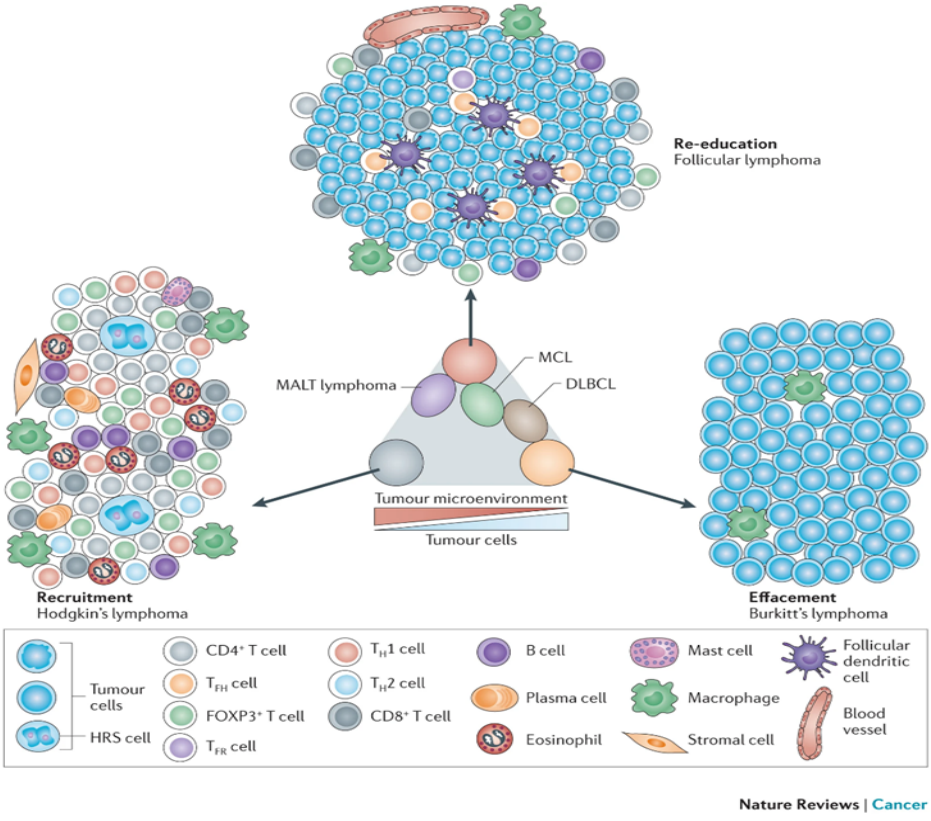


Figure 4. ***TME in B cell lymphomas***

The neoplastic cells influence the composition of the microenvironment, which consists of the remnants of normal lymphoid tissue, effaced, or infiltrated by malignant cells and the host inflammatory response. Thus, neoplastic cell impact on the TME by homing to anatomic sites that contain potentially supportive cells that further alter these environments by recruiting and then further alter these environments by recruiting cells and/or influencing their differentiation.

Lymphoma cells acquire a homing and trafficking mechanism like non-malignant cells to locate tumor-promoting environments. In this scenario, chemokines and adhesion molecules are important in the homing of normal and malignant B-cells, and thus in the dissemination of lymphoma [40].

Chemokines are chemoattractant cytokines that regulate the migration of specific populations of cells during homeostasis and when mounting an immune response; however, this tightly regulated system is often hijacked by lymphomas and associated cells within the microenvironment to attract immunosuppressive cells to the tumor. Emerging results indicate a considerable concentration of CCL2 in tumor tissue, suggesting that the presence of CCL2 may contribute to tumor spreading and pre-metastatic niche formation. high levels of CCL2 promote polarization of TAMs to an immune-regulatory phenotype, which is considered a consequence of tumor cell-mediated immune modulation. In particular, CCL2\CCR2 axis acts as potent factor for M2 polarization contributing to anti-inflammatory response [41].

A pivotal chemokine, CCL22 is mainly expressed by macrophages and dendritic cells, while its receptor C-C motif receptor 4 (CCR4) is mainly expressed by T cells. It has been reported that NHL biopsy specimens also expressed significantly high levels of CCL22, suggesting a possible mechanism for recruitment of immunosuppressive CCR4+ Tregs to the tumours. With regard to the tumour microenvironment, CCL22 polarize TAMs toward M2 macrophages. CCR4 is also known as a marker of regulatory T cells (Tregs) and Th2 cells. CCL22 attracts Th2 cells via CCR4 increasing Th2 cytokines [e.g., interleukin (IL)-4], which leads to the increased expression of CCL22 in M2-like macrophages [42]. High expression of the chemokine CCL17 are found in M2-polarization, being one up-regulated marker of an IL4-polarized M2 macrophages. Indeed, CCL17 and CCL22 are both ligands of the CCR4 receptor and alternatively activated M2 macrophages in response of Th2 cytokines such as IL-4, and IL-13.

2.3 COMPONENT OF TME: ROLE OF MACROPHAGES

TME components, instead of being bystanders, are essential for initiating and maintaining carcinogenesis playing a pivotal role in tumour growth and metastasis. The TME can be divided into two parts: *the non-immune microenvironment*, that express different biomarkers and play a variety of roles in the tumorigenesis and prognosis of B-cell lymphoma, and *the immune microenvironment* that mediate the immunosuppressive microenvironment and escape immunity. The nonimmune microenvironment mainly consists of stromal cells, including cancer-associated fibroblasts (CAFs), extracellular matrix (ECM), pericytes, mesenchymal stromal cells, and other secreted molecules, such as growth factors, cytokines, and chemokines. Both cellular and non-cellular components of the TME The immune microenvironment is composed by T and B lymphocytes, tumour-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), tumour-associated neutrophils (TANs), and natural killer (NK) cells, dendritic cells (DCs) and others. These components mediate the immunosuppressive microenvironment and escape immunity [43].

2.3.1 The "scavengers" of the human body

Macrophages were discovered at the end of the 19th century by Ellie Metchnikoff, who observed phagocytosis and classified the phagocytes as "macrophages". As innate differentiated immune cells, macrophages play a key role in tissue development, homeostasis, tissue repair, and pathological conditions. Macrophages act mainly as functional immune effector cells evoking an immunological response to link innate and adaptive immune responses that contribute to a biochemical milieu involving complex inflammatory events. In response to multiple biological stimuli in the TME, macrophages are recruited locally and differentiate in TAMs, thereby affecting tumour progression. TAMs are responsible for the inhibition of recruitment and activation of T cells via secreting various chemoattractants, including chemokines, cytokines and some complement components (Figure 5).

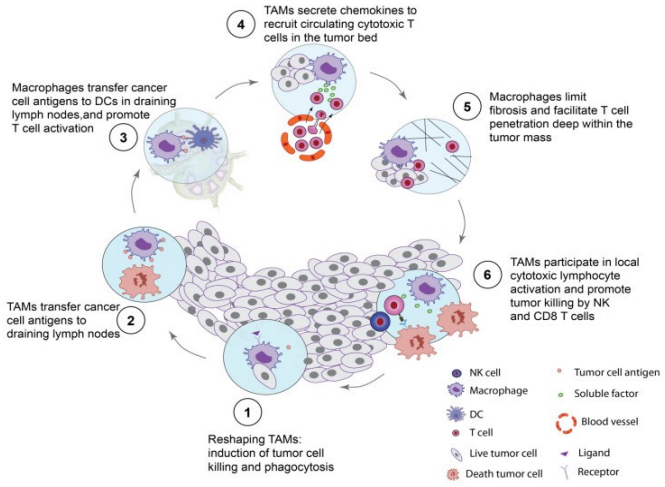


Figure 5. ***Macrophages can act as key effectors in the cancer-immunity cycle***

Owing to the complex tissue niche, macrophages exhibit extreme plasticity, varying their transcriptional landscape and function. Based on this, in the last two decades, macrophage polarization has become increasingly relevant for the role in lymphomagenesis, contributing differently to the immune microenvironment. Macrophage polarization considers the broad outlines of macrophage activation in inflammatory and physiological settings at a given point in time and space. In recent years, a model has been developed to describe the complex mechanism of macrophage activation as polarization towards two opposite states, M1 with proinflammatory properties and M2 with protumoral properties [44]. The M1/M2 nomenclature was inspired by the Th1 versus Th2 nomenclature. M1 macrophages have pro-inflammatory phenotypes and mainly exert pro-inflammatory, anti-bacterial, and antitumor functions by secreting pro-inflammatory cytokines, thus arise in inflammatory settings dominated by Toll-like receptor (TLR) and interferon signalling. M2 macrophages have anti-inflammatory phenotypes and mainly exert pro-repair, pro-tumoral and antiparasitic functions. M2 macrophages are found in settings dominated by Th2 response, such as helminth immunity, asthma, and allergy. Macrophage polarization can occur at any moment during the inflammatory process. The simultaneous presence of T cells producing interferon-gamma and interleukin – 12 (IFN-γ, IL-12) or interleukin 4 – interleukin 10 – interleukin 13 (IL-4, IL-10, IL-13) tilts polarization toward M1 or M2, respectively, depending on the amount of cytokine, time of exposure, and competition for cytokines. This leads to dramatic changes in their physiology, including alterations in the expression of surface proteins as co-stimulatory molecules and the production of cytokines and pro-inflammatory mediators that influence T-cell differentiation. A fundamental phenomenon in M1 / M2 polarization is the differentiation of CD4 + T cells into Th1 and Th2 effector cells; Th1 cells are controlled by the transcription factor T-box protein expressed in T cells (T-bet; also called TBX21) and Th2 cells are controlled by GATA-binding protein 3 (GATA3). M1 macrophages are the primary source of proinflammatory cytokines, which also promote cancer immunosurveillance and cytotoxicity. However, these effects are counterbalanced by M2 macrophages with anti-inflammatory and protumoral effects (Figure 6) [45,46].

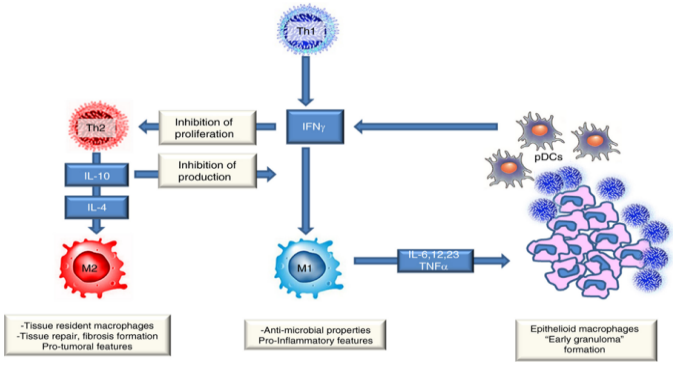


Figure 6. ***The role of Th1 lymphocytes***

In Burkitt Lymphoma, (BL), M2-polarized macrophages are the most prominent TME components. Infiltrating lymphoma-associated macrophages have been described as the classical “starry sky” histological appearance in BL. On the one hand, TAMs play important role in phagocytosis and scavenging of the cytoplasm, promoting the clearance of apoptotic tumor cells and tumor progression. Another important role of TAMs is bind PD-L1 on CD8+ T cells inhibiting inflammatory response and promoting evasion from the anti-tumor immunity of neoplastic cells. It has been reported, in Raji cells that the expression of PD-L1 is facilitated by secretion of cytokines (IL4/IL13) and phosphorylation of STAT3 and STAT6. Recent data suggest that TAMs may secrete cytokines and express immune checkpoints to promote immune suppression. However, the precise mechanism of the pro-tumor effect of TAMs in BL remains poorly understood.

3. BURKITT LYMPHOMA

In 1958 Dennis Burkitt, a surgeon working in Uganda, described for the first time a common cancer affecting children in equatorial Africa regions. This tumour, called Burkitt lymphoma (BL), is an aggressive B cell non- Hodgkin lymphoma (NHL) characterized by the rapidly proliferating tumor involving jaws of African children. The World Health Organization (WHO) classification describes three clinical variants of BL: endemic (eBL), sporadic (sBL) and immunodeficiency-associated (HIV-BL).

Characteristics	Endemic BL (eBL)	Sporadic BL (sBL)	HIV associated BL
Epidemiology	- Equatorial - Median age 7 yrs - Associated with malaria / climate	- Median age 30yrs - Children (30%) - Older adults (1%) - Low Socio Economical Status	- HIV risk groups - Median age 10–19 yrs - Children in Africa?
Clinical Presentation	Facial skeleton (50%) CNS (33%) Other organs also affected	Abdominal, ileo-coecal (80%) Bone marrow (20%) Other organs also affected	Organ and nodal presentation
Geographic regions	Malaria belt	Worldwide	In endemic HIV areas in Africa
Pathology/Morphology	Germinal centre B-cell Monomorphic medium sized B cells with basophilic cytoplasm and multiple mitotic figures		
Chromosomal translocations	t(8;14)(q24;q32) in 60–70% of cases; t(8;22)(q24;q11) in 10–15% cases; t(2;8)(p12;q24) in 2–5% cases		
Ig region involved	Ig heavy chain joining region (early B-cell)	Ig switch region (late stage B-cell)	Not specified in the literature
EBV association	100%	30%	30–50%

Figure 7 . ***Overview of BL clinical variants***

All these subtypes are similar in morphology, immunophenotype, and genetics, but differ in their geographic distribution, epidemiology, risk factors, clinical presentations, and EBV association:

- **eBL** is 100% associated with the presence of EBV and affects African children (50% of all childhood cancer), New Guinea and large areas of South America. This subtype implies an oral presentation, with mandibular and jaw localization. However, the tumour also involves the kidneys.
- **sBL**, which is associated with EBV presence in 10/80% of cases, is usually observed in the USA and Western Europa (three cases per million persons) and is more common in the pediatric population. The sites involved were the abdomen, including the ileum and lymphnodes.
- **Immunodeficiency-associated BL** is most commonly seen in HIV- positive patients and is associated with the presence of EBV in 30/40% of total cases (Figure 7) [47,48].

However, based on recent insights into BL biology, it is recommended by the 5th edition of the WHO classification of hematolymphoid tumors to distinguish EBV-positive and EBV-negative BL based on their molecular features despite the epidemiological context and geographic location [49].

Furthermore, the BL subtypes also differ in terms of EBV association. In particular the virus is detected in 100% of endemic BL cases, 10-80% of sporadic BL cases and 30-40% of Immunodeficiency- associated BL cases. There are still many unsolved questions as to how EBV contributes to BL oncogenesis and the variable association of the BL epidemiologic subtypes with EBV presents a challenge to our understanding of the etiology of this malignancy. One of the paradoxes of determining EBV.s role in BL oncogenesis is the fact that only one EBV latent protein, EBNA1, is consistently expressed in BL. One observation that favoured the carcinogenic role of EBV in BL was the discovery that EBV is an extremely potent transforming virus in culture, as it is capable of converting >50% of B-cells into latently infected, continuously proliferating lymphoblastoid cell lines (LCLs) within days [50]. The link between EBV and human cancer is, however, evolving and may reside in the identification of the type of latency. Indeed, EBV can present three latent gene expression patterns: latency I, II and III. Although EBNA-1 and EBERs (latency I) are thought to be the only EBV genes expressed in endemic lymphomas, with a specific role in preventing apoptosis and neoplastic cell survival, other recent studies have found that a smaller percentage of these tumours present a new form of latency (so-called 'non-canonical' latency) with a different and broader gene expression profile than previously thought, in which LMP1 and LMP2 are expressed in 20% of BLs. Subsequently, further investigations showed that eBLs may consist of tumour cells expressing variable patterns of EBV gene expression, each of which confers a different level of resistance to apoptosis. Thus, EBNA-1, 3A, 3B, 3C and LP-positive and EBNA-2, LMP-negative Burkitt's lymphoma cells were the most resistant to apoptosis, while EBNA-2-positive, LMP1-negative Burkitt's lymphoma cells showed reduced but 'intermediate' resistance. Other studies have suggested that biopsies taken from Burkitt's lymphoma masses may express other latent proteins, including LMP1, LMP2A and EBNA2. In particular, LMP2 appears to play a role in the early stages of BL development, where the survival signal allows the expansion of cells containing a MYC translocation. Expanded cells increase the likelihood of acquiring a p53 mutation, leading to tumour progression. After p53 mutation, tumour cells become less dependent on LMP2A and immune selection may explain the low levels of LMP2A present in tumour biopsies. For EBV-BL subtype, a hit and run mechanism has been proposed where EBV plays an initiating role in oncogenesis, but viral genome is lost. Recent study identified “traces” of EBV infection in BL primary tumors where they detected EBV miRNA but not EBER. Analysis of cellular mutations in BL EBV- positive also has led to the

hypothesis that EBV can lost because compensatory mutations have occurred that substitute for the functions of EBV proteins [51,53].

At the histological level, BL is characterized of medium-sized cells with a germinal center B- cell phenotype CD10+, BCL6+, BCL2-/weak, high Ki67 index (>95%) and an IG::MYC juxtaposition [49]. BL show histiocytes containing abundant cytoplasm dispersed among a background of basophilic tumour cells and a monomorphic population of rapidly proliferating B- cells with low cytoplasm. Normal infiltrating lymphocytes are not present while scattered phagocytic macrophages will give rise to the characteristic histological aspect of BL, known as the "*starry sky" appearance.* These macrophages along with mesenchymal stem cells, stromal cells, immune cells, and soluble factors represents the tumour microenvironment (TME). The activity of macrophages and their impact on the tumour immune response in BL remain unclear. However, TAMs may function as a potential intermediate of tumour advancement due to the secretion of chemokines, cytokines, and the expression of immune checkpoint-associated proteins such as Programmed Death-Ligand 1 (PD-L1). Interestingly, there are few cases of BL characterized by *conspicuous granulomatous reactions*, which can obscure neoplastic proliferation. The granulomatous reaction is a distinctive pattern of chronic inflammation characterized by nodular aggregation of inflammatory cells, predominantly activated macrophages, which are transformed into epithelioid cells. Macrophages in granulomas derive both from circulating monocytes attracted by chemotaxis, and from local, resident macrophages recruited by T cell-derived growth factors. CD4+ T cells accumulate in the center of epithelioid granulomas, whereas the majority of CD8+ T cells are found in their periphery. Usually, such cases are EBV-positive, present in the early stage of disease, and may have spontaneous regression without therapy. The characterization of macrophages shows M1 polarization and pro-inflammatory response which can possibly explain spontaneous regression [46].

3.1 MOLECULAR FEATURES OF BL

All BL types are characterized by a chromosomal translocation juxtaposing MYC and the immunoglobulin heavy or light chain locus (IGH) [t (8;14)], bringing the MYC gene under the transcriptional control of an immunoglobulin locus. Overexpression leads to rapid B cell proliferation and apoptotic cell death. Precisely, most of the cases of Burkitt's lymphomas presented the *MYC* translocation at band 8q24 to the Immunoglobuline heavy chain locus (IGH) (14q32) or, less commonly, at the lambda (22q11) or kappa (2p12) light chain loci (IGL). The reciprocal translocation t(8:14) occurs in approximately 80% of tumours, the remaining 20% being represented by t(2;8) and t(8;22). The first molecular difference in eBL and sBL was the

breakpoint differences in MYC traslocation. In African endemic cases, the breakpoint on chromosome 14 involves the heavy-chain joining region and originate from aberrant somatic hypermutation, whereas in sporadic forms, the translocation involves the heavy chain switch region. Finally, up to 10% of the cases may lack a demonstrable *MYC* translocation by Fluorescence In Situ Hybridation (FISH), otherwise evidenced using other molecular techniques. Translocation and deregulation involving *MYC* gene on chromosome 8 is highly characteristic but not specific for Burkitt's lymphoma [54] . Emerging evidence suggests a dual mechanism of BL pathogenesis: virus-driven versus mutational, depending on EBV status. EBV-positive and EBV-negative BL share evidence of coding mutations affecting pathways such as BCR and PI3K signaling, apoptosis, SWI/SNF complex and GPCR signaling. In comparison with EBV-negative BL, EBV-positive BL show significantly higher levels of somatic hypermutation particularly in noncoding sequences close to the transcription start site, harbours fewer driver mutations, particularly in apoptosis pathway, and show a lower frequency of mutations in the genes encoding the transcription factor TCF3 or its repressor ID3. With the advent of more comprehensive "omic" techniques were identified additional molecular pathway involved in BL oncogenesis. Other common pathways dysregulated in BL include mutation in ARID1A, SMARC4, MCL1, FBX011, DDX3X, CCND3, GNA13 [49,55,56].

4. EPSTEIN BARR VIRUS (EBV)

Fifty years ago, the idea that a virus could cause human cancer was controversial, and to date Epstein-Barr virus (EBV) is the first human tumor virus to have been discovered. EBV (human gamma-1 herpesvirus), is a member of Herpesviridae family, infects over 90% of adults usually without consequences or causing infection mononucleosis. In line with the systematic nomenclature adopted, the formal designation is human herpesvirus 4 (HHV-4). Serendipity event lead to the EBV discovery. A BL biopsy was on a delayed flight that reached the London laboratory later than expected. The microscopic examination showed the presence of transformed cells floating in the tumor mass and infected by small viral molecules. The virologist Anthony Epstein, who performed such analysis, claimed "Exhilarated to observe unequivocal virus particles in a cultured BL cell", and from that moment he established the first isolated cell line of human lymphoma. Later, EBV was classified as oncovirus, showing its ability to quickly convert approximately 3-10 % of lymphoblastoid cell lines (LCLs). The importance of this oncovirus is linked with several human cancers, including lymphoid and epithelial tumors. EBV is associated with about 1.5% of different types of human cancer worldwide, including 100% endemic Burkitt lymphoma (eBL), 30% Hodgkin's lymphoma cases (HL), 10% gastric carcinoma cases (GC), 5-10 % Diffuse large B cell lymphoma (DLBCL) and 1-3% cases of follicular lymphoma (FL) [57-59].

4.1 EBV INFECTION

The infection is asymptomatic, and the virus establishes a life-long infection in the memory B-cell that represents the main reservoirs of the virus. Similarly to Herpesviridae family members, EBV is characterized by three-layer configuration: the outer envelope containing viral glycoproteins for the host recognizing and a double lipoprotein membrane (pericapside), the inner (pseudo)icosahedral nucleocapsid enclosing 172 kb of double-stranded DNA and the middle pleomorphic tegument compartment. During primary infection, after an initial burst of replication burst of replication in the oropharyngeal epithelium, EBV infects the B lymphocytes of the oropharyngeal mucosa. The target cells of the virus are the B-lymphocytes, which present the CD21 receptor on their membrane, that

bind the viral glycoproteins gp350 and gp220 present on the virus surface . The viral infection can be explained by two models: the "germinal center" and the "direct infection" models of virus persistence (Figure 8).

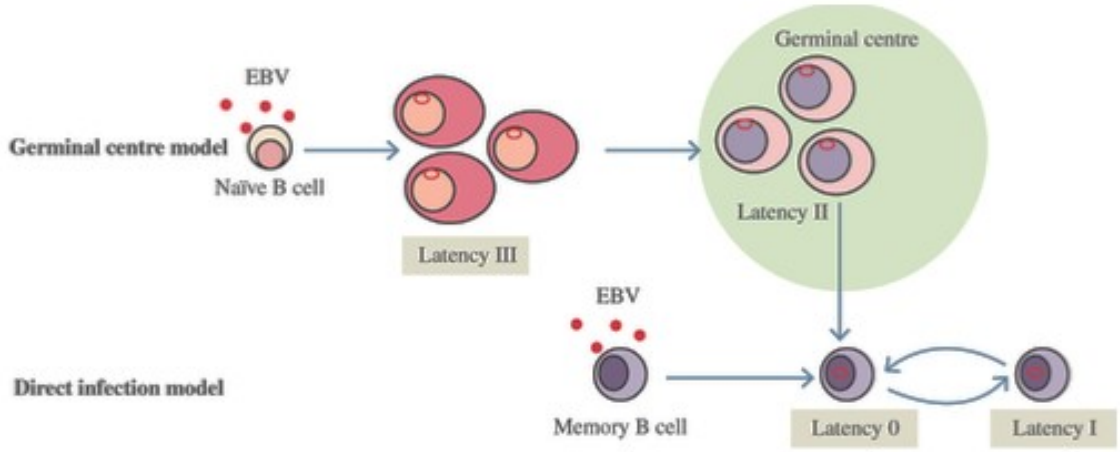


Figure 8. Two different models of EBV life cycle in B cells system

The first model provides an initial infection of naive B cells that guide the expansion of EBV +. This phase is known latency program III and leads to the cell proliferation of infected cells. This latency is characterized by the expression of all latent genes including Epstein- Barr Nuclear Antigen 1\2\3(EBNA1\2\3), latent membrane protein 1\2 (LMP1\2), BZLF1, BRLF1.

Then, the infected cells will go in the germinal center (GC) and switch to a latency program II, which offers the signals for the survival in the GC. This type of latency is characterized by a lower number of viral proteins as EBNA1, LMP1 /2. LMP proteins, homologues to CD40 and BCR signaling respectively, are required for the cell survival and exit of EBV infected cells from the GC. These cells will differentiate in memory B-cell cell and will not express viral proteins (latency 0) to evade immune recognition. EBNA1 only will be expressed because is essential for the segregation of viral episome to daughter cells (latency I). Furthermore, EBNA1 is apparently able to regulate key survival genes in B cells. On the other hand, LMP1 can modify cellular transcription through a multitude of mechanism that include the overexpression of the transcription factors, through DNA methyltransferases and protein arginine methyltransferases. The "direct infection" model suggests two different way of infection: EBV infects directly GC B cells that continue to proliferate, or memory B cells are directly infected by EBV and then will proliferate enter in the GC.

Once inside the host's cells, the viral genome can be observed in an episomal form, in which the viral circular DNA remains separated from the human genomic material. In particular condition, the virus can be virus embedded in host genome, as mainly observed by *in vivo* experiments. After infected the B lymphocytes, EBV governs the synthesis of some viral proteins, which interfere with the cellular

DNA modifying the expression of numerous target genes and leading to B lymphocytes immortalization.

EBV infection presents two different strategies: lytic and latent state. The first, characteristic of most viral infections, consist of a viral replicative cycle (lytic state) where the infected cells will be disrupted and will infect other target cells. The second, a fascinating property of EBV, involves a latency state in which the virus does not multiply inside the cell but transmits its genome to the daughter cells. The switch from latency to the lytic cycle is intermediated by two viral transactivator proteins called BZLF1 and BRLF1, which control the expression of other viral and cellular genes necessary for EBV DNA synthesis. In particular, these two proteins activate a cascade of events, as well as the sequential expression of frequent "early" and "late" viral genes that lead to cell death and the release of other virions.

During latency EBV expresses specific latency genes including EBNA-1, -2, -3A, -3B, -3C, -LP, LMP1 and LMP2 (two isoforms, LMP2A and LMP2B), non-coding RNAs such as EBER-1 and -2, BRLF1, BHRF1-miRNA and BART-miRNA. Latency gene expression program causes proliferation and resistance to cell death in the infected cells. On the other hand, the lytic genes include viral transcription factors (BZLF1), viral DNA polymerase (BALF5) with associated factors, viral glycoproteins such as gp350 and gp110 and structural proteins. Based on the gene expression pattern we can distinguish three types of latency, each of that is associated with different EBV-related tumours. Type III latency is the less restrictive form, its gene expression program (growth program) is characterized by the expression of all of the EBNAs as well as the LMPs and can be appreciated in naïve B-cells of healthy carriers, in post-transplant lymphoproliferative disease (PTLD) and DLBCL. According to the germinal centre model after type III latency program activation, B-cells undergo germinal centre reaction and only three latent EBV proteins, EBNA1, LMP1, LMP2, can be found in centroblasts and centrocytes, this kind of gene expression profile defines the type II latency (default program). Type II latency expression is appreciable in Reed-Sternberg cells in HL, as well as BL, with additional LMPs down-regulation.

During type II latency LMP1 and LMP2A, being CD40 and B-cell receptor (BCR) homologs respectively, provide the necessary signals for survival and post-germinal centre differentiation of the EBV-infected B-cells, allowing them to gain access to the memory B-cell pool, in which EBV will persist in latency 0. No viral proteins are expressed during latency 0 but when cell division occurs, EBNA1 restart to be expressed, this determines the switch to type I latency. BZLF1 and BRLF1 are viral transactivator proteins that allow the switch from latency to lytic cycle. During type I latency the expression is limited to *EBNA-1*, *EBER* and BARTs and occurs during the homeostatic proliferation [60-66].

4.2 MOLECULAR FEATURES ASSOCIATED TO EBV

EBV-associated versus non-EBV-associated malignancies differ regarding their gene expression profiles, metabolism, signal transduction and their immune escape mechanism. It has been reported that EBV may induce a strong up-regulation of PD-L1 expression both directly on the surface on the surface of human primary monocytes, or directly on neoplastic cells, through its viral protein LMP-1 which interfere with downstream cellular signaling to induce an immune tolerant niche for EBV-related tumors. In addition, there is sufficient evidence that tumor cells in EBV-positive lymphomas depend on the TME for certain stimulatory factors. The most important pathways involved in tumor cell promotion in EBV-associated malignancies are the NF-kB and JAK/STAT pathways [67].

- **PD-L1\PD-1 pathway and escape mechanism**

PD-L1 is an immune checkpoint gene that when translated becomes a major regulator of T cell function and, after engaging Programmed cell death protein 1 (PD-1), leads to an altered functional state of T-cells, namely T cell exhaustion. The expression of PD-L1 in B-cell lymphoma remains controversial, especially in BL. Indeed, PD-L1 has been reported in 80% of BL cases (8 out of 10) by Majzner. However, the constitutive association between EBV and BL, especially with endemic Burkitt lymphoma, presents questions regarding the role of the virus in modifying and actively shaping the TME. Indeed, EBV orchestrates a variety of complex mechanisms favouring the escape of lymphoma cells from anti-tumour immune responses while stimulating the creation of niches in which tumour cells may find support for their growth and survival. EBV may induce a strong up-regulation of PD-L1 expression both directly on the surface of human primary monocytes and indirectly on neoplastic cells through its viral protein LMP-1, which interferes with downstream cellular signalling (i.e., JAK/STAT) to induce an immune tolerant niche for EBV- related tumours. LMP-2 may also exert its tolerogenic effect by affecting crucial cell-cycle regulating pathways such as PI3K/Akt, which plays a critical role in PD-L1 expression [69,70].

- **The JAK/STAT pathway**

The Janus Kinases–Signal Transducer and Activator of Transcription (JAK/STAT pathway) is mediated via cytokine receptors. Upon binding of the cytokines (IL-6, IL-8, and IL-10) to the Class I cytokine receptors, dimerization occurs, and JAK proteins are phosphorylated. This leads to phosphorylation and dimerization of STATS, which moves to the nucleus and functions as a transcription factor. Many EBV-related malignancies are characterized by dysregulation of the Jak/STAT pathway. The EBV tegument protein BGLF2 has been identified as a potent suppressor of

JAK-STAT signalling. This is achieved by inhibiting two essential proteins, STAT1 and STAT2, which mediate the antiviral activity of interferons. BGLF2 recruits the host enzyme to remove the phosphate group from STAT1, thereby inactivating its activity. BGLF2 also redirects STAT2 during degradation. Tan et al. discovered that in EBV+ BL, the JAK/STAT pathway is constitutively activated by the amplification of JAK2 or, in some cases, mutations in the inhibitors PTPN1 and SOCS1 [69]. In addition, LMP1 interacts with JAK3 and activates STAT1 in B cells, which promotes corresponding transcriptional regulation in the nucleus and can provide feedback and enhance the regulation of its own expression (Figure 9). LMP1 enhances the transcription of AP-1 through JAK/STAT and promotes the expression of PD-L1 to avoid immune surveillance. LMP1 and LMP2A can activate HLX to inhibit apoptosis [67].

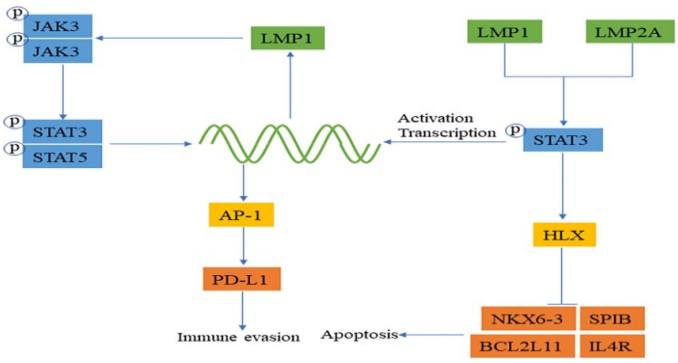


Figure 9. ***JAK-STAT signalling pathway influences EBV oncogenesis***

- **The NF-kB pathway**

The nuclear factor kB (NF-kB) is a critical transcription factor involved in a broad range of biological processes, including immune responses, cell survival, stress response and maturation of various cell type. NF-κB regulates the growth and survival of B cells, interlinking with numerous other pathways, including B cell receptor signaling, PI3K/Akt/mTOR signaling, and toll-like receptor signaling pathways. The BL EBV- positive signature includes increased expression of NF-κB and its activation depend on upstream signaling of phosphatidylinositol 3-kinase (PI3K), which plays a key role in survival signaling. The NF-κB pathway can be divided into canonical and non-canonical pathways. The canonical promotes inflammation, cell proliferation and cell survival and involves BCR. It has been reported that, Decoy receptor 3 (DcR3) in Nasopharyngeal carcinoma (NPC) tissues is upregulated *in vitro* by LMP1, and binding TNSFSF14 can enhances migration and invasion via NF-κB. The non canonical pathway regulates lymphoid development, has anti-inflammatory activity. In EBV-positive Gastric cancer (GC) the virus activates CD40 signaling and promotes survival and proliferation. Thus, signals derived from the TME can stimulate the non-canonical pathway [70,71].

5. *Immune landscape in BL*

Granai *et al. Infectious Agents and Cancer* (2020) 15:28
https://doi.org/10.1186/s13027-020-00292-w

Infectious Agents and Cancer

RESEARCH ARTICLE Open Access

Immune landscape in Burkitt lymphoma reveals M2-macrophage polarization and correlation between PD-L1 expression and non-canonical EBV latency program

Massimo Granai[1,2†], Lucia Mundo[1†], Ayse U. Akarca[3], Maria Chiara Siciliano[1], Hasan Rizvi[4], Virginia Mancini[1], Noel Onyango[5], Joshua Nyagol[6], Nicholas Othieno Abinya[5], Ibrahim Maha[7], Sandra Margielewska[8], Wenbin Wi[8], Michele Bibas[9], Pier Paolo Piccaluga[10], Leticia Quintanilla-Martinez[2], Falko Fend[2], Stefano Lazzi[1], Lorenzo Leoncini[1*] and Teresa Marafioti[11,3]

Abstract

Background: The Tumor Microenviroment (TME) is a complex milieu that is increasingly recognized as a key factor in multiple stages of disease progression and responses to therapy as well as escape from immune surveillance. However, the precise contribution of specific immune effector and immune suppressor components of the TME in Burkitt lymphoma (BL) remains poorly understood.

Methods: In this paper, we applied the computational algorithm CIBERSORT to Gene Expression Profiling (GEP) datasets of 40 BL samples to draw a map of immune and stromal components of TME. Furthermore, by multiple immunohistochemistry (IHC) and multispectral immunofluorescence (IF), we investigated the TME of additional series of 40 BL cases to evaluate the role of the Programmed Death-1 and Programmed Death Ligand-1 (PD-1/PD-L1) immune checkpoint axis.

Results: Our results indicate that M2 polarized macrophages are the most prominent TME component in BL. In addition, we investigated the correlation between PD-L1 and latent membrane protein-2A (LMP2A) expression on tumour cells, highlighting a subgroup of BL cases characterized by a non-canonical latency program of EBV with an activated PD-L1 pathway.

Conclusion: In conclusion, our study analysed the TME in BL and identified a tolerogenic immune signature highlighting new potential therapeutic targets.

Keywords: Burkitt lymphoma, Tumour microenvironment, EBV, PD-L1, Immunotherapy, Immune checkpoint

BACKGROUND

Over recent years, the understanding of the biology of B-cell lymphomas has advanced significantly with the identification of the role played by the tumour microenvironment (TME) in lymphomagenesis [1, 2]. The TME of B-cell lymphomas mainly contains variable numbers of mesenchymal stem cells, immune cells and soluble factors. The complex interplay between tumour cells and TME regulates tumorigenesis and provides novel targets for immunotherapies [3, 4]. In aggressive lymphomas, particularly in BL, due to their high proliferation rate, intensive chemotherapy is required to counteract proliferation and dissemination of neoplastic cells. Unfortunately, these burdensome treatments are not as effective in elderly and immunocompromised patients [5].

Furthermore, in equatorial Africa, where BL is the most common childhood cancer, the prognosis of BL is still poor because the intensive therapeutic regimens often result in a severe neutropenia, with fatal consequences in resource poor settings [6–10]. Shortcomings of current BL therapies make the exploration of new therapeutic avenues a substantial and reasonable aim [7]. Therefore, a proper characterization of the TME in BL might be helpful to identify alternative therapeutic targets. One of the histological hallmarks of BL is the high content of tumour-associated macrophages (TAMs) involved in apoptotic tumour cell clearance that confer the so-called starry-sky appearance [11]. Although little is known about the functional status of macrophages and their impact on tumour immune response in BL, TAMs may function as potential mediator of tumour progression through secretion of chemokines, cytokines and expression of immune checkpoint-associated proteins as PD-L1 [12, 13]. The expression of PD-L1 in B-cell lymphoma remains controversial, especially in BL. Indeed, PD-L1 has been reported in 80% of BL cases (8 out of 10) by Majzner [14]. However, this result was not reproduced by others [15]. Moreover, the role of the antigenic signature of Epstein Barr virus (EBV) in modulating the tumour microenvironment and the expression of immune-tolerant proteins has not been analysed in any of these studies. These different and somehow discordant results may be due to the diverse latency program of EBV infected cells and thus to different patterns of viral genes expression. The constitutive association between EBV and BL, especially with endemic Burkitt lymphoma raises questions regarding the role of the virus in altering and actively shaping the tumour microenvironment [16–20]. Indeed, EBV orchestrates a variety of complex mechanism favouring the escape of lymphoma cells from antitumour immune responses while promoting the creation of niches in which tumour cells may find support for their growth and survival [19–22]. Computational methods such as GEP deconvolution allow high sensitivity discrimination of cell subsets within complex tissues, as tumours [23]. These approaches provide quantitative/ functional information also on rare tumour-infiltrating elements, offering the unprecedented opportunity of reanalysing available genomic data and identifying the immune signature. Here, we applied the computational algorithm CIBERSORT to GEP datasets of 40 BL samples previously published by our group [24], including endemic BL (eBL), sporadic BL (sBL) and immudeficiency associated BL (idBL) cases, to draw a map of immune and stromal components of TME. Finally, in order to validate GEP preliminary data, we applied multiplex immunohistochemistry to an additional cohort of 24 cases. These results were further supported by Vectra analysis of additional 16 BL by immunofluorescence. Thus, a total of 80 BL cases were included in the study. In addition, we investigated the PD-1/PD-L1 pathway activation status and the contribution of EBV in PD-L1 induction as alternative mechanism responsible for immune evasion.

METHODS

CIBERSORT and gene set enrichment analyses

A CIBERSORT-based deconvolution of GEP datasets (GSE26673) from 40 BL samples (13 eBLs, 21 sBLs, 6 idBLs; discovery cohort), previously published [24], was carried out using a 547-gene signature matrix customized for characterizing tissue sample immune cell composition, according to CIBERSORT instructions (https:// cibersort.stanford.edu/) [23]. Briefly, normalized gene expression data were used to infer the relative proportions of 22 types of infiltrating immune cells while gene expression datasets were prepared using standard annotation files and data uploaded to the CIBERSORT web portal (http://cibersort.stanford.edu/), with the algorithm run using the default signature matrix at 1000 permutations. Gene set enrichment analysis (GSEA) was run on GSE26673.

Multiplex immunohistochemistry

Multiplex immunohistochemistry was performed on 24 FFPE BL cases (12 eBL, 8 sBL and 4 idBL; validation cohort 1) which were retrieved from the Departments of Histopathology, University College Hospital, London (UK); Medical Biotechnologies, University of Siena, Siena (Italy); University of Nairobi, Nairobi (Kenya) and Istituto Lazzaro Spallanzani, Rome (Italy). The diagnosis of BL was issued by expert hematopathologists following the criteria described in the revised 4th edition of World Health Organization classification of tumours of Haematopoietic and Lymphoid Tissue [25]. Single immunohistochemistry for the diagnostic antibodies, for EBV antigens and EBV in situ hybridization was carried out on the Bond III Autostainer (Leica, Microsystems, Newcastle upon Tyne, UK) by following the manufacturer's instructions. By applying multiplex immunostaining (IHC), we investigated the simultaneous expression of: a) CD68 (Abcam, ab 955, 1:150) brown, CD-163 (Abcam, Ab87099, 1:100) red and C-Maf (Abcam, Ab243901, 1: 150,) blue; b) PD-1 (Abcam, NAT105, 1:100) brown and CD8 (Leica Biosystem,4B1, 1:200) red and Granzyme B (Abcam134933, marker for T- cell activation) blue c) PD-L1 (ab238697, Abcam, 1:100) brown, CD-163 (Abcam, Ab87099, 1:100) red and MYC (Abcam, Ab32072, Y69 clone, 1:150) in blue;. The triple immunostaining was assessed as previously described [26]. The colour assignment and staining location are: a) PD-L1 brown/membranous; CD-163 red/membranous; C-MYC blue/nuclear b) PD-L1, brown/membranous; CD163 red/ membranous; C-Maf blue/nuclear; c) PD-1 brown/membranous; CD8 red/membranous and Granzyme B blue/ nuclear. Tissue sections from the same set of cases and without antibody/chromogens were used as negative control. The percentage of each cell population characterized by multiplex immunostaining was calculated by counting the individual cell types in 10 hpf using a 40x objective (NIKON Eclipse E400).

Multiplex immunofluorescence staining

Multiplex immunofluorescence (mIF) was carried out on 16 formalin fixed paraffin embedded (FFPE) endemic BL cases (validation cohort 2), belonging to set of samples previously studied and well characterized for EBV latency program [27]. Multiplex IF was applied to simultaneously detect the expression of: a) CD68 (Abcam, ab 955, 1:150) and CD163 (Leica Biosystem, 10D6, 1:200); b) PD-L1 (Dako, clone 22C3, 1:100) and CD163 (Leica Biosystem, 10D6, 1:200); c) PD-L1 and EBV-LMP2A (Abcam, clone 15F9, ab59028, 1:200). These double stainings use red and green or magenta and green chromogens. The colour assignment and staining location are: a) CD68 red/membranous; CD163 green/ membranous; b) PD-L1, green/membranous and CD163, pink/membranous or PD-L1, green/membranous and CD163, red/membranous; c) PD-L1, red; LMP2A green/ membranous. The staining procedure was established according to previously published work [28]. Tissue sections from the same set of cases and without antibody/fluorophore were used as negative control. Multiplex IF staining reaction and image analysis (including quantification of antibodies expression) were performed using the Vectra 2.0 system (PerkinElmer, Waltham, MA) and Tissue FAXSFluo slide scanning system (TissueGnostics, Vienna Austria) based on a Zeiss Axio Imager Z2 upright epifluorescence microscope.

RESULTS

EBV status

EBV status and the viral proteins expression in all the study cases are reported in Table 1. In particular, in the discovery cohort EBV was positive in all of the eBL, in 6 out of 21 sBL and in 5 out of 6 idBL. In the validation cohort 1, all of the eBL cases (12/12) were EBV positive, of which 10 expressed only EBNA1 by IHC while the remaining two were characterized by a non-canonical latency of EBV with the expression of both EBNA1 and LMP2A. Only two out of the 8 sBL cases were EBV positive showing a latency I expression pattern with the sole positivity of EBNA1. All of the idBL cases (4/4) were EBV positive and showed a latency of type I. In the validation cohort 2, all the eBL cases were EBV positive, with 11 out of 16 cases showing an EBV type I latency, while the remaining 5 cases exhibited a noncanonical EBV latency consisting of EBNA1 and LMP1 expression in one case and EBNA1 and LMP2A in the other 4.

Table 1 EBV status and the viral protein expression. Note: NA stands for not available

		EBER/EBNA1+	EBNA1+/LMP1+	EBNA1+/LMP2A+	EBNA1+/LMP1+/LMP2A+	EBV-	Total
Discovery cohort	**eBL**	13	NA	NA	NA	–	13
	sBL	6	NA	NA	NA	15	21
	idBL	5	NA	NA	NA	1	6
Validation cohort 1 (mIHC)	**eBL**	10	–	2	–	–	12
	sBL	2	–	–	–	6	8
	idBL	4	–	–	–	–	4
Validation cohort 2 (mIF)	**eBL**	11	1	4	–	–	16
		51	1	6	–	22	80

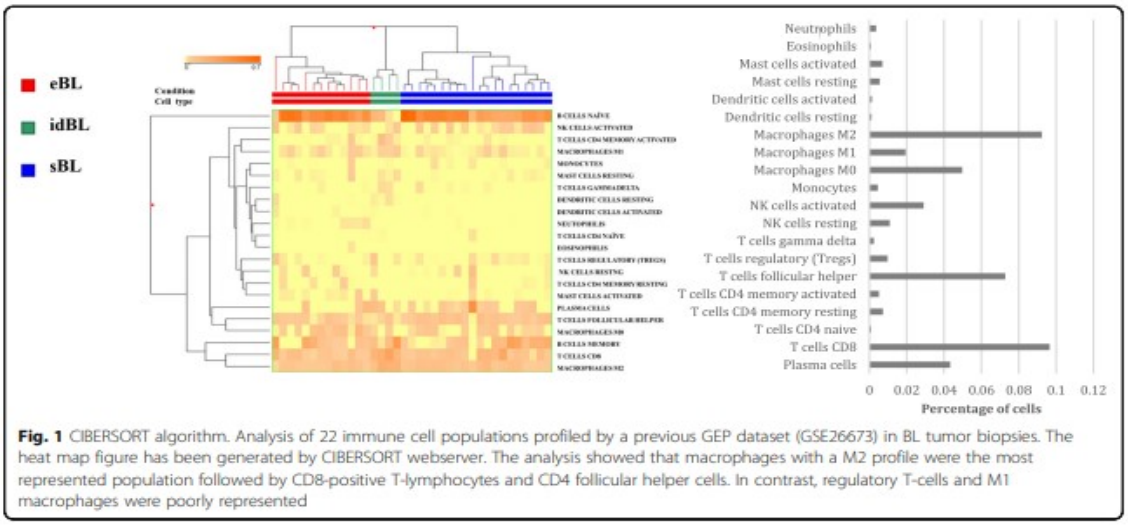


Fig. 1 CIBERSORT algorithm. Analysis of 22 immune cell populations profiled by a previous GEP dataset (GSE26673) in BL tumor biopsies. The heat map figure has been generated by CIBERSORT webserver. The analysis showed that macrophages with a M2 profile were the most represented population followed by CD8-positive T-lymphocytes and CD4 follicular helper cells. In contrast, regulatory T-cells and M1 macrophages were poorly represented

CIBERSORT identifies M2-polarized macrophages as the most representative TME component in BL

The computational algorithm CIBERSORT to GEP datasets from BL samples revealed a heterogeneous reactive milieu with slight differences in tumor microenvironment among the three BL subtypes (eBL, sBL and idBL), most likely reflecting their underlying immunological status. The analysis showed that macrophages with a M2 profile were the most represented population followed by CD8-positive T-lymphocytes and CD4 follicular T helper cells. In contrast, regulatory T-cells and M1 macrophages were poorly represented (Fig. 1).

Multiplex IHC showed M2 macrophage polarization, cytotoxic T cells exhaustion and PD-L1 expression in Burkitt lymphoma cells

The analysis confirmed a shift towards M2 phenotype (CD68 + CD163+/c-maf +) in all BL cases of validation cohort 1 ranging from 60 to 80% of total TAMs (Table 2, Fig. 2a). In particular, the evaluation of M1 macrophages, defined by CD68+, CD163-, c-maf- cells showed very similar values among the series ranging from 20 to 40% of total TAMs. CD8/ PD1/ Granzyme B staining highlighted that the vast majority of CD8+ T cells coexpressed PD-1 ranging from 60 to 80 and 50% to 70% of total tumor infiltrating cytotoxic T cells for eBL and idBL respectively (insert Fig. 2a; Table 2). Interestingly, sBL cases, in which EBV was negative to a greater extent (6 out of 8 cases) showed a markedly lower PD1 expression on CD8 positive T cells (from 35 to 50% of total CD8+ cells; Table 2). The vast majority of TAMs in eBL and idBL cases expressed PD-L1 ranging from 65 to 80% and from 55 to 75% (Fig. 2b, Table 2). Interestingly, PD-L1 expression on TAMs in sBL showed lower values (from 20 to 40%; Table 2). In addition, C-Myc/PD- L1/CD163 triple staining also disclosed

clusters of MYC/PD-L1 double positive cells in 2 eBL cases characterized by expression of LMP2A. PD-L1 expression in these cases was focal and heterogeneous with a degree of intensity from weak to strong in 10–30% of the total tumor cells, clustering with TAMs (Fig. 2b). The co-expression of PD-L1 and LMP2A in scattered neoplastic cells was then confirmed by double IF (insert Fig. 2b), identifying a possible correlation between EBV and LMP2A in PD-L1 induction. Of note, EBV negative BL (6 out of 24 cases) and conventional BL cases with canonical EBV type I latency characterized by the sole expression of EBNA1 (16 out of 24 cases) showed low or absent PD-L1 positivity ranging from 0 to 10% of total tumor cells. These findings indicate that PD-L1 checkpoint activation is more likely related to an unusual latency program of EBV rather than to the EBV presence itself.

Table 2 mIHC for TAMs and PD-L1 expression on 24 BL samples (validation cohort 2) stained for PD-L1, CD68, CD163 and c-maf; mIHC for cytoxic T cells on 24 BL samples (validation cohort 2) stained for PD1, CD8 and granzyme B

mIHC for PD-1/PD-L1 expression and macrophage polarization				
		eBL ($n = 12$)	sBL ($n = 8$)	iBL ($n = 4$)
TAM	M1 (CD68+/CD163-/c-maf–)	20–40%	30–40%	20–30%
	M2 (CD68+/CD163+/c-maf+)	60–80%	60–70%	70–80%
PD-L1	TAMs (PD-L1+/CD163+)	65–80%	20–40%	55–75%
	BL cells (MYC+/PD-L1+)	10–30%	0–10%	0–10%
Exhausted cytotoxic T cells	CD8+/PD1+/granzyme B-	60–80%	20–40%	60–80%

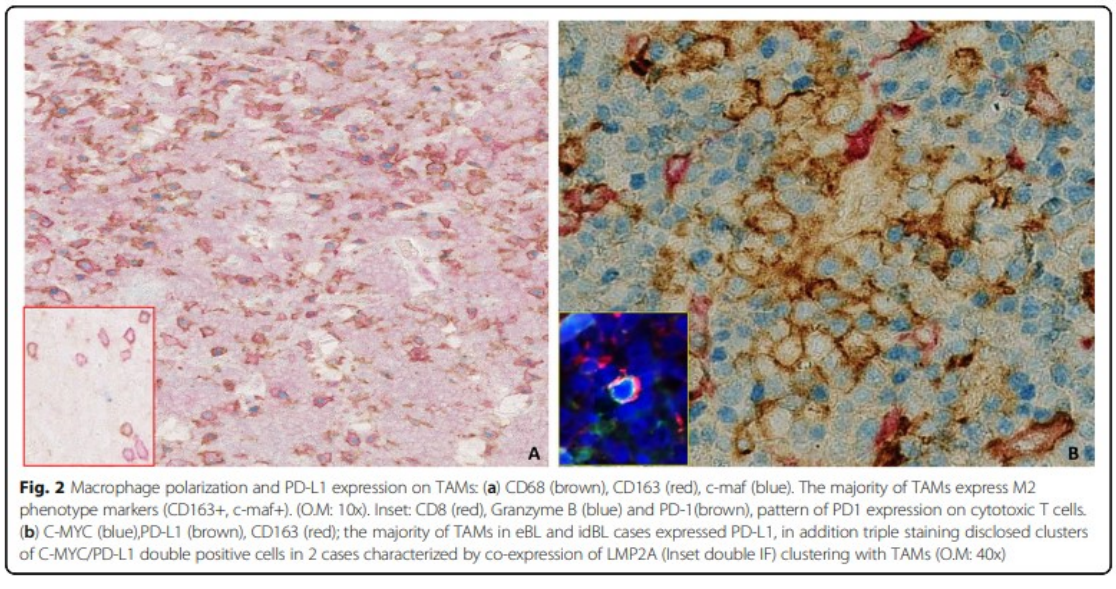


Fig. 2 Macrophage polarization and PD-L1 expression on TAMs: (**a**) CD68 (brown), CD163 (red), c-maf (blue). The majority of TAMs express M2 phenotype markers (CD163+, c-maf+). (O.M: 10x). Inset: CD8 (red), Granzyme B (blue) and PD-1(brown), pattern of PD1 expression on cytotoxic T cells. (**b**) C-MYC (blue),PD-L1 (brown), CD163 (red); the majority of TAMs in eBL and idBL cases expressed PD-L1, in addition triple staining disclosed clusters of C-MYC/PD-L1 double positive cells in 2 cases characterized by co-expression of LMP2A (Inset double IF) clustering with TAMs (O.M: 40x)

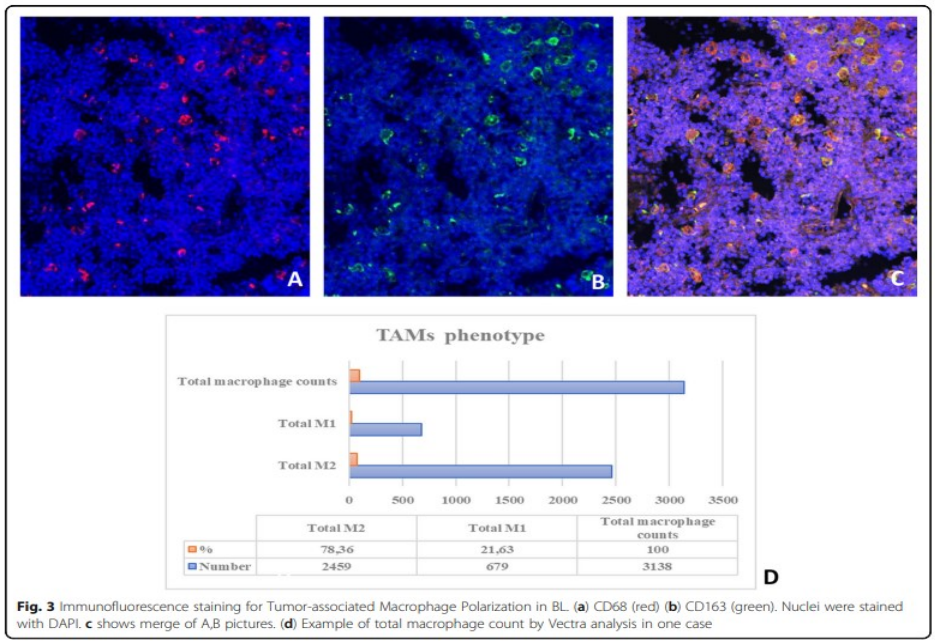


Fig. 3 Immunofluorescence staining for Tumor-associated Macrophage Polarization in BL. (**a**) CD68 (red) (**b**) CD163 (green). Nuclei were stained with DAPI. **c** shows merge of A,B pictures. (**d**) Example of total macrophage count by Vectra analysis in one case

Multiplex immunofluorescence confirmed the prevalence of M2 macrophages and revealed a heterogeneous PD-L1 expression.

Tissue samples were studied by mIF and analyzed by VECTRA to quantify macrophages and PD-L1 expression on 16 BL samples (validation cohort 2) stained for PD-L1, CD68 and CD163. In all the cases in validation cohort 2 the M2 macrophages were the most represented population ranging from 66 to 78% of total TAMs (Fig. 3), thus confirming the CIBERSORT and mIHC results. In addition, the vast majority of them were positive for PD-L1 with a range of expression from 35 to 70% of total macrophages (Fig. 4; Table 3).

Discussion

Although multiple studies have investigated TME and PD-L1 expression in B-cell lymphomas, only limited, small studies have been conducted in BL [14–16]. In the present study, we extensively evaluated the TME composition, activation status and expression of inhibitory immune checkpoints both on the inflammatory infiltrate and neoplastic cells of BL tumors including eBL, sBL and idBL cases. Thus, we investigated PD-L1 expression and the contribution of EBV in fostering the activation of the PD1-PD-L1 axis. The influence of the microenvironment on cell proliferation and destruction varies greatly according to the inherent histotype of the lymphoma cell type [29–31]. In particular, Hodgkin lymphoma (HL) tissue often consists of relatively few monoclonal cancer cells but at least 90% non-malignant cells (e.g., regulatory T cells), contributing to a rather unique surrounding immune ecosystem. On the other hand, BL seems to be largely devoid of such a supportive cellular environment, although the high content of Tumor-Associated Macrophages (TAMs) might play a distinct, specific, important role in neoplastic progression through secretion of chemokines, cytokines and immune checkpoint-associated proteins as PD-L1 [12, 13]. In the recent decade 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 protumoral properties respectively [32–36]. TAM density in particular M2-TAMs have been associated with tumor progression and poor prognosis in DLBCL [37, 38]. In the present work, we identified a polarization towards a M2 phenotype of TAMs in all cases by applying three different approaches (CIBERSORT, mIHC and mIF) regardless of the subtype of BL, and thus, of EBV status. These cells, intimately associated with the neoplastic cells, constituted also the major source of PD- L1, which may inhibit the overall inflammatory response and allow the neoplastic cells to evade antitumor immunity. However, the lower rate of PD-L1 expression on TAMs in sBL, as compared with eBL and idBL, which are frequently associated with EBV, may suggest a role of the virus in inducing PDL1 expression. PD-L1 is a major regulator of T cell function and, after engaging PD-1, leads to an altered functional state of Tcells, namely T cell exhaustion [39]. In this regard, we found that in eBL and idBL the vast majority of the CD8+ infiltrating T cells expressed PD-1 highlighting an adaptive immune response resistance mechanism in such cases. However, PD-L1 limits an antitumor immune response by signalling not only through PD-1 but also with nother receptor, namely CD80 (also named B7–1), expressed on the surface of activated CD8+ T cells [40–42]. The influence of the PD-L1/PD-1 interaction on CD8+ T cell function has been extensively characterized and is known to limit CD8+ T cell responses by inhibiting TCR signalling, thus restricting CD8+ T cell survival, proliferation, and cytokine production. On the other hand, the role of the PD-L1/CD80 pathway on CD8+ T cell functions in BL is unknown, and thus, further studies are necessary. In addition, the role of PD-1 expression in Tumor Infiltrating Lymphocytes (TILs) on both lymphoid and epithelial malignancies is controversial [38]. PD-1 expression in CD8+ cells has been associated with the selective suppression of cytotoxic lymphocytes in EBV positive nasopharyngeal carcinoma [43]. On the other hand, the PD-1+ TILs have also been described to lack Tim-3 expression in papilloma virus positive cancers, and thus possibly representing activated T-cells [44]. Emerging evidence in EBV-related malignancies indicates that the virus possesses the ability to actively shape the tumor microenvironment, and favours its escape from anti-tumor immune responses through a variety of complex mechanisms [18]. EBV may induce a strong upregulation of PD-L1 expression both directly on the surface of human primary monocytes, or indirectly on neoplastic cells, through its viral proteins LMP-1 which interfere with downstream cellular signalling (i.e. AP1; JAK/STAT) [45, 46] to induce an immune tolerant niche for EBV- related tumors [47–49]. LMP-2 may also exert its tolerogenic effect by affecting crucial cell-cycle regulating pathways such as PI3K/Akt wich plays a critical role in PD-L1 expression [50–52]. Although PD-L1 expression has been largely investigated in B cell lymphomas, the distinction of its expression in cellular microenviroment and/or in tumour cells has

not been made in most studies [1–4]. Here we showed that EBV in BL might induce PD-L1 expression on tumor cells in a minority of cases characterized by a non-canonical latency with LMP2A positivity. On the other hand, it might influence PD-L1 upregulation on TAMs also in cases with canonical EBV latency I. However, the prevalence of M2 macrophages as primary constituent of the TME in BL is a constant finding in all BL subtypes and thus, macrophage polarization towards a pro-tumoral state seems an event related to the intrinsic characteristics of the tumor.

Conclusions

In conclusion, although based on a small sample size, our findings may provide insights on BL TME and its underlying mechanisms of immune evasion. The crosstalk between different actors including TAMs, PD-1/PDL1, T-cells, viral antigens and tumor cells may result in the failure of innate immunity in BL which results in M2 polarization. Despite the good response to conventional therapy of BL, our data may provide a rationale for new immunotherapeutic strategies.

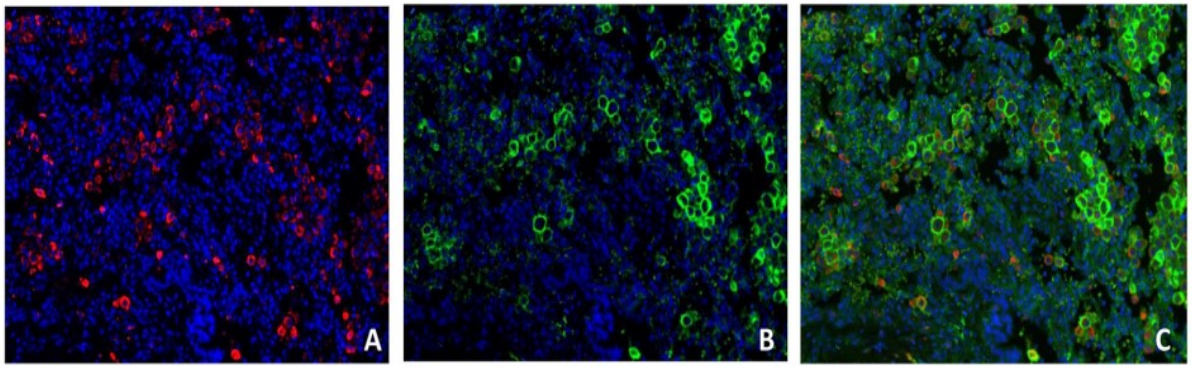


Fig. 4 Immunofluorescence staining for PD-L1 and TAMs. **a** CD163 (red), **b** PDL-1 (green) merge (**c**). The vast majority of TAMs exhibited M2 phenotype markers (CD163+) and expressed PD-L1 to a greater extent

Table 3 mIF and VECTRA analysis of macrophages and PD-L1 expression on 16 BL samples (validation cohort 2) stained for PD-L1, CD68 and CD163

mIF	
Macrophage polarization and PD-L1 expression	n
M1	22–34%
M2	66–78%
n (%) PDL1 (+) CD163(+)	35–70%

REFERENCES

1. Mulders TA, Björn EW. *Targeting the immune microenvironment in lymphomas of B- cell origin*: from biology to clinical application. **Cancers** (Basel). 2019;11:915.

2. Scott DW, Gascoyne RD. *The tumour microenvironment in B cell lymphomas*. **Nat**. 2014;14:517–34.

3. Xu B, Wang T. *Intimate cross-talk between cancer cells and the tumor microenvironment of B-cell lymphomas: the key role of exosomes*. **Tumor Biol.** 2017;39:1–12.

4. Sin Yee Guna SY, Ling Lee SW. *Targeting immune cells for cancer therapy.* **Redox Biol**. 2019;25:101174.

5. Ribrag V, Koscielny S. *Rituximab and dose-dense chemotherapy for adults with Burkitt's lymphoma: a randomised, controlled, open-label, phase 3 trial*. **Lancet**. 2016;387:2402–11.

6. Ngoma T, Adde M. *Treatment of Burkitt lymphoma in equatorial Africa using a simple three-drug combination followed by a salvage regimen for patients with persistent or recurrent disease.* **Br J Haematol**. 2012;158:749–62.

7. God JM, Haque A. *Immune evasion by B-cell lymphoma.* **J Clin Cell Immunol**. 2011;16:1–3.

8. Gopal S, Gross TG. *How I treat Burkitt lymphoma in children, adolescents, and young adults in sub-Saharan Africa.* **Blood**. 2018;132:254–63.

9. Bouda GC, Traorè F. *Advanced Burkitt lymphoma in sub-Saharan Africa pediatric units: results of the third prospective multicenter study of the Groupe Franco-Africain d'Oncologie Pediatrique.* **J Glob Oncol**. 2019;5:1–9.

10. Silva WFD, Garibaldi PMM. *Outcomes of HIV-associated Burkitt lymphoma in Brazil: high treatment toxicity and refractoriness rates - a multicenter cohort study***. Leuk Res**. 2019;10:106287.

11. Ford CA, Petrova S. *Oncogenic properties of apoptotic tumor cells in aggressive B cell lymphoma*. **Curr Biol**. 2015;25:577–88.

12. Pham LV, Pogue E. *The role of macrophage/B-cell interactions in the pathophysiology of B-cell lymphomas.* **Front Oncol**. 2018;8:147.

13. Kumar D, Xu ML. *Microenvironment cell contribution to lymphoma immunity.* **Front Oncol**. 2018;8:288.

14. Majzner RG, Simon JS. *Assessment of programmed death-ligand 1 expression and tumor- associated immune cells in pediatric cancer tissues*. **Cancer**. 2017;123:3807–15.

15. Chen BJ, Chapuy B. *PD-L1 expression is characteristic of a subset of aggressive B-cell lymphomas and virus-associated malignancies.* **Human Cancer Biology**. 2013;19:3462–73.

16. Tan GW, Visser L. *The microenvironment in Epstein–Barr virus-associated malignancies.* **Pathogens.** 2018;7:40.

17. Rowe M, Fitzsimmons L. *Epstein-Barr virus and Burkitt lymphoma*. **Chin J Cancer**. 2014;33:609–19.

18. Dolcetti R. *Cross-talk between Epstein-Barr virus and microenvironment in the pathogenesis of lymphomas.* **Semin Cancer Biol**. 2015;34:58–69.

19. Dojcinov, S.D.; Fend, F. *EBV-positive Lymphoproliferations of B- T- and NKcell derivation in non-Immunocompromised hosts*. **Pathogens.** 2018;7:2–45.

20. Linke-Serinsöz E, Fend F, Quintanilla-Martinez L. *Human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV) related lymphomas, pathology view point*. **Semin Diagn Pathol**. 2017;34:352–63.

21. Granai M, Ambrosio MR. *Role of Epstein Barr virus in transformation of follicular lymphoma to diffuse large B-cell lymphoma: a case report and review of the literature. Haematol.* 2019;104:269–73.

22. Rivera-Soto R, Damania B. *Modulation of Angiogenic processes by the human Gammaherpesviruses, Epstein–Barr Virus and Kaposi's SarcomaAssociated Herpesvirus*. **Front Microbiol**. 2019;10:1544.

23. Chen B, Khodadoust MS. *Profiling tumor infiltrating immune cells with CIBERSORT.* **Methods Mol Biol.** 2018;1711:243–59.

24. Piccaluga PP, De Falco G. *Gene expression analysis uncovers similarity and differences among Burkitt lymphoma subtypes.* **Blood**. 2011;117:3596–608.

25. Swerdlow SH, Campo E. *WHO classification of Tumours of Haematopoietic and lymphoid tissues. 4th ed.* **Lyon: IARC**; 2008.

26. Marafioti T, Paterson JC. *The inducible T-cell co-stimulator molecule is expressed on subsets of T cells and is a new marker of lymphomas of T follicular helper cell-derivation***. Haematol**. 2010;95:432–9.

27. Abate F, Ambrosio MR. *Distinct viral and mutational Spectrum of endemic Burkitt lymphoma*. **PLoS Pathog.** 2015;11.

28. Parra ER, Uraoka N. *Validation of multiplex immunofluorescence panels using multispectral microscopy for immune-profiling of formalin-fixed and paraffin-embedded human tumor tissues*. **Sci Rep**. 2017;7:1–11.

29. Fowler NH, Cheah CY. *Role of the tumor microenvironment in mature B-cell lymphoid malignancies.* **Haematol.** 2016;101:531–40.

30. Shain KH, Dalton WS. *The tumor microenvironment shapes hallmarks of mature B-cell malignancies.* **Oncogene.** 2015;34:4673–82.

31. Wang M, Zhao J. *Role of tumor microenvironment in tumorigenesis***. J Cancer.** 2017;8:761–73.

32. Xue J, Schmidt SV. *Transcriptome-based network analysis reveals a Spectrum model of human macrophage activation. Immunity.* 2014;40:274–88.

33. Steidl C, Lee T. *Tumor-associated macrophages and survival in classic Hodgkin's lymphoma.* **N Engl J Med.** 2010;362:875–85.

34. Li YL, Shi ZH. *Tumor-associated macrophages predict prognosis in diffuse large B-cell lymphoma and correlation with peripheral absolute monocyte count.* **BMC Cancer**. 2019;19:1049.

35. Larionova I, Cherdyntseva N. *Interaction of tumor-associated macrophages and cancer chemotherapy.* **Oncoimmunology.** 2019;8:1596004.

36. Komohara Y, Niino D. *Clinical significance of CD163+ tumor-associated macrophages in patients with adult T-cell leukemia / lymphoma.* **Cancer Sci**. 2013;104:945–51.

37. Bingle L, Brown NJ. *The role of tumour associated macrophages in tumour progression: implications for new anticancer therapies.* **J Pathol.** 2002;196:254–65.

38. Nam SJ, Go H. *An increase of M2 macrophages predicts poor prognosis in patients with diffuse large B-cell lymphoma treated with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone.* **Leuk Lymphoma.** 2014;55:2466–76.

39. Jiang Y, Li Y. *T-cell exhaustion in the tumour microenviroment*. **Cell Death Dis**. 2015;6:1–9.

40. Butte MJ, Keir ME. *Programmed death-1 ligand 1 interacts specifically with the B7–1 costimulatory molecule to inhibit T cell responses.* **Immunity**. 2007; 27(1):111–22.

41. Rollins MR, Gibbons Johnson RM. *CD80 expressed by CD8(+) T cells contributes to PD-L1-induced apoptosis of activated CD8(+) T cells.* **J Immunol Res**. 2017;2017:1–6.

42. Cirone M, Lucania G. *Human Herpesvirus 8 (HHV-8) inhibits monocyte differentiation into dendritic cells and impairs their Immunostimulatory activity.* **Immunol Lett**. 2007;113(1):40–6.

43. Larbcharoensub N, Mahaprom K. *Characterization of PD-L1 and PD-1 expression and CD8+ tumor-infiltrating lymphocyte in Epstein-Barr virusassociated nasopharyngeal carcinoma*. **Am J Clin Oncol.** 2018;41:1204–10.

44. Shayan G, Ferris RL. *PD-1 blockade upregulate TIM-3 expression as a compensatory regulation of immune check point receptors in HNSCC TIL*. **J Immunother Cancer**. 2015;3:196.

45. Bi X, Wang H. *PD-L1 is upregulated by EBV- driven LMP1 through NF-kB pathway and correlates with poor prognosis in natural killer /T-cell.* **J Hematol Oncol**. 2016;9:109–21.

46. Hudnall SD, Küppers R. *Precision molecular pathology of Hodgkin lymphoma*. **1st ed. PLOS Pathogens**; 2018.

47. Wasil LR, Tomaszewski MJ. *The effect of Epstein-Barr virus latent membrane protein 2 expression on the kinetics of early B cell infection*. **PLoS One**. 2013;8:1–14.

48. Deb Pal A, Banerjee S. *Epstein–Barr virus latent membrane protein 2A mediated activation of sonic hedgehog pathway induces HLA class Ia downregulation in gastric cancer cells*. **Virology.** 2015;484:22–32.

49. Rancan C, Schirrmann L. *Latent membrane protein LMP2A impairs recognition of EBV- infected cells by CD8+ T cells*. **PLoS Pathog**. 2015;11:1–23.

50. Yang L, Huang F. *Posttranscriptional control of PD-L1 expression by 17βestradiol via PI3K/Akt signaling pathway in ERα-positive Cancer cell LinesInternational journal of gynecologic*. **Cancer**. 2017;27:196–205.

51. Kristin J. Lastwika, Willie Wilson III, *Control of PD-L1 Expression by Oncogenic Activation of the AKT–mTOR Pathway in Non–Small Cell Lung Cancer*. **Cancer Res January** 15 2016 (76) (2) 227–238;.

52. Gao Y, Yang J. *IFN-γ-mediated inhibition of lung cancer correlates with PD-L1 expression and is regulated by PI3K-AKT signaling.* **Int J Cancer**. 2018;143:931–43.

In preparation for submission

Tumor microenvironment of Burkitt lymphoma: different immune signatures with different clinical behaviors

Maria Chiara Siciliano[1+], Salvatore Tornambè[1+], Marcello Del Corvo[2], Massimo Granai[3], Maria Rosaria Sapienza[2], Felice Arcuri[1], Margherita Vannucchi[1], Virginia Mancini[1], Roberto Boccacci[1], Raffaella Santi[4], Gioia Di Stefano[4], Domenico Ferrara[1], Teresa Marafioti[5], German Ott[6], Reiner Siebert[7], Leticia Quintanilla Fend[3], Falko Fend[3], Cristiana Bellan[1], Stefano Pileri[2], Lorenzo Leoncini[* 1], Stefano Lazzi[1]

+Equally contributed.

*Corrisponding author

[1]Medical Biotechnologies, University of Siena, Siena, [2]Istituto Europeo di Oncologia (IEO), IEO, Milano, Italy, [3]Institut für Pathologie und Neuropathologie, University of Tubingen, Tubingen, Germany, [4]Department of Pathology, University of Florence, Florence, Italy, [5]Department of Cellular Pathology, University College London, London, United Kingdom, [6]Institut für KlinischePathologie, Robert-Bosch- Krankenhaus, Stuttgart, [7]Institut für Humangenetik, Universitätsklinikum Ulm, Ulm, Germany

Background: In Burkitt lymphoma (BL) macrophages represent the main component of TME giving rise to the characteristic histological aspect of the *"starry sky"* pattern. However, in some instances, BL may show a granulomatous reaction and such cases are associated with a favorable prognosis and occasionally spontaneous regression.

Objectives. The aim of the present work was to thoroughly characterize the immune landscape of BL subtypes: 7 EBV-positive BL with granulomatous reaction, 8 EBV-positive BL with the typical starry sky pattern, 8 EBV- negative BL with the typical starry sky pattern.

Design\Methods: Genes expression profiling (GEP) of 23 Formalin-Fixed Paraffin- Embedded (FFPE) samples were performed on the NanoString nCounter using the PanCancer Immune ProfilingPanel consisting of 730 immune-related genes and 40 housekeeping genes.

Results: Based on unsupervised clustering of differentially expressed genes (DEGs) BL samples formed separated clusters, distinguishing EBV-positive BL with granulomatous reaction (cluster 1) from BL with typical starry sky, both EBV-positive and EBV-negative (cluster 2 and 3, respectively). We confirmed previous findings of differently enriched genes in *NF-kB, JAK-STAT, BCR* signaling pathways between EBV positive BL and EBV-negative BL. In addition, differences in immune response signature were identified between BL with granulomatous reaction and BL with typical starry sky. The TME signature in BL with starry sky pattern, both EBV- positive and EBV-negative,

showed an up-regulation of M2 secreted chemokines (*CCL22, CCL17, CCL2, CCR4*), *IL4/ IL13* pathway, M2-immune response genes (*ARG1, ICOS, IDO1, LAG3, CD163, GATA3*), immune checkpoint genes (*CD274, PDCD1, PDCD1LG2, CTLA4*) suggesting a M2- polarization in these cases. Conversely, these signatures were downregulated in BL with granulomatous reaction, resulting in a M1 polarization and a proinflammatory response.

Conclusion: Due to their pivotal role, TAMs are promising targets for additional therapies in BL lymphoma and combining TAM-targeted therapy with conventional treatment may open up novel therapeutic avenues for therapy-refractory patients.

INTRODUCTION

The TME of B-cell lymphomas is highly variable in terms of spatial configuration and composition of cells, including inflammatory cells, blood and lymphatic vascular networks, and extracellular matrix [1]. Although the roles of the TME are not fully understood, increasing evidence suggests that the TME plays an important role in various processes, including the origin and dissemination of B cell lymphomas [2,3]. In aggressive lymphomas, particularly Burkitt Lymphoma (BL), owing to their high proliferation rate, intensive chemotherapy is the standard of care, but unfortunately these treatments are not as effective in elderly and immunocompromised patients [4]. Furthermore, in equatorial Africa, where BL is the most common childhood cancer, the prognosis of BL is still poor because the intensive therapeutic regimens often result in a severe neutropenia, with fatal consequences in resource poor settings. A full understanding of BL TME biology and the interaction between lymphoma cells and TME is expected to provide new strategies for targeted therapies and tumor prognosis prediction.

Historically, three subtypes of BL have been recognized: endemic (eBL), sporadic (sBL), immunodeficiency-associated (HIV-BL). However, based on recent insights into BL biology, it is recommended by the 5th edition of the WHO classification of hematolymphoid tumors to distinguish EBV-positive and EBV-negative BL based on their molecular features despite the epidemiological context and geographic location [5,6].At the histological level, BL shows histiocytes containing abundant cytoplasm dispersed among a background of basophilic tumor cells and a monomorphic population of rapidly proliferating B- cells with a basophilic cytoplasm. Normal infiltrating lymphocytes are few, while scattered phagocytic macrophages give rise to the characteristic histological aspect of BL, known as the *"starry sky"* appearance [7]. These macrophages along with mesenchymal stem cells, stromal cells, immune cells, and soluble factors, represent the main component of TME of BL. The activity of macrophages and their impact on the tumour immune

response in BL remain unclear. However, TAMs may function as a potential mediators of tumor advancement due to the secretion of chemokines and cytokines, and the expression of immune checkpoint-associated proteins such as Programmed Death-Ligand 1 (PD-L1) [8,9,10]. Over the last two decades, macrophage polarization has become increasingly relevant for its role in lymphomagenesis, contributing differently to the immune microenvironment [11,12]. In recent years, a model has been developed to describe the complex mechanism of macrophage activation as polarization towards two opposite states: M1 with proinflammatory properties and M2 with pro-tumoral properties. The M1/M2 nomenclature was inspired by Th1 versus Th2 nomenclature. M1 macrophages are the primary source of pro-inflammatory cytokines, which also promote cancer immunosurveillance and cytotoxicity [13,14]. However, these effects are counterbalanced by M2 macrophages with anti-inflammatory and pro-tumoral effects.

Interestingly, there are few cases of BL characterized by conspicuous granulomatous reactions, which can obscure neoplastic proliferation. The granulomatous reaction is a distinctive pattern of chronic inflammation characterized by nodular aggregation of inflammatory cells, predominantly activated macrophages, which are transformed into epithelioid cells. Macrophages in granulomas derive both from circulating monocytes attracted by chemotaxis, and from local, resident macrophages recruited by T cell-derived growth factors. CD4+ T cells accumulate in the center of epithelioid granulomas, whereas the majority of CD8+ T cells are found in their periphery. Usually, such cases are EBV-positive, present in the early stage of disease, and may have spontaneous regression without therapy [15-19]. Recent data using multiplex immunohistochemistry (mIHC) show that the TME of BL with granulomatous reaction is characterized by the prevalence of M1 macrophages and pro-inflammatory response, which can possibly explain the spontaneous regression of such cases [20]. In addition, the differences in the composition of the cellular and soluble components of the BL TME in both EBV-positive and EBV-negative patients remain unclear.

Therefore, the aim of this study was to further investigate the immune landscape of BL subtypes (7 EBV-positive BL with granulomatous reaction, 8 EBV-positive BL with the typical starry sky pattern, 8 BL EBV with the typical starry sky pattern) by applying NanoString technologies, focusing on the immune gene categories using a large panel of immune-related genes.

MATERIALS AND METHODS

Case Selection

Formalin- fixed paraffin-embedded (FFPE) BL samples was retrieved from the Department of Medical Biotechnologies (University of Siena, Siena, Italy) and University of Nairobi. The diagnosis of BL was issued by expert haematopathologists following the essential criteria reported in the 5Th Edition of the WHO classification of lymphoid tumors. *Diagnostic immunohistochemistry was performed on the Ventana BenchUltra (Roche diagnostic, Monza-Italia), according to the manufacturer's instructions using a large panel of antibodies reported in Table* .....To assess the presence of EBV, *in situ* hybridisation for EBV-encoded small RNAs (EBERs) was performed on all 5 µm FFPE cases by an automated staining system (Ventana BenchMark ULTRA, Roche diagnostic, Monza-Italy), as previously described [10]. All steps were performed inside the instrument, from deparaffinization to counterstaining with appropriate positive and negative controls included in each staining run. A control slide prepared from a paraffin-embedded tissue block containing EBV-positive metastatic nasopharyngeal carcinoma in a lymph node accompanied each hybridization run. The EBER-ISH-stained sections were scanned and analysed by Hamamatsu NanoZoomer-XR digital whole slide scanner.

Fluorescence in situ hybridization for MYC rearrangement and translocationt: (8;14) were performed for each cases, respectivelyusing a break-apart probe (BAP) (Vysis MYC Dual Colour Break Apart Rearrangements Probe; Abbott, Wiesbaden, Germany) and a dual fusion probe (MYC-IgH, ZytoVision GmbH, Bremerhaven, Germany); Bcl6 and Bcl2 rearrangement were performed for each cases, using a break-apart probe (BAP) (VysisBcl2\Bcl6 Dual Colour Break Apart Rearrangements Probe; Abbott, Wiesbaden, Germany) according to the manufacturer's instructions. In addition, we used FISH to evaluate the gain of 11q alterations (ZytoLight SPEC 11q gain\loss Triple Color Probe). For each specimen, a 4- µm-thick tissue section embedded in paraffin was cut. Briefly, the slides were incubated in heat pretreatment solution, washed, digested, dehydrated, and hybridised with probes. At least 100 intact nonoverlapping nuclei were analysed manually on a Leica DM 600B (Leica Microsystems, Heerbrugg Switzerland) fluorescence microscope equipped with 40 ,6-diamidino-2-phenylindole, Spectrum Green and Spectrum Orange filters.

Immune-related gene expression profiling using the NanoString platform.

Digital multiplexed gene expression profiling (GEP) of 730 immune-related genes and 40 housekeeping genes, was performed using the nCounter PanCancer Immune Profiling Panel (Nanostring technologies, Seattle,WA,USA) on primary diagnostic FFPE tumor tissue [21]. Total

RNA from 23 FFPE 10 µm-thick sections from each diagnostic samples was isolated usingRNeasy FFPE kit (Qiagen, Hilden, Germany). The protocol included deparaffinization, proteinase K digestion, extraction, elution or hydration procedures, and DNase treatment to obtain DNase free RNA. The RNA concentration was measured using the Qubit® RNA HS Assay Kit following the manufacturer's instructions.

Pathway and statistical analysis

Gene expression data were normalized using DESeq2 package in R and hierarchical clustering, principal component analysis (PCA) and exploration of differentially expressed genes (DEGs) were performed. Graphs were plotted using ggplot2 and pheatmap packages in R. Gene set enrichment analysis (GSEA) was conducted using GSEA software v4.0.3. The reference data file annotated with immunological functions for 770 genes in the nCounter® PanCancer Immune Profiling Panel was downloaded from the NanoString Technologies website (https://www.nanostring.com/products/gene-expression-panels/gene-expression-panels-overview/hallmarks-cancer-gene-expression-panel-collection/pancancer-immune-profiling-panel?jumpto=SUPPORT). These reference data, which are annotated with the immunological function and biological process categories from the Gene Ontology Consortium, were then processed to gene sets in the gene matrix file format (.gmt) for GSEA. Pathway scores were based on the first principal component of the expression data in each sample, based on the expression levels for the gene sets related to the specific pathway.

The two histotypes were compared using the Mann-Whitney nonparametric rank test, instead of the unpaired t-test, because the data could not be assumed to be normally distributed: the Shapiro-Wilk, Kolmogorov-Smirnov and Anderson-Darling normality tests, applied to the sample data, all yielded p-values < 0.05.Statistical calculations were performed using the freeware software Jamovi, version 2.2, with a statistical significance level of 95% for inferential analyses [22,23]

Multiplex immunohistochemistrystanining to validate immune landscape of BL.

Multiplex immunohistochemistry (mIHC) was performed on 23 FFPE BL cases. The antibody panels used for mIHC consisted of one different triple stains: CD68, CD163, c-Maf. The triple staining was performed with an automated staining system (DISCOVERY ULTRA ICH/ISH research platform; Roche Diagnostics, Indianopolis, USA) for open procedures, according to the manufacturer's protocols [22]. The CD163 +/ CD68+/c-Maf +, CD163+/CD68-/c-Maf + and CD163-/CD68+/c-Maf- cells were evaluated manually and independently by experienced pathologist by counting the individual cell types in 10 high-power fields under a X 40 objective 172 (NOME MICROSCOPIO), and taking into account either nuclear and surface stains, reported as percentages for each cell type in

scores rounded up to the nearest 5%. Only CD163 +/ CD68+/c-Maf + and CD163+/CD68-/c-Maf + cells were considered to be M2 macrophages; CD163-/CD68+/c-Maf- cells were considered to be M1 macrophages. The interobserver reproducibility of each cell count was assessed according to the coefficient of variation as a percentage (CV%), which is the ratio of the standard deviation to the mean of the three percentage observations. For each variable, percentage agreement across the four cases analysed was then expressed as the mean of 100 – CV% and its 95% confidence interval, estimated with the bias-corrected and accelerated bootstrap technique.

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

RESULTS

Patient information

The study cohort includes 23 Formalin-Fixed Paraffin-Embedded (FFPE) samples retrieved by the Department of Medical Biotechnologies (University of Siena, Italy), and University of Nairobi. Specifically, we analyzed three groups of BLs composed of 8 EBV- negative BL with starry sky pattern, 8 EBV- positive BL with starry sky pattern and 7 EBV-positive BL with granulomatous reaction. Among the latter, five out of seven cases were characterized by a diffuse granulomatous reaction that partially obscured the neoplastic proliferation and two out of seven cases were characterized by a partial granulomatous reaction. The diagnosis of BL was issued by expert haematopathologists. All the cases had typical morphology and *immunophenotype features of BL (CD20+, CD10+, BCL6+, LOM2-, BCL2-, Myc<80% and Ki67>95%,) and* harbored only MYC translocation demonstrated by using break-apart or fusion fluorescence in situ hybridization (FISH) probes. All clinicopathological characteristics are summarized in table 1.

The case cohort comprised 10 women and 13 males, with ages ranging from 5 to 70 years. The anatomical localization included the gastrointestinal tract, oral mucosa, bone marrow, lymph nodes, and abdominal mass. The cases with granulomatous reaction were stage I/II and three out of the seven cases showed spontaneous regression. The remaining 4 were characterized by a good outcome and remained relapse-free in a follow-up of 5 years. On the other hand, cases with typical starry sky, EBV positive or EBV negative, were mainly stage III or IV, with bulky disease, significantly different from cases with granulomatous reaction ($p<0.001$) (Fig.1).

Case	Sex	Age	Location	Stage (Murphy Staging System)	EBV status	Hystology	Follow up
1411	M	11	Oral mucose	II	-	"Starry sky" pattern	Complete remission
3616	M	14	Abdominal mass	III	-	"Starry sky" pattern	Complete remission
837_02	M	8	Oral mucose	II	-	"Starry sky" pattern	Complete remission
2215	F	9	Abdominal mass	II	+	"Starry sky" pattern	Complete remission
11459_20_LN1	F	5	Gastro Intestinal tract	III	-	"Starry sky" pattern	Complete Remission
345	M	7	Not available	III	+	"Starry sky" pattern	Complete Remission
11459_20	M	9	Lymph node	III	+	"Starry sky" pattern	Complete Remission
95_2_2016	M	50	Not available	III	+	"Starry sky" pattern	Complete Remission
14196	M	8	Gastro Intestinal tract	III	-	"Starry sky" pattern	Complete Remission
14I_14352_A2	M	6	Gastro Intestinal tract	III	-	"Starry sky" pattern	Complete Remission
E4452_18	F	6	Not available	III	-	"Starry sky" pattern	Complete Remission
1586	M	12	Not available	III	+	"Starry sky" pattern	Complete Remission
9622_1_2018	F	63	Not available	III	+	"Starry sky" pattern	Relapse
17_7935	M	70	Bone marrow	IV	-	"Starry sky" pattern	Relapse
9780_A11_2019	M	3	Not available	II	+	"Starry sky" pattern	Partial remission
1102	F	10	Not available	II	+	Granoulomatous reaction	Partial remission
SR_90	M	10	Lymph node	II	+	Granoulomatous reaction	Partial remission
HP_38864	F	8	Gastro Intestinal tract	II	+	Granoulomatous reaction	Partial remission
457_11	F	26	Oral cavity	II	+	Granoulomatous reaction	Partial remission
11743_20	F	4	Abdominal mass	II	+	Granoulomatous reaction	Partial remission
12_26555_B4	F	65	Lymph node	I	+	Granoulomatous reaction	Spontaneous regression
663_06	M	12	Oral Cavity	I	+	Granoulomatous reaction	Spontaneous regression
V215_9203_2	F	47	Lymph node	I	+	Granoulomatous reaction	Spontaneous regression

Table 1. ***Clinical pathological characteristic of BL samples****.*

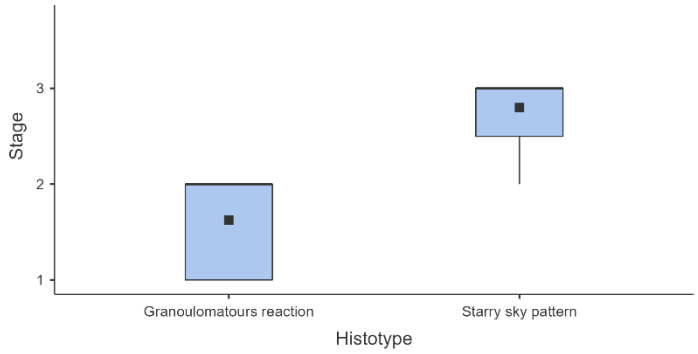


Figure 1. ***Statistical calculation for stage difference***

Principal component analysis and heatmap graph identify three groups among BL cases.

Principal component analysis (PCA) and heatmap graph suggest that BL with granulomatous reaction (cluster 1) and BL with “starry sky” pattern, EBV- positive (cluster 2) and EBV-negative(cluster 3), exhibit different immune-related gene expression profiles. Yet, EBV-positive and EBV- negative BL with “starry sky” pattern (cluster2 and 3, respectively) show similarities but also differences in Gene Expression Profile (GEP) (Figure 1 A-B). Cluster 1 is composed of the five BL with a diffuse granulomatous reaction; cluster 2 is composed of 5 EBV-positive BL with typical “starry sky” and cluster 3 in addition to EBV-negative BL with typical “starry sky” also includes two cases of BL with a partial granulomatous reaction and three cases of EBV positive BL with typical starry sky.

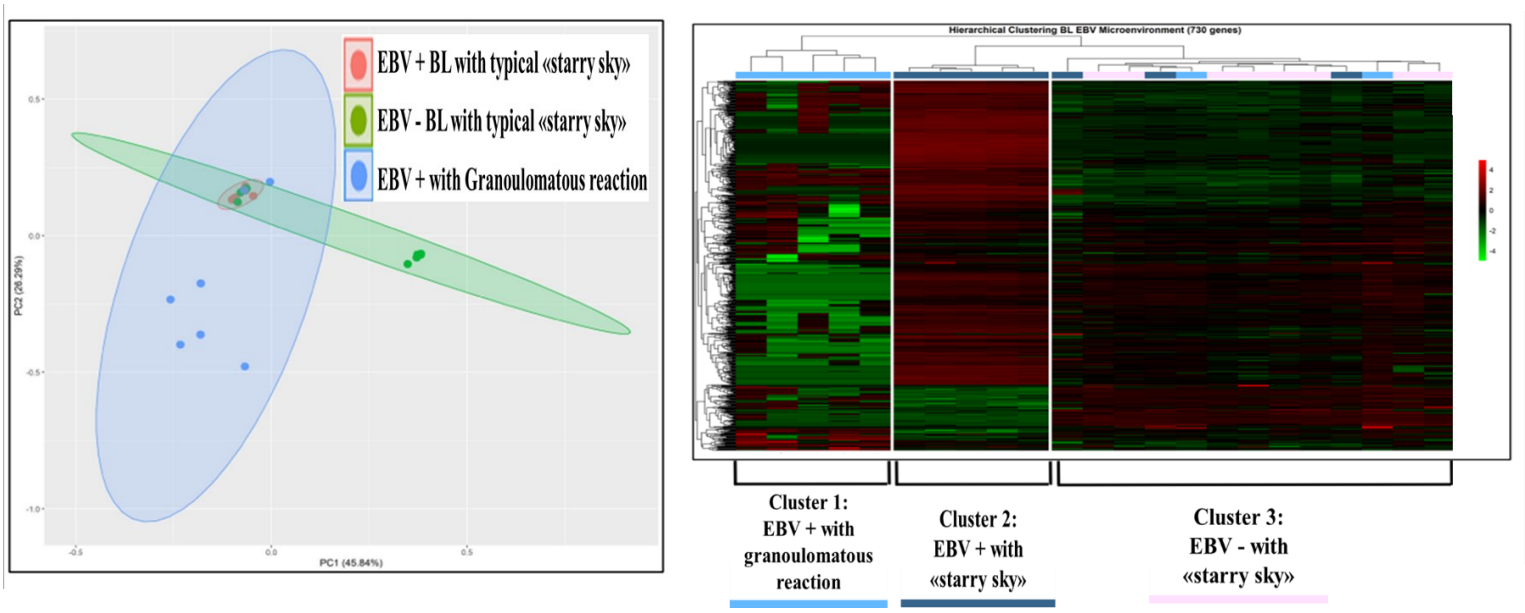


Figure 6. ***PCA and Heatmap graph showed three clusters of BL***": BL EBV positive with granulomatous reaction (Cluster 1), BL EBV positive with the typical starry sky pattern (cluster 2), BL EBV negative with the typical starry sky pattern (cluster 3)

Some features could be linked exclusively to the presence of EBV. Indeed, by comparing Cluster 1 and 2 with Cluster 3, our results showed differentially enriched genes between EBV-positive BL and EBV- negative BL in the NF-kB, JAK-STAT, and BCR signaling pathways (Figure 3- A, B, C, respectively). However, the GEP of BL samples revealed also a high degree of heterogeneity in the functional categories of immune response, showing that BL with granulomatous reaction has a unique immune landscape characterized by down regulation of immune related genes (Table 2)

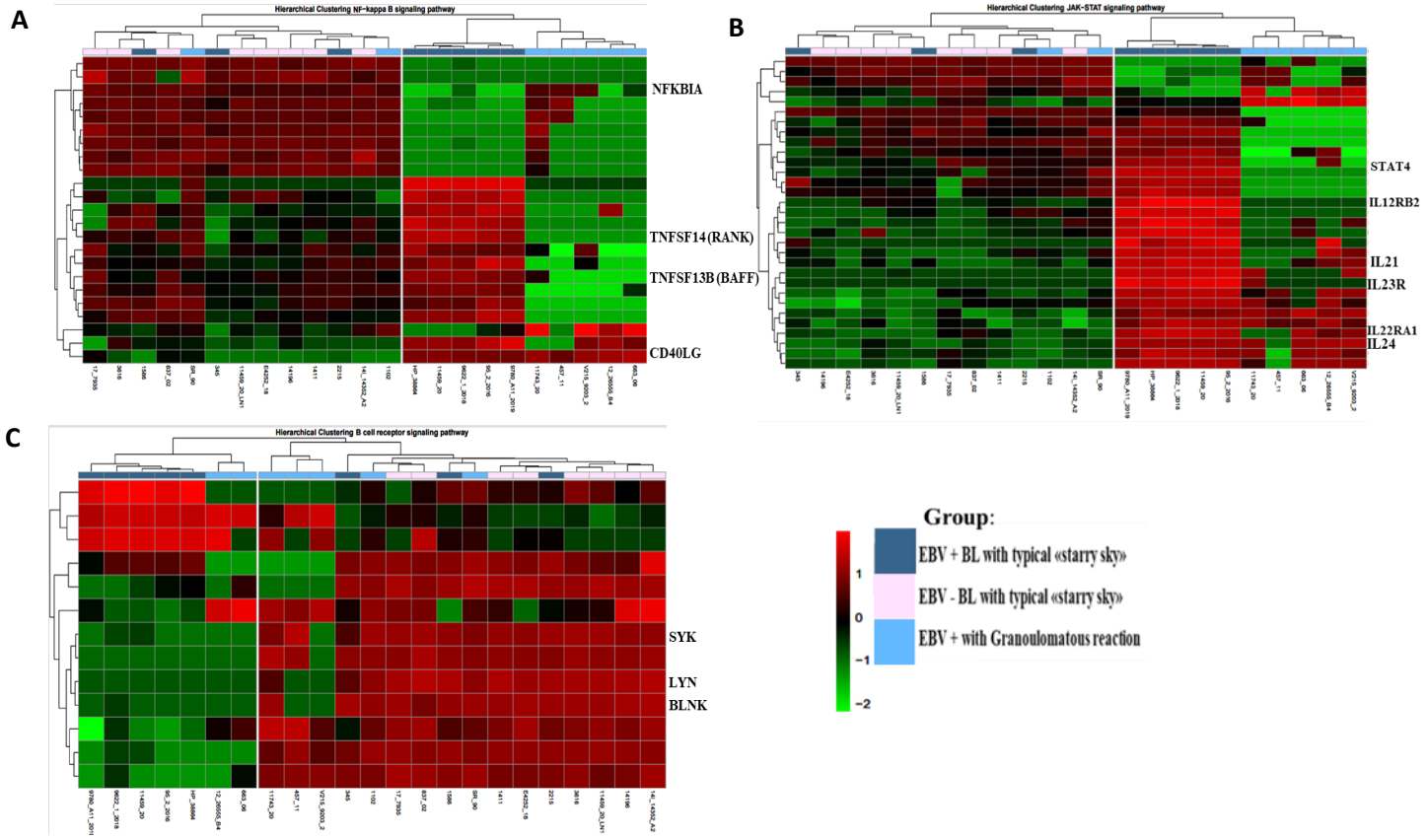


Figure 7. ***Differences between EBV-positive and EBV- negative****. Heatmap graphs show differences in NFkB pathway (A), JAK-STAT pathway (B), BCR signalling.*

Contrast	N.Genes	Up	Down	Immune Response	Immune Response - Checkpoint
EBV+ BL with Granulo vs EBV +	185	10	175	130	5
EBV+ Granulo vs EBV -	43	10	33	33	1
EBV + vs EBV -	203	155	48	152	2

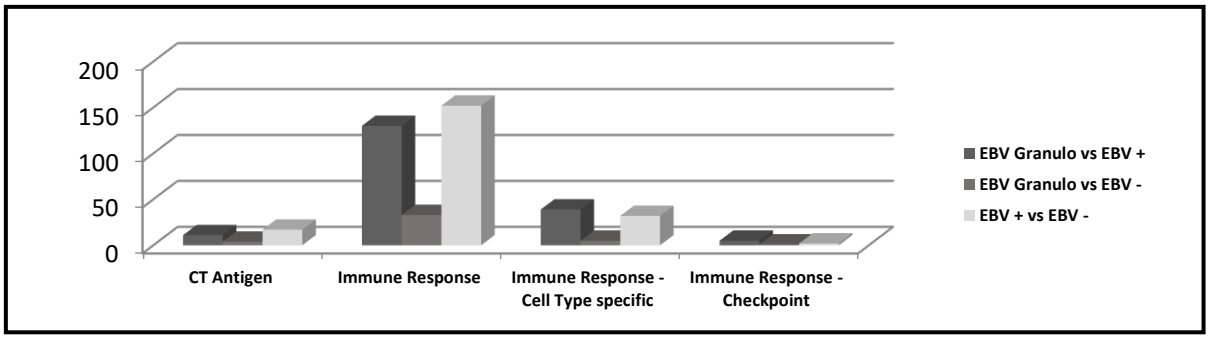


Table 2. ***Graphic representation of the number of genes differentially expressed****.* GEP identify differences in terms of immune-related genes among the 3 different groups (EBV Granulo vs EBV+, EBV Granulo vs EBV-, EBV + vs EBV-)

GEP and GSEA of immune related genes among BL cases show differences in terms of cytokines, chemokines, macrophage polarization and immune checkpoint molecules.

BL with granulomatous reaction has a unique immune landscape characterized by down regulation of genes related to M2 polarization and immune check point. In particular, GEP and GSEA of immune-related genes in BL with starry sky pattern, both EBV positive and EBV negative, (Clusters 2 and 3) showed an upregulation of IL4-IL13 pathway, M2- secreted chemokinesandM2-immune response genes. Specifically, clusters 2 and 3 were enriched in IL4/13 pathway showing an up-regulation of *CD36* and *IL13RA2* (Figure 4-A). In addition, cluster 2 and 3 are characterized of an over-expression of *CCL17, CCL22, CCL2*, and *CCR4*, which are M2- chemokines highly expressed on T cells polarized towards Th2 responses and contributes to M2-macrophage polarization typically associated with Th2 responses, suggesting a M2-polarization in these cases (Figure 4-B). In fact, Cluster 2 and 3 also showed up-regulation in M2- immune response genes (*ARG1, ICOS, IDO1, LAG3, GATA3, CD163, TIM3/HAVCR2)* in comparison with cluster 3 (Figure 4-C).

Furthermore, our results demonstrated the upregulation of several immune checkpoint molecules (*CD274, PDCD1, PDCD1LG2,* and *CTLA4*) in clusters 2 and 3 as compared to cluster 1, facilitating the escape of tumor cells from the T cell-mediated immune response (Figure 4-D).

On the other hand, cluster 1 was characterized by the downregulation of all of the above-mentioned genes(*ARG1, ICOS, IDO1, LAG3, CD163, GATA3,CD36, IL13RA2, CCL17, CCL22, CCL2,CCR4, TIM3/HAVCR2, CD274,PDCD1, PDCD1LG2,* and *CTLA4*), which were upregulated in clusters 2

and 3. Of interest, we could also demonstrate the upregulation of *IRF3* and *IFNG1* in cluster 1, which contributes to the induction of M1 polarization.

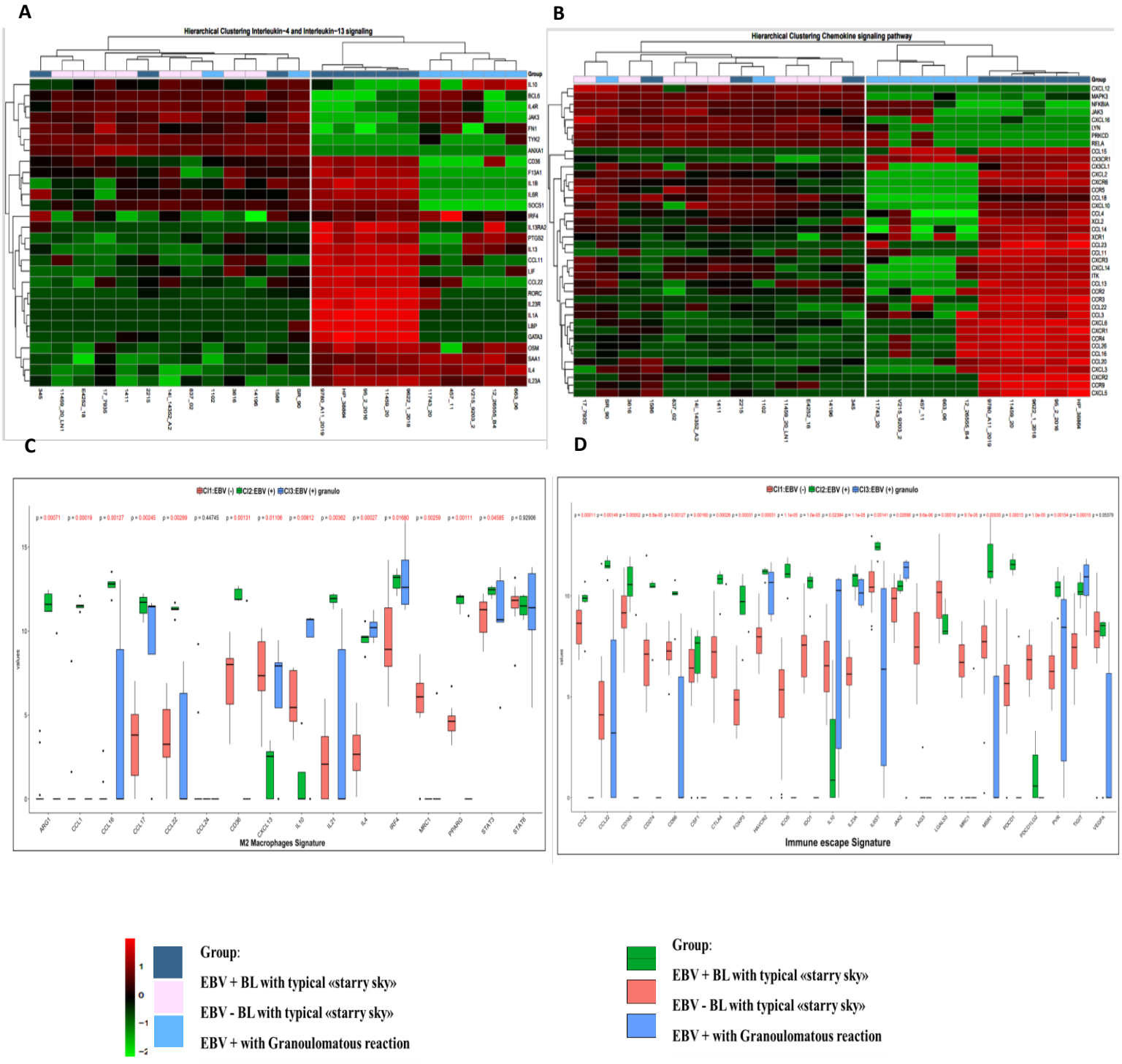


Figure 8. ***Immune different between BL with granulomatous reaction and BL with typical starry sky.*** GEP show difference in terms of IL4-IL13 pathways (A) and M2-chemokines (B). In addition, GSEA show differences in M2-immune response signature (C) and immune checkpoint signaling (D)

Multiplex immunofluorescence showed enhanced M1-polarization in BL with granoulomatousreaction.

Multiplex immunofluorescence confirmed significant heterogeneity presence of macrophage phenotypes. To further characterize the immune cell TME in BL, we performed mIF focusing on the distinct macrophage phenotypes that showed comparable characteristics with GEP analysis. Overall, in all BL with granulomatous reaction, mIHC showed a prevalence of M1 macrophages defined as CD68+/CD163-/C-Maf – cells accounting for 80-95% of the total macrophages (Figure 5-A,B). Most of the macrophages in BL with a typical starry sky were M2, defined as CD68+\CD163+\C-Maf + (Figure 5-C)

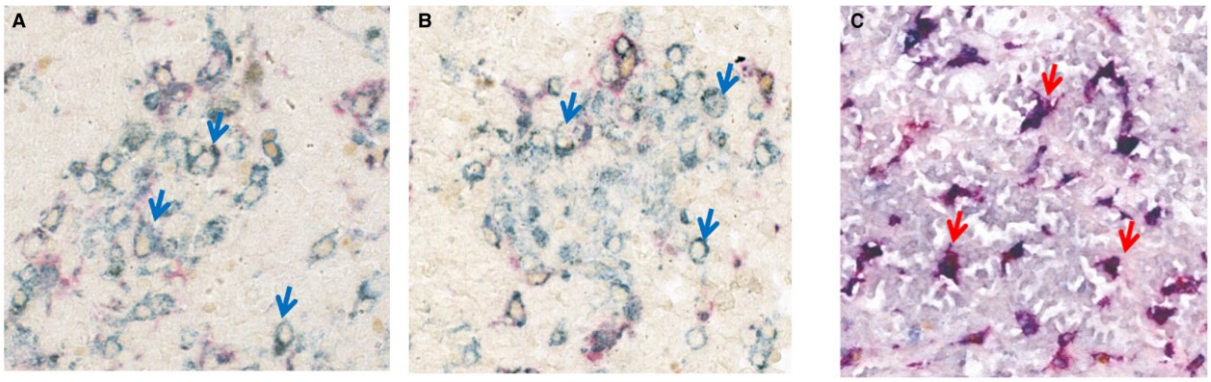


Figure 9. ***Confirmation by multiplex immunohistochemistry****. A high number of M1 (CD68+/CD163–/c-Maf–) macrophages (blue arrows) were seen in the granulomas surrounding the clusters of Burkitt cells in case 1 (A) and case 2 (B); Predominant macrophage M2 population (CD68+/CD163+/c-Maf+) macrophages (red arrows)*

DISCUSSION

The lymphoma microenvironment is increasingly being recognized as a dynamic and interactive supporting network, including immune cells, stromal cells, cytokines, blood vessels, and extracellular matrix components, whose composition is guided by neoplastic cells, which influences tumor initiation, progression, resistance to cell death, evasion of growth suppressors, and drug resistance [24]. Although multiple studies have investigated TME expression in B-cell lymphomas, only a limited number of studies have been conducted on BL. Recent data using mIHC show that the TME of BL with granulomatous reaction is characterized by the prevalence ofM1 macrophages and pro-inflammatory response, which could possibly explain the spontaneous regression in some cases [9]. In this study we further characterize the immune landscape of BL subtypes by GEP with Nanostring technologies focusing on the immune gene categories using a 730 immune-related genes panel.

GEP analysis of immune gene categories could separate the BL into three clusters. Cluster 1 comprised the five cases of EBV-positive BL with diffuse granulomatous reaction and clusters 2 and 3 BL with typical starry sky pattern both EBV-positive and EBV-negative, respectively. However, some overlap was observed. In particular, cluster 3 included also the two cases of BL with a partial granulomatous and three cases of BL-EBV positive with starry sky.

Some features could be linked exclusively to EBV presence by comparing the TME of EBV-positive BL (Cluster 1 and 2) and EBV-negative (Cluster 3). Indeed, our results confirm previous findings for up-regulation of the NF-kB and JAK-STAT pathways in EBV-positive BL, while a "tonic" activation of BCR signaling was more enriched in EBV-negative BL [25,26]. In particular, we could confirm the up-regulation of NF-kB pathways in EBV-positive BL (Cluster 1 and 2) in comparison with BL EBV- negative (Cluster 3). On the other hand, we found up-regulation of NF-kB Inhibitor Alpha (NFKBIA) in BL EBV-negative. The NF-kB pathway is known to play an essential role in tumor progression and can be divided into canonical and non-canonical pathways. In particular, it seems that the TNF family (BAFF, RANK) and CD40L activate the non-canonical NF-κB pathway, influencing B cell biology, proliferation, and maturation [27,28,29].

Our results showed the up-regulation of the JAK-STAT signaling pathway (STAT4, IL12RB2, IL22RA1, IL21, IL21R, IL23R, and IL24) in EBV-positive BL (cluster 1 and cluster 2) in comparison with BL EBV-negative (cluster 3), confirming the hypothesis that EBV induces oncogenesis through the JAK-STAT pathway. The JAK-STAT pathway is stimulated by cytokine receptors and is involved in cell proliferation, survival, invasion, and inflammation [30]. In particular, IL21 is a pleiotropic cytokine that exerts diverse regulatory effects on tumor cells, depending on the type of cell, stage differentiation, stimuli, and EBV status. Recently, it was reported that IL-21 is correlated with the presence of EBV and may also induce a tumor-promoting microenvironment [31]. Finally, we could confirm a different pattern of activation of the BCR between EBV-positive (cluster 1 and 2) and EBV-negative cases (cluster 3). Indeed, all genes involved in the TCF3\ID3 pathway (LYN, SYK, BLNK) were downregulated in clusters 1 and 2 in comparison with cluster 3, confirming that the TCF3\ID3 signature is more enriched in BL EBV negative than BL EBV positive [32,33]

Although some features are related exclusively to EBV presence, clustering analysis focusing on the immune gene categories, revealed that BL with a granulomatous reaction has a unique immune landscape significantly different from BL with starry sky both EBV- negative and EBV- positive.

In fact, the immune cell composition overlaps between EBV-positive BL and EBV-negative BLwith starry sky, showing an up-regulation of IL4/IL13 pathway (*CD36, IL13RA2*), M2-secreted chemokines (*CCL17, CCL22, CCL2, CCR4*), M2-immune response genes (*ARG1, ICOS, IDO1, LAG3, GATA3, CD163,TIM3/HAVCR2*), suggesting a M2- polarization in these cases [34].

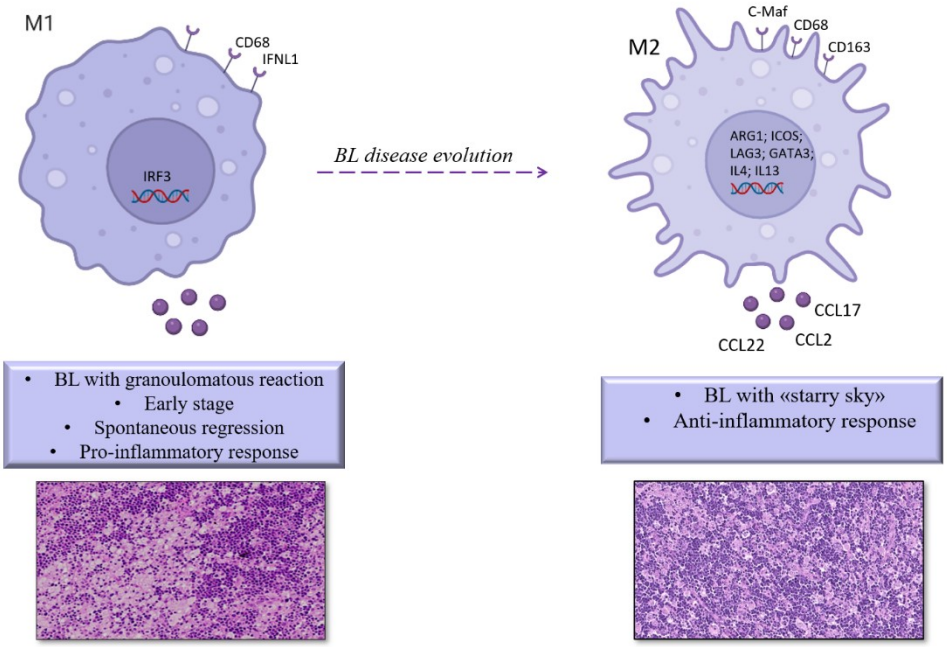
Specifically, the anti-inflammatory cytokines *IL-4 and IL-13* are two closely related cytokines that play both overlapping and distinct roles in type M2- polarization. *CD36* is a macrophage scavenger receptor activated by *IL13,* and *IL13RA2* is a subunit of the interleukin 13 receptor complex [35,36]. Regarding M2-secreted chemokines, it has been established that *CCR4* is highly expressed on T cells polarized towards Th2 responses. *CCL17*, *CCL2*, and *CCL22* signaling contributes to M2-

macrophage polarization typically associated with Th2 responses. CCL17 and CCL2 induce migration of T reg cells via interaction with the CCR4. Thus, the attraction of immunosuppressive cells through chemokine production is one of the pro-tumoral characteristics of the M2 type [37,38,39]. Finally, the enzyme *ARG1* is regulated by the IL13 and IL4 pathways and activates an M2-polarized immune response [40]. High expression of the enzyme *IDO1* has been found to be associated with M2- macrophages, in correlation with high immune infiltration, tumor mutational burden (TMB), and expression of immune checkpoints. *ICOS* has significant homology to the co-stimulatory molecule *CD28* and the immune-attenuator *CTLA-4*. Indeed, ICOS is often tied to M2-macrophages and is characterized by an anti-inflammatory polarization that promotes immune tolerance.*HAVCR2* is a transmembrane protein expressed on the diverse cell types of the immune system and can regulate different immune type. Although *TIM-3* was initially identified as a membrane marker specific for M1, today we know that it is expressed in M2 macrophage, promoting immunological tolerance [40]. Finally, *CD163* is a specific marker of M2 macrophage [42]. In addition, BL with starry sky showed an over-expression of immune checkpoint genes (*CD274, PDCD1, PDCD1LG2, CTLA4*) which favor the immune escape of tumor [43]. *PD-L1 (CD274)* is part of a complex system of immune checkpoint molecules that, by binding to *PD-1 (PDCD1)* on T cells, attenuates antitumor immunity, facilitating the escape of tumor cells from the T cell-mediated immune response [43-46]. *CTLA4* expression is noted in a variety of B-cell lymphomas and drives immune escape strategies [47-50]. High levels of *PD-L2 (PDCD1LG2)* expression are correlated with T cell exhaustion and immune tolerance [51,52].

Conversely, BL with diffuse granulomatous reaction (Cluster 1) is characterized by the down-regulation of all above mentioned genes (*CCL17, CCL22, CCL2, CCR4, CD36, IL13RA2, ARG1, ICOS, IDO1, LAG3, GATA3, CD163, TIM3/ HAVCR2, CD274, PDCD1, PDCD1LG2, CTLA4*). Furthermore, our results showed the up-regulation of M1- signature genes (*IRF3, INFG1*) in BL with granulomatous reaction [53].*IRF3* is constitutively expressed in various cell types, and it resides in an inactive form in the cytoplasm. After stimulation, *IRF3* is phosphorylated on specific serine residues, resulting in translocation of *IRF3* into the nucleus, where it induces the transcription of *type I IFN* genes upon binding to conserved sequences known as IFN-stimulated response elements [54]. *IFNG1* contributes to the induction of hallmark M1-associated gene expression, suggesting a pro-inflammatory response in these cases [55]. In fact, it is of particular interest that BL with a diffuse granulomatous reaction in our series typically occurs at an early stage of the disease, stage I or II, with three out of five cases showing spontaneous regression. In contrast, BLs with a starry sky pattern, both EBV-positive and EBV-negative, were significantly characterized by advanced stage III or IV and bulky disease. Based on our results, we can identify two biological settings with two different

clinical behavior. An early stage of disease where M1 polarized macrophages prevail and the immune response may be able to control the neoplastic growth. Indeed, in the advanced stage of diseases, characterized by the starry sky pattern, M2 macrophages dominated and may be responsible of a pro-tumor immune response resulting in disease progression and dissemination. We can hypothesize that in EBV positive BL with starry sky the virus itself may triggers T-cell exhaustion and immune evasion, while in EBV-negative BL with starry sky other mechanisms of immune tolerance may be at play. Possibly, the overexpression of TIGIT/PVR axis is one of the immune pathways exploited by EBV-negative BL with starry sky to evade immune surveillance [56,57]. In fact, it has been reported that activation of the TIGIT/PVR pathway in macrophages switch towards anti-inflammatory M2 profiles, suggesting an analogous role of the PD-1/PD-L1 axis in tumor immunosuppression.

Due to their pivotal role, TAMs are promising targets for additional therapies in BL lymphoma and combining TAM-targeted therapy with conventional treatment may open up novel therapeutic avenues for therapy-refractory patients.



REFERENCES

1. Anderson NM, Simon MC. *The tumor microenvironment*. **Curr Biol**. 2020 Aug 17;30(16):R921-R925. doi: 10.1016/j.cub.2020.06.081. PMID: 32810447; PMCID: PMC8194051.
2. Nathan H. Fowler, Chan Yoon Cheah, Randy D. Gascoyne, John Gribben, Sattva S. Neelapu, Paolo Ghia, Catherine Bollard, Stephen Ansell, Michael Curran, Wyndham H. Wilson, Susan O'Brien, Cliona Grant, Richard Little, Thorsten Zenz, Loretta J. Nastoupil, Kieron Dunleavy. *Role of the tumor microenvironment in mature B-cell lymphoid malignancies.* **Haematologica** 2016;101(5):531-540; https://doi.org/10.3324/haematol.2015.139493.
3. Scott, D., Gascoyne, R. *The tumour microenvironment in B cell lymphomas*. **Nat Rev Cancer** 14, 517–534 (2014). https://doi.org/10.1038/nrc3774
4. Gopal S, Gross TG. *How I treat Burkitt lymphoma in children, adolescents, and young adults in sub-Saharan Africa.* **Blood**. 2018 Jul 19;132(3):254-263.
5. Alaggio, R., Amador, C., Anagnostopoulos, I. et al. *The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms*. **Leukemia** 36, 1720–1748 (2022).
6. Yu, H.; Robertson, E.S. *Epstein–Barr Virus History and Pathogenesis*. **Viruses 2023**, *15*, 714.
7. Ford CA, Petrova S, Pound JD, Voss JJ, Melville L, Paterson M, Farnworth SL, Gallimore AM, Cuff S, Wheadon H, Dobbin E, Ogden CA, Dumitriu IE, Dunbar DR, Murray PG, Ruckerl D, Allen JE, Hume DA, van Rooijen N, Goodlad JR, Freeman TC, Gregory CD. *Oncogenic properties of apoptotic tumor cells in aggressive B cell lymphoma*. **Curr Biol**. 2015 Mar 2;25(5):577-88
8. Pham LV, Pogue E. *The role of macrophage/B-cell interactions in the pathophysiology of B-cell lymphomas.* ***Front Oncol***. 2018;8:147. doi: 10.3389/fonc.2018.00147.
9. Majzner RG, Simon JS. *Assessment of programmed death-ligand 1 expression and tumor- associated immune cells in pediatric cancer tissues*. ***Cancer***. 2017;123:3807–3815. doi: 10.1002/cncr.30724.
10. Granai M, Mundo L, Akarca AU *et al*. *Immune landscape in Burkitt lymphoma reveals M2-macrophage polarization and correlation between PD-L1 expression and non-canonical EBV latency program.* **Infect. Agents Cancer** 2020; 15; 28
11. Peter J. Murray. *Macrophage Polarization*. **Annual Review of Physiology** 2017 79:1, 541-566
12. Xiong, X.; Xie, X.; Wang, Z.; Zhang, Y.; Wang, L. *Tumor-associated macrophages in lymphoma: From mechanisms to therapy.* **Int. Immunopharmacol**. **2022**, *112*, 109235.
13. Mills CD, Kincaid K, Alt JM *et al*. *M-1/M-2 macrophages and the Th1/Th2 paradigm*. *J.* **Immunol**. 2000; 164; 6166–6173.
14. Wang N, Liang H, Zen K. *Molecular mechanisms that influence the macrophage M1–M2 polarization balance*. **Front. Immunol**. 2014; 5; 614
15. Hollingsworth HC, Longo DL, Jaffe ES. Small *Non-cleaved cell lymphoma associated with florid epithelioid granulomatous response. A clinicopathologic study of seven patients.* **Am. J. Surg. Pathol**. 1993; 17; 51–59.

16. Haralambieva E, Rosati S, van Noesel C *et al*. *Florid granulomatous reaction in Epstein-Barr virus-positive non-endemic Burkitt lymphomas: report of four cases*. **Am. J. Surg. Pathol**. 2004; 28; 379–383.
17. Schrager JA, Pittaluga S, Raffeld M *et al*. *Granulomatous reaction in Burkitt lymphoma: correlation with EBV positivity and clinical outcome.* **Am. J. Surg. Pathol**. 2005; 29; 1115–1116.
18. Janegová A, Janega P, Ilencíková D *et al*. *Burkitt lymphoma with unusual granulomatous reaction. A case report.* **Cesk. Patol**. 2011; 47; 19–22. [
19. Li JN, Gao LM, Wang WY *et al*. *HIV-related Burkitt lymphoma with florid granulomatous reaction: an unusual case with good outcome.* **Int. J. Clin. Exp. Pathol**. 2014; 7; 7049–7053.
20. Granai M, Lazzi S, Mancini V, Akarca A, Santi R, Vergoni F, Sorrentino E., et al. *Burkitt lymphoma with a granulomatous reaction: an M1/Th1-polarised microenvironment is associated with controlled growth and spontaneous regression.* **Histopathology**. 2022 Jan;80(2):430-442.
21. Cesano A. *nCounter(®) PanCancer Immune Profiling Panel (NanoString Technologies, Inc., Seattle, WA).* **J Immunother Cancer**. 2015 Dec 15; 3:42.
22. *The jamovi project* (2021). **jamovi**. (Version 2.2) [Computer Software]. Retrieved from https://www.jamovi.org.
23. R Core Team (2021). *R: A Language and environment for statistical computing*. (Version 4.0) [Computer software]. Retrieved from https://cran.r-project.org. (R packages retrieved from MRAN snapshot 2021-04-01).
24. Barros MHM, Hauck F, Dreyer JH *et al*. *Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages*. **PLoS One** 2013; 8; e80908
25. Steele KE, Brown C. *Multiplex immunohistochemistry for image analysis of tertiary lymphoid structures in cancer.* **Methods Mol. Biol**. 2018; 1845; 87–98
26. Mulders TA, Björn EW. *Targeting the immune microenvironment in lymphomas of B- cell origin: from biology to clinical application.* **Cancers (Basel***)* 2019;11:915. doi: 10.3390/cancers11070915.
27. Abate F, Ambrosio MR, et al. *Distinct Viral and Mutational Spectrum of Endemic Burkitt Lymphoma*. **PLoSPathog**. 2015 Oct 15;11(10):e1005158. doi: 10.1371/journal.ppat.1005158. PMID: 26468873; PMCID: PMC4607508.
28. Rohde, M., Bonn, B. R., Zimmermann, M., Lange, J., Möricke, A., Klapper, W., Oschlies, I., Szczepanowski, M., Nagel, I., Schrappe, M., Project, S., MMML-Seq Project, I., Loeffler, M., Siebert, R., Reiter, A., & Burkhardt, B. (2017). *Relevance of ID3-TCF3-CCND3 pathway mutations in pediatric aggressive B-cell lymphoma treated according to the non-Hodgkin Lymphoma Berlin-Frankfurt-Münster protocols.* **Haematologica,** 102(6), 1091-1098. https://doi.org/10.3324/haematol.2016.156885
29. Poirel, H.A., Ambrosio, M.R., Piccaluga, P.P., & Leoncini, L. (2018). "*Pathology and Molecular Pathogenesis of Burkitt Lymphoma and Lymphoblastic Lymphoma"*.
30. Piccaluga, P. P. et al. *Gene expression analysis uncovers similarity and differences among Burkitt lymphoma subtypes.* **Blood** 117, 3596–3608 (2011).
31. Tan GW, Visser L, Tan LP, van den Berg A, Diepstra A. *The Microenvironment in Epstein-Barr Virus-Associated Malignancies*. **Pathogens**. 2018 Apr 13;7(2):40

32. Nepomuceno RR, Snow AL, Robert Beatty P, Krams SM, Martinez OM. *Constitutive activation of Jak/STAT proteins in Epstein-Barr virus-infected B-cell lines from patients with posttransplant lymphoproliferative disorder.* **Transplantation.** 2002 Aug 15;74(3):396-402.
33. Williams MV, Mena-Palomo I, Cox B, Ariza ME. EBV dUTPase: *A Novel Modulator of Inflammation and the Tumor Microenvironment in EBV-Associated Malignancies.* **Cancers** (Basel). 2023 Jan 30;15(3):855
34. Chen L, Monti S, Juszczynski P, Daley J, Chen W, Witzig TE, Habermann TM, Kutok JL, Shipp MA. *SYK-dependent tonic B-cell receptor signaling is a rational treatment target in diffuse large B-cell lymphoma.* **Blood.** 2008 Feb 15;111(4):2230-7.
35. Vaclav Seda, Marek Mraz1. *B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells*. **European Journal of Haematology**. Volume 94, Issue 3Mar 2015 Pages 189-282
36. Reis-Sobreiro M, Teixeira da Mota A, Jardim C, Serre K. *Bringing Macrophages to the Frontline against Cancer: Current Immunotherapies Targeting Macrophages*. **Cells**. 2021 Sep 9;10(9):2364.
37. Stein M., Keshav S., Harris N., Gordon S. *Interleukin 4 potently enhances murine macrophage mannose receptor activity: A marker of alternative immunologic macrophage activation*. J. Exp. Med. 1992;176:287–292.
38. Helming L, Winter J, Gordon S. *The scavenger receptor CD36 plays a role in cytokine-induced macrophage fusion.* **J Cell Sci**. 2009 Feb 15;122(Pt 4):453-9.
39. Wang D., Yang L., Yue D., Cao L., Li L., Wang D., Ping Y., Shen Z., Zheng Y., Wang L., et al. *Macrophage-derived CCL22 promotes an immunosuppressive tumor microenvironment via IL-8 in malignant pleural effusion.* **Cancer Lett.** 2019;452:244–253. doi: 10.1016/j.canlet.2019.03.040.
40. Elena Sierra-Filardi, Concha Nieto, Ángeles Domínguez-Soto, Rubén Barroso, Paloma Sánchez-Mateos, Amaya Puig-Kroger, María López-Bravo, Jorge Joven, Carlos Ardavín, José L. Rodríguez-Fernández, Carmen Sánchez-Torres, Mario Mellado, Ángel L. Corbí; *CCL2 Shapes Macrophage Polarization by GM-CSF and M-CSF: Identification of CCL2/CCR2-Dependent Gene Expression Profile*. **J Immunol** 15 April 2014; 192 (8): 3858–3867.
41. Ruytinx P, Proost P, Van Damme J and Struyf S (2018) *Chemokine-Induced Macrophage Polarization in Inflammatory Conditions.* **Front. Immunol**. 9:1930. doi: 10.3389/fimmu.2018.01930
42. Junttila IS, Mizukami K, Dickensheets H, Meier-Schellersheim M, Yamane H, Donnelly RP, Paul WE. *Tuning sensitivity to IL-4 and IL-13: differential expression of IL-4Ralpha, IL-13Ralpha1, and gammac regulates relative cytokine sensitivity*. **J Exp Med**. 2008 Oct 27;205(11):2595-608.
43. Jiang X, Zhou T, Xiao Y, Yu J, Dou S, Chen G, Wang R, Xiao H, Hou C, Wang W, Shi Q, Feng J, Ma Y, Shen B, Li Y, Han G. *Tim-3 promotes tumor-promoting M2 macrophage polarization by binding to STAT1 and suppressing the STAT1-miR-155 signaling axis***. Oncoimmunology**. 2016 Aug 3;5(9):e1211219.
44. Buechler C, Ritter M, OrsóE *et al*. *Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli*. **J. Leukoc**. *Biol.* 2000; 67; 97–103.
45. Upadhyay R, Hammerich L, Peng P, Brown B, Merad M, Brody JD. *Lymphoma: immune evasion strategies.* **Cancers (Basel).** 2015 Apr 30;7(2):736-62.

46. Andorsky, D.J.; Yamada, R.E.; Said, J.; Pinkus, G.S.; Betting, D.J.; Timmerman, J.M. *Programmed death ligand 1 is expressed by non-hodgkin lymphomas and inhibits the activity of tumor associated T cells.* **Clin. Cancer Res.** 2011, 17, 4232–4244.

47. Jiang Y, Li Y. *T-cellexhaustion in the tumourmicroenviroment*. **Cell Death** Dis. 2015;6:1–9.

48. Butte MJ, Keir ME. *Programmed death-1 ligand 1 interacts specifically with the B7–1 costimulatory molecule to inhibit T cell responses.* **Immunity.** 2007;**27**(1):111–122. doi: 10.1016/j.immuni.2007.05.016.

49. Rollins MR, Gibbons Johnson RM. *CD80 expressed by CD8(+) T cells contributes to PD-L1-induced apoptosis of activated CD8(+) T cells.* **J Immunol Res**. 2017;2017:1–6

50. Probst, H. C., McCoy, K., Okazaki, T., Honjo, T. & van den Broek, *M. Resting dendritic cells induce peripheral CD8^{+} T cell tolerance through PD-1 and CTLA-4.* **Nature Immunol**. **6**, 280–286 (2005)

51. Wang, Y., Du, J., Gao, Z. *et al. Evolving landscape of PD-L2: bring new light to checkpoint immunotherapy.* **Br J Cancer** (2022).

52. Günthner R, Anders HJ. *Interferon-regulatory factors determine macrophage phenotype polarization*. **Mediators Inflamm.** 2013;2013:731023.

53. Yanai H, Chiba S, Hangai S, Kometani K, Inoue A, Kimura Y, Abe T, Kiyonari H, Nishio J, Taguchi-Atarashi N, Mizushima Y, Negishi H, Grosschedl R, Taniguchi T. *Revisiting the role of IRF3 in inflammation and immunity by conditional and specifically targeted gene ablation in mice.* **Proc Natl Acad** Sci U S A. 2018 May 15;115(20):5253-5258.

54. Martinez FO, Gordon S. *The M1 and M2 paradigm of macrophage activation: time for reassessment***.** *Prime Rep*. 2014 Mar 3;6:13.

55. Gorvel L, Olive D. Targeting the *"PVR-TIGIT axis" with immune checkpoint therapies. Res*. 2020 May 13;9:F1000 Faculty Rev-354.

56. Harjunpää H, Guillerey C. *TIGIT as an emerging immune checkpoint*. **Clin Exp Immunol.** 2020 May;200(2):108-119.

Work in progress. Abstract published in LEUKEMIA Research.

"Burkitt-like lymphoma with 11q aberration": neither Burkitt-lymphoma nor diffuse large B-cell lymphoma. What the microenvironment tells us.

Marcello Del Corvo[1], Maria Rosaria Sapienza[1], Maria Chiara Siciliano[2], Salvatore Tornambè[2], Saveria Mazzara[1], Massimo Granai[3], Felice Arcuri[2], Roberto Boccacci[2], Virginia Mancini[2], Domenico Ferrara[2], German Ott[4], Reiner Siebert[5], Leticia Quintanilla Fend[3], Falko Fend[3], Stefano Pileri[1], Stefano Lazzi[2], Lorenzo Leoncini *[2].

[1]Istituto Europeo di Oncologia (IEO), Milan, Italy

[2]Medical Biotechnology, UNIVERSITY OF SIENA, Siena, Italy

[3]Institut für Pathologie und Neuropathologie, University of Tubingen, Tubingen, Germany

[4]Institut für KlinischePathologie, Robert-Bosch-Krankenhaus, Stuttgart

[5]Institut für Humangenetik, Universitätsklinikum Ulm, Ulm, Germany

*Correspondence to: Professor Lorenzo Leoncini, Department of Medical Biotechnology,

University of Siena, Siena, Italy; e-mail: lorenzo.leoncini@unisi.it

Background. High grade B-cell lymphomas with a morphology similar to Burkitt lymphoma (BL) or showing an intermediate or blastoid appearance, but lacking IG-MYC translocation and carrying a peculiar pattern of an 11q aberration have been described as a new provisional entity called "Burkitt-like lymphoma with 11q aberration" (BLL,11q) in the revised 4th edition of the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Such cases also show additional characteristics of BL as diffuse lymphomatous infiltrate sometimes with a starry sky pattern and a cohesive growth. In particular, a recent paper aiming to describe the morphology of BLL11q, reported that the starry sky macrophages with enhanced number of phagocytized apoptotic bodies were a striking characteristic of this lymphoma type. The functional status of macrophages may impact on tumour immune response, and recent studies have reported differences in tumor microenvironment (TME) and in particular macrophage polarization in different subtypes of BL.

Objectives. Here we aim to compare the TME in BLL11q, BL EBV-negative and DLBCL EBV-negative.

Design/Methods. Genes expression profiling (GEP) of 770 immune-related genes in Formalin-Fixed Paraffin-Embedded (FFPE) samples was performed using the NanoString nCounter PanCancer Immune Profiling Panel.

Results. Based on principal component analysis (PCA), three clusters were identified with some overlap among the cases. However, hierarchical clustering could better show that BL and BLL11q grouped together as compared to DLBCL. Yet, some differences were also found between BL and BLL11q.

Differential expression analysis and Gene Set Enrichment analysis (GSEA) revealed a degree of heterogeneity in several immune cell categories. With an adjusted p-value threshold of 0.05, BLL11q showed only 18 significantly differentially expressed genes (DEGs) in comparison with BL, while DLBCL vs BL11q and DLBCL vs BL reported 32 and 151 DEGs, respectively.

The immune gene profiling of DLBCL, BLL11q and BL cases were also analyzed by cell-type profiling revealing a higher infiltration of T-cell CD8 exhausted in DLBCL vs BLL11q and BL.

Conclusion. In conclusion, the TME in BLL11q differs from that of DLBCL and BL. Our findings further support that BLL11q does represent a distinct lymphoma category.

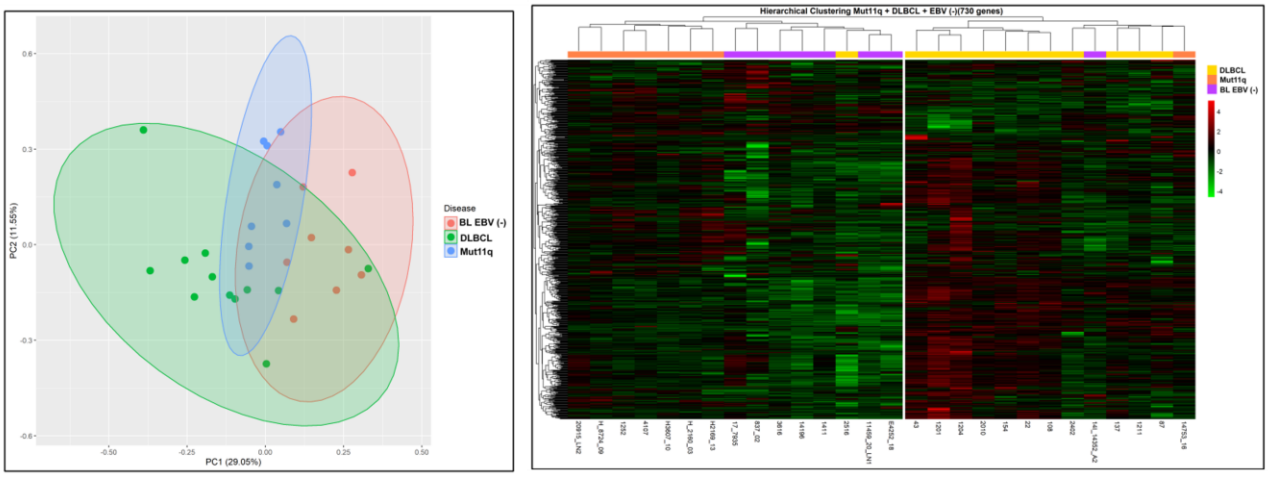


Figure 1. ***PCA and heatmap identify differences between DLBCL and BL***

Work in progress. Abstract published in LEUKEMIA Research

"BCR EXPRESSION IN BURKITT LYMPHOMA: NEW INSIGHT IN MUTATIONAL LANDSCAPE"

Maria Chiara Siciliano[1*], Margherita Vannucchi[1*], Salvatore Tornambè[1], Massimo Granai[2], Felice Paolo Arcuri[1], Ester Sorrentino[1], Teresa Amato[1], Nicola De Leone[1], Roberto Boccacci[1], Stefano Lazzi[1], Reiner Siebert[3], Lorenzo Leoncini[1], Cristiana Bellan[1]

[1]Deapartment of Medical Biotechnologies, University of siena, Siena, [2] Institut für Pathologie und Neuropathologie, University of Tubingen, Tubingen, [3]Institut für Humangenetik, Universitätsklinikum Ulm, Ulm

*Equally contributed

Background: BCR activation is an important step in lymphomagenesis. In different subtype of BL there is a combined mechanism of tonic and extrinsic BCR signaling activation. In eBL BCR activation can be related to the chronic Plasmodium falciparum stimulation which can reactivate latently EBV-infected memory B cells. Yet, whether coincidental infections with additional pathogens may occur in eBL remains to be addressed. On the other hand, in sBL the progressive acquisition of mutations in the TCF3/ID3 genes results in intrinsic tonic activation of BCR signaling. Therefore, neoplastic cells may grow in an EBV (and/or other antigen) independent way. Usually in Burkitt lymphomas surface IgM represents functional BCR. However high number of IgA transcript have recently reported.1 This extend previous studies showing recurrent MYC translocation breakpoints in IGHA in BL suggesting that the cell of origin of BL is a germinal center experienced B-cell primed to switch to and to express IgA. Furthermore, IgM/D/G/A-negative cases have also been reported [1]. Interestingly, heterozygous NRAS and KRAS gain of function mutations have been identified in one sample of such cases, suggesting that BL cells acquiring RAS mutations may lose the selective pressure to express a functional BCR. The aim of the present study is: a) to determines the expression of BCR by immunohistochemistry in a large series of BL b) to investigate the possible constitutive activation of the MAPK pathway through RAS mutations and c) to better understand the genetic landscape of these cases.

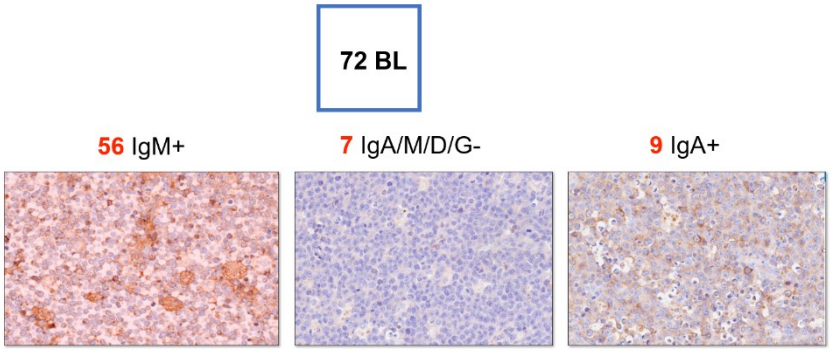
Materials and Methods: We performed an immunohistochemistry analysis to detect IgA/IgM/IgD/IgG expression in 72 Formalin-Fixed Paraffin-Embedded (FFPE) BL samples. We performed qPCR for RAS hotspot mutations and an NGS analysis (Illumina platform) with a capture-base custom panel covering 83 genes known to be involved in lymphomagenesis.

Results: 56/72 cases expressed surface IgM but we also found 9/72 expressing IgA. Interestingly, 7/72 lacked BCR expression, being negative for all surface immunoglobulins. BL expressing surface IgA were localized in the oral and gastrointestinal mucosa, in line with the observation that BL frequently presents in sites of the human body particularly important for IgA production.

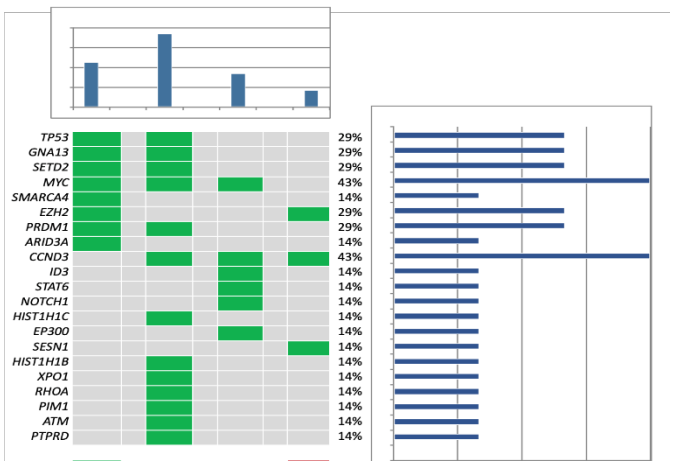
We founded KRAS somatic mutations in the classic hotspots of the gene by qPCR in 2/5 cases lacking expression of BCR. No NRAS single nucleotide variants were identified in our cohort. NGS analysis, in addition to the typical BL's mutations (ID3,GNA13,CCND3,MYC) showed recurrent mutations in genes not usually affected in classic BL like TET2, EZH2, HIST1H1C, HIST1H1B, PRDM1 and PIM1. Interestingly, the cases with KRAS gain of function were those with a high mutational burden, carrying more than 5 mutation and, moreover, the only two cases of our cohort showing MYC gene mutation. EBV infection as detected in 1/2 KRAS mutated samples.

Conclusion: In this study, we confirmed the presence of RAS mutations in IgM/D/G/A-negative Burkitt lymphoma supporting the previous hypothesis2 that the constitutive RAS/MAPK activation can bypass the requirement for a functional BCR/PI3Kδ axis to sustain lymphoma fitness. Furthermore, we had demonstrated that mutation in genes related to epigenetic mechanism and histone remodeling seems to be more frequently involved in such cases.

References: 1. Varano G, Raffel S, Sormani M, Zanardi F, et al. *The B-cell receptor controls fitness of MYC-driven lymphoma cells via GSK3β inhibition.* **Nature.** 2017 Jun 8;546(7657):302-306.



Sample	NRAS status	KRAS status	EBV infection
# 1	Wt	MUT (p.G12X)	Positive
# 2	Wt	MUT (p.G12X)	Negative
# 3	Wt	MUT (p.A59X)	Negative
# 4	Wt	Wt	Negative



Work in progress. Abstract published in Leukemia Research

"SOX11 AND EPSTEIN–BARR VIRUS MAY SUBSTITUTE EACH OTHER IN THE PATHOGENESIS OF BURKITT LYMPHOMA"

Marta Sureda-Gómez[1*], Ingram Iaccarino[2*], Patricia Balsas[1,3], Julia Richter[2], Selina Glaser[4], Marta-Leonor Rodríguez[1], Anna De Bolòs[1], Ferran Nadeu[1,3], Elias Campo[1,3,5], Maria Chiara Siciliano[6], Salvatore Tornambè [6], Cristiana Bellan[6], Lorenzo Leoncini[6], Reiner Siebert[4], Virginia Amador[1,3Ŧ], Wolfram Klapper[2Ŧ].

Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain [1],Department of Pathology, Hematopathology Section and Lymph Node Registry, University of Kiel, Kiel, Germany [2], Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain [3], Instuitute of Human Genetics, University of Ulm, Germany [4], Department of Hematology Hospital Clinic of Barcelona, University of Barcelona, Barcelona, Spain [5], Department of Medical Biotechnologies, Section of Pathological Anatomy, University of Siena, Italy [6]

[*]These authors have contributed equally to this study.

[Ŧ]Dr. V. Amador: vamador@clinic.cat. Dr. W. Klapper: wklapper@path.uni-kiel.de.

Background. Burkitt lymphoma (BL) is an aggressive germinal center-derived B-cell non-Hodgkin lymphoma and the most frequent tumor of children and adolescents but infrequent in adults. Epstein-Barr-Virus (EBV) is found in so called "endemic" BL mostly affecting children in central Africa. In so called "sporadic" BL EBV is detected more frequently in adult compared to pediatric patients. Expression of the transcription factor SRY-related HMG-box gene 11 (SOX11) is confined to precursor cell neoplasms, mantle cell lymphoma (MCL) and BL. In BL, SOX11 expression is more frequent in pediatric than in adult sporadic BL cases. The pathogenic role of SOX11 has been studied in MCL. However, the contribution of SOX11 to BL pathogenesis remains unknown.

Objectives design/methods. To elucidate the role of SOX11 in BL pathogenesis, we ectopically overexpressed SOX11 in SOX11-, and knocked out SOX11 (SOX11 KO) in a SOX11-positive BL cell lines and developed integrative omics analyses, to elucidate different mechanisms leading to oncogenic transformation in sporadic BLs.

To understand the role of SOX11in different clinical presentations of BL, we analyzed the association of SOX11 and SOX11-downstream target with different clinical information, such as age, EBV infection, MYC break and mutational status, diagnosis and response to therapy, in a large series of BL patients.

Results. RNA sequencing upon SOX11 knockout or ectopic overexpression in BL cell lines showed changes in genes associated with cell motility and cancer stemness-related. However, viability of BL cells was largely unaffected.

In a large cohort of clinical specimen SOX11 expression is limited to Epstein Barr Virus negative (EBV-) sporadic BL (sBL) cases. In fact, SOX11 and EBV by EBER in situ hybridization were virtually exclusive. The mechanism underlying the MYC: IGH fusions in BL is due to classes switch recombination in 100% of the

EBV-\SOX11+ sBL cases, while somatic hypermutation as the pathogenic mechanism of MYC-traslocations was confirmed to SOX11- cases (50%). Moreover, SOX11 was associated with mutations in ID3, CCND3, TP53 and apoptotic-related genes.

Conclusion. We propose a model in which SOX11 expression and EBV-association reflect a molecular dichotomy of BL. The association of either SOX11 or EBV with the pathogenic mechanism of MYC-translocations but little effect of knock-out in cell lined suggests that both EBV and SOX11, play a major role in early pathogenesis but may be less relevant in a fully developed BL. Our data support the concept that virus-associated pathogenesis rather than historical subtypes (“endemic”, “sporadic”) determines the biology of BL.

6. *Conclusions*

The lymphoma microenvironment is increasingly being recognized as a dynamic and interactive supporting network, including immune cells, stromal cells, cytokines, blood vessels, and extracellular matrix components, whose composition is guided by neoplastic cells, which influences tumor initiation, progression, resistance to cell death, evasion of growth suppressors, and drug resistance [72]. Although multiple studies have investigated TME expression in B-cell lymphomas, only a limited number of studies have been conducted on BL. In our work we identified a polarization towards M2 phenotype of TAMs in all cases by applying three different approaches (CIBERSORT, mIHC and mIF) regardless of the subtype of BL, and thus, of EBV status. These cells, intimately associated with the neoplastic cells, constituted also the major source of PD- L1, which may inhibit the overall inflammatory response and allow the neoplastic cells to evade antitumor immunity. However, the lower rate of PD-L1 expression on TAMs in sBL, as compared with eBL and idBL, which are frequently associated with EBV, may suggest a role of the virus in inducing PDL1 expression. Infact, we found that the majority of the CD8+ infiltrating T cells expressed PD-1 highlighting ad adaptive immune response resistance mechanism, supporting the idea that EBV in BL might induce PD-L1 expression on tumor cells. We concluded that the prevalence of M2 macrophages are the primary constituent of the TME in BL, leading to a pro-tumoral state in these cases.

Recent data using mIHC show that the TME of BL with granulomatous reaction is characterized by the prevalence of M1 macrophages and pro-inflammatory response, which could possibly explain the spontaneous regression in some cases [46]. Based on that, we decided to further characterize the immune landscape of BL subtypes by GEP with Nanostring technologies focusing on the immune gene categories using a 730 immune-related genes panel.

GEP analysis of immune gene categories could separate the BL into three clusters. Cluster 1 comprised the five cases of EBV-positive BL with diffuse granulomatous reaction and clusters 2 and 3 BL with typical starry sky pattern both EBV-positive and EBV-negative, respectively. However, some overlap was observed. In particular, cluster 3 included also the two cases of BL with a partial granulomatous and three cases of BL-EBV positive with starry sky.

Some features could be linked exclusively to EBV presence by comparing the TME of EBV-positive BL and EBV-negative. Indeed, our results confirm previous findings for up-regulation of the NF-kB and JAK-STAT pathways in EBV-positive BL, while a “tonic” activation of BCR signaling was more

enriched in EBV-negative BL [29]. Although some features are related exclusively to EBV presence, clustering analysis focusing on the immune gene categories, revealed that BL with a granulomatous reaction has a unique immune landscape significantly different from BL with starry sky both EBV-negative and EBV- positive.

In fact, the immune cell composition overlaps between EBV-positive BL and EBV-negative BL with starry sky, showing an up-regulation of IL4/IL13 pathway (*CD36, IL13RA2*), M2-secreted chemokines (*CCL17, CCL22, CCL2, CCR4*), M2-immune response genes (*ARG1, ICOS, IDO1, LAG3, GATA3, CD163,TIM3/HAVCR2*), suggesting a M2- polarization in these cases [72-77].

In addition, as previously demonstrated, BL with starry sky showed an over-expression of immune checkpoint genes (*CD274, PDCD1, PDCD1LG2, CTLA4*) which favor the immune escape of tumor *PD-L1 (CD274)* is part of a complex system of immune checkpoint molecules that, by binding to *PD-1 (PDCD1)* on T cells, attenuates antitumor immunity, facilitating the escape of tumor cells from the T cell-mediated immune response. Conversely, BL with diffuse granulomatous reaction is characterized by the down-regulation of all above mentioned genes (*CCL17, CCL22, CCL2, CCR4, CD36, IL13RA2, ARG1, ICOS, IDO1, LAG3, GATA3, CD163, TIM3/ HAVCR2, CD274, PDCD1, PDCD1LG2, CTLA4*) [78-86]. Furthermore, our results showed the up-regulation of M1- signature genes (*IRF3, INFG1*) in BL with granulomatous reaction [87,88]. In fact, it is of particular interest that BL with a diffuse granulomatous reaction in our series typically occurs at an early stage of the disease, stage I or II, with three out of five cases showing spontaneous regression. In contrast, BLs with a starry sky pattern, both EBV-positive and EBV-negative, were significantly characterized by advanced stage III or IV and bulky disease. Based on our results, we can identify two biological settings with two different clinical behavior. An early stage of disease where M1 polarized macrophages prevail and the immune response may be able to control the neoplastic growth. Indeed, in the advanced stage of diseases, characterized by the starry sky pattern, M2 macrophages dominated and may be responsible of a pro-tumor immune response resulting in disease progression and dissemination. We can hypothesize that in EBV positive BL with starry sky the virus itself may triggers T-cell exhaustion and immune evasion, while in EBV-negative BL with starry sky other mechanisms of immune tolerance may be at play. Possibly, the overexpression of TIGIT/PVR axis is one of the immune pathways exploited by EBV-negative BL with starry sky to evade immune surveillance. In fact, it has been reported that activation of the TIGIT/PVR pathway in macrophages switch towards anti-inflammatory M2 profiles, suggesting an analogous role of the PD-1/PD-L1 axis in tumor immunosuppression [88-91]. Due to their pivotal role, TAMs are promising targets for additional therapies in BL lymphoma and combining TAM-targeted therapy with conventional treatment may open up novel therapeutic avenues for therapy-refractory patients.

Finally, the relationship of B cells to their microenvironment is crucial for the establishment of altered proliferative/apoptotic pathways, which are hallmarks of lymphoma pathogenesis. The generation of Ig-negative B cells during tumor progression should result in these cells lacking essential survival and proliferation signals provided through BCR signaling. This selective pressure for BCR-negative cells means that only those that receive sufficient compensatory signals can survive and possibly persist. Following genetic alterations accumulated by tumor cells before the loss of the BCR, compensatory signals are activated, which lead these cells to resistance to apoptotic stimuli and to better proliferation [31,32]. In a scenario like that neoplastic cells may grow in an EBV (and/or other antigen) independent way. Usually in Burkitt lymphomas surface IgM represents functional BCR. However high number of IgA transcript have recently reported. This extend previous studies showing recurrent MYC translocation breakpoints in IGHA in BL suggesting that the cell of origin of BL is a germinal center experienced B-cell primed to switch to and to express IgA. Furthermore, IgM/D/G/A-negative cases have also been reported Interestingly, heterozygous NRAS and KRAS gain of function mutations have been identified in one sample of such cases, suggesting that BL cells acquiring RAS mutations may lose the selective pressure to express a functional BCR [92]. Based on that, we decided to

performed an immunohistochemistry analysis to detect IgA/IgM/IgD/IgG expression in 72 Formalin-Fixed Paraffin-Embedded (FFPE) BL samples. Additionally, we performed qPCR/ddPCR for RAS hotspot mutations and an NGS analysis (Illumina platform) with a capture-base custom panel covering 83 genes known to be involved in lymphomagenesis. Our results showed that 56/72 cases expressed surface IgM but we also found 9/72 expressing IgA. Interestingly, 7/72 lacked BCR expression, being negative for all surface immunoglobulins. BL expressing surface IgA were localized in the oral and gastrointestinal mucosa, in line with the observation that BL frequently presents in sites of the human body particularly important for IgA production.

We founded KRAS somatic mutations in the classic hotspots of the gene by qPCR/ddPCR in 2/5 cases lacking expression of BCR. No NRAS single nucleotide variants were identified in our cohort. NGS analysis, in addition to the typical BL's mutations (ID3,GNA13,CCND3,MYC) showed recurrent mutations in genes not usually affected in classic BL like TET2, EZH2, HIST1H1C, HIST1H1B, PRDM1 and PIM1. Interestingly, the cases with KRAS gain of function were those with a high mutational burden, carrying more than 5 mutation and, moreover, the only two cases of our cohort showing MYC gene mutation. EBV infection as detected in 1/2 KRAS mutated samples.

In this study, we confirmed the presence of RAS mutations in IgM/D/G/A-negative Burkitt lymphoma supporting the previous hypothesis that the constitutive RAS/MAPK activation can bypass the requirement for a functional BCR/PI3Kδ axis to sustain lymphoma fitness. Furthermore, we had

demonstrated that mutation in genes related to epigenetic mechanism and histone remodeling seems to be more frequently involved in such cases.

REFERENCES

1. Chaplin DD. *Overview of the immune response*. **J Allergy Clin Immunol**. 2010.
2. Ollila J, Vihinen M. *B cells*. **Int J Biochem Cell Biol**. 2005 Mar;37(3):518-23.
3. Xie, W., Wucherpfennig, K. & Patel, D.J. *A structural platform for B cell receptor signaling.* **Cell Res** 33, 95–96 (2023).
4. Lockey C, Young H, Brown J, et al. *Characterization of interactions within the Igα/Igβ transmembrane domains of the human B-cell receptor provides insights into receptor assembly*. **Journal of Biological Chemistry**, Volume 298, Issue 5,2022
5. Treanor B. *B-cell receptor: from resting state to activate*. **Immunology** (2012) 136(1):21–7.
6. Janeway CA Jr, Travers P, Walport M, et al. *Immunobiology: The Immune System in Health and Disease. 5th edition.* **New York: Garland Science**; 2001.
7. Wang LD, Clark MR. *B-cell antigen-receptor signaling in lymphocyte development.* **Immunology.** 2003 Dec;110(4):411-20.
8. Yuekang Xu, Kenneth W. Harder, Nicholas D. Huntington, et al. *Lyn Tyrosine Kinase: Accentuating the Positive and the Negative*. **Immunity**, Volume 22, Issue 1,2005. Pages 9-18
9. Geahlen RL. *Getting Syk: spleen tyrosine kinase as a therapeutic target.* **Trends Pharmacol Sci**. 2014;35(8):414-22.
10. Diop A, Santorelli D, Malagrinò F, et al. *SH2 Domains: Folding, Binding and Therapeutical Approaches.* **Int J Mol Sci**. 2022;23(24):15944.
11. Oh-hora M, Rao A. *The calcium/NFAT pathway: role in development and function of regulatory T cells.* **Microbes Infect**. 2009 Apr;11(5):612-9.
12. Chen Zhangguo, Wang Jing H., et al. *How the Signaling Crosstalk of B Cell Receptor (BCR) and Co-Receptors Regulates Antibody Class Switch Recombination: A New Perspective of Checkpoints of BCR Signaling,* **Frontiers in Immunology**, 2021.
13. Anshuman Bhanja, Ivan Rey-Suarez, Wenxia Song, Arpita Upadhyaya. *Bidirectional feedback between BCR signaling and actin cytoskeletal dynamics*. **The FEBS Journal**, 2021.
14. Young RM, Staudt LM. *Targeting pathological B cell receptor signalling in lymphoid malignancies.* **Nat Rev Drug Discov**. 2013 Mar;12(3):229-43.
15. Mlynarczyk C, Fontán L, Melnick A. *Germinal center-derived lymphomas: The darkest side of humoral immunity*. **Immunol Rev**. 2019 Mar;288(1):214-239.
16. Rossi D, Ciardullo C, Gaidano G. *Genetic aberrations of signaling pathway in lymphomagenesis: revelations from next generation sequencing studies*. **Semin Cancer Biol**. 2013;23:422-430.
17. Pereira MI, Medeiros JA. *Role of Helicobacter pylori in gastric mucosa–associated lymphoid tissue lymphomas.* **World J Gastroenterol**. 2014;20:684-698.
18. Pannekoek Y, van der Ende A. *Chlamydia psittaci infection in nongastrointestinal MALT lymphomas and their precursor lesions.* **Am J Clin Pathol**. 2011;136:480. - PubMed
19. Carugi A, Onnis A, Antonicelli G, et al. *Geographic variation and environmental conditions as cofactors in Chlamydia psittaci association with ocular adnexal lymphomas: a comparison between Italian and African samples.* **Hematol Oncol**. 2010;28:20-26.
20. Davis RE, Hgo VN, Lenz G, et al. *Chronic active B cell receptor signaling in diffuse large B cell lymphoma.* **Nature**. 2010;463:88-92.
21. Richter J, Schlesner M, Hoffmann S, et al. *Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcription sequencing.* **Nature** Gen. 2012;44:1316-1322.

22. Schmitz R, Young RM, Ceribelli M, et al. *Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics.* **Nature**. 2012;490:116-120.
23. Love C, Sun Z, Jima D, et al. *The genetic landscape of mutations in Burkitt lymphoma.* **Nat Genet**. 2012;44:1321-1325.
24. van den Bosch CA. *Is endemic Burkitt's lymphoma an alliance between three infections and a tumour promoter?* **Lancet Oncol**. 2004;5:738-746.
25. Mancao C, Hammerschmidt W. *Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival.* **Blood**. 2007 Nov 15;110(10):3715-21.
26. Portis, T., Longnecker, R. *Epstein–Barr virus (EBV) LMP2A mediates B-lymphocyte survival through constitutive activation of the Ras/PI3K/Akt pathway.* **Oncogene** 23, 8619–8628.
27. Rohde, M., Bonn, B. R., Zimmermann, M., Lange, J., Möricke, A., Klapper, W., et al. *Relevance of ID3-TCF3-CCND3 pathway mutations in pediatric aggressive B-cell lymphoma treated according to the non-Hodgkin Lymphoma Berlin-Frankfurt-Münster protocols.* **Haematologica**. 2017, 102(6), 1091-1098.
28. Amato T, Abate F, Piccaluga P, Iacono M, et al. *Clonality Analysis of Immunoglobulin Gene Rearrangement by Next-Generation Sequencing in Endemic Burkitt Lymphoma Suggests Antigen Drive Activation of BCR as Opposed to Sporadic Burkitt Lymphoma*. **Am J Clin Pathol**. 2016 Jan;145(1):116-27.
29. Abate F, Ambrosio MR, et al. *Distinct Viral and Mutational Spectrum of Endemic Burkitt Lymphoma.* **PLoS Pathog**. 2015
30. Ingravallo G, Tamma R, Opinto G, et al. *The Effect of the Tumor Microenvironment on Lymphoid Neoplasms Derived from B Cells*. **Diagnostics (Basel).** 2022 Feb 23;12(3):573.
31. Casola S, Perucho L, Tripodo C, et al. *The B-Cell Receptor in Control of Tumor B-Cell Fitness: Biology and Clinical Relevance.* **Immunol Rev** (2019) 288:198–213
32. Shain KH, Dalton WS, Tao J. *The tumor microenvironment shapes hallmarks of mature B-cell malignancies.* **Oncogene**. 2015 Sep 3;34(36):4673-82.
33. Linfeng Chen, Jing Ouyang, Kirsty Wienand, et al. *CXCR4 upregulation is an indicator of sensitivity to B-cell receptor/PI3K blockade and a potential resistance mechanism in B-cell receptor-dependent diffuse large B-cell lymphomas.* **Haematologica** 2020;105(5):1361-1368;
34. Mezzapelle R, Leo M, Caprioglio F. et al. *CXCL12 Activities in the Tumor Microenvironment and Implications for Tumor Immunotherapy.* **Cancers (Basel).** 2022 May 6;14(9):2314.
35. Anderson NM, Simon MC. *The tumor microenvironment*. **Curr Biol**. 2020 Aug 17;30(16).
36. Martin JD, Fukumura D, Duda DG, Boucher Y, Jain RK. *Reengineering the Tumor Microenvironment to Alleviate Hypoxia and Overcome Cancer Heterogeneity*. **Cold Spring Harb Perspect Med**. 2016 Dec 1;6(12):a027094.
37. Nathan H. Fowler, Chan Yoon Cheah, Randy D. Gascoyne, et al. *Role of the tumor microenvironment in mature B-cell lymphoid malignancies.* **Haematologica** 2016;101(5):531-540;
38. Liu, Y., Zhou, X. & Wang, X. *Targeting the tumor microenvironment in B-cell lymphoma: challenges and opportunities*. **J Hematol Oncol** 14, 125 (2021).
39. Scott, D., Gascoyne, R. *The tumour microenvironment in B cell lymphomas.* **Nat Rev Cancer** 14, 517–534 (2014).
40. Ng, W.L., Ansell, S.M. & Mondello, P. *Insights into the tumor microenvironment of B cell lymphoma.* **J Exp Clin Cancer Res** 41, 362 (2022).
41. Maosen Xu, Yang Wang, Ruolan Xia, Yuquan Wei, Xiawei Wei. *Role of the CCL2-CCR2 signalling axis in cancer: Mechanisms and therapeutic targeting.* **Cell proliferation in basic and clinical sciences**. 2021
42. Rapp M, Wintergerst MWM, Kunz WG, et al. *CCL22 controls immunity by promoting regulatory T cell communication with dendritic cells in lymph nodes*. **J Exp Med**. 2019

43. Pham LV, Pogue E, Ford RJ. *The Role of Macrophage/B-Cell Interactions in the Pathophysiology of B-Cell Lymphomas.* **Front Oncol**. 2018
44. Xiong X, Xie X, Wang Z, Zhang Y, Wang L. *Tumor-associated macrophages in lymphoma: From mechanisms to therapy.* **Int Immunopharmacol.** 2022.
45. Peter J. Murray. *Macrophage Polarization*. **Annual Review of Physiology**. 2017 79:1, 541-566
46. Granai M, Lazzi S, Mancini V, Akarca A, et al. *Burkitt lymphoma with a granulomatous reaction: an M1/Th1-polarised microenvironment is associated with controlled growth and spontaneous regression.* **Histopathology**. 2022 Jan;80(2):430-442.
47. Orem, J., Mbidde, E. K., Lambert, B., et al. *Burkitt's lymphoma in Africa, a review of the epidemiology and etiology.* **African Health Sciences**, 2007, 7(3), 166-175.
48. Kalisz, K., Alessandrino, F., Beck, R. et al. *An update on Burkitt lymphoma: a review of pathogenesis and multimodality imaging assessment of disease presentation, treatment response, and recurrence.* **Insights Imaging** 2019 May 21;10(1):56.
49. Alaggio, R., Amador, C., Anagnostopoulos, I. et al. *The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms.* **Leukemia** 36, 1720–1748 (2022).
50. Shannon-Lowe C, Rickinson A. *The Global Landscape of EBV-Associated Tumors*. **Front Oncol**. 2019 Aug 6;9:713.
51. G Kennedy, J Koman, B Sugden. *Epstein-barr virus provides a survival factor to Burkitt's lymphomas.* **Proc Natl Acad Sci**. 2003.
52. Saha, A., & Robertson, E. S. *Epstein-Barr virus–associated B-cell lymphomas: pathogenesis and clinical outcomes*. **Clinical Cancer Research.** 2011.17(10), 3056-3063.
53. Shannon-Lowe, C., Rickinson, A. B., & Bell, A. I. *Epstein–Barr virus-associated lymphomas*. **Philosophical Transactions of the Royal Society B: Biological Sciences.** 2017. *372*(1732), 20160271.
54. Nguyen, L.; Papenhausen, P.; Shao, H. *The Role of c-MYC in B-Cell Lymphomas: Diagnostic and Molecular Aspects.* **Genes** 2017, *8*, 116
55. Schmitz R, Ceribelli M, Pittaluga S, Wright G, Staudt LM. *Oncogenic mechanisms in Burkitt lymphoma.* **Cold Spring Harb Perspect Med**. 2014
56. Dozzo M, Carobolante F, Donisi PM, Scattolin A, et al. *Burkitt lymphoma in adolescents and young adults: management challenges*. **Adolesc Health Med Ther**. 2017;8:11-29
57. Young, L. S., Yap, L. F., & Murray, P. G. *Epstein–Barr virus: more than 50 years old and still providing surprises.* **Nature Reviews Cancer.** 2016. *16*(12), 789-802
58. Young LS, Yap FL, Murray PG. *Epstein–Barr virus: more than 50 years old and still providing surprises*. **Cancers**, 2016. 16: 789- 802.
59. Andersson, J. *An overview of Epstein-Barr virus: from discovery to future directions for treatment and prevention.* **Herpes: the journal of the IHMF**. 2000. *7*(3), 76-82.
60. Gequelin, L. C. F., Riediger, I. N., Nakatani, S. M. et al. *Epstein-Barr virus: general factors, virus-related diseases and measurement of viral load after transplant*. **Revista brasileira de hematologia e hemoterapia.** 2011. *33*(5), 383-388.
61. Kieff, E. R. A. B. *Epstein-Barr virus and its replication*. **Fields' virology.** 2001. 2575-2627.
62. Münz C. *Latency and lytic replication in Epstein-Barr virus-associated oncogenesis*. **Nat Rev Microbiol**. 2019 Nov;17(11):691-700.
63. Longnecker, R. M., Kieff, E., & Cohen, J. I. *Epstein-Barr virus.* **In Fields Virology: Sixth Edition.** 2013. Wolters Kluwer Health Adis (ESP).
64. Babcock, G. J., Hochberg, D., & Thorley-Lawson, D. A. *The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B-cell*. **Immunity**, 2000. *13*(4), 497-506.

65. Li, Z., Zhang, X., Dong, L., et al. *CryoEM structure of the tegumented capsid of Epstein-Barr virus.* **Cell research**,2020. *30*(10), 873-884.
66. Kenney, S. C., & Mertz, J. E. *Regulation of the latent-lytic switch in Epstein–Barr virus*. **In Seminars in cancer biology**. 2014, 26, 60-68
67. Tan GW, Visser L, Tan LP, van den Berg A, Diepstra A. *The Microenvironment in Epstein-Barr Virus-Associated Malignancies.* **Pathogens.** 2018 Apr 13;7(2):40.
68. Münz C, Moormann A. *Immune escape by Epstein-Barr virus associated malignancies*. **Semin Cancer Biol.** 2008 Dec;18(6):381-7.
69. Majzner RG, Simon JS. *Assessment of programmed death-ligand 1 expression and tumour-associated immune cells in pediatric cancer tissues*. **Cancer**. 2017;123:3807–3815.
70. Krappmann D., Emmerich F., Kordes U., et al. *Molecular mechanisms of constitutive NF-kB/Rel activation in Hodgkin*. **Oncogene**. 1999;18:943.
71. Chung G.T.Y., Lou W.P.K., Chow C., et al. *Constitutive activation of distinct NF-κB signals in EBV-associated nasopharyngeal carcinoma*. **J. Pathol.** 2013;231:311–322
72. Stein M., Keshav S., Harris N., Gordon S. *Interleukin 4 potently enhances murine macrophage mannose receptor activity: A marker of alternative immunologic macrophage activation*. J. **Exp. Med**. 1992;176:287–292.
73. Helming L, Winter J, Gordon S. *The scavenger receptor CD36 plays a role in cytokine-induced macrophage fusion.* **J Cell Sci**. 2009 Feb 15;122(Pt 4):453-9.
74. Wang D., Yang L., Yue D., Cao L., Li L., Wang D., Ping Y., Shen Z., Zheng Y., Wang L., et al. *Macrophage-derived CCL22 promotes an immunosuppressive tumor microenvironment via IL-8 in malignant pleural effusion.* **Cancer Lett.** 2019;452:244–253. doi: 10.1016/j.canlet.2019.03.040.
75. Elena Sierra-Filardi, Concha Nieto, Ángeles Domínguez-Soto, Rubén Barroso, Paloma Sánchez-Mateos, Amaya Puig-Kroger, María López-Bravo, Jorge Joven, Carlos Ardavín, José L. Rodríguez-Fernández, Carmen Sánchez-Torres, Mario Mellado, Ángel L. Corbí; *CCL2 Shapes Macrophage Polarization by GM-CSF and M-CSF: Identification of CCL2/CCR2-Dependent Gene Expression Profile*. ***J Immunol*** 15 April 2014; 192 (8): 3858–3867.
76. Ruytinx P, Proost P, Van Damme J and Struyf S (2018) *Chemokine-Induced Macrophage Polarization in Inflammatory Conditions.* **Front. Immunol**. 9:1930. doi: 10.3389/fimmu.2018.01930
77. Junttila IS, Mizukami K, Dickensheets H, Meier-Schellersheim M, Yamane H, Donnelly RP, Paul WE. *Tuning sensitivity to IL-4 and IL-13: differential expression of IL-4Ralpha, IL-13Ralpha1, and gammac regulates relative cytokine sensitivity*. **J Exp Med**. 2008 Oct 27;205(11):2595-608.
78. Jiang X, Zhou T, Xiao Y, Yu J, Dou S, Chen G, Wang R, Xiao H, Hou C, Wang W, Shi Q, Feng J, Ma Y, Shen B, Li Y, Han G. *Tim-3 promotes tumor-promoting M2 macrophage polarization by binding to STAT1 and suppressing the STAT1-miR-155 signaling axis***. Oncoimmunology**. 2016 Aug 3;5(9):e1211219.
79. Buechler C, Ritter M, OrsóE *et al*. *Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli*. **J. Leukoc**. *Biol.* 2000; 67; 97–103.
80. Upadhyay R, Hammerich L, Peng P, Brown B, Merad M, Brody JD. *Lymphoma: immune evasion strategies.* **Cancers (Basel).** 2015 Apr 30;7(2):736-62.
81. Andorsky, D.J.; Yamada, R.E.; Said, J.; Pinkus, G.S.; Betting, D.J.; Timmerman, J.M. *Programmed death ligand 1 is expressed by non-hodgkin lymphomas and inhibits the activity of tumor associated T cells.* **Clin. Cancer Res.** 2011, 17, 4232–4244.
82. Jiang Y, Li Y. *T-cellexhaustion in the tumourmicroenviroment*. **Cell Death** Dis. 2015;6:1–9.

83. Butte MJ, Keir ME. *Programmed death-1 ligand 1 interacts specifically with the B7–1 costimulatory molecule to inhibit T cell responses.* **Immunity.** 2007;**27**(1):111–122. doi: 10.1016/j.immuni.2007.05.016.
84. Rollins MR, Gibbons Johnson RM. *CD80 expressed by CD8(+) T cells contributes to PD-L1-induced apoptosis of activated CD8(+) T cells.* **J Immunol Res**. 2017;2017:1–6
85. Probst, H. C., McCoy, K., Okazaki, T., Honjo, T. & van den Broek, *M. Resting dendritic cells induce peripheral CD8^{+} T cell tolerance through PD-1 and CTLA-4.* **Nature Immunol**. **6**, 280–286 (2005)
86. Wang, Y., Du, J., Gao, Z. *et al. Evolving landscape of PD-L2: bring new light to checkpoint immunotherapy.* **Br J Cancer** (2022).
87. Günthner R, Anders HJ. *Interferon-regulatory factors determine macrophage phenotype polarization*. **Mediators Inflamm.** 2013;2013:731023.
88. Yanai H, Chiba S, Hangai S, Kometani K, Inoue A, Kimura Y, Abe T, Kiyonari H, Nishio J, Taguchi-Atarashi N, Mizushima Y, Negishi H, Grosschedl R, Taniguchi T. *Revisiting the role of IRF3 in inflammation and immunity by conditional and specifically targeted gene ablation in mice.* **Proc Natl Acad** Sci U S A. 2018 May 15;115(20):5253-5258.
89. Martinez FO, Gordon S. *The M1 and M2 paradigm of macrophage activation: time for reassessment***.** *Prime Rep*. 2014 Mar 3;6:13.
90. Gorvel L, Olive D. Targeting the *"PVR-TIGIT axis" with immune checkpoint therapies. Res*. 2020 May 13;9:F1000 Faculty Rev-354.
91. Harjunpää H, Guillerey C. *TIGIT as an emerging immune checkpoint*. **Clin Exp Immunol.** 2020 May;200(2):108-119.
92. . Varano G, Raffel S, Sormani M, Zanardi F, et al. *The B-cell receptor controls fitness of MYC-driven lymphoma cells via GSK3β inhibition.* **Nature.** 2017 Jun 8;546(7657):302-306.