

# Phenotypes of B Cells Differ in EBV-positive Burkitt's lymphoma Derived Cell Lines

Irina Spaka, Rita Birkenfelde, Svetlana Kozireva, Jevgenija Osmjana, Madara Upmane, Elena Kashuba, and Irina Kholodnyuk Holodnuka

**Abstract**—Epstein-Barr virus (EBV) is implicated in the pathogenesis of the endemic Burkitt's lymphoma (BL). The EBV-positive BL-derived cell lines initially maintain the original tumor phenotype of EBV infection (latency I, LatI), but most of them drift toward a lymphoblast phenotype of EBV latency III (LatIII) during *in vitro* culturing. The aim of the present work was to characterize the B-cell subsets in EBV-positive BL cell lines and to verify whether a particular cell subset correlates with the type of EBV infection. The phenotype analysis of two EBV-negative and eleven EBV-positive (three of LatI and eight of LatIII) BL cell lines was performed by polychromatic flow cytometry, based on expression pattern of CD19, CD10, CD38, CD27, and CD5 markers. Two cell subsets, CD19<sup>+</sup>CD10<sup>+</sup> and CD19<sup>+</sup>CD10<sup>-</sup>, were defined in LatIII BL cell lines. In both subsets, the CD27 and CD5 cell surface expression was detected in a proportion of the cells.

**Keywords**—B-cell subsets, Burkitt's lymphoma cell lines, EBV latency, phenotype profiles.

## I. INTRODUCTION

**B**URKITT'S lymphoma (BL) is an aggressive B-cell lymphoma, characterized by a high degree of proliferation of malignant cells and deregulation of the c-MYC gene. BL can be classified into three forms which differ in geographic distribution and Epstein-Barr virus (EBV)

association: endemic (eBL), sporadic (sBL) and HIV-associated BL. There is a low background incidence of BL worldwide (sBL), which is rarely associated with EBV and accounts for 1–2% of adult lymphoma in Western Europe and America, but eBL is associated with EBV in over 95% of cases and is predominant in the equatorial Africa and other parts of the world where malaria is hyperendemic. Endemic EBV-associated BL has an incidence of 5–10/100 000 children and accounts for up to 74% of childhood malignancies in the equatorial African [1]–[3]. Detection of somatic mutations in the rearranged immunoglobulin variable-region genes suggested that both sporadic and endemic BL represent a B-cell malignancy originating from germinal center (GC) B cells or their descendants [4], [5]. BL tumor cells usually express IgM, B-cell markers such as CD19, CD20 and CD22, and markers of GC centroblasts, such as CD10, BCL6 and the GC-expressed protein GCSAM [2].

EBV is implicated in the pathogenesis of the endemic BL and posttransplant lymphomas in immunosuppressed individuals [6], [7]. The EBV-carrying posttransplant and AIDS-associated lymphoma cells express the full set of EBV latent proteins (the latency III form of infection, LatIII): six EBV nuclear antigens (EBNA 1–6) and three latent membrane proteins (LMP1, 2A, and 2B). The vast majority of EBV-positive (EBV+) BL tumors display a restricted latency I (LatI) express only EBNA-1) form of infection. EBV has the unique ability to transform normal resting B-lymphocytes *in vitro* into permanent, latently infected lymphoblastoid cells, in which every cell constitutively expresses viral latent proteins, six EBNAs and three LMPs (Lat III) [6], [8].

The EBV infection leads to B cell activation, inducing the interferon pathway, cell-surface adhesion molecules and receptors (such as CD23, CD40, and CD38), and chemokines (CXCL8 / IL8, CXCL10 / IP10, CCL5 / RANTES, CCL17 / TARC, and CCL22 / MDC) [9]. The upregulation of CCR6 and CCR10 was observed in B cells immortalized with EBV *in vitro* [10]; the increase of mRNA expression for CCR2 and CCR9 was detected in tonsil B cells upon EBV infection [11]. One proposed model suggests that the infected B cell, in the establishment of EBV latency *in vivo*, follows the same path as the antigen-activated B cell, which proliferates, enters the follicle and expands to form a germinal centre. In the peripheral blood (PB) EBV persists in memory B-cell population [12].

BL-derived cell lines are commonly used as model systems to study the biology of cancer [8]. It is known that EBV+ BL

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cell lines initially maintain the original tumor phenotype of EBV LatI, but most of them drift toward a lymphoblastoid phenotype during *in vitro* culturing and express all nine EBV latent proteins (LatIII) [13], [14].

The aim of the present work was to characterize by polychromatic flow cytometry (pFC) the phenotypes of cells in EBV-positive BL cell lines and to verify whether a particular cell subset correlates with the type of EBV infection (LatI and LatIII).

## II. METHODS

### A. Cell Lines Analysis by Polychromatic Flow Cytometry

The original characteristics of the BL cell lines that were analyzed in this study, have been described previously [15], [16]. Cells of BL lines were immunofluorescently stained for cell surface markers using the standard protocol "Cell Surface Staining of Human PBMCs and Suspension Cell Lines" (BD Biosciences, San Jose CA USA). Fluorochrome-conjugated mouse anti-human monoclonal antibodies (mAbs) CD19-PE-Cy5 (clone HIB19), CD10-PE (clone HI10a), CD38-Horizon V450 (clone HB8), CD27-FITC (clone M-T271), and CD5-PE-Cy7 (clone L17F12), and nonspecific mouse IgG1 and IgG2b, matching the respective fluorochrome and Ig isotype of CD19, CD10, CD38, CD27, and CD5, were purchased from BD Biosciences. The Annexin V-APC (BD Biosciences) stained cells (apoptotic cells) were excluded from each analysis by gating on viable cells (APC-negative). Autofluorescent cells were excluded from each analysis in 2 ways: using unstained cells and using an empty fluorescence channel in each staining experiment. Five-color polychromatic flow cytometry (pFC) was performed, cells were scored using a FACSARIAII analyzer equipped with 3 lasers (BD; Becton, Dickinson and Company, NJ, USA), and data were processed using Diva6.2 software (BD).

Negative controls included substitution of the relevant mAb with a murine Ig of the same isotype and showed no significant positivity. In the fluorescence-activated cell analysis, 3000-9000 events were acquired. The threshold line was based on the staining obtained with irrelevant isotype-matched mAb; the cell line was defined as negative if <3% of cells stained positive with mAbs.

### B. RT-PCR and PCR Analyses

Reverse transcription (RT)-duplex-PCR analysis was carried out as reported by us previously; duplex-PCR was performed using two pairs of primers simultaneously (30 cycles, 55°C of the annealing) with the one pair of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) [17]. The primers used to define the EBV EBNA1 transcripts derived from Qp and from Wp/Cp promoters were 5'-gtgcgctaccggatggcg-3' (Q), 5'-cgtgtgacgtggtgtaaagt-3' (Y), and 5'-ggctccggacaccatctct-3' (K1) [16]. The EBNA2 primers that recognize the EBV type-1 sequences and the LMP1 primers are published by [18].

## III. RESULTS

We analyzed B cell phenotypes in 13 BL cell lines, two EBV negative and eleven EBV positive (three of LatI and eight of LatIII) by pFC. These BL cell lines are well characterized for the EBV latency form of infection. Seven BL cell lines in the study were established by a single cell cloning of the original EBV+ BL-tumor derived cell lines: Oma cl.4 (EBV-) and Oma cl.6 (LatI); Mutu cl.30 (EBV-), Mutu cl.148 (LatI) and Mutu cl.99 (LatIII); Jijoye M13 (LatI/III) and Jijoye P79 (LatIII) [15]. We confirmed the presence of EBV and the latency type in all cell lines, using RT-duplex-PCR of EBV genes, EBNA1 (derived from Qp and from Wp/Cp promoters) and LMP1. The results are shown on Fig. 1.

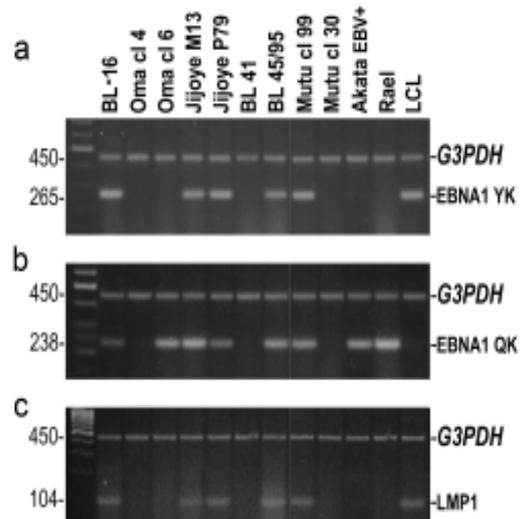


Fig. 1 RT-duplex-PCR analysis of EBV latent genes transcripts in Burkitt's lymphoma cell lines: (a,b,c) the EBNA1 and LMP1 RT-PCR products in the representative cell lines. The EBNA1 transcripts derived from Qp and from Wp/Cp promoters, respectively, are referred to as QK and YK splices. G3PDH, the PCR product of the glyceraldehyde-3-phosphate dehydrogenase gene; LCL, the LatIII LCL cell lines; BL41 and Rael, the LatI BL cell lines used as the controls. Duplex-PCR was conducted using 2 pairs of primers simultaneously. Representative data from 2 independent experiments are shown

For a phenotype profiling of BL cell lines, we analyzed the cell surface expression of CD19, CD10, CD38, and CD27; CD5 was assayed in the LatIII BL cell lines. The results are presented in Fig. 2. In EBV- and LatI BL cell lines, all cells showed the CD19+CD10+CD38+ phenotype. Only a small fraction of the CD19+CD10- cells (about 4%) was found in EBV- cell line Oma cl.4. In contrast, more than 90% of the cells were CD19+CD10-CD38+ in three LatIII cell lines: Mutu cl.99 and Jijoye P79 that were established by a single cell cloning, and BL41/95 that was generated by the EBV (B95-8) infection of EBV- BL41 cells (Fig. 2). The rest of the cells were CD19+CD10+CD38+. In all BL cell lines, the CD19+CD10+ cells expressed CD38.

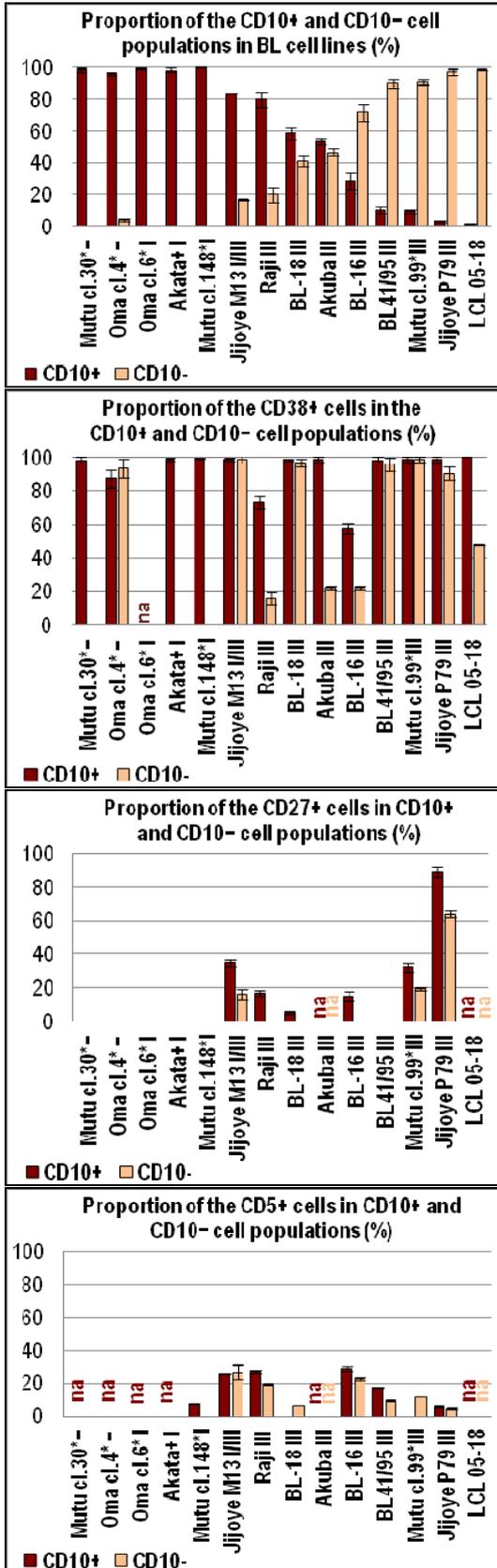


Fig. 2 B cell phenotype profiles in Burkitt's lymphoma cell lines. Bars show percentage of positive cells. The CD38, CD27, and CD5 stained cells were defined in 2 cell subsets, CD19+CD10+ (dark brown bars) and CD19+CD10- (beige bars) for each BL cell line. \*, the EBV+ and EBV- cell lines were established by a single cell cloning of the original EBV+ BL-derived cell lines; Mutu cl.30- and Oma cl.4-, the EBV-negative BL cell lines; BL41/95 was generated by infection of the EBV- BL41 cell line cells with B95-8 EBV; Jijoye M13 I/III, BL cell line of EBV LatI originally that acquired the phenotype of EBV LatIII during the in vitro culturing: the mRNA expression of EBV latent genes LMP1 and EBNA1 Qp was detected (Fig. 1); LCL 05-18, the LCL cell line generated by B95-8 EBV infection of B cells from PB of EBV- healthy adult donors; na, not analyzed. The percentage of cells expressing the respective B-cell surface CD marker is averaged over both experiments with mean  $\pm$  SD shown. Percentage of positive cells based on quadrant encompassing 3% of isotype control-stained cells in dot blots ( $0 < 3\%$  of cells). Data are representative of two independent experiments with 2 tubes per experiment

The original BL-tumor derived cell lines of LatIII, i.e. Jijoye M13, Raji, BL18, Akuba, and BL16, consisted of the CD19+CD10+ and CD19+CD10- cell subsets in proportion of 84%-28% and 17%-72% of the cells, respectively (Fig. 2). The CD19+CD10- cells expressed the cell surface CD38 in 6 out of the 9 BL cell lines that contained the CD19+CD10- cell subset.

Of note is that CD27, the marker of B cell maturation, was expressed on the CD19+ CD10+ cells in six LatIII BL cell lines but not in EBV- and LatI cell lines. Moreover, in three LatIII BL cell lines, two with the highest proportion of the CD19+ CD10- cells (Mutu cl.99 and Jijoye P79) and in Jijoye M13, the CD27 cell surface expression was detected in 20%, 64%, and 16% of the CD19+CD10- cells, respectively (Fig. 2).

In conclusion, the phenotype analysis of cells in EBV-negative and EBV-positive (both of LatI and LatIII) Burkitt's lymphoma derived cell lines defined two cell subsets, CD19+CD10+ and CD19+CD10-, in LatIII cell lines; in both subsets, the CD27 and CD5 cell surface expression was detected in a proportion of the cells.

IV. DISCUSSION

Epstein-Barr virus (EBV), a ubiquitous B-lymphotropic human gamma-herpesvirus, infects nearly all human populations. Moreover, a great majority of adults produce antibodies to the virus. EBV in a form of episome persists in peripheral blood memory B cells [6], [8]. It was demonstrated that EBV can establish infection even in the absence of a classical GC memory B-cell population, in the small proportion of GC-independent memory B cells (IgD+, CD27+) [19]. Naïve and memory B cells are distinguished by the absence and presence of CD27 expression, respectively: most IgA+ and IgG+ B-cells co-express CD27, confirming their memory cell status [20].

Recently it was shown that CD19, CD10, CD27, and CD38 is the minimal combination of subsetting markers allowing

unequivocal identification of immature (CD10+ CD38+ CD27-), naïve (CD10- CD38-/+ CD27-), and memory B lymphocytes (CD10- CD38-/+ CD27+), and plasma cells (CD10- CD38++ CD27++) within PB circulating CD19+ cells. A population-based cohort of 600 healthy adults aged from 20 to 80 years [21] and 106 healthy adults [22] were analyzed, using pFC, in these comprehensive studies. In our experiments we have also assessed expression of these CD markers to characterize the immunophenotypes of BL cell lines.

The phenotype analysis of cells in EBV-negative and EBV-positive (both of latencies I and III) Burkitt lymphoma derived cell lines revealed that a proportion of the cells in LatIII BL cell lines display the phenotype of mature B cells (CD19+ CD10- CD38+ CD27+ CD5-). This observation may suggest that expression of the EBV LatIII genes may promote the differentiation of malignant cells.

#### APPENDIX

Authors' contributions: I. Kholodnyuk Holodnuka and E. Kashuba have made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data. I. Kholodnyuk Holodnuka, S. Kozireva, and E. Kashuba have been involved in drafting the manuscript and revising it critically for important intellectual content. I. Kholodnyuk Holodnuka, S. Kozireva, and J. Osmjana carried out in vitro studies with cell culture. S. Kozireva, R. Birkenfelde, and M. Upmane carried out RT and PCR analyses. I. Kholodnyuk Holodnuka and I. Spaka carried out polychromatic flow cytometry analysis of cell lines. All authors read and approved the final manuscript.

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