CD19 signalling improves the Epstein–Barr virus-induced immortalization of human B cell

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Abstract. Epstein–Barr virus (EBV) infection *in vitro* immortalizes primary B cells and generates B lymphoblastoid cell lines (LCLs). These EBV-LCLs have been used for several purposes in immunological and genetic studies, but some trials involving these transformations fail for unknown reasons, and several EBV-LCLs do not grow in normal culture. In this study, we improved the immortalization method by CD19 and B-cell receptor (BCR) co-ligation. This method shortens the time required for the immortalization and generation of EBV-LCLs but does not alter the cell phenotype of the LCLs nor the expression of the EBV genes. In particular, the CD19 and BCR co-ligation method was found to be the most effective method examined. EBV-infected B cells induced by CD19 and/or BCR ligation expressed the intracellular latent membrane protein LMP-1 earlier than EBV-infected B cells, and the expression of intracellular LMP-1 was found to be closely related to the time of immortalization. These results suggest that the modified method, using CD19 and/or BCR ligation, may efficiently generate EBV-LCLs, by expressing intracellular LMP-1 at an early stage.

INTRODUCTION

Epstein–Barr virus (EBV) is routinely used in the laboratory setting to generate B lymphoblastoid cell lines (LCLs) (Neitzel 1986). EBV-LCLs have served a variety of purposes in immunological and genetic studies. Although several methods have been developed for the generation of B LCLs by EBV (Werner *et al.* 1972; Schneider & zur Hausen 1975; Tohda *et al.* 1978; Neitzel 1986), these methods are protracted. Recently, a modified method that reduces the duration time required to generate LCLs was introduced (Oh *et al.* 2003). In the banking of cells for genome research, reducing the generation time is a significant concern. In addition, some EBV-LCLs grow too slowly to expand and some trials fail for unknown reasons. Thus researchers working on genomes need an efficient solution.

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EBV-LCLs express some nuclear proteins [Epstein–Barr nuclear antigen (EBNA)-1, -2, -3A, -3B, -3C, and -LP] and some integral latent membrane proteins (LMP-1, LMP-2A, and LMP-2B). Among these, EBNA-1, EBNA-2 and LMP-1 seem to be required constitutively for immortalization of B cells by EBV (Bornkamm & Hammerschmidt 2001). Therefore, we studied the expression of these molecules for the generation of B LCLs by EBV.

CD19 is a 95-kDa transmembrane protein bearing two extracellular immunoglobulin domains and an extensive cytoplasmic tail (Stamenkovic & Seed 1988). In mature B cells, CD19 is found in a complex with CD21 (complement receptor, receptor for EBV). Moreover, CD19 associates with members of the B-cell receptor (BCR) complex and is rapidly phosphorylated following BCR cross-linking (Matsumoto *et al.* 1991; Bradbury *et al.* 1992). However, the CD19–CD21 and –BCR complexes can function and be regulated independently (Tedder *et al.* 2002). CD19 co-ligation with surface immunoglobulin greatly augments B-cell activation (Carter & Fearon 1992). Thus, we hypothesized that CD19 and/or BCR ligation might influence the EBV transformation of B cells; however, CD19 is used to positively select B cells for EBV infection.

In this study, we observed that B-cell pre-activation by CD19 and/or BCR ligation shortened the time required for immortalization through the expression of intracellular LMP-1 earlier than 2 weeks. These results suggest that the method used in this study may provide an effective method for generating a B LCL using EBV.

MATERIALS AND METHODS

EBV infectious culture supernatant preparation

EBV stock was prepared from an EBV-transformed B95-8 marmoset cell line. These cells were grown in RPMI-1640 medium (Gibco/BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (BioWhittaker, Walkerville, MD, USA) and infectious culture supernatants were harvested and stored at -80°C until needed.

B-cell purification and generation of EBV-transformed LCLs

Ten volunteers participated in this study, and informed consent was obtained from all participants. Sixty millilitres of peripheral blood were collected in a syringe containing heparin. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll–Hypaque gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). B cells were purified ($>95\%$ CD20⁺) using a B-cell isolation kit and MACS separators (Miltyeni Biotec, Bergisch Gladbach, Germany). Purified B cells were stimulated with $10\text{ }\mu\text{g/mL}$ mouse anti-human CD19 (BD Pharmingen, San Diego, CA, USA) and/or $40\text{ }\mu\text{g/mL}$ rabbit anti-human immunoglobulin M (IgM) F(ab')₂ monoclonal antibody (mAb; DakoCytomation, DK-2600 Glostrup, Denmark), and added to B95-8 supernatant in a culture flask (Neitzel 1986). Following 2-h incubation at 37°C , the same volume of complete medium, and $0.5\text{ }\mu\text{g/mL}$ cyclosporin A (Pelloquin *et al.* 1986) was added (1×10^6 cell/mL). The cultures were incubated for 2–4 weeks until clumps of EBV-infected B cells were visible and the medium had turned yellow.

Flow cytometric analysis for immunophenotyping and the expression of intracellular LMP-1

Resting B cells and EBV-transformed LCLs were tested for cell surface antigen expression by immunofluorescence and flow cytometric analysis. As required, resting B cells incubated with

purified mouse IgG anti-human CD19 mAb (BD Pharmingen) and/or purified rabbit anti-human IgM F(ab')₂ mAb (DakoCytomation). Fluorescein isothiocyanate or phycoerythrin (PE)-conjugated mouse mAb to human CD20, human IgD, human CD38, human CD21, human CD23, human CD54, human CD30, human CD69, human CD71, human CD77 and human CD95 were purchased from BD-Pharmingen. For single-colour or two-colour immunofluorescence analysis, cells (5×10^5 to 1×10^6) were stained at 4 °C as described below. Briefly, cells (5×10^5 to 1×10^6) were pelleted in 1.5 mL tubes and washed twice in cold washing buffer (phosphate-buffered saline containing 0.5% fetal bovine serum and 0.03% sodium azide). Cells were re-suspended in 80 µL fluorescence-activated cell sorter (FACS) buffer, 10 µL antibody was added, and cells were incubated for 30 min at 4 °C, washed twice in cold washing buffer and fixed in 4% formaldehyde. All fixed cells were analysed on a FACS Vantage SE flow cytometer (BD Pharmingen).

An LMP-1 PE-conjugated antibody set containing mouse IgG2A isotype control was used to analyse intracellular LMP-1. At first, cells were washed in FACS buffer, and then cell pellets were re-suspended in permeabilization buffer (phosphate-buffered saline containing 0.1% saponin and 0.05% sodium azide) and incubated for 10 min at room temperature in the dark. Cells were then washed twice in FACS buffer and PE-conjugated mouse anti-human LMP-1 was added, and the mixture was incubated for 30 min at room temperature in the dark. The cells were then re-suspended in 500 µL of 4% formaldehyde and incubated for 30 min. All fixed cells were analysed on a FACS Vantage SE flow cytometer (BD Pharmingen).

Reverse transcription-polymerase chain reaction (RT-PCR) for EBNA-1, EBNA-2, and LMP-1 and the image analysis of the PCR products

One microgram of total RNA from EBV-transformed B cells was reverse transcribed in a 25-µL reaction mixture containing 5× reverse transcriptase buffer, 25 mM dNTPs, random hexamers (0.5 µg/µL), 20 units RNase inhibitor and 200 units Moloney-murine leukaemia virus reverse transcriptase at 37 °C for 1 h. This was followed by incubation for 5 min at 70 °C. PCR was performed in a 50 µL volume using 2 µL of the reverse transcription reaction plus 1 unit *Taq* polymerase, 10× buffer, 0.2 mM dNTPs, 2 mM MgCl₂ and 0.2 µM of each primer using the following conditions; 95 °C for 2 min, then 34 cycles of 95 °C for 20 s, 58 °C for 40 s (EBNA-2) or 60 °C for 40 s (EBNA-1 and LMP-1), and 68 °C for 2 min; followed by 10 min at 68 °C. The sequence-specific primers used for EBNA-1, EBNA-2 and LMP-1 were as follows: EBNA-1, forward primer 5'-GAG CGG GGA GAT AAT GTA CA-3' and backward primer 5'-TAA AAG ATG GCC GGA CAA GG-3' (217-base-pair product); EBNA-2, forward primer 5'-AAC CCT CTA AGA CTC AAG GC-3' and backward primer 5'-ACT TTC GTC TAA GTC TGC GG-3' (377-base-pair product); LMP-1, forward primer 5'-GGA TCC ATG GAA CAC GAC CTT GAG AG-3' and backward primer 5'-GAA TTC TTA GTC ATA GTA GCT TAG CT-3' (1149-base-pair product). β-actin was used as a control. Aliquots of the PCR reactions were loaded into a 1.2% agarose gel containing ethidium bromide, electrophoresed, visualized, and quantified by densitometry using an image analyser (IMAGE-PRO PLUS version 3.0).

RESULTS

CD19 ligation increased expression of EBNA-1 and EBNA-2 genes in EBV-transformed B cells at an early stage

To examine the EBV transformation of B cells by CD19 ligation, B cells were selected negatively using a B-cell isolation kit (Miltenyi Biotec, GmbH). CD19 co-ligation with surface

Figure 1. Image analysis results of RT-PCR for EBNA-1 and EBNA-2 mRNA 30 min after EBV infection. Both CD19 ligation and BCR ligation increased the expression of EBNA-1 and EBNA-2 mRNA levels 30 min after EBV infection. Co-ligation with two molecules gave a simple additive pattern.

immunoglobulin is known to greatly augment B-cell activation (Carter *et al.* 1992), so we tested the effect of the CD19 and BCR co-ligation signalling with RT-PCR and image analysis. CD19 ligation and BCR ligation increased the expression of EBNA-1 and EBNA-2 mRNA at 30 min after EBV infection, and CD19 and BCR co-ligation showed an additive effect (Fig. 1). Differences in EBNA-1 and EBNA-2 mRNA expressions were most prominent 30 min after EBV infection, but from the second day after EBV infection no significant differences were found between EBV-infected B cells with CD19 and/or BCR ligation and EBV-infected B cells without other pretreatments (data not shown). These results indicate that CD19 and BCR ligation enhanced the efficiency of EBV infection in B cells.

CD19 and BCR co-ligation synergistically shortens the time from culture initiation to immortalization

Generally the EBV transformation process is established at almost 4 weeks and the early signs of transformation (cell aggregation, increased cell size and a sudden increase in growth) are observed after 1–3 weeks (Tohda *et al.* 1978; Neitzel 1986). EBV-infected B cells induced by CD19 ligation or BCR ligation aggregated at least within 14 days and showed a sudden increase in growth at the same time. In addition, B cells induced by CD19 and BCR co-ligation aggregated more rapidly (within 10 days) and produced larger aggregates than other B cells (Fig. 2). There was also a distinctive difference in the number of B-cell clumps. EBV-infected B cells induced by CD19 ligation increased more than three times faster than non-CD19-ligated, EBV-infected B cells, as determined from the number of clumps, whereas EBV-infected B cells induced by BCR ligation showed only a 1.5-fold increase. However, EBV-infected B cells induced by CD19 and BCR co-ligation showed more than a 6-fold increase in terms of clump numbers, which showed a synergistic increase pattern (Fig. 3).

Figure 2. Visualization of cell aggregation of EBV-infected B cells on the 14th day. EBV-infected B cells without other treatment did not show massive cell aggregation at this time (a), but EBV-infected B cells induced by CD19 ligation (b) or BCR ligation (c) aggregated markedly. In addition, co-ligation with CD19 and BCR increased substantially and rapidly the sizes and numbers of cell clumps (d). Note these are cell clumps of proliferative lymphoblast cells, indicating successful transformation.

Figure 3. Measurement of cell clumps of EBV-infected B cells on the 14th day. Number of clumps were equivalent to those of Fig. 2. EBV-infected B cells induced by CD19 ligation showed more than three or four times as many clumps as control EBV-infected B cells. BCR ligation increased only 2-fold the numbers of clumps versus those of the EBV-infected control. EBV-infected B cells induced by CD19 and BCR co-ligation showed a synergistic increase.

Figure 4. FACS analyses of IgD and CD38 (widely used to identify the B-cell phenotype) on EBV-infected B cells at different times. Purified resting B cells were tested at the baseline for the B-cell phenotype (a). After EBV transformation, resting B cells ($\text{IgD}^+ \text{CD38}^-$) changed to $\text{IgD}^- \text{CD38}^+$ cells (germinal centre B-cell phenotype) (b–e). EBV-infected B cells induced by CD19 and BCR co-ligation became $\text{IgD}^- \text{CD38}^+$ cells on the 14th day (b), EBV-infected B cells induced by BCR ligation (40 $\mu\text{g/mL}$) showed the $\text{IgD}^- \text{CD38}^+$ cell phenotype on the 21st day (c), and cells induced by CD19 ligation (10 $\mu\text{g/mL}$) changed on the 25th day (d). Only EBV-infected B cells showed this germinal centre B-cell phenotype on the 35th day (e).

IgD and CD38 cell surface molecules have been widely used for B-cell phenotyping in association with B-cell development (Pascual *et al.* 1994; Hur *et al.* 2000), and EBV-transformed B cells were found to be mainly positive for only CD38 (like germinal centre B cells). EBV-infected B cells were tested for the expression of CD38 and IgD when the cells showed signs of transformation (cell aggregation, increased cell size and a sudden increase in growth). EBV-infected B cells induced by CD19 and BCR co-ligation became $\text{IgD}^- \text{CD38}^+$ cells earliest (2.5 weeks), B cells induced by BCR ligation became $\text{IgD}^- \text{CD38}^+$ second, B cells induced by CD19 ligation came third, and the EBV-only infected B cells became $\text{IgD}^- \text{CD38}^+$ last (after 4 or 5 weeks) (Fig. 4). This result shows that CD19 and BCR ligation enhanced the surface expression of CD38 in EBV-infected B cells.

EBV-infected B cells were characterized for 10 cell surface markers (including IgD and CD38) by FACS analysis. The markers chosen have been previously used for EBV transformation cell surface phenotyping (Wroblewski *et al.* 2002; Middeldorp *et al.* 2003). Results of the FACS analysis obtained using the 10 cell surface markers are presented in Table 1. After completion of the EBV transformation process (at 5 weeks), group B cells had similar cell phenotypes. We also observed the expressions of EBV genes in association with immortalization (EBNA-1, EBNA-2, LMP-1) at the time when the cells were analysed for surface markers. Of

Table 1. Immunophenotyping of EBV-infected B cells (percentage of cells expressing cell surface markers)

	Resting B cells	No ligation	CD19 ligation	BCR ligation	CD19 and BCR co-ligation
CD21	90.50	63.2	66	54.3	52.7
CD23	41	73.9	78.2	60	51
CD30	< 0.2	66.1	64	57	45.3
CD54	43	98.1	99.43	96.7	97.6
CD69	< 0.2	28.6	36.3	33.7	26
CD71	43	98.1	99.43	96.7	97.6
CD77	< 1	28.7	25.1	18.5	29.2
CD95	24	98.9	99.5	96.9	98.9
IgD	64.3	17.1	12.9	14.8	16.3
CD38	< 10	94.4	88.5	93.4	97.1

The results of cell surface marker immunophenotyping by FACS analyses. After EBV infection, cells induced by CD19 and BCR co-ligation were tested on the 14th day, cells induced by BCR ligation were stained on the 21st day, and those induced by CD19 ligation were stained on the 25th day. EBV-infected B cells were tested on the 35th day. No apparent difference was observed in the expressions of the cell surface markers of the EBV-transformed B cells.

Figure 5. RT-PCR results for the expression of the EBV genes, EBNA-1, EBNA-2 and LMP-1, associated with immortalization at 14 days after EBV infection. An EBV-transformed lymphoblastoid cell line was used as a positive control. All EBV-infected B cells with/without CD19 (10 µg/mL) and/or BCR ligation (40 µg/mL) expressed EBV genes highly, to the same extent as the EBV-transformed cell line.

the EBV genes, EBNA-1, EBNA-2 and LMP-1 are critical B-cell transformation factors (Cohen *et al.* 1989; Young *et al.* 2000). No significant differences were observed in the expressions of EBV genes between EBV-infected B cells induced by CD19 and/or BCR ligation and EBV-infected B cells without other pretreatments (Fig. 5).

Figure 6. Intracellular FACS analysis for LMP-1 on EBV-infected B cells on the 14th day. Cells were harvested (10^6 cells/200 μ L) and then stained with an LMP-1 staining set, as described in the Materials and Methods section. The isotype control profiles depicted by the thin line were generated from cells stained with mouse isotype PE control IgG₂ and samples stained with LMP-1 PE are depicted by a solid line. Intracellular LMP-1 was mildly expressed on EBV-infected B cells without other treatments (a), whereas LMP-1 was expressed markedly on EBV-infected B cells induced by CD19 (10 μ g/mL) and BCR co-ligation (40 μ g/mL) (d). EBV-infected B cells induced by CD19 (b) or by BCR ligation (c) expressed LMP-1 moderately.

CD19 and BCR ligation enhanced the expression of LMP-1 at the intracellular level at an earlier stage

Two weeks after EBV infection, EBV-infected B cells induced by CD19 and/or BCR ligation were phenotypically different from EBV-infected B cells in terms of cell morphology (Fig. 2). Expression of EBV genes however, including LMP-1, showed no significant difference between the EBV-infected B-cell groups (Fig. 5). Variations in cell morphology (homotypic aggregation) are related to the expression of intracellular adhesion molecule-1 (ICAM-1; Rothlein & Springer 1986). LMP-1 up-regulates ICAM-1 (Young *et al.* 2000), and we observed the expression of LMP-1 at the intracellular level by FACS. Two weeks after EBV infection, we compared the expression of intracellular LMP-1 in the EBV-infected B cells. CD19 or BCR ligation significantly up-regulated intracellular LMP-1 (more than three times as much as EBV-infected B cells without other treatments), but the difference between CD19 and BCR ligation was not significant (Fig. 6).

These results are in accord with previous results in terms of the shortened time to immortalization, and suggest that CD19 and BCR ligation enhance the expression of intracellular LMP-1 protein at an earlier stage.

DISCUSSION

Initially, we observed B cells sorted in one of two ways. One group of B cells was sorted positively using CD19 microbeads (Miltenyi Biotec), and the other was sorted negatively using a B-cell isolation kit. After EBV infection, expression of the EBV gene and the immortalization process were somewhat different between the positively and negatively selected B cells (data not shown). These results suggested that CD19 signalling influenced the EBV transformation process; thereafter, all B cells studied were selected negatively using the B-cell isolation kit. This kit contained Fc receptor-blocking reagents, and therefore, we ruled out the effects of the Fc receptor on B cells (effects of the Fc receptor are mainly associated with the inactivation of B cells). CD19 and BCR co-ligation dramatically lowered the threshold for B-cell activation *in vitro* (Cater & Fearon 1992; Dempsey *et al.* 1996). Thus, we observed the effects of CD19 co-ligation with BCR. For BCR ligation (cross-linking), we used anti-IgM F(ab')₂ antibody without secondary antibody.

CD19 ligation increased the expression of EBNA-1 and EBNA-2 mRNA in EBV-transformed B cells at an early stage, which is in accord with that observed in the positively selected B cells. Moreover, BCR ligation also increased the expression of EBNA-1 and EBNA-2 mRNA at 30 min after EBV infection, whereas CD19 and BCR co-ligation showed a simple additive effect (Fig. 1). From the second day after EBV infection however, there were no significant differences between EBV-infected B cells induced by CD19 and/or BCR ligation and EBV-infected B cells without other treatment (data not shown). This result suggests that the effect of ligation could be a temporary triggering event in EBV infection.

Both EBNA-1 and EBNA-2 seem to be constitutively required for B-cell immortalization by EBV (Bornkamm & Hammerschmidt 2001). But LMP-1 is a direct target gene of EBNA-2, so we thought that EBNA-2 might be a more important factor than EBNA-1.

CD19 is a B-cell surface molecule that functions as a general response regulator, and the cytoplasmic domain of CD19 is critical for normal B-cell signalling (Bradbury *et al.* 1992; Sato *et al.* 1997). In addition, CD19 makes a complex with the CD21 molecule, a receptor for EBV. However, in many studies, including EBV infection protocols, CD19 has been used as a selection molecule for the purification of B cells. Recent studies suggest that CD19/21 may be required for sustained signalling by BCR (Engel *et al.* 1995; Fujimoto *et al.* 1999). This result suggests that CD19 ligation, including in a positive selection process, may influence EBV infection.

CD19 and BCR co-ligation synergistically lowers the threshold for B-cell activation and proliferation (Cater & Fearon 1992; Dempsey *et al.* 1996). CD19 and BCR co-ligation showed an additive effect in only Fig. 1. Other results, however, indicate that CD19 and BCR co-ligation produces synergistic effects (Figs 2 and 3). CD19–CD21 and BCR complexes can be regulated independently up-stream of Src PTK, but share overlapping pathways down-stream (Tedder *et al.* 2002). The properties of signalling pathways may account for the unexpected result shown in Fig. 1.

CD19 and BCR ligation shorten the time taken from the initiation of culture to immortalization, and in this respect co-ligation showed a synergic pattern. In addition, a distinctive difference was observed in the number of clumps of cells counted (Fig. 3). In the banking of many numbers of cells for genome research, reducing the generation time is of significant concern. Recently EBV stocks prepared from TPA-activated B95-8 cells have shortened the time between initiation and immortalization, and have increased the success rate of immortalization (Oh *et al.* 2003). Our results on time-reducing effects were equivalent to those of previous studies. Although the method of Oh's group is very simple and concerns pre-activated B95-8 cells (for

supernatant containing viral particles), the method used here is more complex and concerns pre-activated B cells.

In the present study, no overall difference was observed in cell phenotype after complete EBV transformation (Fig. 4 and Table. 1), and the EBV-mRNA-associated immortalizations were also similar (Fig. 5). Of the latency EBV proteins, EBNA-1, EBNA-2 and LMP-1 are critical B-cell transformation factors (Cohen *et al.* 1989; Young *et al.* 2000).

Figure 2 shows the homotypic aggregation of EBV infection; this aggregation is related to the expression of ICAM-1 (Rothlein & Springer 1986). LMP-1 up-regulates B-cell activation markers including ICAM-1 (CD54) (Young *et al.* 2000), but the expression of LMP-1 mRNA in EBV-infected B cells induced by CD19 and BCR ligation is not up-regulated compared to EBV-infected B cells without other treatment. Thus we examined the expression of intracellular LMP-1 protein and found a difference in the expression of intracellular LMP-1 in EBV-infected B cells induced by CD19 and/or BCR ligation, and in EBV-infected B cells, at 2 weeks after EBV infection (Fig. 6). We re-evaluated the expression of membrane LMP-1, but membrane LMP-1 was not expressed up to 14 days (data not shown). These results suggest that expression of intracellular LMP-1 protein might be a more efficient indicator of immortalization than the expression of mRNA. Thus this may be one of the necessary quality controls when banking cells for genomic gene preservation.

Recently, Ohtani *et al.* reported that EBV LMP-1 blocks the p16^{INK4a}-RB pathway by promoting nuclear export of E2F4/5 (Ohtani *et al.* 2003). Their studies concerning signalling pathways may be a guideline for future work, i.e. determination of the mechanism of the LMP-1 signalling pathway to improve the process of cell immortalization.

In summary, the pre-activation method using CD19 and BCR co-ligation can shorten the time required from initiation to immortalization by EBV, by enhancing the expression of intracellular LMP-1 to within 2 weeks. However, this does not alter the expression of cell surface molecules on EBV-transformed B cells. Thus, we suggest that the method described may be used effectively to generate lymphoblastoid B-cell lines induced by EBV.

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REFERENCES

- BORNKAMM GW, HAMMERSCHMIDT W (2001) Molecular virology of Epstein–Barr virus. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* **356**, 437.
- BRADBURY LE, KANSAS GS, LEVY S, EVANS RL, TEDDER TF (1992) The CD19/21 signal transducing complex of human B lymphocytes includes the target of antiproliferative antibody-1 and Leu-13 molecules. *J. Immunol.* **149**, 2841.
- CARTER RH, FEARON DT (1992) CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* **256**, 105.
- COHEN JI, WANG F, MANNICK J, KIEFF E (1989) Epstein–Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. *Proc. Natl Acad. Sci. USA* **86**, 9558.
- DEMPSEY PW, ALLISON ME, AKKARAJU S, GOODNOW CC, FEARON DT (1996) C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* **271**, 348.

- ENGEL P, ZHOU LJ, ORD DC, SATO S, KOLLER B, TEDDER TF (1995) Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity* **3**, 39.
- FUJIMOTO M, BRADNEY AP, POE JC, STEEBER DA, TEDDER TF (1999) Modulation of B lymphocyte antigen receptor signal transduction by a CD19/CD22 regulatory loop. *Immunity* **11**, 191.
- HUR DY, KIM S, KIM YI, MIN HY, KIM DJ, LEE DS, CHO D, HWANG YI, HWANG DH, PARK SH, AHN HK, CHANG KY, KIM YB, LEE WJ (2000) CM1, a possible novel activation molecule on human lymphocytes. *Immunol. Lett.* **74**, 95.
- MATSUMOTO AK, KOPICKY-BURD J, CARTER RH, TUVESON DA, TEDDER TF, FEARON DT (1991) Intersection of the complement and immune system: a signal transduction complex of the B lymphocyte-containing complement receptor type 2 and CD19. *J. Exp. Med.* **173**, 55.
- MIDDELDORP JM, BRINK AA, VAN DEN BRULE AJ, MEIJER CJ (2003) Pathogenic roles for Epstein–Barr virus (EBV) gene products in EBV-associated proliferative disorders. *Crit. Rev. Oncol. Hematol.* **45**, 1.
- NEITZEL H (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum. Genet.* **73**, 320.
- OH HM, OH JM, CHOI SC, KIM SW, HAN WC, KIM TH, PARK DS, JUN CD (2003) An efficient method for the rapid establishment of Epstein–Barr virus immortalization of human B lymphocytes. *Cell Prolif.* **36**, 191.
- OHTANI N, BRENNAN P, GAUBATZ S, SANIJ E, HERTZOG P, WOLVETANG E, GHYSDAEL J, ROWE M, HARA E (2003) Epstein–Barr virus LMP1 blocks p16INK4a-RB pathway by promoting nuclear export of E2F4/5. *J. Cell Biol.* **162**, 173.
- PASCUAL V, LIU YJ, MAGALSKI A, DE BOUTEILLER O, BANCHEREAU J, CAPRA JD (1994) Analysis of somatic mutation in five B cell subsets of human tonsil. *J. Exp. Med.* **180**, 329.
- PELLOQUIN F, LAMELIN JP, LENOIR GM (1986) Human B lymphocytes immortalization by Epstein–Barr virus in the presence of cyclosporine A. *In Vitro Cell Dev. Biol.* **22**, 689.
- ROTHLEIN R, SPRINGER TA (1986) The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. *J. Exp. Med.* **163**, 1132.
- SATO S, MILLER AS, HOWARD MC, TEDDER TF (1997) Regulation of B lymphocyte development and activation by the CD19/CD21/CD81 Leu13 complex requires the cytoplasmic domain of CD19. *J. Immunol.* **159**, 3278.
- SCHNEIDER U, ZUR HAUSEN H (1975) Epstein–Barr virus-induced transformation of human leukocytes after cell fractionation. *Int. J. Cancer* **15**, 59.
- STAMENKOVIC I, SEED B (1988) CD19, the earliest differentiation antigen of the B cell lineage, bears three extracellular immunoglobulin-like domains and an Epstein–Barr virus-related cytoplasmic tail. *J. Exp. Med.* **168**, 1205.
- TEDDER TF, HAAS KM, POE JC (2002) CD19–CD21 complex regulates an intrinsic Src family kinase amplification loop that links innate immunity with B lymphocyte intracellular calcium responses. *Biochem. Soc. Trans.* **30**, 807.
- TOHDA H, OIKAWA A, KUDO T, TACHIBANA T (1978) A greatly simplified method of establishing B-lymphoblastoid cell lines. *Cancer Res.* **38**, 3560.
- WERNER J, HENLE G, PINTO CA, HAFF RF, HENLE W (1972) Establishment of continuous lymphoblast cultures from leukocytes of gibbons (*Hylobates lar*). *Int. J. Cancer* **10**, 557.
- WROBLEWSKI JM, COPPLE A, BATSON LP, LANDERS CD, YANNELLI JR (2002) Cell surface phenotyping and cytokine production of Epstein–Barr Virus (EBV)-transformed lymphoblastoid cell lines (LCLs). *Immunol. Meth.* **264**, 19.
- YOUNG LS, DAWSON CW, ELIOPOULOS AG (2000) The expression and function of Epstein–Barr virus encoded latent genes. *J. Clin. Pathol.: Mol. Pathol.* **53**, 238.