EBV infection in treatment-naïve patients with chronic lymphocytic leukemia and mobility of the leukemic cells.

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- ✓ **Chronic lymphocytic leukemia (CLL)** is the most common type of leukemia in adults and accounts for about 25% of the all new cases of leukemia.

- ✓ CLL remains incurable despite the available targeted therapies

- ✓ The World Health Organization (WHO) classifies CLL as a **B-cell** neoplasm since 2008 [Alaggio R, et al., Review, *Leukemia*, 2022].

- ✓ CLL, in its **clinical behavior**, can be divided into two main types:
  1. **indolent (low risk)** slowly-progressing disease: ~30% of patients survive to more than 15 years, including patients who do not require therapy for many years and may never need any CLL-directed treatment;
  2. **aggressive (high risk)** rapidly-progressing disease: patients survive no more than 3 years after the diagnosis and require therapy urgently.

➢ The **high risk** CLL disease (with the late stages) is characterized by the presence of pronounced **anemia** and/or **thrombocytopenia**.

Transformation of CLL to an aggressive lymphoma (Richter transformation) occurs in approximately **5%** of patients and the majority of these cases represents diffuse large B-cell lymphoma (**DLBCL**),

according to an electronic database search of 2042 patients with CLL at The University of Texas M. D. Anderson Cancer Center (Houston, TX) between 1992 and 2002 [Tsimberidou et al, *Cancer*, 2005].

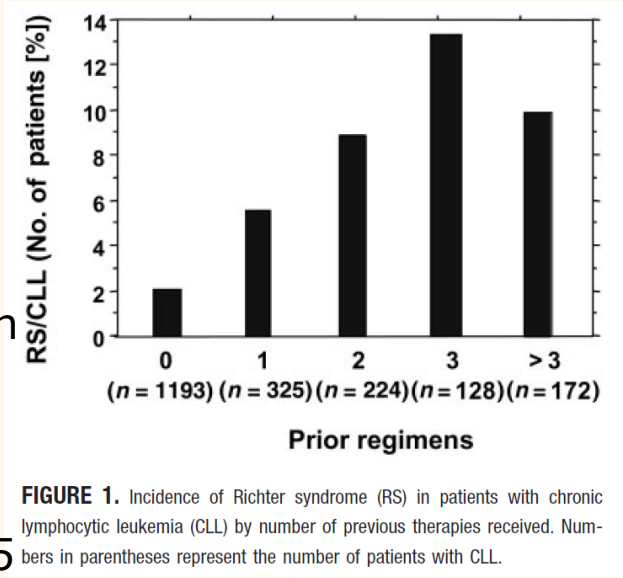
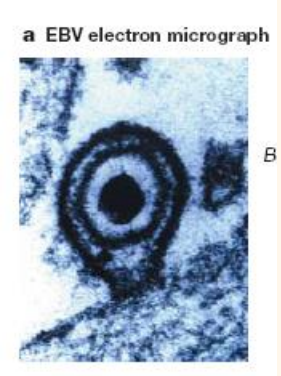


FIGURE 1. Incidence of Richter syndrome (RS) in patients with chronic lymphocytic leukemia (CLL) by number of previous therapies received. Numbers in parentheses represent the number of patients with CLL.

- ✓ In a retrospective study of DLBCL Richter transformation (**DLBCL-RT**), 58% of cases were clonally related to the initial CLL clone and the presence of the **Epstein Barr virus (EBV)** encoded RNA (EBER) was documented in 5 of 85 cases (**5,9%**) [Rossi et al., *Blood,* 2011].

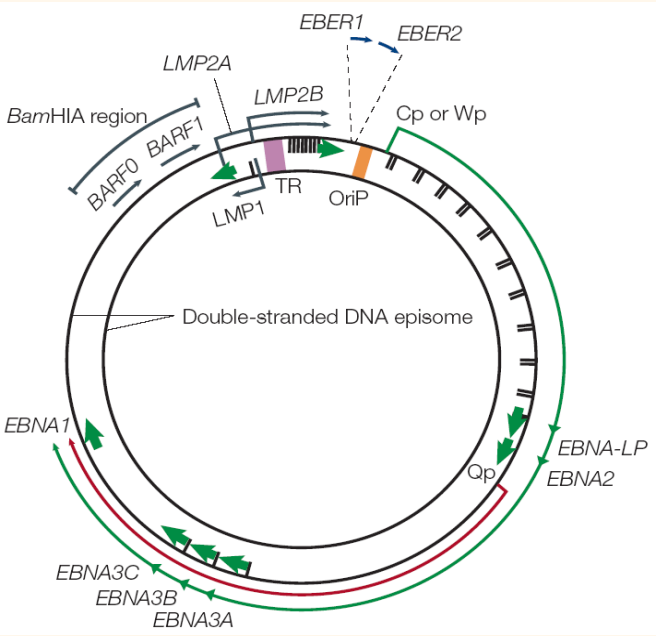
- ➢ Less than 1% (0.7%) of CLL patients developed biopsy-proven **Hodgkin lymphoma** (HL) in a retrospective study of 3887 CLL patients [Parikh et al., *Am. J. Hematol.,* 2015].

- ➢ In patients with HL of Richter transformation (HL-RT), **63–71%** of cases were **EBV-positive** [Xiao et al., Hum. Pathol., 2016; King et al, *Blood Cancer J.*, 2022].



Epstein–Barr virus (EBV) is a human gammaherpesvirus

- ✓ Since its discovery in 1964 (Epstein, Achong, and Barr, 1964), EBV was found to be widespread in all human populations and to persist in the vast majority of individuals as **a lifelong, asymptomatic infection** of the B-lymphocyte pool. More than 90% of adults carry the virus (de-Thé, 1975).
- ✓ EBV infects **B lymphocytes** through the binding of the major viral envelope glycoprotein gp350 to the CD21 receptor on the surface of B cells (Nemerow et al., 1987).
- ✓ During primary EBV infection of the immunocompetent host, the infected **B cells** transform into **lymphoblasts**

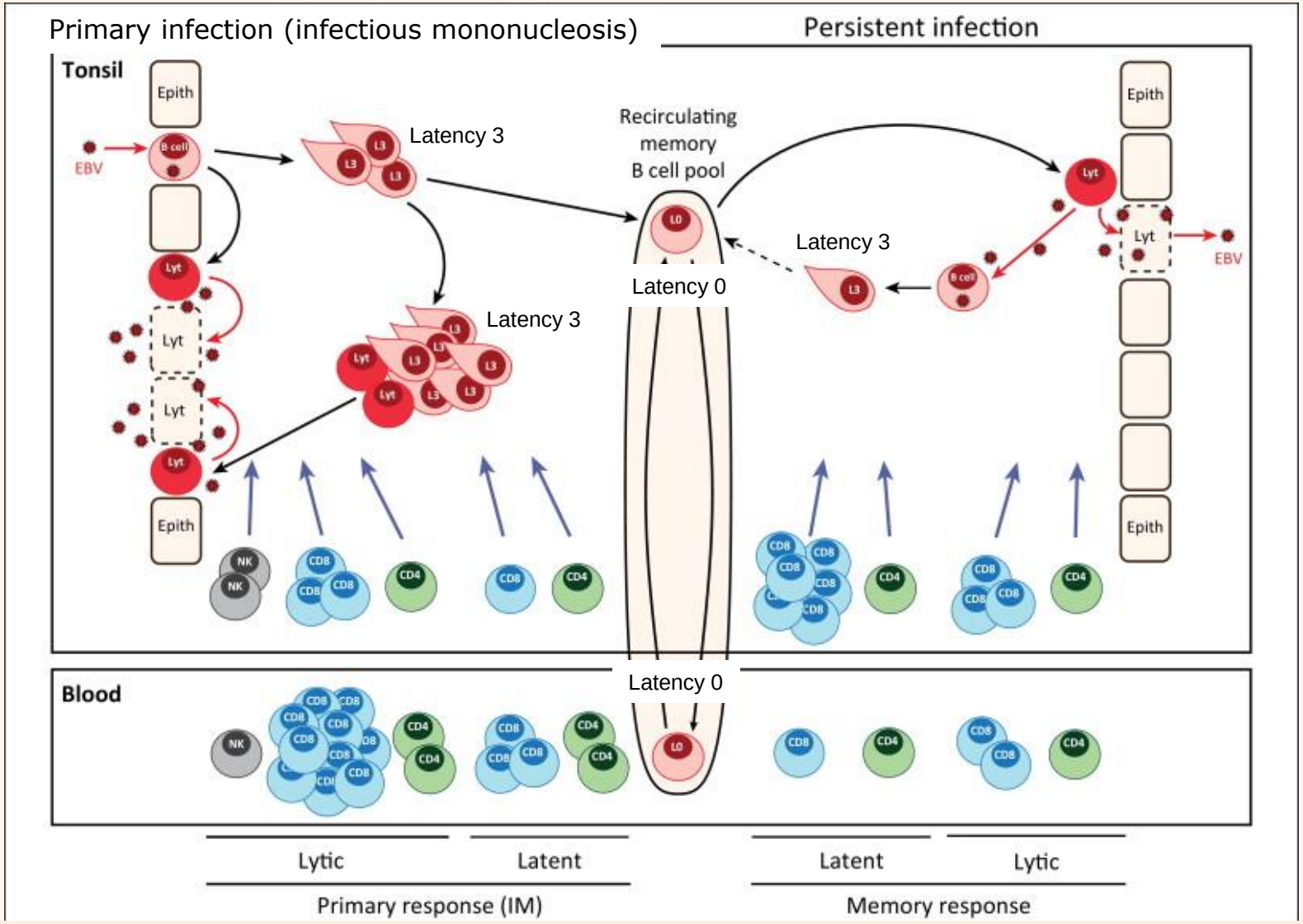


The EBV genome: latent genes

The Figure is adopted from Young & Rickinson, 2004.

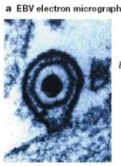
Despite such ubiquity, EBV infection is implicated in the aetiology of several different lymphoid and epithelial malignancies (reviewed in Farrell, 1995; Young & Rickinson, 2004; Claire Shannon-Lowe, Alan B. Rickinson and Andrew I. Bell, 2017).

- ✓ **The current model of the EBV persistence in immunocompetent hosts**
- ✓ In the EBV-infected B cells, virus down-regulate expression of the EBV proteins and acquire a virus antigen-negative form of latency (**Latency 0**), thereby escaping the elimination by immune cells and establishing an asymptomatic lifelong latent infection.



(Reproduced from Alan B. Rickinson, Heather M. Long, Umaimainthan Palendira, Christian Munz, and Andrew D. Hislop. Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the laboratory. *Trends Immunol.* 2014.)

Epstein–Barr virus (EBV) is a human gamma-herpesvirus (Epstein, Achong, and Barr, 1964) with the **high tropism for B-lymphocytes** (CD21).



EBV is associated with different lymphoid malignancies

Disease	Cellular origin	% EBV association	EBV latency	Latent EBV protein expression
B-lymphoproliferative disease				
post-transplant	naive or memory	>90	III	EBNAs 1, -2, -3A, -3B, -3C, -LP, LMPs 1, 2
HIV-related				
Burkitt lymphoma				
endemic		100		
sporadic	GC centroblast	10–80	I	EBNA1
HIV-related				
classical Hodgkin lymphoma				
nodular sclerosis		10–40		
mixed cellularity		70–80		
lymphocyte depleted	post-GC centroblast	10–50	II	EBNA1, LMPs 1, 2
lymphocyte rich		30–60		
HIV-related				
Diffuse large B cell lymphoma				
NOS		10	II/III	EBNA1, LMPs 1, 2/all EBNAs, LMPs 1, 2
PAL	post-GC centroblast	100	III	EBNA1, -2, -3A, -3B, -3C, -LP, LMPs 1, 2
HIV-related				
Rare immunocompromised B lymphomas				
plasmablastic lymphoma	plasmablast	75–90	I	EBNA1
primary effusion lymphoma				
T/NK lymphoproliferations				
CAEBV	T/NK/B	100		
extra-nodal T/NK lymphoma	T/NK	100	II	EBNA1, LMPs 1, 2
aggressive NK lymphoma	T/NK	100		

(Reproduced from C. Shannon-Lowe, A.B. Rickinson and A.I. Bell. Epstein–Barr virus-associated lymphomas. *Phil. Trans. R. Soc* B 372: 2017.)

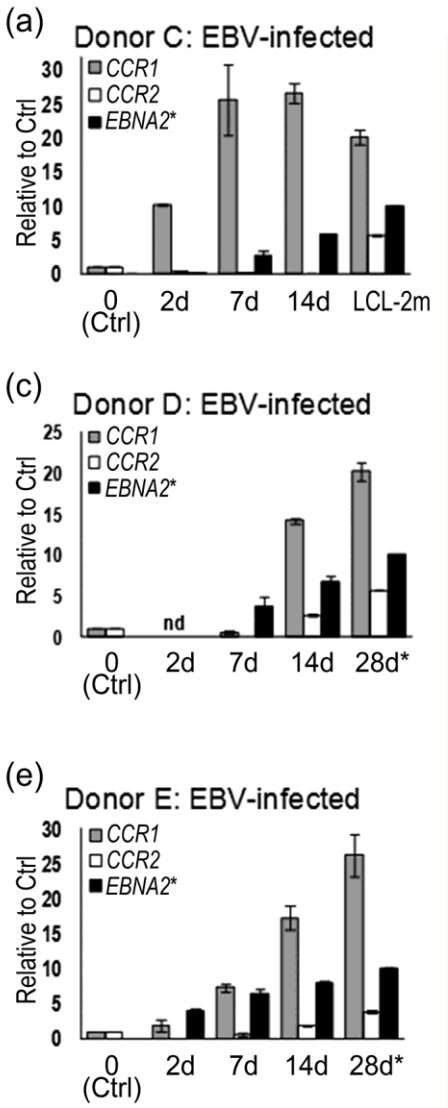
- ✓ **EBV is not implicated** in the development of chronic lymphocytic leukemia (CLL).

- ✓ However, CLL is frequently complicated due to **immune dysregulation** that leads to autoimmune complications and increased incidence of infections [reviewed in: Kipps et al., 2017; Vitale et al., 2021].
- Autoimmune complications (AIC) have been reported in up to 25% of CLL patients [*Hamblin, 2006; Fattizzo & Barcellini, 2020*].

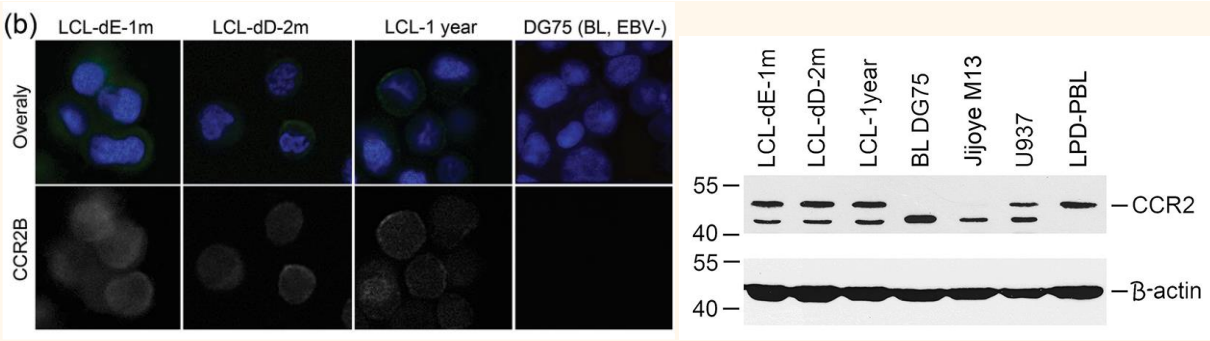
- ➢ Three independent studies reported **associations** of **high EBV DNA loads** in peripheral blood (PB) cells of CLL patients with the poor overall survival of patients and with the more aggressive course of the disease *[Grywalska et al., PLoS One, 2015; Visco et al., Oncotarget, 2015; Liang et al., Oncotarget, 2016]*,

- Previosly, we have demonstrated that **the *in vitro* EBV infection of B lymphocytes,** isolated from the PB of healthy donors, **up-regulated** the inflammatory chemokine receptors CCR1 and CCR2, but not CCR3 or CCR5.

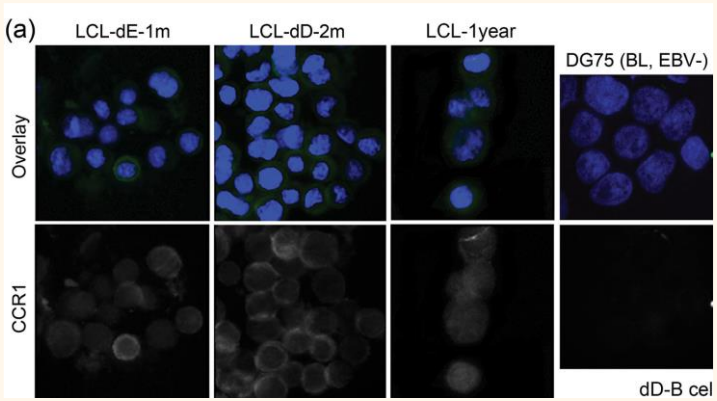
mRNA expression in B cells from 3 healthy donors *in vitro* **infected** with EBV:

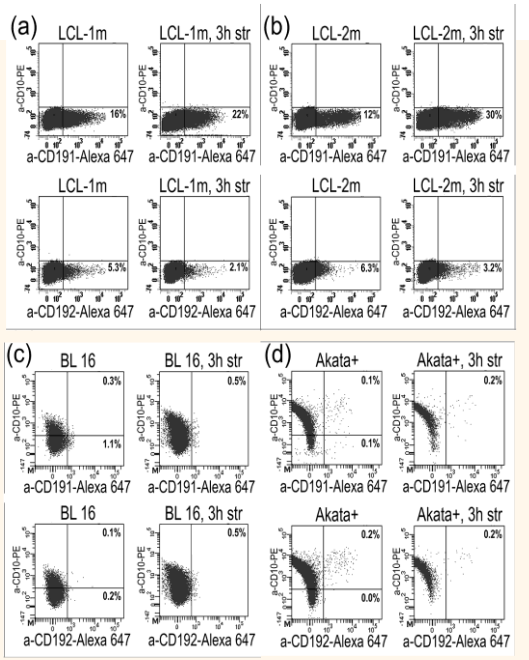


The presence of the CCR2 protein in LCL and in the type III BL cell lines, we have shown using immunostaining and immunoblotting analyses:



Expression of the CCR1 protein in LCL we have demonstrated using immunostaining and flow cytometry analyses:





(*Kholodnyuk et al., Virology, 2017; Kozireva et al., Viruses, 2018; Zvejniece, Kozireva et al., IJMC, 2022*)

- **The aim** of our study was to investigate whether the presence of **EBV DNA** and/or **EBV transcripts** in PB mononuclear cells (PBMCs) of treatment-naïve CLL patients **is associated** with the cell-surface expression of **CCR1** and/or **CCR2** on circulating **CLL (CD19+CD5+) cells.**

- **Materials and Methods**

- The number of the EBV DNA copies was determined using a commercial quantitative real-time PCR kit (Sacace Biotechnologies, Italy) with the detection limit of 5 copies per 10^5 cells (or ~ 8.3 EBV DNA copies per 1 ug of PBMC DNA).
- The presence of EBV transcripts, the early lytic gene BZLF1 and the latent genes, LMP1, LMP2A, and EBNA2, was assessed by RT-nested-PCR.
- Expression of the cell-surface markers was analyzed using multi-parameter flow cytometry and the BD FACSAria IIIu equipment.
- The Mann–Whitney U test, a nonparametric unpaired 2-tailed rank sum test including outliers, was applied to estimate associations.

➢ I. Results

- **EBV DNA** has been detected in PBMCs of 21 (**38.2%**) out of the 55 treatment-naïve CLL patients in the study.

✓ We found **no association** between the EBV-positive (≥5 copies/10^5 PBMCs; n=21) and EBV-negative (<5 copies/10^5 PBMCs; n=34) patients regarding the proportions of the CD19+CD5+ lymphocytes expressing CCR1, CCR2, or CD38.

- **High EBV DNA load** (>200 copies/10^5 PBMCs) was determined **in only three** patients (out of the 128 tested), all of them displayed two variants of IGHV, unmutated and mutated. In 18 EBV-positive patients (out of 55 in the study), EBV DNA load ranged 5–46 copies in 10^5 PBMCs.
- **The EBV transcripts** was found **in 3 other cases** (**with 5–6 EBV DNA copies**): two co-expressed **LMP1** and **EBNA2**, while the third co-expressed **LMP1** and **LMP2A**.

➢ II. Results

- Both, the **high EBV DNA load** and the **co-expression of LMP1 and EBNA2** in PBMCs of treatment-naïve CLL patients, were accompanied by a considerable increase in the number of leukemic cells (CD19+CD5+) that expressed CCR1, CCR2, and the negative prognostic marker CD38.

✓ Chemokine receptors and their ligands regulate **migration** of the immune cells and **dissemination** of malignant cells:

- *Stone M. et al., 2017. Int J Mol Sci. doi: 10.3390/ijms18020342*
- *Zabel B. et al., 2015. Annu.Rev.Pathol. doi: 10.1146/annurev-pathol-012513-104640*

✓ We earlier demonstrated the *in vitro* **migration** of the newly established lymphoblastoid (LCL) **B cells** (EBV latency III) and BL B cells (with EBV type II and type III growth phenotype), which expressed CCR1 and CCR2, towards the CCR2-ligand chemokine CCL2 (is also referred to as monocyte chemoattractant protein 1, MCP1), the only unique chemokine to the inflammatory chemokine receptor CCR2 [*Kholodnyuk et al., Virology, 2017; Kozireva et al., Viruses, 2018].*

➢ **Conclusions**

✓ Expression of CCR1 and/or CCR2 on CLL (CD19+CD5+) cells can promote migration of these cells into secondary lymphoid organs, which are enriched with the chemokine ligands, - thus contributing to progression of the disease.

✓ Detection of CCR1 and CCR2 on circulating CLL (CD19+CD5+) cells can be suggested to assure accurate prognoses and personalized treatment selection.

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