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THE ROLE OF THE MICROENVIRONMENT ON THE REGULATION OF EPSTEIN-BARR VIRUS LATENT GENE EXPRESSION

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To my beloved parents
Szeretö szüleimnek

ABSTRACT

Epstein-Barr virus (EBV) is a human-specific gamma-herpes virus of the *Lymphocryptovirus* genus that has succeeded to colonize more than 90% of the adult population. EBV's co-evolution with humans has established a largely harmless co-existence that depends on the variability of viral gene expression and on the immunological host response. Though infection with EBV is generally harmless, the virus is associated with multiple human tumors, such as Burkitt lymphoma, classical Hodgkin lymphoma, nasopharyngeal carcinoma, post-transplant lymphoproliferative disorders, and AIDS lymphomas. The role of EBV in the malignant transformation is still enigmatic even after more than 40 years of research on this virus.

EBV readily infects B-lymphocytes *in vitro*. After infection the virus establishes a latent infection and expresses 9 viral proteins. The concerted effect of these EBV proteins will be the activation and proliferation of the infected B cells. This viral gene expression pattern was named type III latency. As the EBV-infected B cells with type III latency are highly immunogenic they are readily detected and killed by the specific cytotoxic T cells. In contrast to the EBV-infected normal B cells, the majority of the EBV-carrying tumors do not express all the nine proteins; rather they express only EBNA-1 or EBNA-1 together with the latent membrane proteins (LMP-1 and LMP-2). The factors that determine what viral genes EBV will express in the different normal and malignant cells are only partially known.

Motivated by the lack of in vitro models in which to study the interaction of EBV with the malignant Hodgkin/Reed-Sternberg (HRS) cells, we infected with EBV one of the Hodgkin lymphoma-derived cell lines and studied the viral gene expression in this EBV-converted subline. In this system we identified two cytokines, IL-4 and IL-13, that could modulate the viral gene expression (specifically, induce the expression of LMP-1) and with their help we could for the first time reconstitute *in vitro* the EBV gene expression seen in the classical Hodgkin lymphomas *in vivo*. We have also studied the molecular mechanisms that are responsible for the induction of LMP-1 by IL-4 and IL-13. Through these studies we identified STAT6 as an important inducer of LMP-1 expression. As STAT6 is constitutively activated in the majority of Hodgkin lymphomas, our results not only provides an explanation how LMP-1 is expressed in the EBV-carrying HRS cells, but might also have future therapeutic implications.

Further work identified two additional cytokines, IL-10 and IL-21, which could induce the expression of LMP-1 in EBV-positive B cell and NK cell lymphoma-derived cell lines. The effect of IL-21 was pleiotropic: it could induce LMP-1 in cells that did not express it, and it induced the plasma cell differentiation and down-regulation of expression of the EBV nuclear antigens (EBNA-1 to -6) in type III lymphoblastoid and Burkitt lymphoma cell lines.
Furthermore, when isolated human CD4+ T cells were co-cultured with different EBV-carrying lymphoma cell lines, we found that upon activation they were capable of inducing the expression of LMP-1, just as the recombinant cytokines did.

Altogether our results provide evidence for an important role for the cytokines secreted by CD4+ T or other inflammatory cells in the modulation of EBV latent gene expression.

LIST OF PUBLICATIONS INCLUDED IN THE THESIS

I. **Kis LL**, Nagy N, Klein G, Klein E. Expression of SH2D1A in five classical Hodgkin's disease-derived cell lines. *Int J Cancer*. 2003 May 1;104(5):658-61.

II. Nishikawa J, **Kis LL**, Liu A, Zhang X, Takahara M, Bandobashi K, Kiss C, Nagy N, Okita K, Klein G, Klein E. Upregulation of LMP1 expression by histone deacetylase inhibitors in an EBV carrying NPC cell line. *Virus Genes*. 2004 Jan;28(1):121-8.

III. **Kis LL**, Nishikawa J, Takahara M, Nagy N, Matskova L, Takada K, Elmberger PG, Ohlsson A, Klein G, Klein E. In vitro EBV-infected subline of KMH2, derived from Hodgkin lymphoma, expresses only EBNA-1, while CD40 ligand and IL-4 induce LMP-1 but not EBNA-2. *Int J Cancer*. 2005 Mar 1;113(6):937-45.

IV. **Kis LL**, Takahara M, Nagy N, Klein G, and Klein E. IL-10 can induce the expression of EBV-encoded latent membrane protein-1 (LMP-1) in the absence of EBNA-2 in B-lymphocytes and in Burkitt lymphoma-, and NK lymphoma-derived cell lines. *Blood.* 2006 Apr 1; 107(7): 2928-2935.

V. Takahara M., **Kis LL**, Nagy N, Liu A, Harabuchi Y, Klein G, and Klein E. Concomitant increase of LMP1 and CD25 (IL-2-receptor alpha) expression induced by IL-10 in the EBV-positive NK lines SNK6 and KAI3. *Int J Cancer*. 2006 Dec 15;119(12):2775-83.

VI. **Kis LL**, Salamon D, Persson EK, Nagy N, Scheeren FA, Spits H, Klein G, Klein E. “IL-21 imposes a type II EBV gene expression on both type III and type I B cells by the repression of the C-promoter and activation of the LMP-1 promoter” – manuscript-

VII. **Kis LL**, Gerasimčik N, Salamon D, Persson EK, Nagy N, Klein G, Severinson E, Klein E. “A new STAT6 signaling pathway activated by the cytokines IL-4 and IL-13 induces the expression of the EBV-encoded protein LMP-1 in absence of EBNA-2: implications for the type II EBV latent gene expression in Hodgkin lymphoma” – manuscript-

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

1. **Kis LL**, Takahara M, Nagy N, Klein G, and Klein E. Cytokine mediated induction of the major Epstein-Barr virus (EBV)- encoded transforming protein, LMP-1. *Immunol Lett.* 2006 Apr 15;104(1-2):83-8. Review.
2. Klein E, **Kis LL**, Takahara M. Pathogenesis of Epstein-Barr virus (EBV)-carrying lymphomas. *Acta Microbiologica et Immunologica* 2006, 53(4):439-455. Review.
3. Klein E, **Kis LL**, Klein G. Epstein-Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions. *Oncogene*. 2007 Feb 26;26(9):1297-305. Review.
4. Klein E, Takahara M, and **Kis LL** (2008). "Contribution of the microenvironment to the pathogenesis of EBV-positive Hodgkin and nasal NK/T-cell lymphomas", in "Innate and Adaptive Immunity in the Tumor Microenvironment", ed. Eitan Yefenof (Springer, Heidelberg) pp. 195-209

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AITL	Angioimmunoblastic T cell lymphoma
AT	Ataxia telangiectasia
5-AzaC	5-Azacytidine
BART	BamHI-A rightward transcript
BCR	B cell receptor
BL	Burkitt lymphoma
BM	Bone marrow
CalHV-3	Callitrichine herpesvirus 3
CHIP	Chromatin immunoprecipitation
cHL	Classical Hodgkin lymphoma
CLL	Chronic lymphocytic lymphoma
CRE	Cyclic AMP response element
CSR	Class-switch recombination
CTL	Cytotoxic T-lymphocyte
cHL	Classical Hodgkin lymphoma
CVID	Common variable immunodeficiency
CysLT	Cyteinyl leukotrien
EBNA	EBV-Nuclear Antigen
EBV	Epstein-Barr virus
ED-L1	EcoRI D leftward 1
EMSA	Electrophoretic mobility shift assay
CSR	Class-switch recombination
DLBCL	Diffuse large B cell lymphoma
GC	Germinal center
HRS	Hodgkin/Reed-Sternberg
HSC	Hematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IM	Infectious mononucleosis
IRF	Interferon regulatory factor
IRS	Insulin receptor substrate

ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LMP	Latent membrane protein
MAPK	Mitogen-activated protein kinase
NB	Sodium butyrate
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NPC	Nasopharyngeal carcinoma
NRTK	Non-receptor tyrosine kinase
oriP	Origin of replication
PC	Plasma cell
PD-1	Programmed cell death protein-1
PEL	Primary effusion lymphoma
PHA	Phytohemagglutinin
PI3K	Phosphatidyl-inositol 3-kinase
PTLD	Post-transplant lymphoproliferative disease
SAP	SLAM-associated protein
SCID	Severe combined immunodeficiency
SEB	Staphylococcus enterotoxin B
SH2	Src homology 2
SHM	Somatic hypermutation
siRNA	Small interfering RNA
SLAM	Signaling lymphocytic activation molecule
STAT	Signal transducer and activator of transcription
TNFR	Tumor necrosis factor receptor
TR	Terminal repeat
TRADD	TNFR-associated death-domain containing protein
TRAF	TNFR-associated factor
Treg	Regulatory T cell
TSA	Trichostatin A
TYK2	Tyrosine kinase 2
WAS	Wiskott-Aldrich syndrome
XLP	X-linked lymphoproliferative disease

1 BACKGROUND

1.1 EBV-general introduction

Epstein-Barr virus (EBV) (also human herpesvirus 4, HHV4) is a human-specific gamma-herpes virus of the *Lymphocryptovirus* (LCV) genus that has succeeded to colonize more than 90% of the adult population. As a general characteristic of the viruses of this genus, they can all infect and immortalize B-lymphocytes *in vitro* and *in vivo*.

EBV and man have a common history that reaches back to our primate ancestors. After its discovery, EBV-like viruses were found also in Old world primates. It was believed that New World primates do not harbor such viruses, until 2000 when Ramer *et al.* reported the identification of novel viral DNA sequences from a spontaneous B cell lymphoma of the New World primate *Callithrix jacchus* (common marmoset)[1]. Subsequently in 2001 Cho *et al.* isolated and sequenced the Callitrichine herpesvirus 3 (CalHV-3) from the same species[2], and also showed that the newly isolated virus was present in other New World primates. Even more challenging were the findings of Ehlers *et al.* who, using a panherpesvirus PCR assay, identified 26 novel LCVs in multiple primates[3]. While the majority of the viral sequences were closely related to EBV or CalHV-3, a LCV from gorillas and three LCVs from orangutans and gibbons were only distantly related, raising the possibility that a yet unknown human LCV might exist[3].

EBV's co-evolution with humans has established a largely harmless co-existence that depends on the variability of viral gene expression and on the immunological host response. Similarly to other herpes-viral infections, after the primary infection with EBV humans become virus carriers for lifetime. The maintenance of the virus in face of the immune response is secured by the cell-virus interactions that vary with the differentiation and maturation state of the target cells.

EBV was first seen in a B cell-derived lymphoma[4] with characteristic epidemiological, clinical, and histopathological features that after its discoverer, Dennis Burkitt, was named as Burkitt lymphoma (BL)[5]. Soon thereafter the *in vitro* transforming capacity of the virus for B-lymphocytes was shown[6,7]. This *in vitro* system became widely used for analysis of the viral transforming mechanism and of the immune response against the virus carrier cells. The virus-induced B cell proliferation *in vitro* was taken as strong evidence for a similar mechanism in the development of BL. However the discovery of EBV-negative BL lymphomas with similar pathology and with identical cytogenetical changes indicated that the virus alone is not responsible, but contributes to the development of BL.

The primary infection generally occurs during early childhood and is asymptomatic. If the infection is delayed in about half of the individuals it can result in a benign, self-limiting disease, called infectious mononucleosis (IM) or kissing disease. The symptoms of IM are the manifestation of the immune response, the atypical lymphocytosis, which is made up by virus specific $CD8^+$ and $CD4^+$T lymphocytes. IM is easily diagnosed by detection of IgM antibodies against the virus capsid antigen (VCA). Antibodies against virus-encoded proteins associated with lytic and latent infection appear in a sequencial manner. EBV can also be detected by the presence of virus in the saliva, by the establishment of LCLs in explanted lymphocyte populations, or by PCR techniques in the peripheral blood mononuclear cells (PBMC).

It is important to note that despite the largely harmless outcome of the EBV infection, certain immuno-deficient patients cannot control the infection and develop fatal IM. The prominent example of this state is the X-linked lymphoproliferative disease (XLP)[8,9], caused by mutations in the *SH2D1A (SAP)* gene[10-12]. Interestingly, these patients can control the infections caused by other viruses. Conversely, EBV was not reported to cause disease in immuno-deficient patients in the IFN-α/β pathway, in whom HSV-1 causes encephalitis[13].

1.2 EBV latent gene expression

1.2.1 The type III latency program

Upon *in vitro* infection of the B cells with EBV, the virus will establish a latent infection and by this it will impose the continuous proliferation of the B-lymphocytes, giving rise to lymphoblastoid cell lines (LCLs). The EBV-encoded genes expressed in non-virus producing cells were defined in these virus transformed LCLs. In such cells one of the two alternative viral promoters (Wp or Cp) is used to generate a giant mRNA, out of which six nuclear protein-encoding mRNAs (EBV-Nuclear Antigen = EBNA1-6, also called EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-LP, EBNA-3C) are spliced. In addition 3 membrane proteins Latent Membrane Protein (LMP)-1, -2A, and -2B are expressed. This latent gene expression pattern is referred to type III latency (or growth program). Except for these protein-coding RNAs, type III latent cells express also three different sets of EBV non-coding RNAs: EBER-1and EBER-2, the highly spliced *Bam*HI-A rightward transcripts (BARTs), and the recently discovered EBV microRNAs (miRNAs). EBERs are believed to be expressed in all EBV-carrying normal or malignant cells in which the virus is maintained in a latent state. Furthermore, at 24 hrs after infection of the B cells the two Bcl-2 homologs, BALF1 and BHRF1 are also expressed[14].

EBV encodes for at least 23 miRNAs that are expressed in the latently infected cells. These are arranged in two clusters: the BART cluster encodes 20 miRNAs (miR-BART1-20), while the BHRF1 cluster encodes 3 miRNAs (miR-BHRF1 1-3) (Fig.1A). The BART miRNAs are expressed at high levels in latently infected epithelial cells and at lower levels in B cells, whereas the BHRF1 miRNAs are found at high levels in type III latent B cells and are essentially undetectable in type I/II latent B or epithelial cells. Importantly, the EBV-like Rhesus LCV also expresses at least 16 distinct miRNAs, seven of which are closely related to EBV miRNAs. The complexity of the viral gene expression and its effect on the expression of cellular genes is thus increased through the expression of EBV-miRNAs that can have multiple targets (both cellular and viral). Furthermore, the EBV-encoded latent proteins can modulate the expression of cellular miRNAs, as shown for the expression of miR-155 being induced in an NF- κB-dependent manner by LMP-1[15].

The linear viral genome in the infected cell circularizes and is maintained as an extra-chromosomally replicating episome. EBNA-1, by binding to the origin of replication of the viral genome and by anchoring the episome to the chromosomes during mitosis, is pivotal for the replication and partitioning of the EBV genomes during cell division. Recent work showed that this process is not perfect and EBV genomes are lost during replication[16]. Interestingly EBNA-1's transcriptional activator function was also shown to be important for the EBV-induced B cell immortalization[17]. Furthermore, recently Dresang *et al.* reported the identification of sites bound by EBNA1 in the human genome[18].

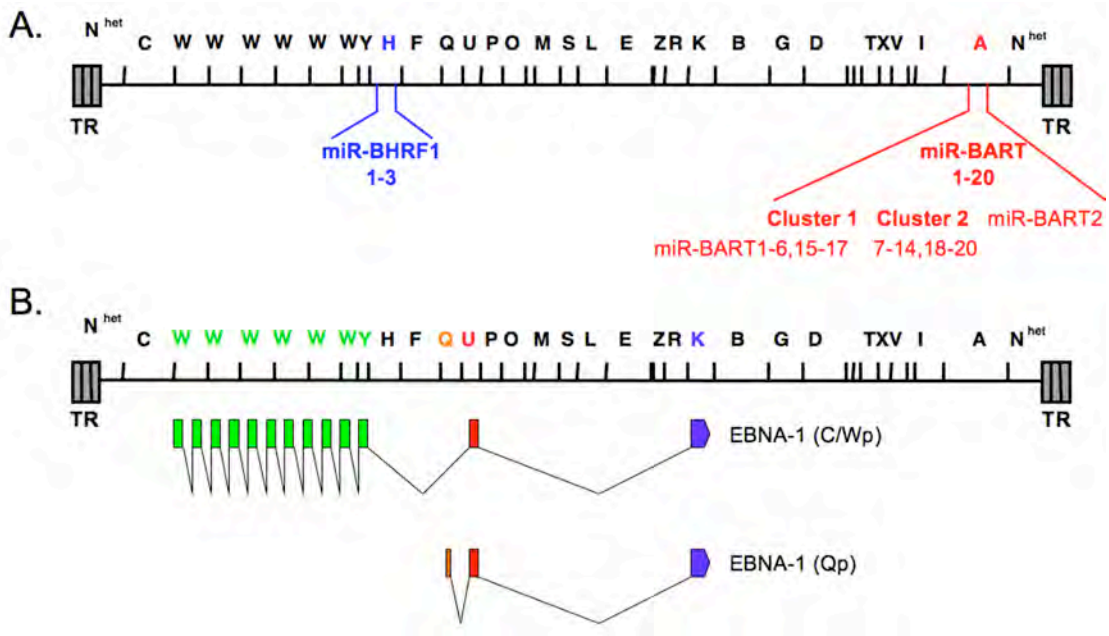


Figure 1. Physical location of the two EBV miRNA clusters (A) and the structure of the Cp/Wp- or Qp-derived EBNA-1 mRNAs (B) shown on the linearized BamHI restriction map of the EBV genome.

Among the nuclear proteins EBNA-2, -3, -5, and -6, while among the membrane proteins only LMP-1 is required for the transformation of the B cells *in vitro*. Furthermore, the two Bcl-2 homologs (BALF1 and BHRF1)[14] and EBER-2 (but not EBER-1)[19] were found to play a critical role in the EBV-induced B cell growth transformation. Though LMP-2A was originally found to be dispensable for the transformation of B cells[20], a later report showed the contrary[21]. Recently Mancao and Hammerschmidt provided evidence that in spite of the fact that B cell receptor (BCR)-positive and BCR-negative germinal center (GC) B cells could readily be transformed *in vitro* with a recombinant EBV encoding a conditional, floxed LMP2A allele, their survival and continued proliferation were strictly dependent on LMP2A[22].

Table I. EBV latent gene expression patterns in tumors∗

Latency type	Viral genes expressed	EBV-associated tumors
Type I	EBNA-1 (Qp)	BL, PEL, NPC, PTLD
Type II	EBNA-1 (Qp), LMP-1, LMP-2A, LMP-2B	cHL, NPC, nasal NK/T lymphoma, PTLD
Type III	EBNA-1 (Cp), -2, -3, -4, -5, -6; LMP-1, LMP-2A, LMP-2B	PTLD, AIDS-DLBCL
Type IIB	EBNA-1 (Cp), -2, -3, -4, -5, -6	PTLD
Wp-restricted	EBNA-1 (Wp), -3, -4, -5, -6, BHRF1	rare BLs

BL-Burkitt lymphoma, PEL-primary effusion lymphoma, NPC-nasopharyngeal carcinoma, PTLD-post-transplant lymphoproliferative disorders, cHL-classical Hodgkin lymphoma

∗ Important to note that:

- Type I and type II different category, but it could be regrouped as typeI/II "restricted" latency.
- The viral gene expression within the tumors can be heterogeneous: not all EBV-positive NPC or NK/T-lymphoma cells express LMP-1 i.e. tumors are a mixture of type I and type II cells; not all PTLDs express type III latency.
- EBERs are expressed in all tumors, irrespective of their tissue origin or of co-expression of other EBV genes.
- Epithelial tumors express in addition to the listed latent genes also BARF1.
- Two clusters of EBV miRNAs were discovered encoding for at least 23 mature miRNAs. Because the miR-BHRF1 cluster is encoded in the introns of the *Bam*HI H region, they are expressed in type III latency when the Cp/Wp promoters drive the expression of a primary RNA that encompasses the H region as well. The expression of BART miRNAs parallels the expression of the BART RNAs being highly expressed in epithelial cells. Because the data on the expression of EBV miRNAs in tumors is just emerging and only NPCs were studied in more details, they are not included in this table.
- Wp is seen only if the viral genome contains the deletion of the EBNA-2 gene.

In B cells EBNA-2 activates the LMP-1[23], LMP-2[24], and Cp viral promoters[25-28], and by this its expression is instrumental for the expression of type III latency. EBNA-2 does not directly bind to DNA but acts as a trans-activator through interactions with other DNA-

binding proteins. More specifically, EBNA-2 hijacks the major transcription factor of the Notch pathway, the Jκ recombination signal-binding protein RBP-jκ (also CBF-1) protein[29-32]. Through this interaction EBNA-2 trans-activates the Cp, the bidirectional LMP-1- LMP-2B[33], and the LMP-2A promoters[24]. Other interaction partners of EBNA-2 are the PU.1[34,35] and the ATF-2/c-Jun heterodimers[36].

EBV-carrying type III latent cells occur also in the lymphoid tissues of IM patients, tonsillar B cells of healthy virus carriers, and in the lymphoproliferations arising in transplanted patients (post-transplant lymphoproliferative disease, PTLD) and lymphomas in AIDS patients (Table I). Because of their immunogenic phenotype the type III B cells in the first two *in vivo* situations probably survive and proliferate only transiently, as being recognized and killed by the cytotoxic T-lymphocytes (CTLs)[37], while in the last two examples they survive and expand because of the lack of functional CTL control.

1.2.1.1 Signaling by LMP-1

LMP-1 is a 386-amino-acid (aa) transmembrane protein with a 25-aa intracellular amino terminus, followed by six membrane-spanning domains (160 aa), and an intracellular carboxyl-terminal domain of approximately 200-aa[23,24]. LMP-1 signals in a constitutive active manner and self-aggregation is needed its signaling capacity[25]. Furthermore, LMP-1 is targeted to the lipid rafts (cholesterol- and spingolipid-rich microdomains also known as detergent-resistant membranes) and this localization is important for its signaling capability[26].

LMP-1 is a viral mimic of CD40, a glycoprotein belonging to the tumor necrosis factor receptor (TNFR) family. LMP-1 activates the NF-κB (both the classical and non-classical)[27-29], stress-activated MAP kinase[30], phosphatidylinositol 3-kinase (PI3K)[31], and extracellular-regulated kinase (ERK)-MAPK signaling pathways through its C terminus by binding to TNFR-associated factors (TRAF)[32] and/or TNFR-associated death domain-containing protein (TRADD)[33]. As foreseen from its ability to engage multiple signaling pathways, LMP-1 induces the expression of multiple genes involved in protection from apoptosis (c-FLIP, A20, Bfl-1, cIAP1, cIAP2, Mcl-1), cytokines (IL-6, IL-10, IL-8, IL-18, TNF, BAFF, APRIL, CCL17, CCL22), adhesion and activation markers (ICAM-1, SLAM, CD83, CD48, CD40, EGFR, c-Met, LFA-1, LFA-3), gene involved in metastasis (MMP-1, MMP-9, VEGF, Cox-2)[34]. LMP-1 can also repress the expression of certain genes, and this effect seems indirect as shown for the repression of E-cadherin expression (by the induction of DNMT1 expression) and BCL6 (possibly through the induction of IRF4 expression). Though a comprehensive comparison of the LMP-1 regulated genes in epithelial and in

lymphoid cells is still missing, it is important to note that the effect of LMP-1 and the signaling pathways engaged have certain tissue specificity.

LMP-1 is the major transforming protein of EBV and behaves as a classical oncogenic protein in rodent fibroblast transformation assays[35]. Furthermore, in monolayer keratinocytes LMP-1 alters cell morphology and cytokeratin expression[36], inhibits cell differentiation of immortalized epithelial cells in raft cultures[37], while when expressed in the skin of transgenic mice it induces epidermal hyperplasia[38].

Genetic studies indicate that LMP-1 signaling is not only required for efficient immortalization of the B lymphocytes *in vitro*[39], but its continuous expression is needed for maintaining B cell proliferation[40,41]. Furthermore, when LMP-1 was expressed from the immunoglobulin (Ig) heavy chain promoter/ enhancer transgenic mice developed lymphomas with an increased incidence, especially at old age (41,8% in LMP-1-trangenics versus 11,6% in controls in mice older than 18 months)[42].

In order to test the effect of LMP-1 on the activation and differentiation of normal B cells *in vivo*, and to study how well LMP-1 mimics the CD40 receptor, Uchida *et al.* generated transgenic mice expressing LMP-1 in B cells under the control of immunoglobulin promoter/enhancer both on CD40-sufficient and -deficient backgrounds[43]. The LMP-1 expressing B cells displayed enhanced expression of activation antigens, spontaneously proliferated, and produced antibodies. LMP-1 mimicked CD40 signals to induce extrafollicular B cell differentiation (including antibody class-switching to IgG1) but, unlike CD40, it blocked GC formation. The inhibition of GC formation was seen on both mouse backgrounds[43].

Later the Mosialos group generated transgenic mice expressing either LMP1 or a chimeric LMP1CD40 molecule, which constitutively activates the CD40 pathway, under the control of the CD19 promoter[44]. LMP1CD40 suppressed GC formation and antibody production in response to thymus-dependent antigens to a greater extent than LMP1. GC suppression was linked to the ability of LMP1CD40 and LMP1 to down-regulate the expression of BCL6. No tumor development was observed up to 5 months. In line with the *in vivo* studies that showed that LMP-1 inhibits the GC reaction by repressing the expression of BCL6[44], LMP-1 was shown to inhibit the proliferation of BL cell lines when expressed in an inducible manner[45]. This latter *in vitro* effect might be also due to inhibition of BCL6 that is known to be required for the proliferation of BL cells[46,47].

To directly compare the signaling of the cytoplasmic tails of LMP1 and CD40 within B cells, Rastelli *et al.* generated transgenic mice conditionally expressing a CD40/LMP1

fusion protein, which retained the LMP1 cytoplasmic tail but has lost the constitutive activity of LMP1 and needs to be activated by the CD40 ligand[48]. These authors showed that the LMP1 cytoplasmic tail can substitute CD40 signaling in B cells, leading to normal B-cell development, activation, and immune responses including class-switch recombination (CSR), GC formation, and somatic hypermutation (SHM) of the immunoglobulin variable regions. Furthermore, the LMP1-signaling domain was found to have a unique property in that it could induce class-switch recombination to IgG1 in a cytokine-independent manner[48].

As a general conclusion for the transgenic mice studies on the effect of LMP-1 in B cells it is evident that its expression resulted in the activation of B cells, it inhibited the formation of GCs, and was not enough for lymphoma development in young animals.

1.2.1.1 Signaling by LMP-2A

LMP-2A and LMP-2B are also transmembrane proteins containing 12 membrane-spanning domains. The difference between them resides in the first exon that encodes a signaling domain and is part of LMP-2A, but is lacking from LMP-2B. The intracellular N-terminal signaling domain of LMP-2A contains a functional immunoreceptor tyrosine-based activation motif (ITAM) motif, similar to those found in the CD79α and CD79β, the signal transducing components of the BCR[49]. The phosphorylated tyrosines 74 and 85 in the ITAM motif are required for the binding of the non-receptor tyrosine kinase (NRTK), Syk, while the tyrosine 112 acts as a docking site for the NRTK Lyn[50,51]. Additionally, LMP2A contains two polyproline motifs that have been shown to recruit Nedd4/AIP4 ubiquitin ligases, shown to mediate polyubiquitination of the LMP-2A-associated Lyn and Syk, and also of LMP-2A itself[52,53]. Furthermore, LMP-2A was shown to activate the survival kinase Akt in a PI3K-dependent fashion, through its interaction with the signaling scaffold Shb[54].

The biochemical data on LMP-2A suggested that LMP-2A interferes with the BCR signaling by sequestering the NRTKs Syk and Lyn from the BCR and by this acting as an inhibitor of BCR signaling. Contrary to this conclusion, *in vivo* transgenic mice studies, in which LMP-2A was expressed from the Ig heavy chain promoter/enhancer, showed that LMP-2A could actually mimic the BCR signals[55]. The LMP-2A-trangenic mice showed high proportion of the surface IgM-negative CD19+ B cells in the spleens[55]. The non-expression of the surface Ig was due to the uncompleted V-DJ_H Ig gene rearrangement, while the Ig light chain genes were rearranged. Furthermore, when the Eμ-LMP2A transgene was expressed in a recombinase activating gene 1 (RAG-1) null animals, known to be unable to rearrange their Ig genes and characterized by a block in B cell development at the $CD43^+$ pro-B stage, significant numbers of CD19+ cells could be identified in the spleen of Eμ-$LMP2A^+RAG\text{-}1^{-/-}$

mice, while this population was absent in spleens of nontransgenic RAG-1$^{-/-}$ animals[55]. Based on these *in vivo* evidences it was concluded that LMP-2A is capable of providing signals that allowed the developing B cells in the bone marrow to bypass the requirement for the expression of a functional pre-BCR and provided a survival signal to the progenitor and peripheral B cells. Interestingly, no lymphoma development was found in the aforementioned LMP-2A transgenic mice. In a follow up study it was shown that the survival of the Ig-negative B cells was dependent on the ITAM motif of the LMP-2A[56].

Interestingly, when LMP-2A was expressed in epidermis of transgenic mice by a keratin 14 promoter cassette it did not alter the normal epithelial differentiation program in the epithelia of K14–LMP2A transgenic mice[57]. In sharp contrast to these *in vivo* findings Scholle *et al.* reported that expression of LMP-2A in the human keratinocyte cell line HaCaT, resulted in inhibition of epithelial differentiation, anchorage independent growth, and colony formation in soft agar[58]. Furthermore, the LMP-2A-expressing HaCaT cells were highly tumorigenic, forming aggressive tumors in nude mice and developing frequent metastases. Inhibition of the PI3-kinase-Akt pathway in the LMP2A-expressing cells blocked their growth in soft agar[58]. In line with a potential role of LMP-2A and LMP-2B in epithelial cell growth transformation, expression of LMP-2A or LMP-2B in the epithelial cell lines A431, SCC12F, and HaCaT was found to increase their capacity to spread and migrate on extracellular matrix[59]. These effects of LMP-2s were dependent on some unidentified tyrosine kinase activity different from the phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), and protein kinase C[59]. Unexpectedly, expression of LMP-2B induced a phenotype that was virtually indistinguishable from that of LMP2A, suggesting that regions of the LMP2 protein in addition to the cytosolic amino terminus are capable of inducing phenotypic effects in epithelial cells, and that LMP2B may directly engage signaling pathways to influence epithelial cell behavior such as cell adhesion and motility[59].

1.2.2 The type II latency program

This restricted EBV gene expression pattern, in which EBNA-1 is co-expressed with LMP-1 and LMP-2, was first seen in nasopharyngeal carcinoma (NPC)[60,61], and was later designated as type II latency[62]. The typical malignancies associated with this viral gene expression are the classical Hodgkin (cHL)-[63-65], T-[66], and NK-lymphomas[67,68], and some NPCs (Table I). Though at first glance it might appear that cHL is the only B cell-derived

tumor with a type II EBV latency, there are several reports in the literature describing B cell lymphomas or lymphoproliferations with this viral gene expression (Table II).

Table II. Presence of LMP-1-positive, EBNA-2-negative (type II) B cells in lymphomas/lymphoproliferations

Malignancy/lymphoproliferation	Comments	Reference
1. Infectious mononucleosis	tonsillar sections	J Pathol. 1997;182:151-9
	tonsillar sections	PNAS 2003;100:4730-35
2. PTLD	1 P-PTLD, 1 DLBCL	Am J Pathol. 1995;146:1113-20
	2 cases, BM transpl. for ALL	Am J Pathol. 1995;147:923-33
	8 cases, solid organ transpl.	Blood 1993;81:1393-1403
	2 cases, solid organ transpl.	Transplantation 1994;58:317-324
	1 HL, 1 P-PTLD, 6 DLBCL	Transplantation 2003;76:988-994
3. AITL	3 cases	J Exp Med. 2001;194:927-40
	6 cases	Am J Clin Pathol. 2002;117:368-79
	3 cases	Leukemia 2002;16:2134-41
4. Gastric MALT	2 cases	J Med Virol. 1998;56:342-50
5. Ulcerative colitis-associated colorectal NHL	1 case DLBCL 1 case CD30+, CD20-, CD3-	J Pathol. 2003;201:312-318
6. BL	2 cases of eBL	Blood 1995;86:659-65
	5 cases of sBL	Blood. 1996;87:1202-4
	2 cases BL in Brazil	Blood 1996;87:5279-86
7. AIDS lymphomas	5 cases of systemic IB/CB LCL	Leuk Lymph. 1998;30:515-24.
	4 cases of BL 13 cases IB/LC	Blood 1995;86:432-5
	3 cases of BL 7 cases of IB/LC	Am J Pathol.1993;143:1072-85
8. T-cell/Histiocyte-rich large B cell lymphoma	1 case	Am J Surg Pathol. 2002;26:1458-66
9. B-CLL	1 case	Int J Cancer 1995;63:486-90
10. Primary bone lymphoma associated with chronic osteomyelitis	1 case	J Pathol. 1997;183:287-92
11. Plasmablastic lymphomas of the oral cavity	5 cases	Blood 1997;89:1413-20

The enigma of EBNA-1 expression in the absence of other EBNAs was solved by the identification of a different promoter located in the BamHI Q-region (Qp) of the genome that drives only the expression of EBNA-1[69-71]. Thus the structure of the EBNA-1 mRNA originating from the Cp/Wp or Qp is different (Fig.1B), while the encoded EBNA-1 proteins are identical.

With regard to the mechanism governing the expression of LMP-1 and LMP-2 in type II latency it is important to note that because EBNA-2 is not expressed in this state therefore other cellular or viral proteins must be involved in the induction of their expression ("EBNA-2-independent LMP regulation") (Fig.2).

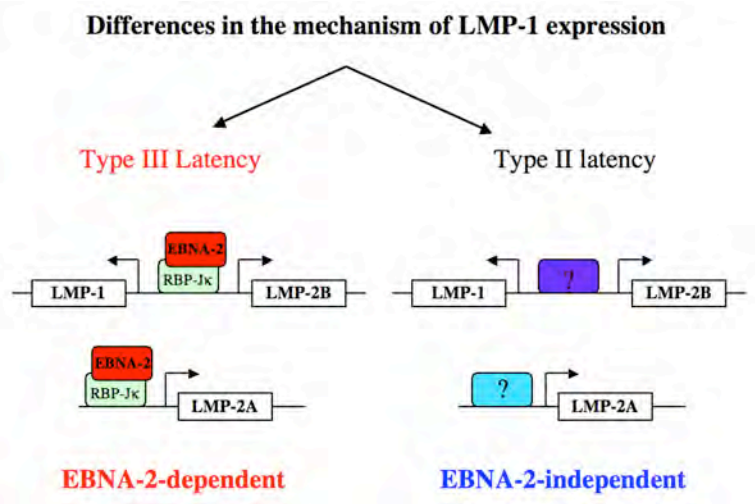


Figure 2. Differences in the regulation of LMPs expression in type III and type II EBV latency.

Based on this knowledge it appears that in order to define the type II viral gene expression of the EBV-carrying normal or malignant cells they should show the expression of Qp-derived EBNA-1 mRNA together with the expression of LMP-1 and LMP2. A further question is whether the expression of LMP-1 and LMP-2 mRNAs is sufficient or proof of their protein expression is necessary. This latter question becomes timely in the new world of cellular and viral miRNAs that can inhibit the expression of their targets at post-transcriptional level (LMP-1 targeting EBV miRNAs were recently identified, see later). The investigation of LMP-2 protein expression is still problematic because only few laboratories succeeded to study the expression of LMP-2 protein with the presently available antibodies. Another problem arises by the heterogeneous expression of the LMP-1 within the same EBV-carrying tumor population, some cells being LMP-1-negative while others LMP-1-positive. And what if a cell expresses only LMP-1 but not LMP-2, or only expresses LMP-2A and not LMP-1 (as seen in EBV-positive gastric carcinomas[72,73]), can it still be regarded as type II? Is the expression of LMP-1 more important than the LMP-2 in order to be considered type II latent?

In summary, the type II gene expression is more difficult to define, especially at the protein level, and only part of the EBV-carrying cHLs and NPCs show this viral gene expression pattern. As discussed later we believe that the type I and type II latent gene expression can be regarded as a single category of "restricted" EBV latency.

1.2.3 The type I ("EBNA-1 only") latency program

During the type I latent gene expression the only EBV-encoded protein that is expressed is EBNA-1 and, as mentioned above, its expression is driven by the Qp and includes the Q, U, and K exons. The classical example of a tumor with a type I EBV latent gene expression is the endemic BLs[74]. Furthermore, all EBV-carrying primary effusion lymphomas (PELs)[75], and some NPCs, DLBCLs[76,77], and PTLDs[78] also show type I expression (Table I).

1.2.4 "Non-classical" EBV gene expression programs

Recently two unusual combination of EBV-encoded protein expression received attention. The first type was described in the *in vitro* EBV-infected B- chronic lymphocytic leulemia (B-CLL) cells, while the second in rare EBV-carrying BL cell lines.

The *in vitro* infected B-CLL cells express the 6 nuclear proteins, EBNA1-6, but lack LMP-1[79-81]. This type of viral gene expression was termed type IIB latency[82,83]. Consistently with the requirement of LMP-1 for immortalization of B cells B-CLL clones only rarely yield LCLs when infected *in vitro* with EBV, and when LCL grow out (so called CLL-LCLs) they express LMP-1. EBV is not involved in the pathogenesis of B-CLL and *in vivo* EBV is very rarely found in the malignant B-CLL clone. Because the infected B-CLL cells express EBNA-2 that together with cellular transcription factors activate the LMP-1 promoter in normal B-lymphocytes, the lack of LMP-1 expression in the EBNA-2-positive B-CLL cells is noteworthy. The molecular mechanisms that determine the inadequacy of the EBNA-2 protein in these B-lymphocytes have not been clarified. Importantly, EBV-positive cells with a type IIB viral gene expression were found not only in the *in vitro* infected B-CLL cells, but also in the lymphoid tissues of IM patients[84], and in the lymphoproliferations of PTLD[84] and of EBV-infected "humanized" mice[85].

Rare BL lines and primary tumors can carry EBV genomes that have deletion in the EBNA-2 gene[86-89]. Interestingly, in these cases the virus does not express the typical type I latency pattern, rather the Wp-promoter is used to give rise to the primary RNA from which EBNA-5, -3, -4, -6, -1 are spliced (because EBNA-5 gene is located in the vicinity of EBNA-2 it is truncated and its size depends on the size of the deletion)[89]. LMPs are not expressed because of the lack of EBNA-2 expression. This viral gene expression is termed the "Wp-restricted" latency and was first seen in the BL lines P3HR-1 and Daudi[90,91], and later described in some primary endemic BLs[89]. One of the important implications of this viral

gene expression is that it provides evidence that the routinely used way of assessing the EBV latent gene expression (EBER in situ hybridization followed by EBNA-2 and LMP-1 immunohistochemical staining) is inadequate because these tumors would be categorized as type I (Fig.3).

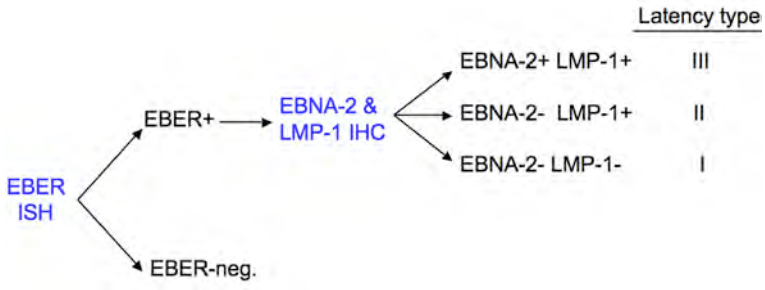


Figure 3. Assessment of EBV latent gene expression in a clinical setting.

1.3 The mature life of a B cell

Because the main targets of EBV are the mature B cells, here I shortly review the differentiation of normal B cells. The earliest steps of B cell development takes place in the bone marrow where the hematopoietic stem cells through sequential differentiation give rise to common lymphoid progenitors, pre-B cells, pro-B cells, and finally to immature B cells[92]. The immature B cells leave the bone marrow and enter the secondary lymphoid organs where they further differentiate through several transitional stages into IgM^+, IgD^+ naïve (antigen-inexperienced) B cells. Upon encounter with its cognate T cell-dependent antigen and receiving $CD4^+$ T cell help, the naïve B cell will clonally expand and differentiate either to extrafollicular plasma cell (PC), or it will enter a primary B cell follicle and differentiate to a highly proliferating centroblast. What determines exactly the differentiation pathway of the naïve B cell (extrafollicular PC versus GC B cell) is currently unknown, but in mouse models its was shown that B cells with a high affinity BCR will preferentially differentiate into PC[93].

After a few days of proliferation, the characteristic structure of the GC develops: a dark zone consisting almost exclusively of densely packed proliferating centroblasts, and a light zone comprised of smaller, non-dividing centrocytes situated within a mesh of follicular dendritic cells, T cells, and macrophages[94]. GC B cells can be distinguished from naïve B cells by the differential expression of multiple genes, but the most commonly used ones are the positivity for CD10 and CD77, lack of IgD, and the expression of the transcription factors BCL6 and activation-induced cytidine deaminase (AID).

Centroblasts diversify their IgV genes by SHM, and those cells that express newly generated high affinity antibodies are selected for survival in the light zone[94]. The centroblasts that do not succeeded to re-express a surface Ig or express a BCR with low affinity die by apoptosis. Some of the centrocytes undergo a second type of recombination, i.e. CSR, by which they switch their Ig class expression from IgM and IgD to IgG, IgA, or IgE. CSR requires the activity of AID and it is thought that CSR-inducing signals are transmitted to B cells by GC T cells that are present in the light zone. One of these signals is the CD40–CD154 (CD40-ligand) interaction that is responsible for the induction of AID and IRF4 expression[94]. The exact isotype to which the switch will occur is determined by the specific cytokines and co-stimulatory signals provided by the follicular T-helper cells (T_{FH}).

Finally the centrocytes differentiate into memory B cells or plasma cells (Fig. 4). Again it is still a question what governs the differentiation of a centrocyte into PC versus memory B cell, but it seems that the affinity of the newly expressed Ig plays a critical role, because in mouse models high affinity GC B cells are actively selected into the plasma cell compartment[95].

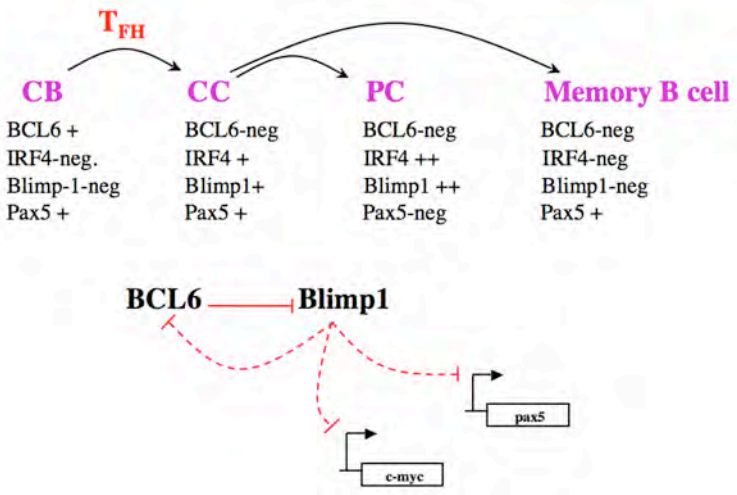


Figure 4. Changes in the expression of transcription factors during the differentiation of GC B cells into memory B cell or plasma cell.

PC differentiation is better understood than the memory B cell differentiation in terms of the mechanisms involved. PC differentiation occurs in a stepwise manner and the master key regulator of this process is the transcription factor Blimp-1[96]. Blimp-1 is responsible for the down-regulation of many of the mature B cell-specific genes (partially through the direct repression of the *PAX5* gene), and also for the growth arrest of the terminally differentiated PCs (through direct repression of the *C-MYC* gene)[97]. A negative regulatory loop exists between BCL6 and Blimp-1: in GC B cells BCL6 represses the expression of Blimp-1, while

in PCs Blimp-1 is repressing the expression of BCL6 (Fig.4). The balance between BCL6 and Blimp-1 can be tilted in the favor of Blimp-1 by multiple extracellular signals, most of which can be provided by the T_{FH} cells. Thus BCL6 expression can be down-regulated at the transcriptional level by the NF-κB-inducible IRF4[90], while BCR-activated signals result in the proteasome-mediated degradation of BCL6 protein[98]. In addition cytokines, such as IL-21, can induce the expression of Blimp-1 through the activation of STAT3[94,99].

Memory B cells are formed from activated B cells that are specific to the antigen encountered during the primary response. They are long-lived and can mount an enhanced response compared to the naïve B cells. Upon Ag re-encounter the memory B cells will preferentially differentiate to PC[100]. CD27 was identified as a marker of human memory B cells based on the finding that the CD27-positive PB B cells carry somatically hypermutated Ig variable genes[101]. Two major categories of CD27-positive memory B cells were identified: the class-switched, IgD-negative (i.e. IgG-, or IgA-positive) and the IgM-positive IgD-negative (also called "IgM memory B cells"). Besides the positivity for CD27, these two memory B cell types also carry somatically hypermutated Ig variable genes, with the notable difference that the class-switched ones have more mutations (6%) than the IgM memory (3%). Thus using IgM and CD27 together with a B cell marker, three populations of B cells can be identified among the human peripheral blood lymphocytes: the CD27-neg. IgM-pos naïve (making up about 60% of the B cells), the CD27-pos. IgM-neg. class-switched memory B cells (25% of the B cells), and the CD27-pos. IgM-pos. memory B cells (15% of the B cells)[102] (Fig. 5).

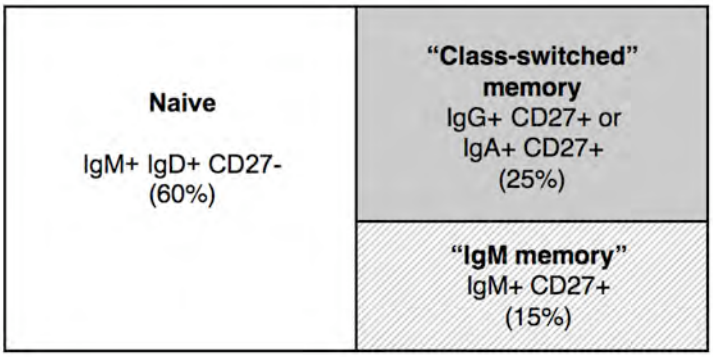


Figure 5. Normal distribution of the major B cell subpopulations in the human peripheral blood.

The CD27-positive Ig class-switched memory B cells are products of the GC reaction, as they are not present in immuno-deficient patients with mutations in genes involved in GC response (mutations in *CD40* or *CD40L* giving rise to hyper-IgM syndrome; mutations in

SH2D1A in XLP patients; mutations in *ICOS* in some common variable immuno-deficiencies, CVID)[102]. Conversely, the CD27-positive, IgM-positive memory B cells are present in the aforementioned immuno-deficient patients, and therefore they are believed to be generated independently of GC and undergone SHM during the generation of the preimmune repertoir. The IgM memory B cells are also believed to be involved in the responses to T cell-independent Ags.

1.4 EBV gene expression in normal B cells

In sharp contrast to the broad latent gene expression seen in LCLs, early work found that in the peripheral blood B cells of EBV-carriers the virus expresses only EBNA-1, LMP2, and EBER RNAs[103-105]. Today we know that the virus establishes a life-long infection in the class-switched memory B cell reservoir[106], from where it is believed to re-activate upon plasma cell differentiation and produce new progeny that are shed in the saliva[107]. The way in which EBV accesses the memory B cell pool is still not known.

The frequency of EBV-carrying B cells in the peripheral blood is 1-50 per 10^6 B cells and their frequency is stable over time (for at least 1–3.5 years).[108] In IM the frequencies of EBV-infected cells can range from 1 in every 2 memory B cells to 1 in >100 memory B cells[109]. The frequency of EBV-infected B cells in IM patients can be up to 1,000-fold greater than in healthy carriers. Interestingly, EBV infection in the blood of IM patients is tightly regulated as the phenotype of the latently infected cells is restricted to IgD-negative, sIg-positive, CD27-positive, CD5-negative, and CD20-positive, just as in the healthy virus carriers[109].

Similarly to the EBV gene expression seen in LCLs, type III latent B cells were found in healthy individuals during the primary infection[110,111] and the virus carrier state[112], but such cells are highly immunogenic and therefore are cleared by the emerging cellular immune responses[113].

In one of the studies performed on FACS-sorted tonsilar B cell subpopulations it was found that the type III latent B cells specifically segregated within the naïve B cell population[114]. Interestingly, in the same study if EBV resided within the GC B cells it expressed a type II latency, similarly to the memory B cells from the same tonsils[114]. Furthermore, the EBV-carrying memory B cells isolated from the PBMC did not express LMP-1 mRNAs and only rarely expressed LMP-2 mRNAs, and therefore shown to express a type I/0 latent viral gene expression pattern.

The switch from latent to lytic EBV infection is mediated by the immediate-early proteins BZLF1 and BRLF1[115]. Both proteins are transcription factors that activate each other's transcription and together are sufficient to activate the entire lytic viral gene expression cascade. Because in latently infected cells the promoters driving BZLF1 and BRLF1 expression are inactive, the activation of their promoters by cellular transcription factors is the crucial initial step required for lytic viral gene expression. BCR engagement activates lytic EBV gene expression in some B cell lines *in vitro* and also the immediate early EBV promoters in reporter gene assays[115].

Laichalk *et al.* showed that in the FACS sorted CD38-high, CD10-negative, CD19-positive, CD20-low, surface Ig-negative, and cytoplasmic Ig-positive PC in the tonsils of healthy carriers EBV gene expression switched from its latent state to the lytic replication, concluding that differentiation into PCs initiates viral replication[116]. This finding is supported by two recent publications that found that the plasma cell-specific spliced-variant of the transcription factor XBP-1, XBP-1s, binds to and transactivates the promoter of the immediate early lytic gene BZLF1[117,118].

Tying all these observations together Thorley-Lawson developed a model to explain how EBV accesses the memory B cell pool[107,119]. According to this model naïve B-cells are infected with EBV and express the type III pattern (Fig.6). In healthy individuals these cells are recognized and eliminated by the cellular immune response. Escaping B cells may enter and differentiate in the GCs of secondary follicles into GC B cells, and concomitantly switch their viral gene expression to type II (also called the “default program”). Because LMP-1 and LMP-2 were known to mimic the CD40 and BCR signaling, respectively, it was proposed that the LMPs provide the signals needed for the survival and differentiation of the EBV-carrying, type II latent GC B cells.

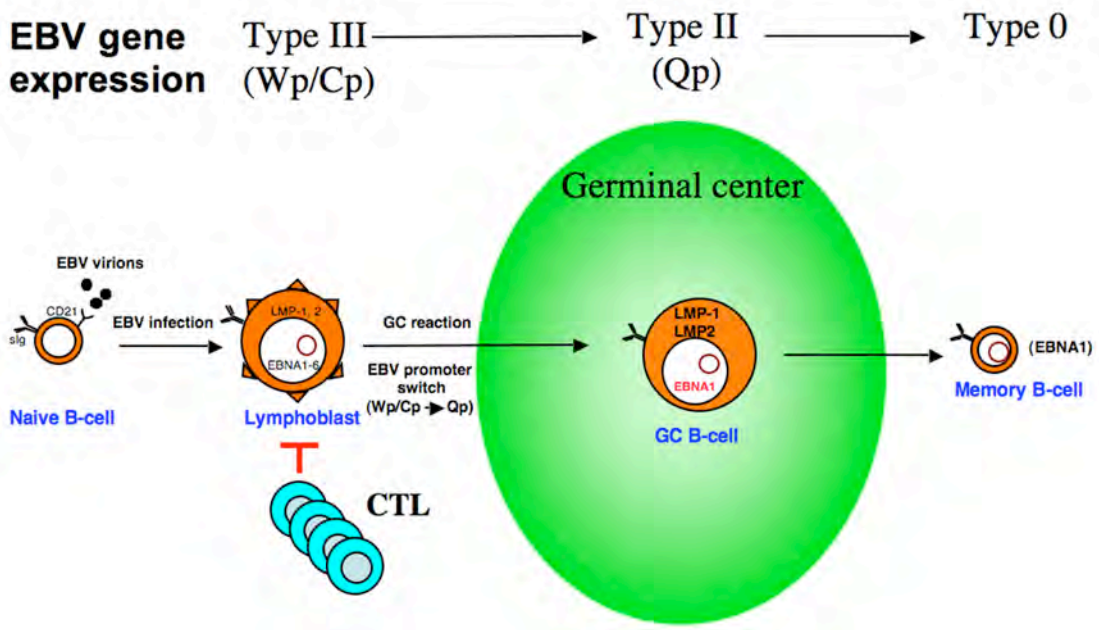


Figure 6. The model of GC-dependent origins of the EBV-carrying memory B cells with type I/type 0 latency.

Furthermore, the model proposed that when the EBV-positive GC B cells leave the GC environment the expression of EBV-encoded proteins would be down-regulated. In the resting memory B-cells the viral genome is retained, but only the EBERs are detectable (type 0 latency), unless the cells become stimulated and enter the cell cycle, when the expression EBNA-1 is induced (type I latency). Upon PC differentiation of the EBV-carrying memory B cells, EBV's lytic replication will be initiated and new virus progeny will be produced in the saliva, helping in its spreading.

Thorley-Lawson also proposed that based on the similarity in EBV latent gene expression between the B cell lymphomas and EBV-carrying normal B cells at different stages of differentiation, one can pinpoint the origin (the precursors) of the tumors (Fig.7A)[107]. Thus, because EBV-positive BL express type I latency and he found type I expression only in EBV-carrying normal memory B cells, he proposed that BLs might have arisen from memory B cells. Similarly, since cHLs express type II latency and he found similar EBV gene expression in GC B cells, his conclusion was that HRS cells arose from the type II GC B cells (Fig.7A). I would argue that such a simplistic view cannot be used to trace the "cell-of-origin" (i.e. the precursors) of EBV-carrying B cell-derived tumors, as the viral gene expression is flexible and can change during the transformation process or it can be influenced by signals from the microenvironment (Fig.7B).

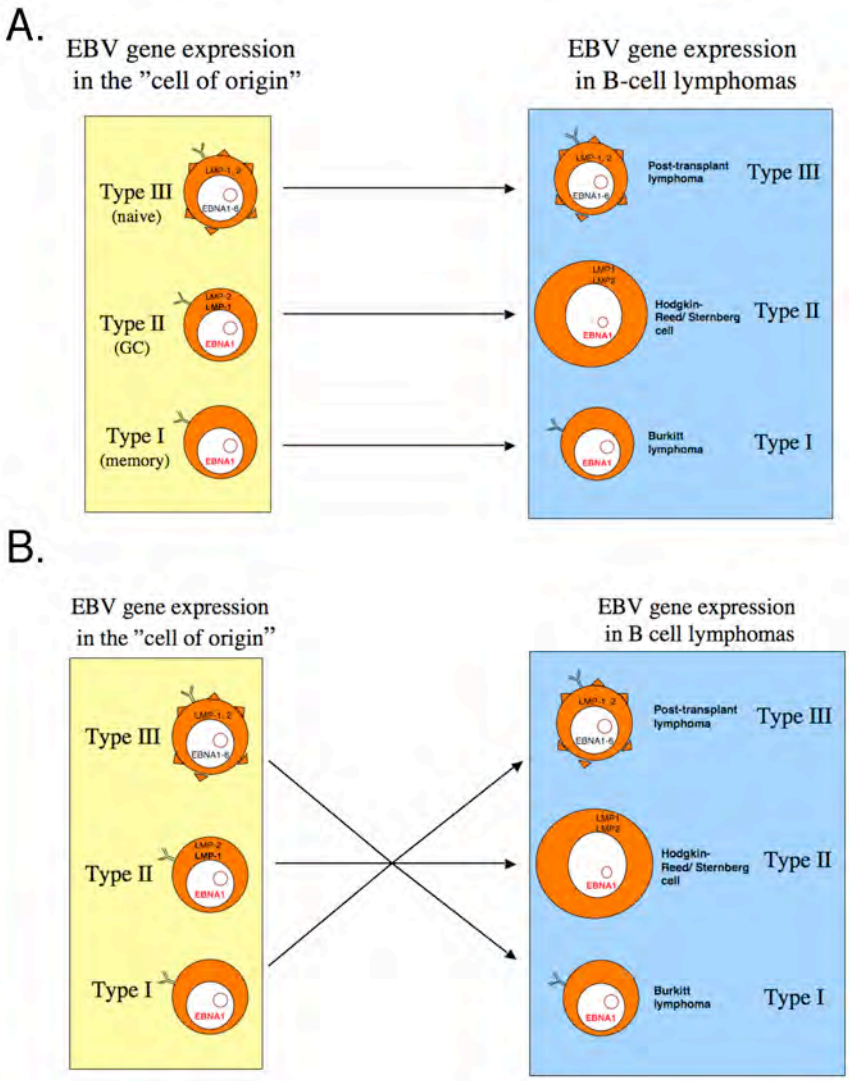


Figure 7. The origin of EBV-carrying B cell-derived lymphomas, based on the EBV latent gene expression in their presumed precursors. A). parallel EBV gene expression in normal B cells and EBV-carrying tumors, pinpointing the cell-of-origin, as proposed by Thorley-Lawson. B). changes in the EBV gene expression during lymphomagenesis.

The strength of the model is that it can potentially give an explanation for:

- Why is EBV specifically residing in the class-switched memory B cells and is excluded from the IgM^+ IgD^+ $CD27^+$ memory B cells? (because the formers are the products of a GC reaction and can be generated only through such a differentiation process)
- Why did EBV evolve to use a type III, B cell proliferation-inducing program? (by this mechanism EBV amplifies the pool of the infected naïve cells with diverse specificities for antigens, to increase the chance of encountering the cognate antigen, as this step is a prerequisite for the differentiation of the EBV-carrying B cell)

- How does EBV down-regulate its type III expression into a type 0/I restricted pattern, the latter being advantageous for its latent persistence? (the repression of Cp happens during the differentiation of naïve B cells to GC B cells and the memory B cells inherit this repressed Cp)
- Why do the GC B cells have active Qp? (after the repression of Cp during the naïve to GC B cell differentiation, the virus still needs the expression of EBNA-1 in order to maintain its viral genome in the proliferating GC B cells and Qp is turned on by default, as Qp is activated upon entry to the S-phase of the cell cycle)
- Why did some investigators found, while others did not find expression of Qp-derived EBNA-1 mRNA in the EBV-carrying memory B cells? (EBV is maintained in the absence of EBNA-1 in the non-proliferating memory B cells-type 0 latency; EBNA-1 is turned on only when these cells start to divide)
- Why do the majority of EBV-carrying B cell-derived lymphomas do not express type III latent gene pattern? (most of the B cell lymphomas originate from GC or post-GC B cells in whose progenitors if EBV was present it already expressed a restricted type I/II latency i.e. they already inactivated their Cp).

The weaknesses of the GC-dependent EBV persistence model are:

- The work investigated the expression of latent EBV genes at the mRNA level and no data was provided if this also occurs at the protein level. This is a major concern, especially with regard to LMP-1, for at least 4 reasons: 1) LMP-1 was shown to repress the expression of BCL6 *in vitro*[44], while in transgenic mouse models LMP-1 expression in B cells inhibited the GC development upon immunization[43,44], and therefore the expression of BCL6 (i.e. GC B cell phenotype) in normal B cells is incompatible with the expression of LMP-1. 2) LMP-1 was shown to induce the expression of miRNA-155[44] that is a negative regulator of the cellular gene AID[120] and by this it might interfere with the SHM and CSR processes. 3) In the new world of miRNAs there might be cellular or viral miRNAs that could prevent the expression of the protein products. In line with this latter assumption LMP-1 expression was shown to be modulated by three BART EBV-miRNAs[121]. 4) LMP-1 was shown to induce the expression of the chemokine receptor CCR7[122] that helps the B cells to remain in the extra-follicular zone, and therefore its down-regulation is needed for the naïve B cells to enter the follicules and differentiate into GC B cells.

- Does not take in consideration that the incoming virions would have greater chance to directly infect memory B cells found in the epithelial-subepithelial areas, rather than specifically infecting naïve B cells. In line with this assumption *in vitro* experiments showed that B-lymphocyte subpopulations are equally susceptible to EBV infection, irrespective of their Ig isotype expression[123].
- The role of the antigen and CD4+ T cell help is left out despite the common knowledge that they are needed for the B cell differentiation and despite their own findings that the EBV-carrying memory B cells have similar pattern of IgV mutation (but with more R mutations and higher R/S in their CDRs) rate as the normal, EBV-negative memory B cells[124], the latter findings arguing for a role of antigen in the selection process.
- Much of the differentiation processes in the model are believed to be EBV driven, while such differentiation does not spontaneously occur in the *in vitro* EBV-infected B cell cultures. Especially the role of the LMP-1- and LMP-2-signalling is overemphasized as contributors to the survival of the EBV-carrying GC B cells. If this would be the case one would expect to observe in healthy virus carriers EBV-carrying surface Ig-negative, "crippled" memory B cells that are saved from apoptosis by LMP-1 and LMP-2, and this is not seen.
- Does not provide explanation why the EBV-carrying GC B cells in healthy virus carriers are very rare, contrary to the common knowledge that GC are oligoclonal in their nature.

The alternative model proposed by Küppers posits that EBV-directly infects the GC or memory B cells without the requirement of GC differentiation of the EBV-carrying naïve B cell ("GC-independent model")[84,111,125]. This model is based on the observations of Kurth *et al.* who micro-dissected EBV-positive GC B cells from the tonsils of IM patients and studied the SHM of their rearranged Ig V genes[84,111]. Interestingly, they found that most of the EBV-carrying GC B cells belonged to clones of B cells harboring somatically mutated Ig V gene rearrangements, but ongoing SHM (the hallmark of the GC reaction) was not present in these B cell clones[84,111].

Further support for the "GC-independent model" was provided by two studies on EBV persistence in immuno-deficient patients whose B cells cannot undergo a GC differentiation and therefore lack conventional, class-switched memory B cells. Namely, Conacher *et al.* studied the EBV infection in patients with X-linked hyper-immunoglobulin M (hyper-IgM)

syndrome due to mutation in *CD40L* gene[126], while more recently Chaganti *et al.* studied XLP patients who survived the primary infection with EBV[127].

In the first study EBV DNA could be detected in peripheral blood B cells of patients suffering from hyper-IgM syndrome, and in the only case analyzed in more details it was shown that the infection was restricted to the small population of non-classical, IgD^+ $CD27^+$, GC-independent memory B cells[126]. Interestingly, when the EBV latent gene expression was analyzed in this B cell population the expression of EBERs, LMP-2 and EBNA-6 could be detected, suggesting type III latency, while in another case only EBERs and LMP-2 were expressed[126]. Analysis of EBV DNA in serial samples showed variability over time, suggesting cycles of infection and loss. These authors concluded that short-term EBV persistence could occur in the absence of a GC reaction and a classical memory B cell population[126].

In the second study the authors found that in XLP patients the circulating EBV load was concentrated within a small population of IgM^+ IgD^+ $CD27^+$ (non-switched) memory cells rather than within the numerically dominant naive IgM^+ IgD^+ $CD27^-$ or transitional $CD10^+$ $CD27^-$ subsets[127]. Furthermore, in 2 prospectively studied patients, the circulating EBV load was stable and markers of virus polymorphism detected the same resident strain over time[127]. As mentioned earlier the IgM^+ IgD^+ $CD27^+$ (non-switched) memory cells are believed to develop through a GC-independent differentiation pathway. Therefore studies in immuno-deficient patients who cannot mount a GC reaction, and as such lack conventional class-switched memory B cells, can be regarded as supportive evidence for the GC-independent persistence of EBV. Unfortunately, the EBV latent gene expression was not studied in this latter study[127], and one cannot be sure that it is similar to the expression seen in healthy EBV carriers. Furthermore, studies on EBV persistence in immuno-deficient states have to be interpreted with precaution, because the results are generated in an immuno-deficient host and therefore cannot be directly translated to the EBV healthy carrier state. EBV might adopt a different persistence strategy in the aforementioned patients that is not at all or not predominantly used in the healthy carriers.

At least two observations exist to which the GC-independent EBV persistence model did not provide explanation. Firstly, how is it possible for EBV to express a restricted (type I/type 0) latent gene expression upon direct infection of GC or memory B cells, when *in vitro* experiments showed that when EBV infected B cells it always expressed a type III latency, irrespective if the target B cell was a naïve, GC, or memory B cell? Though the possibility exists that EBV will directly express a type I or type 0 latent gene expression upon entry in a

B cell, the obvious contra-argument for the importance of such a mechanism for the persistence of EBV is that the type III, proliferation-inducing gene expression would be not needed and the encoding genes should have been lost from the EBV genome. But as we know this is not the case and the type III latent genes and the B cell proliferation capacity are conserved in other LCVs as well. Secondly, why is EBV predominantly found in the class-switched memory B cells, which carry the genotypic signs of a GC-dependent SHM and CSR, in healthy virus carriers?

1.5 EBV gene expression in tumors

1.5.1 Burkitt lymphoma

BL is a mature B cell tumor with typical clinical picture and histopathology. Based on their immunophenotype resembling GC B cells (IgM^+, $CD20^+$ $BCL6^+$, $CD10^+$, $BCL2^-$), genotype carrying rearranged Ig genes with SHMs in the V regions, and the structure of Ig-myc translocations (arising as a mistake of SHM or CSR), BL cells are believed not just to originate from GC B cells but also to be arrested in their differentiation at this stage[128,129]. As GC B cells do not express c-myc, it seems that c-myc expression in BL is not only deregulated, but is also ectopic[130].

Constitutive activation of c-myc, due to a reciprocal chromosomal translocation between chromosome 8 and either chromosome 14, 2 or 22, is the driving force for proliferation. In BL cells the expression of myc oncogene (chr. 8) is constitutive due to its regulation by the juxtaposed immunoglobulin (heavy, chr. 14 or light chains, chr. 2 or 22) regulatory elements[128,129].

Based on differences in clinical presentation, morphology, and biology, 3 clinical variants of BL are recognized: the endemic BL, the sporadic BL, and the AIDS-associated BL. EBV is present in almost all endemic BLs, in 30% of sporadic, and 25-40% of AIDS-BL. Independently of the clinical variant of BL, if EBV is present in the malignant BL cells, it generally expresses only the EBNA-1 protein, thus being type I latent[74]. As already mentioned, other viral gene expression patterns were also seen in BL, such as the "Wp-restricted" latency[89], but also some endemic BL cells were found to be LMP-1-positive, EBNA-2-negative in variable proportions of tumor cells in two cases[131], and EBNA2 was detected in some tumor cells in three other cases, pointing to a more heterogeneous EBV gene expression in BLs[131]. LMP-1-positive, EBNA-2-negative BLs were reported by others as well (see Table II).

Araujo *et al.* in 1996 described two interesting examples of LMP-1-positive, EBNA-2-

negative BL cases[132]. These authors investigated 54 BL cases from Bahia (a tropical region of Northeast Brazil) and found that 87% of the Brazilian BL cases harbored EBV (compared to the 20% of control German cases). All the BL cases expressed type I latency (EBER+, EBNA-2-, LMP-1-), except for two cases, where occasional LMP1-containing tumor cells were found in the neighborhood of small Schistosoma mansoni granulomas and scars[132]. Because chronic schistosomiasis is associated with elevated Th2 cytokine expression resulting in reduced cell-mediated cytotoxicity, the authors speculated that the inability of the cell-mediated cytotoxicity to delete LMPl-expressing cells might have allowed BL cells to acquire an LMP1-positive phenotype[132]. The unlikely scenario that *S. mansoni* directly influences EBV gene expression could not be ruled out either.

After the discovery of type I EBV gene expression in BL it was assumed that this EBV gene expression was the result of immune selection *in vivo*, as the BL cells with type III latency would have been eliminatied by the EBV-specific CD8+ T cells. But because BLs are more common in HIV-infected patients than in other forms of immunosuppression (appearing early in the progression of HIV infection when the CD4+ counts are still high) and they express type I latency even under this condition, the EBV type I expression as a result of immune selection can be dismissed. A more probable explanation for the type I gene expression of BL is their GC B cell phenotype, that imposes this latent viral gene expression (discussed later).

The role of EBV in the pathogenesis of BL is still a mystery. Though comprehensive gene expression profiling studies of EBV-negative and EBV-positive BL are still lacking, genetic studies pointed to a probably different pathogenetic mechanism of the two BL subtypes[133]. More specifically, it was demonstrated that BL cells from patients with EBV-negative tumors (mostly sporadic BL) had lower numbers of somatic mutations in their Ig heavy chain V genes, and lacked ongoing mutations and signs of antigen selection, compared to BL cells from patients with EBV-positive tumors (mostly endemic and HIV-related BL)[133]. Based on these data it was suggested that EBV-negative BLs derive from early centroblasts, whereas EBV-positive cases derive from late or post-germinal center B cells[133].

EBV's contribution to the genesis of BL is difficult to understand because of its type I, non-proliferation-inducing, gene expression in this tumor. Still, EBV-encoded latent genes (EBNA-1, EBERs, EBV miRNAs) could contribute to the development of malignancy through other mechanism than inducing B cell proliferation, such as inhibition of apoptosis, inhibition of DNA repair, induction of genomic instability. *In vitro* anti-apoptotic functions were shown for the non-coding EBERs[134] and the EBNA-1 protein[135], while EBNA-1 was

reported to promote genomic instability *in vitro*[136]. Inhibition of EBNA-1 by a dominant-negative EBNA-1 mutant resulted in reduced *in vitro* and *in vivo* proliferation of type I BL lines[135,137]. Furthermore, EBV's effect might be imprinted at an earlier stage of the transformation, when the EBV-carrying cell still expressed its type III gene expression, as proposed for the epigenetic silencing of the tumor suppressor gene Bim by EBNA-3 and EBNA-6[138]. The different anti-apoptotic functions of EBV are believed to contra-balance the pro-apoptotic functions of the deregulated c-myc in the BL precursor B cells.

1.5.2 Classical Hodgkin lymphoma

Classical Hodgkin lymphoma (cHL) tissue contains the characteristic mononuclear Hodgkin and multinucleated Reed-Sternberg (HRS) cells that make up about 1% in the population of non-neoplastic, inflammatory cells. In the majority of the cases HRS cells are derived from B cells, as shown by their Ig gene rearrangements. During their malignant transformation they down-regulated the expression of several B cell-specific genes ("loss of B cell identity")[139] and express an immunophenotype that does not resemble any normal hematopoietic cell (negativity for surface and cytoplasic Ig, CD20, CD45, J-chain, BCL6, Blimp-1, PU.1, Oct2, and positivity for CD30, CD15, IRF4).

Based on their high load of SHM in the IgV genes, HRS cells are thought to be derived form pre-apoptotic, GC B cells[140,141]. Part of the HRS cells was found to have non-functional Igs due to aberrant SHM and deletions in the rearranged Ig genes[141]. Because the HRS precursor GC B cells probably do not express a functional BCR, they should die by apoptosis. Several mechanisms have been proposed that enables these cells to evade apoptosis, EBV, through its latent gene expression, being one of the possibilities. Constitutive activation of the NF-κB and JAK-STAT pathways are considered to be critical for the survival of HRS cells[142]. Reciprocal interaction between the HRS cells and the inflammatory cells in the tissue, through cytokines and adhesion molecules, seems also to be important for the survival of these cells.

The frequency of EBV-positive HL cases varies between 40-100% depending on the geographical distribution, histological subtype, and immune status of the host[143]. Interestingly, all cHLs arising in AIDS patient carry EBV. Defined by in situ analysis, the EBV latent expression pattern in the HRS cells is type II. The EBV-carrying HRS cells always express LMP-1 protein, while the expression of LMP-2A protein was found only in 52-90% of the cases[144,145]. As mentioned, both LMP-1 and LMP-2A are believed to provide survival signals to B-lymphocytes by mimicking the externally activated CD40 and BCR

pathways, respectively. Therefore it was proposed that in the EBV-positive cHL cases expression of these viral proteins could be responsible for the survival of surface Ig-negative GC B cells, the putative precursors of HRS cells.

HRS cells are dependent on survival signals received from other cells as indicated by the difficulty to grow them in culture and in immunodeficient mice, by the observations that HRS cells are rarely found in the peripheral blood and even when they metastasize into non-lymphoid tissues they are embedded in their typical microenvironment[142]. The key players in providing these pro-survival factors are the inflammatory cells recruited by the HRS cells. The rosetting CD40L-expressing CD4+ T cells could activate the HRS cells through their CD40 receptor[146]. Furthermore, infiltrating neutrophils could activate the TACI- and BCMA-receptors on the HRS cells through production of their ligand APRIL[147], while the CD30L-expressing mast cells and eosinophils could engage the CD30 receptor on the HRS cells[148].

HRS cells secrete CCL5 (RANTES), CCL17 (TARC)[149] and CCL22, which attract T_H2 cells and Treg cells[150,151]. The secretion of IL-5, CCL5, CCL28, and granulocyte–macrophage colony-stimulating factor by HRS cells presumably causes the recruitment of eosinophils into the Hodgkin's lymphoma microenvironment. As HRS cells secrete CCL5 and IL-8, these chemokines could be responsible for the attraction of mast cells and neutrophils, respectively. Chemokines may not only be involved in the attraction of other cells into the lymphoma microenvironment but could also have direct effects on HRS cell survival and proliferation.

HRS cells also produce several immunosuppressive cytokines, such as IL-10, TGFβ, galectin 1[152,153], and prostaglandin E2[154]. Furthermore, recent studies identified the expression of programmed cell death protein 1 (PD-1) ligand in HRS cells that through its interaction with the PD-1 on T cells inhibits the effector functions of T cells[155,156]. The reactive T cells in cHL cases are predominantly positive for T-bet, the master factors regulating induction of T_H1 differentiation[157]. The T cells in cHL include cells expressing IL-2, IL-10, IL-13, IFNγ, TGFβ1,2, and FOXP3, and can be regarded as having a Th2-like or immunoregulatory phenotype. Furthermore, the T cells immediately surrounding the HRS cells are CCR4+, GITR+, and CD25+[158].

Recent IHC studies of cHL biopsies and microarray analysis of microdissected cells revealed that HRS cells express the cysteinyl leukotrienes (CysLT) 1 receptor. Its ligand, LTD(4), stimulated protein release of TNF, IL-6, and IL-8 in HL-derived cell lines. As the HRS cells are surrounded by CysLT-producing eosinophils, macrophages and mast cells,

these results suggest that CysLTs might be mediators in the pathogenesis of cHL by contributing to the aberrant cytokine network of this malignancy[159].

Search for EBV-regulated genes with potential implications for the transformation and survival of HRS precursors identified autotaxin (that increased the generation of lysophosphatidic acid and led to the enhanced growth and survival of HL cells *in vitro*)[160], the polycomb group protein Bmi-1 (up-regulated by LMP-1 in EBV-negative HL cells and needed for their *in vitro* survival)[161], the TGF-β target gene protein tyrosine phosphatase receptor kappa (down-regulated by EBNA-1 and acting as an inhibitor of survival and proliferation in the EBV-positive KMH2 cells when overexpressed)[162], and CCL20 (MIP-3α) (induced by EBNA-1 and potentially responsible for the recruitment of the CCR6+ regulatory T cells in the HL tissue)[163].

Recent work by Chetaille *et al.*, studying the gene expression of EBV-positive and EBV-negative cHL cases, found that the EBV-carrying tissues could be distinguished from the EBV-negative ones by a gene signature characteristic of Th1 and antiviral responses[164]. Furthermore, tumors with high percentage of either TIA-1-positive reactive cells or topoisomerase-2-positive tumor cells had bad prognosis, whereas high numbers of reactive cells positive for BCL11A, FOXP3, or CD20 had a favorable influence[164].

1.5.3 Post-transplant lymphoproliferative disorders

Post-transplant lymphoproliferative disorders (PTLD) comprise a spectrum of B cell lymphoproliferations, ranging from early EBV-driven polyclonal proliferations resembling IM to monoclonal B cell lymphomas, which develop as a consequence of immunosuppression in recipients of solid organ, bone marrow (BM) or hematopoietic stem cell (HSC) allograft[165]. The most important risk factor for EBV-driven PTLD is EBV seronegative status at the time of transplantation. Furthermore, the frequency of PTLD correlates in part with the intensity of immunosuppression, such as adult patients receiving renal allograft have the lowest frequency of PTLD (<1%), while those receiving heart-lung/lung allograft have the highest frequency (5% or greater). BM or HSC allograft recipients in general have a low risk of PTLD (about 1%).

Early lesions often arise within the first 2 years in the oropharynx or lymph nodes, are nearly always polyclonal, and usually contain multiple EBV infection events. EBV gene expression in such B cells is type III. The early lesions tend to regress with the reduction of immunosuppression.

Polymorphic PTLDs (P-PTLDs) develop predominantly in extranodal sites (including the allograft) and in contrast to early PTLD lesions, show effacement of the underlying tissue architecture. Clonal Ig rearrangements and clonal EBV episomes are common. Comparative genomic hybridization studies detected chromosomal abnormalities/imbalances[166]. EBV gene expression is generally type III and the P-PTLDs regress in variable proportion upon reduction in immunosuppression. Interestingly, up to 75% of the P-PTLDs had somatically hypermutated Ig variable genes and 20.0% of them carried crippling mutations in their Ig genes, precluding expression of a functional BCR[167]. Immunohistochemistry showed detectable expression of Ig light chains in only 41.9% of PTLDs, suggesting that a large fraction of PTLDs arise from GC-experienced B-cells that display impaired BCR[167].

Monomorphic PTLDs (M-PTLDs) fulfill the criteria for a B cell lymphoma (or more rarely T/NK lymphoma). They are monoclonal transformed B lymphocytic or plasmacytic proliferations, generally of diffuse large B cell lymphoma (DLBCL) type, or less commonly of BL or of plasma cell neoplasm. As envisaged based on the spectrum of B cell lymphoma types in this PTLD category, EBV gene expression also varies considerably, and can be either type I, type II, or type III.

The fourth subtype of PTLD is the cHL type and is the least common. Interestingly, it occurs most often in renal transplant patients and is always EBV-positive, type II.

Two additional notes on PTLDs:

- up to 20-30% of PTLDs can be EBV-negative and they occur generally late after transplantation[168,169],
- when EBV is present in PTLDs its latent gene expression is not always type III.

1.5.4 NK and T cell lymphoma

Several NK or T cell-derived lymphomas can harbor EBV. These EBV-positive malignancies/lymphoproliferations are: the extranodal NK/T cell lymphomas, nasal type, the aggressive NK cell leukemias, and the EBV-positive T cell lymphoproliferations of childhood.

The extranodal NK/T cell lymphomas, nasal type (known also as “lethal midline granuloma”), are characterized by a very strong association with EBV (basically always EBV-positive), by an angio-infiltrative and angio-destructive histopathology, by an increased prevalence in Asians and native Americans in Central and South America[170,171]. Immunophenotypically the malignant cells express CD2, CD56, cytoplasmic CD3ε, cytotoxic molecules (such as granzyme B, perforin, TIA-1) but not surface CD3[170,171]. Most of the

tumors genotypically resemble NK cells (i.e. have their T cell receptor genes in germline configuration), while a minority seems to be derived from CTL. Therefore these tumors are named "NK/T" in order to include the malignancies with both NK and T cell origin, but should not be interpreted as meaning a CD3+, CD56+ NK-T cell origin.

EBV gene expression is type II, as the malignant cells express Qp-derived EBNA-1, LMP-1 and LMP-2, but no EBNA-2 mRNAs[67,68]. By IHC LMP-1 protein expression is heterogeneous within the tumors[68], while LMP-2 protein expression was not investigated.

Extranodal NK/T cell lymphomas contain a heavy inflammatory infiltrate, composed of lymphocytes, macrophages, eosinophils, plasma cells. The role of the non-malignant infiltrate in the pathogenesis of the tumor is not known and could be either part of a anti-tumor immune response or, as already discussed for cHL and NPC, it could be actively recruited by the malignant cells to be used for their survival and proliferation.

1.5.5 Nasopharyngeal carcinoma

NPC was the first epithelial tumor associated with EBV infection[172]. It originates from the lateral walls of the nasopharynx, especially from the fossa of Rosenmuller and Eustachian tube cushions, and it has a remarkably distinctive ethnic and geographic distribution. NPC is a rare malignancy in Caucasians from North America and Europe (incidence is under 1 per 100,000 persons per year), while Southern Chinese, especially those of Cantonese origin, have the highest incidence (about 25-30 per 100,000 persons per year)[173].

Histopathologically 3 types of NPC are distinguished by the World Health Organization classification based on the degree of differentiation: the type I NPC that is keratinizing squamous cell carcinoma, the type II non-keratinizing carcinoma, and the type III undifferentiated carcinoma. The undifferentiated carcinoma has a prominent lympho-plasmacytic infiltrate, and therefore is also called as "lymphoepithelioma". EBV is present in almost all WHO type III NPCs found in endemic regions, but is absent in WHO type I NPCs from non-endemic regions.

EBV expresses a type II latency pattern in the NPC cells[60,61,174,175]. As already discussed the definition of type II latency still requires improvements. It is important to note that even if EBV is present in all the undifferentiated NPCs, the expression of LMP-1 protein, as studied by IHC, ranges from 20-60%[176]. Furthermore, among those NPCs that are LMP-1-positive, the staining pattern is highly heterogeneous and sometimes only detected in a small subpopulation of the malignant cells. The non-expression of LMP-1 in the EBER-positive NPCs could be attributed to the CpG methylation of the LMP-1-promoter[177].

LMP-2A protein expression is detectable by IHC in about 45% of NPC and tends to be mutually exclusive with the expression of LMP-1[178,179]. Interestingly, antibodies to LMP-2A and LMP-2B were present in virtually all NPC patients and were very specific to NPC when compared to patients with BL, HL, gastric carcinoma, oral hairy leukoplakia, or other head and neck tumors[180].

There are 3 important etiological factors recognized in the pathogenesis of NPC: the genetic factors, the environmental factors, and EBV.

Interestingly, while EBV is present in the high-grade dysplastic lesions and in the developed carcinomas, it is absent in the low-grade dysplastic lesions[181]. In NPC the tumour cells carry monoclonal viral genomes, which indicates that EBV infection must have occurred prior to expansion of the malignant cell clone[181,182]. Taken together with the finding that allelic deletion of chromosome 3 and 9 are present at a significant percentage in nasopharyngeal epithelium of seemingly healthy Cantonese adults[183,184], it seems that EBV infection takes place in an already genetically altered nasopharyngeal epithelium that might help in the establishment of a latent EBV infection, and thereafter the latent genes expressed by EBV might favor the expansion of the malignant clone[35].

As mentioned the characteristic feature of most undifferentiated NPCs, besides its EBV association, is the presence of an intense lymphoid stroma, consisting predominantly of CD4+ and CD8+ T cells. It is believed that the interaction between tumour cells and lymphocytes might be crucial for the continued proliferation of these malignant cells[185,186]. Busson *et al.* found that the C15 nude mice transplantable NPC tumor produced IL-1α. The authors hypothesized that the IL-1α produced by the malignant epithelial cells *in vivo* could stimulate the development of the pronounced T cell infiltrate observed in NPC tumors[185].

Later Agathanggelou and co-workers investigated the expression of immune regulatory receptor/ligand pairs by IHC in biopsies of 20 EBV-positive undifferentiated NPCs, out of which 6 cases expressed LMP-1[186]. CD70 and CD40 were expressed by the malignant cells in 16 and 18 cases, respectively. Interestingly, the infiltrating lymphoid cells expressed CD27, the receptor of CD70, and the CD40 ligand[186]. Furthermore, tumor cells of 5 cases expressed at least one member of the B7 family (CD80, CD86, and B7-3) and many lymphoid cells expressing the corresponding counter-receptor, CD28, were detected in all cases. The expression of B7 correlated with the expression of LMP-1. These results indicated that T cells and carcinoma cells communicate in the microenvironment of undifferentiated NPCs, and suggested that the presence of a lymphoid stroma may be a requirement for the growth of these malignant cells[186].

Thinking along the same lines, Sbih-Lammali *et al.* found that NPC not only that expressed CD40, but activation of CD40 with its ligand protected NPC cells form the CD95-induced apoptosis *in vitro*[187]. Furthermore, most NPC cells expressed the interferon-inducible chemokine IP-10 (CXCL10) and numerous CXCR3-positive lymphocytes were detected in the lymphoid stroma, raising the possibility of a Th1-predominant immune response in these cases[188]. Rare NPC tumors also expressed IL-6 (3 of 43 cases) and IL-8 (2 of 40 cases), while there was no detectable expression of IL-10 and TARC[188].

Furthermore, NPC patients have significant expansions of circulating naïve and memory CD4+CD25 (high) regulatory T cells (Treg), which overexpressed Foxp3 and GITR, and demonstrated enhanced suppressive activities against autologous CD4+CD25- T-cell proliferation in functional studies[189]. Abundant intraepithelial infiltrations of Treg with very high levels of Foxp3 expression and absence of CCR7 expression were also detected in primary tumours[189].

As shortly reviewed above, the expression of multiple receptor-ligand pairs have been detected in the tumor and infiltrating lymphoid cells in NPC, pointing to the possibility that this interaction is specifically established and used by the tumor cells for their growth and survival advantage.

1.5.6 Other EBV-related tumors

Besides the well-known malignancies discussed above there are a number of EBV-associated lymphoproliferative disorders that are "neglected" and rarely mentioned. These are either rare or newly emerging entities, such as the EBV-positive DLBCLs of the Elderly (also known as "Age-related or Senile EBV-associated B cell Lymphoproliferative Disorders)[190,191], the DLBCLs Associated With Chronic Inflammation (Pyothorax-associated Lymphoma)[192,193], and lymphomatoid granulomatosis[194]. In the limited number of studies that investigated the EBV latent gene expression they showed EBER-positive, EBNA-2-positive, LMP-1-positive (type III) viral gene expression in most, but not all, of the cases.

Another interesting group of EBV-associated lymphoproliferations are the ones in which the malignant cells are of T cell origin and do not harbor EBV, but the tumors may contain monoclonal/oligoclonal B cell proliferations that most of the time carry EBV. The two malignancies with such characteristics are the angioimmunoblastic T cell lymphomas (AITL)[195,196] and peripheral T cell lymphomas, not otherwise specified (PTCL-NOS)[197-199].

EBV plays also an important role in the lymphoproliferative disorders associated with many congenital immunodeficiencies, such as Wiskott-Aldrich syndrome (WAS), CVID,

ataxia telangiectasia (AT), severe combined immunodeficiency (SCID), and XLP[200].

1.6 Regulation of the EBV latent gene expression

At first glance it seems that the multiple EBV latent gene expression patterns seen in the *in vitro* established cell lines and tumours is almost incomprehensible. Still certain rules and specificities were learnt during the more than 40 years of EBV research, shortly summarized thereafter.

First of all there is a clear tissue specificity of the EBV latent gene expression, such as type III latency can only be seen in B cells or B cell-derived lymphomas (therefore all the EBV-positive NK, T, or epithelial malignancies can have only type I or type II expression). The direct proof for the B cell specificity of the type III program came from *in vitro* somatic cell hybrid experiments, exemplified by the fusion of an type III LCL (KR4) and an EBV-negative epithelial cell line (HeLa)[201]. The resulting stable hybrids (KH-1 and KH-2) did not express EBNA-2 any longer, while the expression of EBNA-1 was maintained (LMP-1 expression was present, but low)[201]. The loss of EBNA-2 expression was paralleled by the down-regulation of B cell-specific proteins, such as total Ig, CD19, and CD20[201]. Furthermore, S1 nuclease protection analysis provided evidence that the Cp-derived RNAs were not expressed in the KR4-HeLa hybrids, in contrast to the parental KR4 LCL[91]. These experiments showed that the Cp activity is linked to the B cell phenotype (which in turn means B cell-specific transcription factors).

The B cell-specificity of the Wp was shown in similar cell fusion experiments between the EBV-positive, Wp-user BL lines Daudi or P3HR-1 and the EBV-negative human erythroleukaemia cell line K-562[201]. Similarly to the KH-1 and KH-2 hybrids, the resulting DUTKO and PUTKO hybrids did not express the B cell-specific proteins (total Ig, CD20, CD19) and down-regulated the expression of EBNA-3, -4, -6 expression[201]. The down-regulation of EBNA-3, -4, and -6 expression was due to the repression of the Wp[91]. Similar down-regulation of EBNA-3 was reported in the cell fusion experiments between the Daudi and HeLa cells that was paralleled by the down-regulation of expression of Ig κ, Ig μ, CD19, CD20, CD10, HLA cl. II, Oct-2, OBF-1, PU.1, NF-κB, and E47 [202]. Later the B cell specific activator protein (Pax5) was shown to bind to sequences in the Wp[203] and mutations of one of the Pax5-binding sites in the context of the EBV genome abolished its B cell immortalization capabilities[204].

The conclusion from the cell fusion experiments is that the Cp and Wp are B cell specific and if a type III B cell looses its B cells phenotype (in this case through a cell fusion

with non-B cells) the type III gene expression collapses. Another way by which B cells, at least partially, can lose their B cell phenotype is during the physiological process of PC differentiation (discussed previously in more details). In line with the assumption that during the PC differentiation of a type III latent B cell the Cp/Wp would be inactivated/inhibited and the expression of EBNA-2, -3, -4, -5, -6 down-regulated, Wendel-Hansen *et al.* already in 1987 reported that in LCLs a minority of cells were EBNA-negative, contained abundant cytoplasmic immunoglobulin, and were largely non-proliferating[205]. Later Rochford *et al.* reported that when LCLs were transplanted in SCID mice, 3-6 weeks later the cells exhibited predominantly plasmacytoid phenotype (decrease in CD23, CD11a, and CD58 expression, and increase in CD38, CD54, and HLA class I)[206]. Two-color FACS analysis showed that while the predominant population (> 80%) in LCLs was CD23hi/CD38lo, the major population in the LCL-derived tumors recovered from the mice was CD23lo/CD38hi[206]. Furthermore, ribonuclease protection assay on tumor cells showed a reduction in mRNA for the EBNA-2 and LMP-1[206]. Similar PC differentiation and down-regulation of the EBNA-2 and LMP-1 expression was observed in the B cell lymphoproliferations emerging after the transplantation of EBV-seropositive PBMCs in SCID mice (hu-PBL-SCID tumors)[207]. Another *in vitro* study using the IL-6-sensitive, IgG-positive LCL CESS as a model to study the PC differentiation also concluded that when the IL-6-treated cells differentiated into cIg-positive, non-proliferating PCs, the expression of EBNA-2, EBNA-1, and LMP-1 was down-regulated (in the absence of lytic replication)[208]. Co-culture of CD4+ T cells with LCLs also resulted in their PC differentiation with concomitant down-regulation of LMP-1, LMP-2A, EBNA-1, EBNA-2, and EBNA-6 mRNAs, and no signs of lytic replication[209]. These PC cell differentiation and change in EBV gene expression were dependent on cell-cell contact and could not be inhibited by neutralizing anti-CD40L antibodies[209].

In summary, the type III EBV latent gene expression in a B cell (driven by the Cp) can be inhibited by inducing its PC differentiation.

Besides the aforementioned studies in which PC differentiation resulted in down-regulation of type III EBV expression, Pokrovskaja *et al.* published the only study that succeeded in modulating the type III latent gene expression.[210] These authors showed that CD40 crosslinking on LCLs led to a partial downregulation of EBNA-2, EBNA3-6 and LMP-1. These changes in the viral gene expression were paralleled by the downregulation of Cp promoter activity and upregulation of the GC marker CD77[210].

A more puzzling question than the type III EBV program is the expression of restricted

type I or type II EBV latency in B cells or B cell-derived tumors (in the absence of PC differentiation). A typical model to study this question is the EBV-positive, type I BLs and derived cell lines. As mentioned, when EBNA-1 is expressed alone then the promoter that drives is expression is located in *Bam*HI Q-region (the Q-promoter)[211]. The Qp is a TATA-less promoter with architecture similar to the promoters of housekeeping genes, suggesting that Qp may be a default promoter that ensures EBNA-1 expression in cells that cannot express the full viral latency program[69].

Qp is not transcribed in type III B cell lines. Furthermore, in transient transfection experiments with reporter vectors Qp was active in EBV-negative epithelial and BL lines, and in EBV-positive type I BL lines, while it was repressed in type III BL lines and LCLs (irrespective if they used Wp or Cp). Two low-affinity EBNA-1 binding sites were identified in the Qp, just down-stream of the transcription initiation site[212]. Later it was shown that EBNA-1 could repress the Qp through the aforementioned two binding sites[213]. More specifically, transient transfection of EBNA-1 together with a Qp-reporter vector in the EBV-negative BL line Loucke resulted in the inhibition of Qp-activity, while EBNA-1 could not inhibit the activity of the Qp-reporter when the EBNA-1 binding sites were deleted[213]. At first glance it seems that EBNA-1 is suppressing its own expression in type III cells, but put in a different way the Cp or Wp represses the Qp, using EBNA-1.

Besides EBNA-1, the transcription factor interferon regulatory factor 7 (IRF7) and IRF2 could repress the Qp in transient transfection experiments through an ISRE (interferon-regulated response element)-like sequence located at position -5-18 to the Qp initiation site[214,215]. Because LMP-1 was shown to induce the expression of IRF7 and by this IRF7 is highly expressed in type III cells[216], IRF7 was proposed to be responsible for the repression of Qp in type III latency. However, mutations in the IRF binding sites partially inhibited Qp activity and over-expression of IRF-7 in the EBV-negative Loucke BL and BAJB B cell lines did not repress a Qp-driven reporter construct[217], arguing against a role for IRF7 in the Qp-repression in type III latency.

At least 3 positive regulators of the Qp were proposed: two IRF members (IRF1 and IRF2)[218], the E2F-1[219], and the STATs transcription factors[220]. Sung *et al*. reported that transfection of the EBV-positive Raji cells or the EBV-negative SVK keratinocyte cell lines with E2F-1 expression vector could activate a co-transfected Qp-driven reporter construct[219]. The two E2F-1 sites in the Qp are overlapping with the EBNA-1 binding sites[219]. E2F-1 was shown not only to bind to these binding sites *in vitro*, but a purified GST-E2F-1 protein could displace the pre-bound GST-EBNA-1 to a Qp double stranded DNA probe[219]. Furthermore,

mutations within one of the E2F-1 binding sites abolished the E2F-1 responsiveness of a Qp reporter contruct[219].

Chen *et al.* reported the existence of potential STAT binding sites in the Qp[220]. The authors found that a Qp-reporter could be activated in HeLa cells by transfection of JAK-1 or by exposure to IL-6[220]. Furthermore, purified STAT4 could bind to Qp-derived DNA probes *in vitro*, and STAT4 was found to localize to the nucleus in the Type I BL Rael and EBV-positive NPC C666-1 lines[220].

To summarize, Qp in type III cells seems to be repressed by EBNA-1 (originating from Cp or Wp), while in type I/II cells its expression is presumably driven by E2F-1.

Still the main question with regard to the EBV latency choice in B cells is the mechanism of repression of the Wp and Cp. Cp is positively regulated by EBNA-2 through its interaction with an EBNA-2-reponsive element (containing binding sites for RBP-jκ and CBF2)[221,222], while both Cp and Wp are activated by EBNA-1 through its binding to the multiple EBNA-1 binding sites within the plasmid origin of replication (*ori*P)[223,224] (Fig. 8A). It is also known that the CpG dinucleotides in the Cp and Wp are hyper-methylated in cell lines in which these promoters are repressed, while the region around Qp is not methylated[225,226]. Similar hypermethylation of the Cp/Wp was shown in peripheral B cells of healthy virus carriers, arguing for role of the DNA methylation in the life cycle of EBV *in vivo*[227].

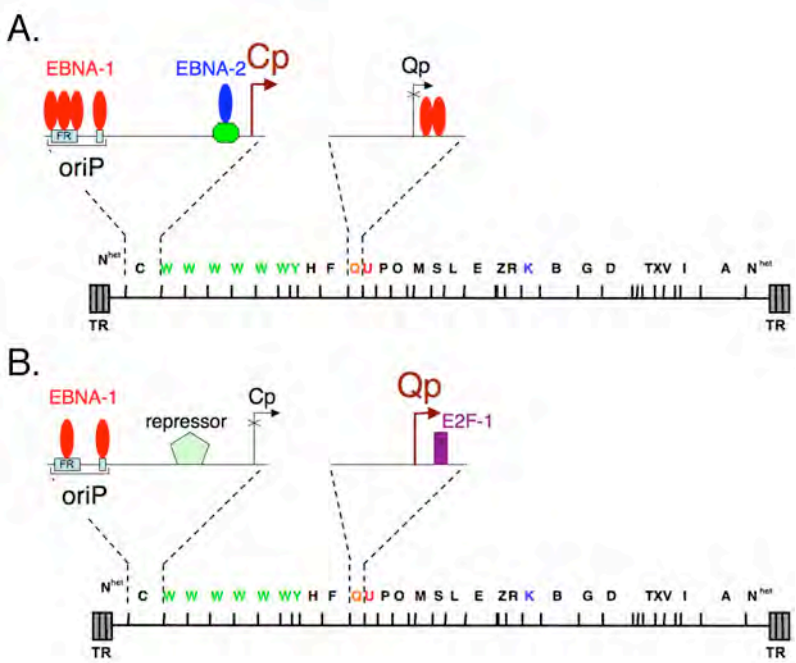


Figure 8. Hypothetical model on the regulation of Cp and Qp activities in type III (A) and type I (B) EBV latency.

In line with the inactivity of Cp and Wp in type I BL lines their DNA regions contained high levels of histone H3 K9 dimethylation, a mark typically associated with transcriptional repression.[228] The same authors found that the DNA binding protein CTCF (CCCTC-binding factor), which is involved in transcriptional repression and in blocking enhancer-promoter interactions, can bind to EBV sequences between the *ori*P and the RBP-Jκ response elements of Cp, and regulates the transcription levels of EBNA-2 mRNA[229]. Transfection of type I Mutu BL cells with CTCF siRNA resulted in 1.9-fold increase in the expression of EBNA-2 mRNA levels, while over-expression of CTCF in the Raji BL cells resulted in a 4.1-fold decrease in EBNA-2 mRNA levels[229], arguing for a potential repressor function on the activity of Cp. Binding of CTCF to the region located between Rep* and the Cp in type I BL (Mutu I cl.216, Rael), type III BL Mutu III cl.99 and LCL CBM1-Ral-STO, and EBV-carrying NPC line C666-1 was confirmed in a recent study, but its binding did not correlate with the activity of Cp[230].

Besides CTCF the only transcription factor reported to have potential inhibitory function on the Cp activity is Oct-2. Oct-2 was found to bind *in vitro* to oligonucleotides with sequences from the FR of the oriP[231]. Furthermore, it was highly expressed in type I BLs, when compared to type III BLs and LCLs[231], and could act as a repressor in reporter gene assays when interacted with members of the Grg/TLE family of co-repressors[232]. The *in vivo* binding of Oct-2 to the FR was also shown[233].

The inactivity of Cp in BL lines is not due to the lack of transcription factors involved in its activation, but rather to epigenetic silencing mechanisms, because transfected Cp reporters are active in these cells and upon super-infection of type I BL the incoming EBV expressed EBNA-2 and LMP-1, and activated the Cp promoter (while, interestingly, the Cp of the endogenous virus could not be activated)[234]. These latter experiments provided evidence that the epigenetically repressed Cp cannot be directly activated by EBNA-2 together with its partner, EBNA-5[234]. In line with the epigenetic inhibition of Cp/Wp in BLs, treatment of the type I Rael cells with the demethylating agent 5-Azacytidine resulted in the activation of Cp and Wp, and expression of EBNA-2[91,235].

Based on this wealth of information a simplified scenario for the restricted EBV gene expression in B cells can be envisaged, as follows: if the activity of the Cp and Wp promoters is suppressed by a putative repressor protein, the expression of EBNAs and LMPs are down-regulated (Fig. 8B). As the level of EBNA-1 decreases the repression of Qp is relieved and taken over by the E2F-1. This latter mechanism ensures that the virus in any proliferating cell can express EBNA-1 that is instrumental for the replication and partitioning of its genome.

1.6.1 Regulation of the LMP-1 expression

The LMP-1 (BNLF1) gene has 3 promoters: the EBNA-2-responsive *Eco*RI D leftward promoter (ED-L1)[236], the terminal repeat L1-TR[237], and the ED-L1A promoter[236]. The LMP-1 mRNA expressed from the ED-L1 promoter has a size of about 2.5 kbp, while the one originating from the TR-LMP-1 is 3.5 kbp (Fig.9). Because the translation of the LMP-1 protein starts at the same ATG, both of these LMP-1 mRNAs give rise to a protein with identical molecular weight (in SDS-PAGE and western blotts 57-66 kDa)[238].

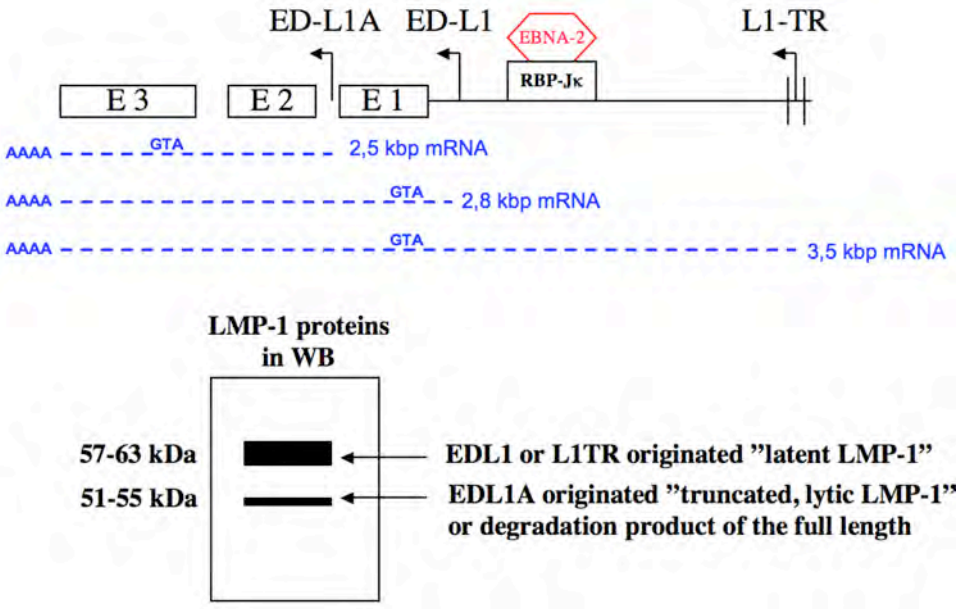


Figure 9. Structure of the LMP-1 gene, mRNAs, and protein products.

The ED-L1A promoter is located in the first intron and gives rise to a truncated transcript from which a 51-55 kDa "truncated" LMP-1 protein is translated[236]. The ED-L1A is activated upon lytic replication and therefore the truncated LMP-1 is sometimes referred to as "lytic" LMP-1. It is important to note that the "truncated" LMP-1 can be expressed in multiple cell lines in the absence of lytic replication. Furthermore, because it can also be found in total cell lysates from cells infected with the Akata EBV strain, in which the ATG used for initiation of translation of the ED-L1A-originated LMP-1 mRNA is mutated[239], is evident that the "truncated" LMP-1 is a degradation product of the full length 63 kDa LMP-1 protein in such cells. It is also important to note that the 55 kDa "truncated" LMP-1 is non-functional because it lacks the first 4 trans-membrane domains required for its self-aggregation.

As mentioned, in type III latency EBNA-2 together with EBNA-5 are responsible for the expression of LMP-1 from the ED-L1 promoter[221,240,241]. Elegant molecular studies defined multiple regulatory elements in the LMP1 ED-L1 promoter region. Two RBP-Jκ

sites[242,243], a PU-box[243,244], a ATF/CRE[245], and an AP-2[246] consensus site confer activation of the promoter in an EBNA2-dependent manner.

Detailed molecular studies of the EBNA-2-independent regulation of the LMP-1 promoters showed that this effect could be mediated by multiple regulatory elements, such as an Sp factor-binding site[245], a cyclic AMP response element (CRE)[247], an E-box element[248], an octamer-binding site, and an interferon-stimulated response element (ISRE)[242] (Fig.10). Because most of the aforementioned studies did not investigate the involvement of these regulatory elements in type II latent cells, the mechanism responsible for the expression of LMP-1 in the absence of EBNA-2 is still unknown.

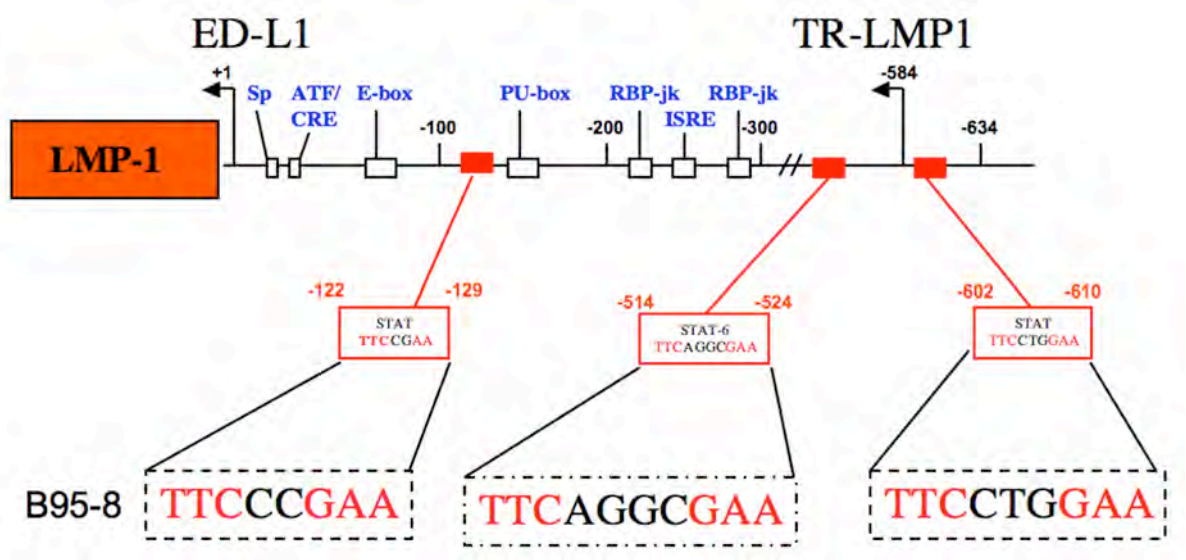


Figure 10. Structure of the LMP-1 promoters with their multiple transcription binding sites.

Identification of a 3rd promoter, the L1-TR, from which LMP-1 mRNAs could be expressed, added a further layer of complexity to the regulation of LMP-1 gene expression. The original cDNA originating from the TR-LMP1 promoter was isolated from a cDNA library derived from the NPC established in nude mice, C15[249]. Later the 5' sequence of the 3.5-kb LMP-1 mRNA was determined and found to initiate from heterogeneous start sites within the first TR of the viral genome[237]. The L1-TR promoter is TATA-less and contains multiple GC-rich elements, which potentially interact with the Sp1 transcription factor[237].

Chen *et al.* in 2001 reported the identification of two STAT binding sites (one in the ED-L1 and one in the TR-LMP-1 promoters)[250] (Fig.10). These authors found that oligonucleotide probes with the STAT binding sites bound purified, activated STAT1 and STAT4 *in vitro*[250]. IL-6 activated the TR-LMP1 promoter in reporter assyas in HeLa cells and the endogeneous L1-TR in the marmoset LCL B958. Furthermore, both the ED-L1 and the

L1-TR promoter were activated by v-Src (known to activate STAT1, STAT3, and STAT5) in reporter assays, and the activation of the L1-TR, but not ED-L1, could be inhibited by the dominant-negative isoform of STAT3, STAT3β[250]. IHC stainings in one HL case showed nuclear localization for STAT3 (but not STAT1), while in one NPC case STAT1, STAT3, and STAT5 (but not STAT4) were activated[250]. Using a sensitive PCR method with one of the primers located upstream of the TSS of the EDL1 promoter, the authors also found in one EBV-positive HL case LMP-1 mRNAs originating upstream of the ED-L1 promoter. Based on these results it was proposed that STATs may be the regulators of LMP-1 expression in type II latency in NPC and cHL. It is important to note that Chen *et al*. carried out their STAT binding analysis only in the B958 EBV strain and no proof was provided whether the STAT binding sites are conserved in other EBV strains. Furthermore, the end-point PCR used to detect LMP-1 mRNAs upstream of the ED-L1 promoter could only show that some transcription occured from promoter(s) upstream of the ED-L1 promoter, but it could not be concluded that the LMP-1 transcripts originated from the L1-TR, neither that L1-TR is the only promoter that is active (i.e. how much of the total LMP-1 transcripts originate from the ED-L1 promoter if any).

In a follow up study Chen *et al.* showed that LMP-1 expression in NPC cell line CNE-2 or EBV-infection of HeLa cells resulted in the activation of STAT3 and STAT5[251]. STAT3 activation was shown to be due to the activity of IL-6, induced in turn by LMP-1, as neutralizing anti-IL-6 antibodies decreased STAT3 phosphorylation in the LMP-1-transfected CNE-2 cells. Furthermore, transfection of a constitutively active STAT3 mutant upregulated the expression of LMP-1 protein and mRNAs (presumably TR-LMP1 originating as well) in the Akata EBV-converted HeLa subline[251]. Stewart *et al*. also reported the induction of LMP-1 by IL-6 in 2 EBV-carrying NPC lines (C666-1 and Akata-Neo-converted HONE-1)[252]. These authors also showed that LMP2A could interfere with the activation of LMP-1 by IL-6 in NPCs, because LMP-2A could down-regulate the expression of IL-6[252].

The constitutive activation of the JAK-STAT pathways was investigated also in a recent study in 61 NPC cases[253]. The authors found that constitutive activation of STAT3 and STAT5 was detected in 70.5 and 62.3%, respectively, of the tumour specimens. Furthermore, activated STAT3 and STAT5 were co-expressed in 54.1% of the tumors, while STAT1 was activated in 13.1% of the cases[253].

Just about the time that evidence accumulated for a possible role of IL-6 and STAT3 in the expression of LMP-1 in NPC, Niedobitek and his co-workers reinvestigated this question and found that STAT3 activation was a consistent feature of NPC tumour cells, but in most

cases this was not accompanied by detectable expression of LMP1[179]. The expression of LMP1 and LMP2A tended to be mutually exclusive in NPCs[179].

As already mentioned Chen *et al.* proposed that STATs might be responsible for the EBNA-2-independent expression of LMP-1 not only in NPC, but also in cHLs. In line with this assumption HRS cells showed activated STAT1[254], STAT3[254-256], STAT5[257], and STAT6[256]. Still the direct demonstration that any of these factors can induce LMP-1 in cHLs is lacking.

Besides IL-6 and STAT3, three factors were shown to induce LMP-1 expression in the absence of EBNA-2 in the context of whole viral genome: surface IgM crosslinking, and overexpression of the transcription factors IRF7[258] and NF-κB[259]. Rowe *et al.* found that crosslinking of surface IgM in the type I BL line Eli induced the expression of LMP-1 in the absence of EBNA-2 and lytic replication[62].

To summarize in a sentence: today the best-documented factor shown to induce LMP-1 expression in the absence of EBNA-2 is STAT3.

1.7 Signaling through cytokine receptors

Cells of the immune system communicate with each other to initiate, establish and maintain immune responses. The communication occurs through cell-to-cell contact or through a variety of intercellular mediators that include cytokines, chemokines, growth factors and hormones[260]. In the case of cytokines, the signal is transmitted from the outside to the inside of a cell through cell surface receptors specific for each cytokine. At this step the signal is also decoded and amplified: ligand binding causes recruitment and/or activation of numerous cytoplasmic proteins. One cytokine can activate a number of signal transduction pathways leading to regulation of a wide array of biological activities[260]. As in the present work IL-10, IL4/IL-13, and IL-21-signaling was studied in some details, here I review their signaling properties.

The γ_c cytokine family comprises interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21, named after the γ_c subunit (CD132) shared by receptor complexes of these cytokines. IL-4, IL-7, IL-9, and IL-21 bind heterodimeric receptors comprised of the γ_c and their specific receptor subunits, IL-4Rα, IL-7Rα (CD127), IL-9Rα, and IL-21Rα chain, respectively. The heterotrimeric receptors of IL-2 and IL-15 are composed of the specific IL-2Rα (CD25) or IL-15Rα chain, and the shared IL-2/15Rβ (CD122) and γ_c chains.

Type I cytokine receptors are defined by several features, including 4 conserved cysteine residues, a W-S-X-W-S motif, fibronectin type II modules in the extracellular domain, and proline-rich box regions in the intracellular domain that are important for binding of JAKs. IL-13 signals through a type I cytokine heterodimeric receptor composed of the IL-13-specific IL-13Rα1 and the IL-4Rα chains[261] (Fig.11A). The complex of IL-4Rα and γ_c is known as the type I receptor complex and only binds IL-4, whereas, in the type II receptor, IL-4Rα dimerizes with IL-13Rα1 upon either IL-4 or IL-13 binding. Another ligand binding chain potentially implicated in the IL-4/IL-13 receptor has been described, the IL-13Rα2, that is believed to be a decoy receptor.

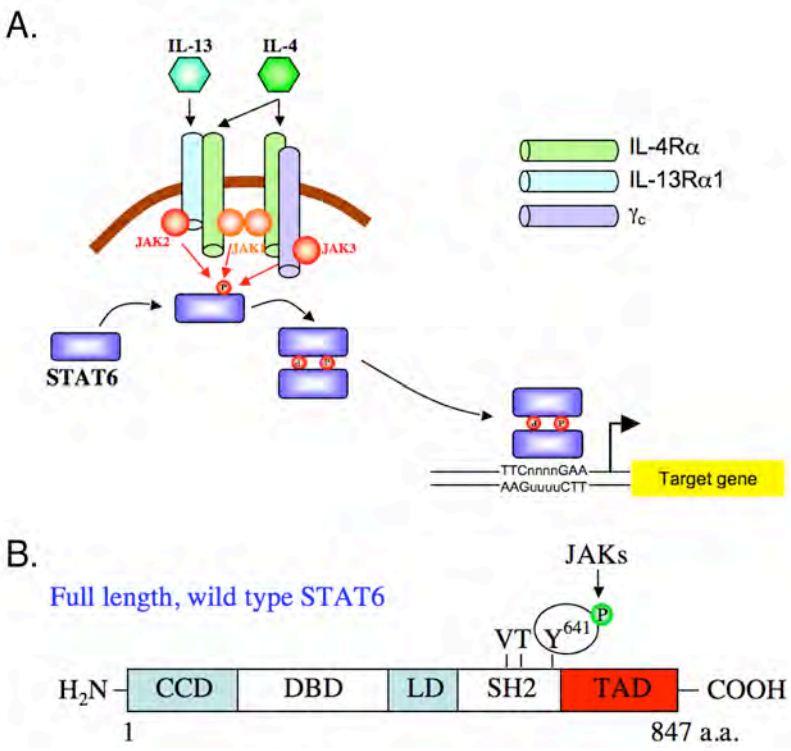


Figure 11. A. Schematic illustration of the IL-4/IL-13-STAT6 signaling pathway. B. Structure of the STAT6. CCD-coild-coild domain, DBD-DNA binding domain, SH2-SH2-domain, TAD-transactivation domain

Studies on gene induction by IFNs led to the discovery of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, which later was found to be a common signalling pathway used by many cytokines. The binding of a cytokine to its cell-surface receptor results in receptor dimerization and the subsequent activation of JAK tyrosine kinases, which are constitutively associated with the receptor. Specific tyrosine residues on the receptor are then phosphorylated by activated JAKs and serve as docking sites for a family of latent cytoplasmic transcription factors known as STATs. STATs are phosphorylated by JAKs, then dimerize and subsequently leave the receptor and translocate to the nucleus, where they activate gene transcription (shown for IL-4/IL-13 in Fig. 11A).

The mammalian JAK family has four members: JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2), all containing a conserved kinase domain. There are seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. In their structure STATs have highly homologous regions, such as a SRC homology 2 (SH2) domain, which is involved in the activation and dimerization of STATs, a DNA-binding domain, and a transactivation domain, which is located at the carboxyl terminus (exemplified by STAT6 in Fig. 11B). The amino-terminal region of STATs is involved in the formation of STAT tetramers.

While both IL-4 and IL-13 activate predominantly STAT6, IL-21 depending on the cell type can activate to different degree STAT1, STAT3, and STAT5.

After cytokine-receptor stimulation, STATs are tyrosine phosphorylated on a single conserved residue. The tyrosine phosphorylation of STATs is required for their dimerization, nuclear translocation, and DNA binding. Furthermore, STATs also modified by serine phosphorylation and mutational analysis of STAT1 provided evidence that the serine phosphorylation of STAT1 is required for the maximum induction of IFN-responsive genes.

Class II cytokine receptors were originally defined on the basis of sequence homologies in the extracellular domains of receptors for interferons (IFNs) and interleukin-10 (IL-10), and the ligands, known as class II cytokines, also have a common structure[262].

The IL-10 receptor complex on cells is composed of four transmembrane polypeptides: two chains of IL-10R1 that bind ligand and two chains of IL-10R2 that initiate signal transduction. Owing to its structural homology to the IFN-γ receptor complex, shown to be pre-assembled, the IL-10 receptor complex is predicted to be preassembled as well. Jak1 is constitutively bound to IL-10R1, while TYK2 constitutively associates with the IL-10R2 chain. Upon activation of the IL-10 receptor complex by IL-10, JAK1 and TYK2 are activated, that leads to the phosphorylation of STAT3, STAT1, and STAT5. Subsequently homodimers and heterodimers of these STAT proteins form and translocate to the nucleus to drive transcription of STAT3-responsive genes.

It is important to note that the cytokines activate not only the JAK-STAT pathway, but other pathways are also activated down-stream of the cytokine receptors. Binding of the cytokine to its specific receptor leads to JAK activation and phosphorylation of critical tyrosine residues in the receptor-specific chains. The tyrosine residues phosphorylated by the JAKs serve as docking sites also for the Insulin Receptor Substrate (IRS) proteins and Shc adapter protein, and by this it results in the activation of the phosphoinositide 3-kinase (PI3K)/Akt and RAS-mitogen-activated protein kinase (MAPK) pathways.

2 AIMS OF THE THESIS

The primary goals of our work were to:

- study the interaction of HRS cells with EBV *in vitro*,

- study the regulation of EBV gene expression in different *in vitro* cell culture models in order to establish models of type II EBV latency, with special emphasis on the EBNA-2-independent regulation of LMP-1 expression,

- study the interaction between EBV-carrying malignant cells with normal hematopoietic cells that are known to be present in these tumors *in vivo*.

3 MATERIALS AND METHODS

The methods used in our studies are described in details in the published articles and in the manuscripts, and therefore I do not consider it necessarily to repeat them again. But I think it would be helpful to summarize in a table the multiple cell lines and EBV strains used in our studies.

Table III. List of the cell lines used in our studies

A). Burkitt lymphoma-derived cell lines

1). EBV-negative and -converted sublines:
- DG75/ DG75 infected with Akata Neo^R virus –DG75-Akata cl.3, cl.6
- Ramos/Ramos infected with B958-EBNA2KO virus: Ramos-EB2KO cl. 3 and cl.6

2). EBV-positive type I: Mutu I cl.148, 216, 59; Rael; Jijoye M13; Akata

3). EBV-positive type III: Mutu III cl.99, 176; Jijoye P79; AG876; BL16; Raji

4). EBV-positive with EBNA-2 deletion: P3HR1; Daudi; Sal

B). Hodgkin lymphoma-derived cell lines

1). EBV-negative: L428, L1236, HDLM-2, L540, KMH2

2). KMH2 infected with Akata Neo^R virus: KMH2-EBV (our lab)
KMH2-Akata (Paul Murray's lab)

3). KMH2 infected with recombinant wild-type B958-EGFP virus

4). KMH2 infected with recombinant EBNA-2-deleted B958-EGFP virus (B958-EBNA2KO virus)

5). KMH2 infected with recombinant BZLF1-deleted B958-EGFP virus (B958-BZKO virus)

C). EREB2-5 and its derivates

ER/EB2-5- conditional LCL obtained form B cells infected with P3HR1 virus and a recombinant maxi-EBV expressing an estrogen-receptor-EBNA-2 fusion gene

A1- c-myc-transfected ER/EB2-5 constitutively expressed from regulatory elements derived from the Igκ locus

P493- tetracycline-regulated c-myc-transfected ER/EB2-5

D). Cell lines with epithelial origin

TWO3 and TWO3-EBV-NPC line and its Akata-Neo-converted subline

C666-1- the only EBV-positive NPC line that maintained the viral genome

AGS-Akata, AGS-Akata-GFP- gastric carcinoma cell line infected with Akata-Neo or Akata-GFP EBV strains

NK/T lymphoma-derived cell lines (IL-2-dependent)

1). EBV-negative: NK-L, KHYG-1

2). EBV-positive: SNK-6, KAI-3

Table IV. List of the EBV strains used in our studies

Name of the strain	Way of lytic cycle induction	Origin	Comments
B95-8	spontaneous, serum starvation	this lab	transforming, from marmoset LCL[263]
P3HR1	TPA + sodium butyrate treatment	this lab	non-transforming, EBNA-2 deleted[264]
Akata-Neo	produced from Akata BL by anti-human IgG treatment	K. Takada	recombinant Akata strain with neomycin-resistance gene under SV40 early promoter[265]
	produced from AGS-Akata cells by TPA treatment	this lab	the same[266]
Akata-GFP	produced from Akata BL by anti-human IgG treatment	L. M. Hutt-Fletcher	recombinant Akata strain with GFP and neomycin-resistance cassette driven by CMV and TK promoters, respectively[267]
	produced from AGS-Akata GFP cells by TPA treatment	L. M. Hutt-Fletcher	the same[267]
B958-EGFP (maxi EBV 2089)	produced from HEK293 cells by BZLF1+BALF4 transfection	W. Hammerschmidt	recomb. B858 strain with EGFP and hygromycin cassette driven by SV40 promoter[268]
B958-EBNA2KO (maxi EBV 2491)	produced from HEK293 cells by BZLF1+BALF4 transfection	W. Hammerschmidt	the same as B958-EGFP+ deleted for EBNA-2[14]
B958-BZKO (2809.13)	produced from HEK293 cells by BZLF1+BALF4 transfection	W. Hammerschmidt	the same as B958-EGFP+ deleted for BZLF1[269]

4 RESULTS AND THEIR POTENTIAL IMPLICATIONS

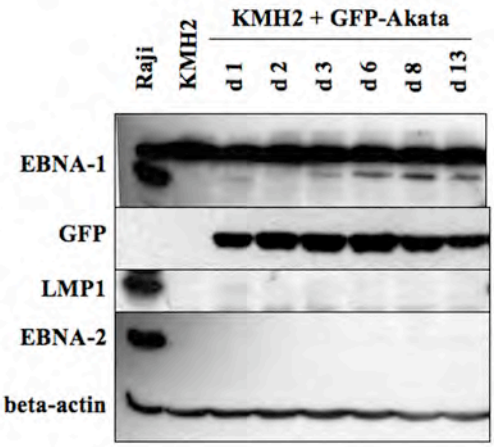
A) *IN VITRO* STUDIES ON THE INTERACTION OF EBV WITH CHL-DERIVED CELLS (PAPERS I, III, VII)

Our work was motivated by the lack of *in vitro* or *in vivo* models in which to study the interaction of EBV with the HRS cells. As a continuation of the work in our laboratory that found a direct correlation between the expression of the SAP (SH2D1A) protein and the presence of EBV in EBV-negative and EBV-positive, type I BL lines[270], we investigated the expression of this protein in cHL-derived cell lines (**Paper I**). Furthermore, we confirmed the EBV-negative status of these cell lines and characterized their immunophenotype. We found that SAP (for SLAM-associated protein) and one of its interacting cell surface receptors, SLAM (for Signaling Lymphocytic Activation Molecule) were co-expressed in 4 of the 5 cHL-derived cell lines (the DEV cell line used in our study found to be SAP- and SLAM-negative was later shown to be derived from nodular lymphocyte predominant HL[271]). The cHL-derived cell lines were the first cell lines of hematopoietic origin that co-expressed SAP and SLAM, as their expression was mutually exclusive in LCLs, BL, and NK lines. The role of SAP and SLAM in the pathogenesis of cHL remains unknown. Based on our findings Mikhalap *et al*. investigated the expression of SAP and SLAM in cHLs *in vivo* and found to be co-expressed in the HRS cells [272]. Furthermore, these authors also found that cross-linking of SLAM activated the Akt pathway in the KMH2 and L1236 cell lines, while in the KMH2 cells signaling via SLAM also activated ERK1/2[272]. The expression of SAP in the HRS cells might be either due to the origin of HRS cell from a SAP-positive GC B cell or it might be ectopically expressed as a result of malignant transfomation and ectopic expression of T cell-associated genes, as shown for CD3, CD4, and granzyme B[142]**.**

During our immunophenotypic studies on the cHL-derived cell lines we noted that the KMH2 cell line expressed high level of CD21 and therefore we tested if it could be infected with EBV **(Paper III**). The first experiments were done with the B958 EBV virus and showed that about 1% of the cells could be infected. In order to have the possibility to select the EBV-infected cells in the KMH2 cells, we used for infection the recombinant Akata strain that carried a neomycin-resistance gene cassette. With this viral strain we succeeded to established an EBV-converted subline of the KMH2 line, that we named KMH2-EBV. Interestingly, when EBV latent gene expression was studied at early timepoints after infection

or in the converted subline, the cells expressed EBNA-1 protein, but not EBNA-2 or LMP-1. The LMP-1 gene was still present in the converted subline and could be induced by treatment with the phorbol ester TPA, the histone-deactylase inhibitors trichostatin A (TSA) and sodium-butyrate, or the demethylating agent 5-Azacytidine (5-AzaC).

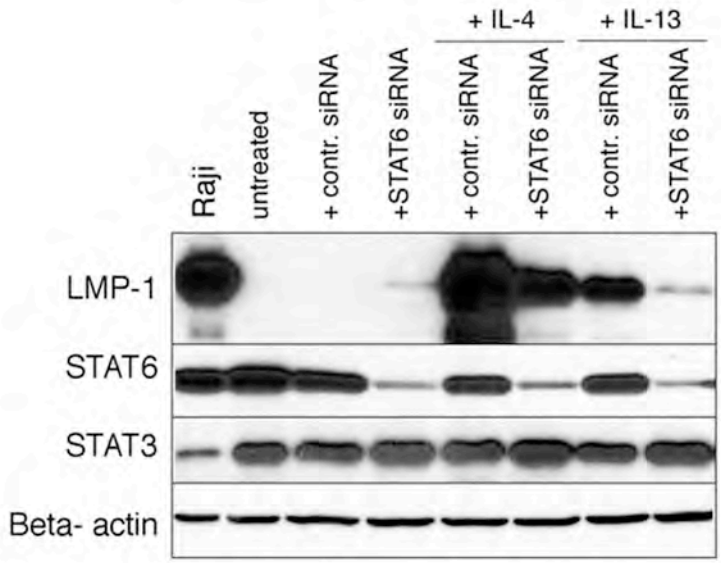
Expression of EBV latent genes in the GFP-tagged, Akata EBV-infected KMH2 cells at different timepoints after infection



As LMP-1 was not expressed in the EBV-infected KMH2 cells neither at early timepoint after infection nor in the stable converted subline, and as CD4+ T cells with a predominantly Th2 phenotype closely surround the HRS cells in the tumor tissues, we wondered whether T cell-derived signals might be involved in the induction of LMP-1 expression in EBV-carrying HRS cells. When we exposed the KMH2-EBV cells to membrane-bound CD40L and IL-4, LMP-1 expression was induced in the absence of EBNA-2 and lytic replication. The expression of LMP-1 was transient as it was down-regulated if the CD40L-IL-4-activated KMH2-EBV cells were re-plated for culturing in the absence of the stimuli.

Further work defined IL-4 as the active stimulus in our original CD40L-IL-4 mixture (**Paper VII**). In a cytokine screening we identified IL-13 as another cytokine with LMP-1-inducing effect on the KMH2-EBV cells. As both IL-4 and IL-13 signal through STAT6 we focused our attention on this signaling pathway as the primary candidate to mediate the induction of LMP-1. In line with this assumption, 1-2 hrs of IL-4 exposure was enough to induce the expression of LMP-1 protein in total cell lysates of the KMH2-EBV cells. The direct proof that STAT6 mediates the LMP-1-inducing effect of IL-4 and IL-13 came from the experiments in which STAT6 expression was transiently down-regulated in the KMH2-EBV cells using STAT6-specific small interfering RNAs (siRNAs). Exposure of the KMH2-EBV cells with low STAT6 expression to IL-4 or IL-13 resulted in lower level of LMP-1 induction.

STAT6 is required for the induction of LMP-1 expression by IL-4 and IL-13

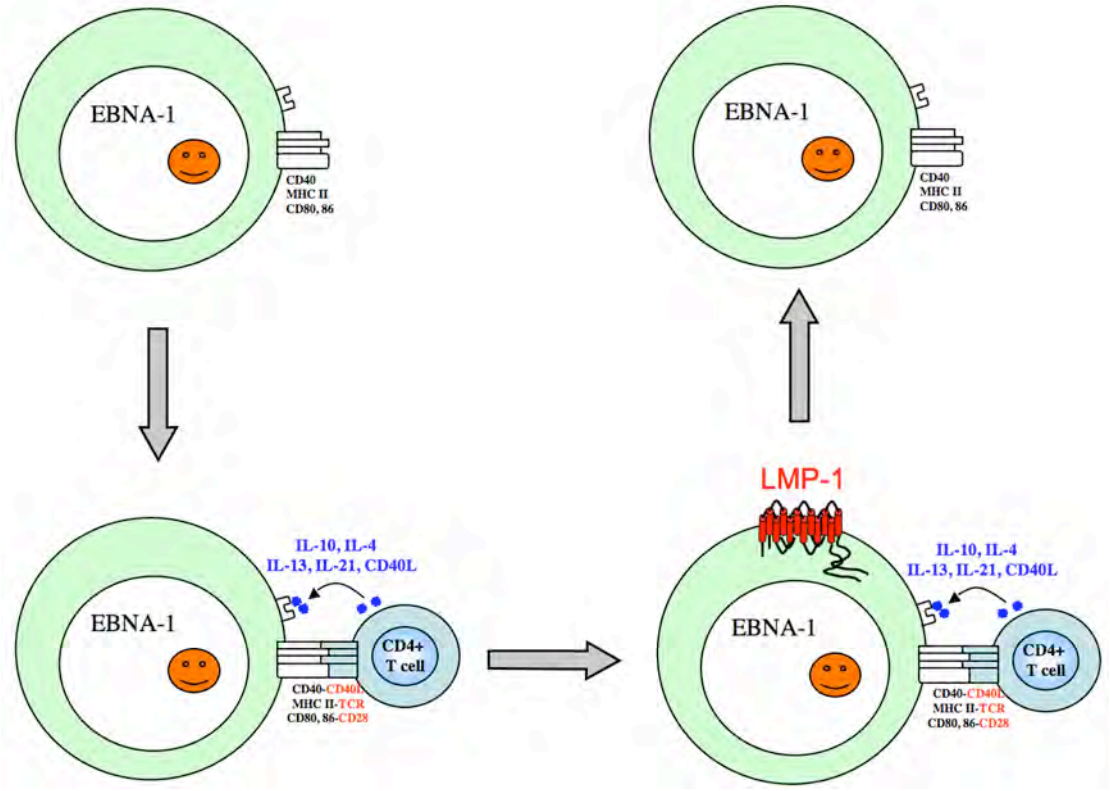


Though two STAT binding sites were defined in the LMP-1 promoter, none of them concurred in their sequence with a high affinity STAT6 binding site. Upon re-analysis of the sequence of LMP-1 promoter of the B95-8 EBV strain, at positions -514-524 upstream of the transcriptional start site we identified a new STAT binding site with the TTCAGGCGAA sequence that resembled a high affinity STAT6 binding site. The functionality of this new STAT6 binding site was confirmed in EMSA and luciferase reporter gene assays. Furthermore, the in vivo binding of STAT6 to the LMP-1 promoter was shown by CHIP.

Exposure of EBV-positive BL lines to either IL-4 or CD40L resulted in the induction of LMP-1 expression, while when the 2 stimuli were combined LMP-1 was strongly induced. As a final proof that such signals would induce LMP-1 not only in EBV-carrying lymphoma cell lines, but also in EBV-infected normal B cells, we infected isolated B cells with the EBNA-2-deleted P3HR1 virus and tested their LMP-1 expression after activation with IL-4, CD40L, or IL-4+CD40L. Similarly to the BL lines, LMP-1 was induced by the cytokines in the P3HR1-infected normal B cells.

As the cytokines found to induce LMP-1 are secreted by CD4+ T cells, we studied the effect of allogeneic CD4+ T cells on the LMP-1 expression in EBV-carrying BL lines. To this end the Daudi BL line, harboring a deletion in the EBNA-2 gene, was co-cultured with isolated CD4+ T cells. LMP-1 was induced in such co-cultures only if the T cells were activated (either with phytohemagglutinin, Staphylococcus enterotoxin B, or anti-human CD3- and CD28-conjugated beads).

Transient induction of LMP-1 in EBV-carrying type I/0 B cells by CD4+ T cell-derived signals



Collectively the results on the EBV-infected KMH2 cells helped in the identification of 2 cytokines that could be involved in the EBNA-2-independent expression of LMP-1 in cHL, in EBV-carrying normal GC B cells, and other pathological interactions between T cells and EBV-positive B cells. They also helped in the identification of a previously unknown signaling pathway, namely JAK-STAT6, involved in the regulation of LMP-1 expression in the EBV-carrying HRS cells. Furthermore, these studies established for the first time a link on the involvement of CD4+ T cell-derived signals in the induction of LMP-1 expression. During the interaction of CD4+ T cells and the EBV-carrying type 0/I B cells LMP-1 could be transiently expressed and later down-regulated as this interaction subsides. This transient and signal-induced LMP-1 expression brings the type I and type II cells in a common latency category, as type I cells could easily become LMP-1-positive under the influences of the microenvironmental signals.

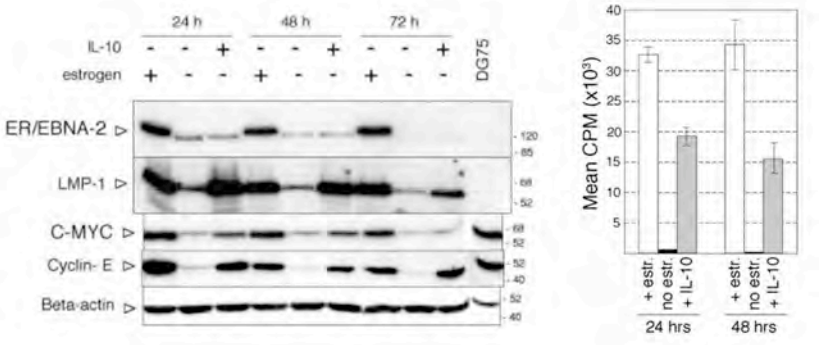
B) STUDIES ON THE REGULATION OF EBV LATENT GENE EXPRESSION BY HDAC-INHIBITORS, IL-10, AND IL-21 (PAPERS II, IV, V, VI)

In our following studies we found that exposure of the EBV-converted subline of the TWO3 NPC line to the histone deacetylase (HDAC) inhibitors TSA and sodium butyrate

(NB) resulted in the induction of LMP-1 (**Paper II**). Interestingly, LMP-1 induction was accompanied by entry into the lytic cycle upon NB, but not TSA treatment. Luciferase reporter assays provided evidence that the TSA-induced LMP-1 was functional as it could activate the NFκB signaling pathway.

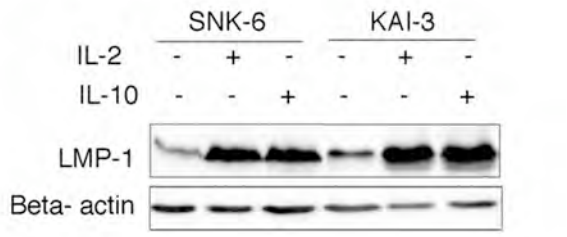
While the effect of TSA and NB was studied in the TWO3-EBV NPC cells, we also studied the effect of IL-10 in BL lines. We found that both the human and the EBV-encoded IL-10 could induce the expression of LMP-1 in the multiple EBV-carrying BL cells (**Paper IV**). The LMP-1 induced by IL-10 seemed to be fully functional as expression of LMP-1 was accompanied by the upregulation of CD80, CD83, HLA II, and CD54 (ICAM-1) expression. Furthermore, IL-10 treatment could induce not only LMP-1 expression in the estrogen-starved ER/EB2-5 cells, but also resulted in the transient proliferation of these cells.

Exposure of the estrogen-straved ER/EB2-5 cells to IL-10 results in the induction of LMP-1 expression and transient proliferation



The EBV-carrying NK lines SNK6 and KAI3 studied in the **Papers IV** and **V** require the addition of exogenous IL-2 for their *in vitro* growth. We found that upon removal of IL-2 from their cell culture medium they stopped to proliferate and this was paralleled by the down-regulation of LMP-1 expression. As these cells expressed functional IL-10 receptors, we wondered whether replacing IL-2 with IL-10 would maintain the LMP-1 expression and *in vitro* proliferation. As predicted based on the effect of IL-10 on the BL and ER/EB2-5 cells, exposure of the SNK6 and KAI3 NK lines to IL-10 resulted in LMP-1 induction, but interestingly not proliferation.

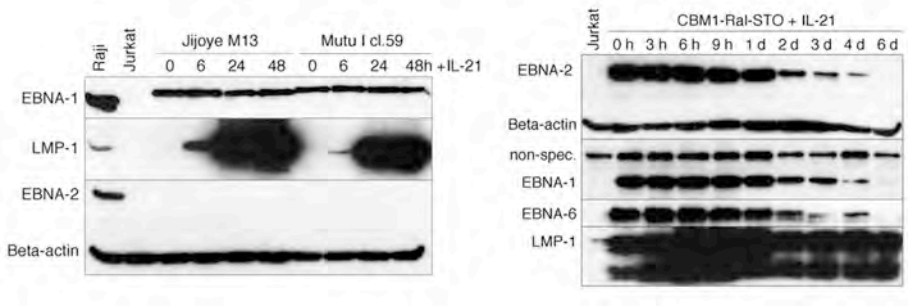
Treatment of the EBV-carrying lymphoma-derived cell lines SNK-6 and KAI-3 with IL-10 or IL-2 results in the induction of LMP-1 expression



Interestingly, the EBV-carrying NK lines secreted large amounts of IFN-γ and IL-10 when cultured in IL-2-containing medium. As both of these cytokines could induce LMP-1 in the absence of IL-2, the possibility emerged that the IL-2 induces the expression of LMP-1 indirectly through the autocrine secretion of IFN-γ and IL-10. In line with this assumption neutralizing antibodies to these cytokine could decrease the expression of LMP-1 when the cells were cultured in IL-2-containing medium. Collectively, the results with the EBV-carrying NK lines defined an important role for the cytokines in the expression of LMP-1 in this malignancy as well. As the tumor cells *in vivo* are admixed with a inflammatory infiltrate, the studied cytokines might be provided by the inflammatory cells, and by this the infiltrating normal cells might contribute to the expression of LMP-1 and proliferation of the EBV-carrying malignant NK cells. The inflammation in this case might precede the tumor development and lay the grounds for increased susceptibility of NK cells to EBV infection at this location.

Based on our previous results on the induction of LMP-1 by IL-10, on the role of STAT3 in the induction of LMP-1 by IL-6, and on the strong PC differentiating effect of IL-21 on normal B cells, we became interested in the effect of IL-21 on the EBV gene expression. Interestingly, IL-21 had pleiotropic effects on the EBV-carrying B cell lines: it induced LMP-1 expression in the absence of EBNA-2 in type I BL lines, while in the type III BL and LCL lines it resulted in the down-regulation of EBNA-1, -2, -6 expression (**Paper VI**).

Pleiotropic effect of IL-21 on the EBV gene expression: EBNA-2-independent induction of LMP-1 expression and down-regulation of EBNA-1, EBNA-2, EBNA-6 expression

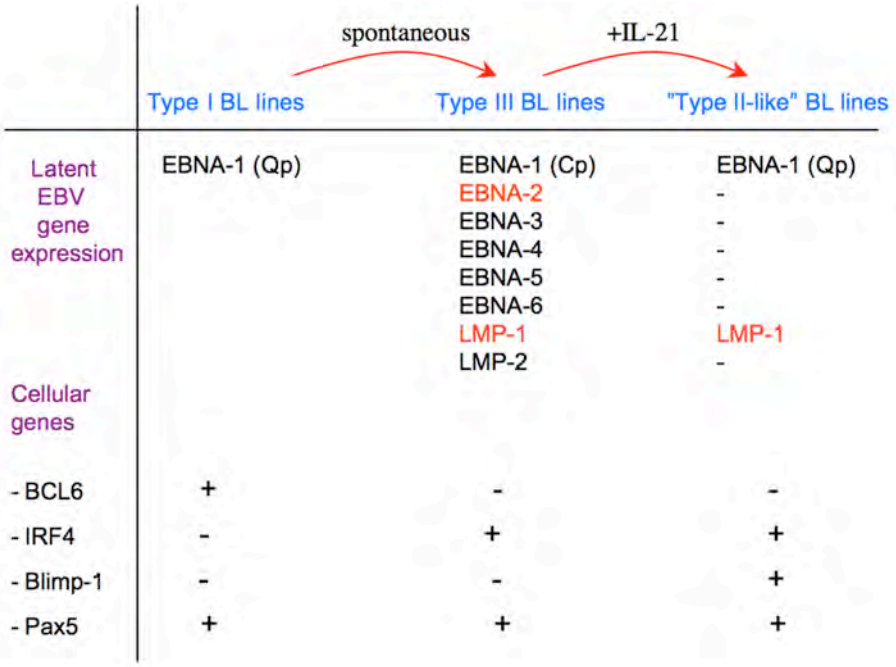


The molecular mechanism of the down-regulation of EBNAs by IL-21 involved the repression of transcription from the Cp, while the induction of LMP-1 was probably mediated by STAT3. IL-21 strongly induced the expression of the master key regulator of PC differentiation, Blimp-1, in the type III LCLs and BL lines. Interestingly, the IL-21-treated LCLs maintained their proliferation and continued LMP-1 expression even after 8-10 days of *in vitro* culturing. Though the expression of EBNA-1 in the IL-21-treated LCLs decreased dramatically, it never became totally down-regulated, probably as a result of the activation of the Qp. IL-21 did not directly effect the Qp activity in type I BL or LCLs, but upon continued IL-21 exposure the Qp was activated in LCLs. The molecular details of the repression of Cp and activation of Qp in the IL-21-treated LCLs remains to be investigated.

In summary IL-21 had dramatic effect both on the phenotype and on the EBV gene expression in the EBV-positive BL and LCL lines. With regard to the EBV gene expression IL-21 treatment of both type I BL lines and LCLs resulted in a "type II- like" gene expression, as the cells became EBNA-2-negative, LMP-1-positive. But it is important to note that phenotypically the two IL-21-treated cell lines were very different, as PC differentiation (as evidence by Blimp-1 expression) could be induced only in the type III lines. Interestingly, the switch of EBV gene expression in BL lines from type I to type III rendered the cells susceptible to the PC-differentiating effect of IL-21.

We believe that our results on the modulation of EBV latent gene expression by cytokines might also have therapeutic implications. If *in vivo* the major mechanism of LMP-1 induction is the JAK-STAT signaling pathways, and if continuous expression of LMP-1 is required for the proliferation and survival of the HRS cells, inhibiting the signaling through these pathways would have beneficial effect in the EBV-carrying HRS cells by the inhibition of LMP-1 expression. Such inhibition could be achieved by neutralizing antibodies directed against IL-13 or CD40L, or by the use of selective chemical inhibitors of the JAKs or STATs.

IL-21-induced changes in the phenotype and EBV gene expression of BL lines



With regard to the potential therapeutic implications of the modulation of EBV gene expression by IL-21 it is important to note that this cytokine is already tested in clinical trials in patients with advanced melanoma, renal cell carcinoma, and non-Hodgkin B cell lymphoma[273]. Based on our results, IL-21 could be useful in the immunotherapy of EBV-carrying B cell lymphomas or lymphoproliferations. In type I latent B cell malignancies IL-21 treatment could induce the expression of LMP-1 that would provide new viral peptides that can be recognized by both CD4+ and CD8+ T cells. Furthermore, the IL-21-induced LMP-1 would make the EBV-carrying tumor cells more immunogenic[274,275] and would directly inhibit the proliferation of BL cells[45]. Taken together with the direct effect of IL-21 on the maturation and enhanced cytotoxicity of NK and CD8+ T cells, IL-21 has therapeutic potential in EBV-carrying lymphomas. Still care must be taken when it comes to the treatment of B cell lymphomas with a type III latent gene expression, because, as we showed, long term continuous IL-21 exposure repressed the expression of the EBNAs that are the main targets of CTL responses[113]. Even in spite of this potentially negative effect a short IL-21 treatment could have beneficial effects in type III PTLD and AIDS-lymphomas, since it will up-regulate the expression of LMP-1 that in turn will enhance the immunogenicity and antigen-processing capacity of the malignant cells as recently shown for the killing of LCLs by CD8+ T cells *in vitro*[276].

5 CONCLUSIONS AND FUTURE PERSPECTIVES

One limitation of our studies is the extensive use of *in vitro* models. As there are no EBV-positive HRS cell lines or *in vivo* models of cHL, and as there are no *in vivo* models of EBV infection in which to study EBV's role in BL or cHL genesis, the aforementioned limitation is more general for the scientific community studying this questions. We tried to overcome the limitations of our *in vitro* systems, by using multiple EBV-carrying cells lines (both with regard to their increased number and also with regard to their tissue origin, using BL-, cHL-, NK lymphoma-, and NPC-derived cell lines) and EBV-infected normal B cells. The lack of *in vivo* model to study EBV-induced lymphomagenesis might soon change with the more refined use of immunodeficient mice with reconstituted human hematopoietic system in EBV research[85,277,278].

As could be seen in our work not all the cell lines studied reacted to the cytokine treatments. In some instances this non-responsiveness could be traced back to the lack of expression of the specific cytokine receptor (IL-13Rα1 on BL lines), while in other cells the signal transduction pathways were defective in spite of the presence of the cytokine receptor (phosphorylation of STAT3 in the IL-21R-positive Mutu I cl.216). Another level of regulation, not studied in this thesis, but under investigation, is the accessibility of the LMP-1 promoter to extra-cellular signals. The epigenetic status of the LMP-1 promoter is an important factor in determining if the extracellular signals will succeed in its transcriptional activation.

Our results together with the other studies on the modulation of EBV gene expression by extracellular signals raises also the possibility that the timing of expression of EBV latent genes *in vivo* might be different from the one seen in the *in vitro* EBV-infected B cells. More specifically LMP-1 expression *in vivo* might occur concomitantly with the expression of EBNA-5 and EBNA-2 and might be driven at this time by the extracellular signals that the infected B cells receive, rather than being induced by EBNA-2.

Based on the findings that:

1) EBV-carrying GC and memory B cells express Qp-derived EBNA-1 and Cp is inactive,
2) EBV-positive GC-derived B cell lymphomas (BL, cHL, PEL), just as their GC B cell precursors, express Qp-derived EBNA-1 and their Cp is inactive,
3) EBV-positive B cells in the GC rarely express LMP-1 protein when studied by IHC,

4) LMP-1 protein expression in EBV-carrying B cell lymphomas is heterogeneous, and
5) our *in vitro* findings LMP-1 can be induced by cytokines and activated CD4+ T cells, we modified the scenario proposed by Thorley-Lawson in the following way.

In order to reconcile the incompatibility between the expression of LMP-1 and the GC B cell differentiation, we assume that LMP-1-positive, EBNA-2-negative type II cells in the GC develop from type I cells upon receipt of signals (ligands and cytokines) from the microenvironment, most probably originating from T_{FH} cells (Fig. 12).

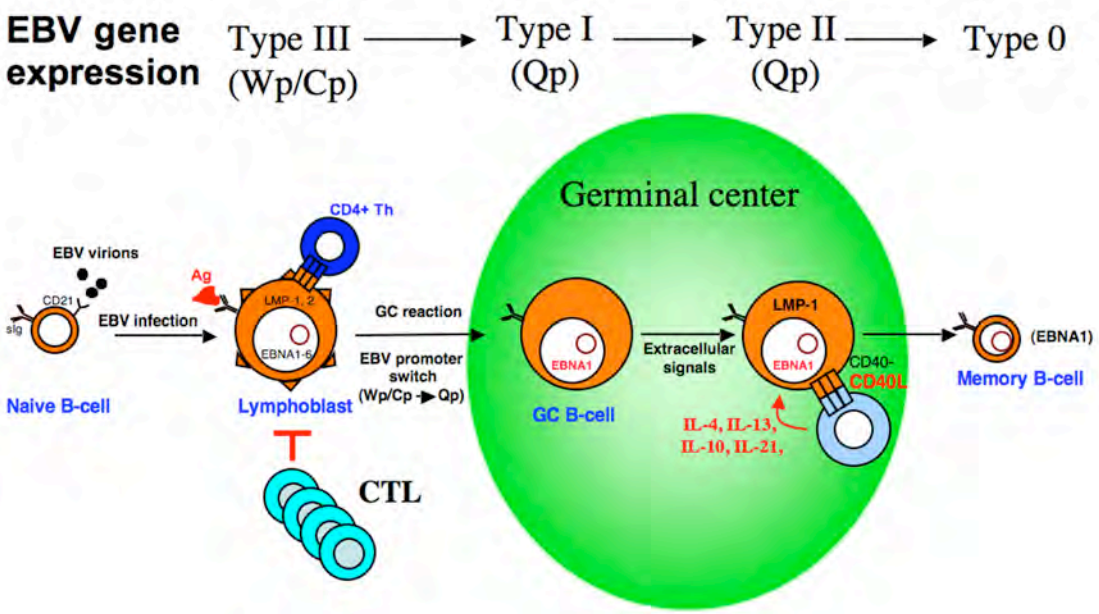


Figure 12. Our model on the entry of EBV in the memory B cell pool.

Thus our model incorporates an additional step in which the EBV-carrying GC B cells express only Qp-derived EBNA-1 (type I latency), but no LMP-1. Qp activity in the proliferating cells could be a default mechanism by which EBV expresses EBNA-1 when the C- or W-promoters are inactive, and is in line with the *in vitro* observations that the activation of the Qp occurs upon entry to the cell cycle[279]. The existence of the type I latent EBV-carrying GC B cells is purely hypothetical and is indirectly deduced from the listed evidences. Its purpose is to solve the incompatibility between LMP-1 expression and GC B cell differentiation. Importantly, LMP-1 in our model is induced upon the encounter of antigen-specific GC B cells and CD4+ T cells, and therefore its expression is transient.

When the EBV-positive cells leave the GC and differentiate into memory B cells, they leave the cytokine-providing environment and therefore they do not express LMP-1.

What would our model help to explain?

1). It would explain the apparent contradiction of Thorley-Lawson's findings that memory B cells isolated from tonsils were LMP-1-positive[280], while the ones isolated from the peripheral blood were LMP-1-negative (cytokines and other T cell-derived signals responsible for the LMP-1 expression act in the lymphoid organs and not in the circulating blood).
2). It could also give explanation for the difference in EBV-gene expression of BL and cHL, both originating form GC B cells but having type I and type II EBV gene expression, respectively. The BLs originate from the type I GC B cells and do not contain infiltrating CD4+ T cells or constitutively activated JAK-STAT pathways, while the HRS cells originate either from the type I or from the LMP-1-positive GC B cells, but maintain a continuous interaction with the CD4+ cells, and also acquire during their malignant transformation constitutive activity in multiple JAK-STAT pathways, both of which could induce/maintain the EBNA-2-independent expression of LMP-1. Similarly, PELs arising in the visceral cavities in the absence of $CD4^+$ T cell infiltrate do not express LMP-1, while infiltrating B cells in AITL express LMP-1 in the absence of EBNA-2 expression.
3). It could explain the heterogeneous expression of LMP-1 in tumors, as only the EBV-carrying tumor cells that receive cytokine signals or have JAK-STAT pathways active would express LMP-1.
4). As already mentioned, it would overcome the inherent problem of interference of LMP-1 with the GC B cell differentiation, as the type I GC B cells would not interfere with this process and LMP-1 would be expressed only at later stages of the differentiation, at the centrocyte stage, when the T_{FH} cells interact with the EBV-carrying B cells.
5). It might help to explain the rather strange observation that none of the existing cHL-derived cell lines are EBV-positive, as these cells might need the continuous expression of LMP-1 that is sustained *in vivo* by cytokines or by other signals engaged through cell-cell contact.

EBV is one of the 6 human viruses accepted to have a causal role in human cancers[281]. EBV is a lymphocryptovirus with a fantastic inbuilt mechanism that allows the colonization of its host and its spreading. Its main objective is to fulfill its life cycle and not to induce cancer or kill its host. Still as at certain moments during its life cycle it expresses viral genes that dramatically influence the behavior of the infected target cell, EBV puts its host at risk for uncontrolled cell division with the development of a malignant tumors. Several pro- and contra-arguments for EBV's role in malignant transformation are listed in table V.

EBV's role in the malignant transformation of cells is complex and is probably unique in the different cell types. I will finish by citing Ronald T. Javier and Janet S. Butel who wrote in their recent review on the history of tumor virology that

"...the fundamental concept of tumor virology that, as opposed to representing complete carcinogens, viruses generally act as initiating or promoting factors of the carcinogenic process, consistent with the principle that cancer development occurs not by a single event but rather by the accumulation of cooperating events."

Table V. Pro- and contra-arguments for a role of EBV as a tumor virus

Pro-arguments

1. Epidemiological evidence for: the presence of EBV in tumors; EBV infection precedes the development of the tumors; primary EBV infection manifested as IM increases the risk of developing EBV-positive classical Hodgkin lymphoma
2. EBV is the most potent B-cell transforming (immortalizing) agent.
3. EBV latent genes have transforming potential *in vitro* and *in vivo*.
4. The EBV episomes in the tumors are monoclonal.
5. Tumor cells maintain the EBV genome even though the viral episome replication in not perfect.
6. EBV is associated only with certain B cell-derived lymphoma subtypes (i.e. EBV is not randomly distributed among the B cell lymphomas) (summarized in table VI).
7. Classical HL originating from "Ig crippled" germinal center B cells almost always carry EBV.
8. EBV infection causes lymphomas in cottontop tamarins, common marmosets, and owl monkeys.
9. EBV-related LCVs also immortalize B cells *in vitro* and cause lymphomas *in vivo*.

Contra-arguments

1. The widespread nature of the EBV infection: many EBV carriers while only few persons develop EBV-related tumors.
2. EBV alone is not sufficient for tumorigenesis.
3. The same type of lymphoma can be EBV-positive or EBV-negative.
4. Most of the lymphomas do not express the proliferation-inducing type III latency.
5. EBV latent and lytic proteins are highly immunogenic, the immune response eliminates the emerging pre-malignat, EBV-carrying cells.
6. No support from population vaccination studies that tumorigenesis can be prevented.

Table VI. Association of EBV with mature B cell lymphomas

Mature B cell neoplasms	EBV association
1. Chronic lymphocytic leukaemia/ small lymphocytic lymphoma	rare
2. B-cell prolymphocytic leukaemia	negative
3. Lymphoplasmacytic lymphoma	negative
4. Splenic marginal zone lymphoma	negative
5. Hairy cell leukaemia	negative
6. Plasma cell neoplasms	rare
7. Extranodal marginal zone B-cell lymphoma	rare
8. Nodal marginal zone B-cell lymphoma	negative
9. Follicular lymphoma	negative
10. Mantle cell lymphoma	negative
11. Diffuse large B-cell lymphoma	10%
12. Primary mediastinal (thymic) large B-cell lymphoma	negative
13. Intravascular large B-cell lymphoma	negative
14. ALK-positive DLBCL	negative
15. Primary DLBCL of the CNS	negative
16. T cell/histiocyte-rich large B cell lymphoma	negative
17. Primary cutaneous DLBCL, leg type	negative
18. Burkitt lymphoma/leukaemia	
- sporadic	30%
- endemic	100%
- immunodeficiency-associated	25-40%
19. Lymphomatoid granulomatosis	100%
20. Hodgkin lymphoma (HL)	
- Nodular lymphocyte predominant HL	negative
- Classical Hodgkin lymphoma	40-100%
▪ Nodular sclerosis	10-40%
▪ Mixed cellularity	75%
▪ Lymphocyte-rich	50%
21. HIV-associated lymphomas	
- Lymphomas also occurring in immunocompetent patients	
▪ BL	25-40%
▪ DLBCL- centroblastic	30%
▪ DLBCL- immunoblastic	90%
▪ cHL	100%
- Lymphomas occurring more specifically in HIV-positive patients	
▪ Primary effusion lymphoma	80-90%
▪ Plasmablastic lymphoma	60-75%
▪ Lymphoma in multicentric Castleman disease	negative
- Lymphomas occurring in other immnuodeficient states	
22. PTLD	80-100%
23. EBV-positive DLBCL of the elderly	100%
24. DLBCL associated with chronic inflammation	60-80%
(Angioimmunoblastic T-cell lymphoma	variable)

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