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CHARACTERIZATION OF EPSTEIN-BARR VIRUS-ENCODED LATENT MEMBRANE PROTEIN 1

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Prof. Romano Regazzi

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SUMMARY

Epstein-Barr virus (EBV), a member of the gammaherpesvirus family, infects about 95% of the human population worldwide. EBV is associated with several kinds of cancers such as Hodgkin's lymphoma (HL), Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). Latent membrane protein 1 (LMP1), EBV's major oncogene, is an integral membrane protein composed of a short cytoplasmic N-terminal domain, six transmembrane spanning segments (TMs) linked by small loops and a long cytoplasmic C-terminal domain. LMP1 gene, *BNLF-1*, is highly polymorphic and several LMP1 variants were described. One significant difference found among LMP1 variants is their ability to activate NF-κB transcription factor. We mapped polymorphisms responsible for increased NF-κB activation levels by natural LMP1 variants when compared to B95-8 LMP1 prototype. All critical polymorphisms reside in LMP1 TMs 4 and 5. We studied the involvement of each pair of TMs for membrane association, self aggregation, binding of LMP1's cellular partners TRAF3 and β-TrCP, as well as for NF-κB activation. Moreover, we described a role for LMP1 in the inhibition of MAVS-mediated activation of ISRE and IFNβ-promoter activation. In summary, we observed that the different TMs pairs as well as the two intracellular loops are not equivalent. Overall, the study showed that TMs play key roles in protein-protein interactions and signaling and can be considered as essential regulators of LMP1's activities.

RÉSUMÉ

Le virus d'Epstein-Barr (EBV), un virus de la famille des gammaherpesvirus, infecte plus de 95% de la population adulte mondiale. EBV est associé à plusieurs types de cancers dont le lymphome de Hodgkin, le lymphome de Burkitt et le carcinome nasopharyngé. La protéine membranaire de latence 1 (LMP1), l'oncogène principal d'EBV, est une protéine membranaire intégrale composée d'une petite extrémité N-terminale cytoplasmique, six segments transmembranaires (TMs) lié par de petites boucles et un long domaine C-terminale cytoplasmique. Le gène de LMP1, *BNLF-1*, est très polymorphe et plusieurs variants de la protéine LMP1 ont été décrits. Parmi les variants de LMP1 la majeure différence décrite est leur capacité à activer le facteur de transcription NF-κB. Nous avons défini des polymorphismes permettant aux variants d'avoir une activation accrue de NF-κB comparé au prototype B95-8 LMP1. Tous les polymorphismes cruciaux identifiés dans notre étude se trouvent dans les TMs 4 et 5 de LMP1. Nous avons étudié l'implication de chaque paire de TMs dans l'association à la membrane, l'auto-agrégation, la liaison aux partenaires cellulaires de LMP1 TRAF3 et β-TrCP, ainsi que pour NF-κB. De plus, nous avons décrit un nouveau rôle pour LMP1 consistant à inhiber l'activation contrôlée par MAVS de ISRE et du promoteur d'IFNβ. En résumé, nous avons observé que les différentes paires de TMs, ainsi que les deux boucles intracellulaires, ne sont pas équivalents. Dans l'ensemble, notre étude a montré que les TMs jouent un rôle clé dans les interactions protéine-protéine et la signalisation et qu'ils peuvent être considérés comme des régulateurs essentiels des activités de LMP1.

ABBREVIATIONS

aa	amino acid
β-TrCP	Beta-transducin repeat-containing protein
BL	Burkitt's lymphoma
CTAR	C-terminal activating region
Co-IP	Co-immunoprecipitation
EBER	EBV encoded RNAs
EBERs	EBER1 and EBER2
EBV	Epstein-Barr virus (HHV-4)
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HIV-HL	HL arising in HIV-infected individuals
HL	Hodgkin's lymphoma
HPV	Human Papilloma Virus
HTLV	Human T-cell Lymphotropic virus
HHV	Human herpes virus
IARC	International Agency for Research on Cancer
IB	Immunoblotting
IFN	Interferon
IM	Infectious Mononucleosis
IP	Immunoprecipitation
KSHV	Kaposi's sarcoma-associated Herpesvirus (HHV-8)
LCL	Lymphoblastoid cell line
LMP1	Latent Membrane Protein 1
MCPyV	Merkel cell Polyomavirus
NF-κB	Nuclear Factor-kappaB
NPC	Nasopharyngeal carcinoma
PCR	Polymerase chain reaction
PTLD	Post transplant lymphoproliferative disease
SAP	SLAM associated protein
SLAM	Signaling lymphocytic-activation molecule

SHCS	Swiss HIV Cohort Study
TM	Transmembrane domain
TMs	Transmembrane segments
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAF	TNF Receptor Associated Factor
vFLIP	viral FLICE (caspases-8)-inhibitory protein
XLP	X-linked lymphoproliferative disease

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1 GENERAL INTRODUCTION

1.1 HUMAN TUMOR VIRUSES

For the year 2002, 1.9 million of cancers were attributable to infectious agents, such as viruses, Helicobacter pylori or parasites, associated with 17.8% of all cancers. Developing countries are more affected with respect to cancers attributable to infectious agents. Viruses contribute to the major part of human cancer worldwide, 12.1% [1]. The International Agency for Research on Cancer (IARC) classified all these agents as group 1 carcinogens (for classification see: http://monographs.iarc.fr/ENG/Classification/index.php). Several viruses are classified as group 1 carcinogens: Epstein-Barr virus (EBV - HHV-4), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human Immunodeficiency virus type 1 (HIV-1), Human papilloma virus (HPV) high risk 16-18-31-33-35-39-45-51-52-56-58-59, Human T-cell lymphotropic virus type 1 (HTLV-1) and Kaposi sarcoma-associated herpesvirus (KSHV - HHV-8). Other viruses are classified as probably carcinogenic to humans (group 2A): HPV 68; or possibly carcinogenic to humans (group 2B): HIV-2, HPV low risk 5-8-26-30-34-53-66-67-69-70-73-82-85-97. In 2008, a new polyomavirus has been found in Merkel cell carcinoma by Feng et al. and named Merkel cell polyomavirus (MCPyV) [2]. This virus was not yet classified by the IARC, but is in the list of priority agents for future IARC monographs [3].

A major advance in the fight against cancer would be to understand the oncogenic mechanisms of viruses in order to develop specific treatments and vaccines [4,5]. The first vaccine against a tumor-associated virus was developed in the late 1970s against HBV. More recently, in the mid 2000’s, two vaccines against HPV were available on the market: Gardasil (MERK) containing HPV genotypes 6, 11, 16 and 18, and Cervarix (GlaxoSmithKline) containing genotypes 16 and 18.

1.1.1 IARC GROUP 1 CARCINOGENS

Epstein-Barr virus (EBV) or Human herpes virus 4 (HHV-4) [Herpesviridae family, Gammaherpesvirinea subfamily, Lymphocryptovirus genus] is an enveloped double stranded DNA virus, which causes infectious mononucleosis and is associated with several tumors in humans such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, B and T cell lymphoma, post-transplant lymphoproliferative disease, leiomyosarcomas, gastric carcinomas. Patients with immunodeficiency as transplanted or AIDS patients are more susceptible to develop EBV-associated cancers. EBV was the first human tumor virus described.

Hepatitis B virus (HBV) [Hepadnaviridae family, Orthohepadnavirus genus] is an enveloped double stranded DNA virus, which causes acute and chronic hepatitis. If the infection lasts more than 3 months it can lead to cirrhosis or liver failure and to hepatocellular carcinoma.

Hepatitis C virus (HCV) [Flaviviridae family, Hepacivirus genus] is an enveloped ssRNA(+) virus, which causes acute and chronic hepatitis which can result in cirrhosis and hepatocellular carcinoma.

Human imunodeficiency virus type 1 (HIV-1) [Retroviridae family, Orthoretrovirinae subfamily, Lentivirus genus] is an enveloped ssRNA(+) virus, which acts mainly through immunosuppression to increase the risk of several cancers such as Kaposi sarcoma, non-Hodgkin lymphoma, cancer of the cervix – these three cancers are "AIDS-defining conditions" –, Hodgkin's lymphoma, anal cancer, seminoma, myeloma, and less certainly, cancers of the lip, brain and lung. Even if HIV-1 does not have proper oncogenic properties it was classified as group 1 carcinogen by the IARC in 1996.

Human papilloma virus (HPV) [Papillomaviridae family] is a small non-enveloped double stranded DNA virus, which causes benign papillomas or warts in humans by infecting

epithelial cells. It causes cervical cancer, genital cancers, skin cancer and cancers of the oral cavity and pharynx.

Human T-cell Leukemia virus type 1 (HTLV-1) [Retroviridae family, Orthoretrovirinae subfamily, Deltavirus genus] is a single stranded ssRNA(+) virus, which causes adult T-cell leukemia/lymphoma. It has a slow transforming potency and a long latency, but once tumor formation begins the progression is rapid

Kaposi's sarcoma-associated Virus (KSHV) or Human herpes virus 8 (HHV-8) [Herpesvirus family, Gammaherpesvirinea subfamily, Rhadinovirus genus] is a double stranded DNA virus. KSHV is a cofactor in the development of Kaposi sarcoma, multicentric Castelman's disease and pleural effusion lymphoma.

More details about carcinogenic viruses can be found in the following book and reviews [1,4,5,6,7].

1.2 HUMAN GAMMA-HERPESVIRUSES

Gamma-herpesvirus subfamily includes two genera: the gamma 1 or lymphocryptovirus genus to which belongs EBV also named HHV-4 and the gamma 2 or rhadinovirus genus to which belongs KSHV also named HHV-8. EBV and KSHV, as all herpesviruses, have a life cycle divided into two main phases: a lytic and a latent cycle. In the lytic phase new virions are produced in infected cells and viruses can spread in new cells or hosts. In latent phase viral genome is kept in host nucleus as an episome and few viral genes are expressed [7,8]. Both viruses are activators of the NF-κB pathway, which is involved in the viral life cycle of EBV and KSHV and is constitutively active in tumor cells from malignancies associated with these viruses. EBV-encoded latent membrane protein 1 (LMP1) and KSHV-encoded viral

FLICE (caspases-8)-inhibitory protein (vFLIP) are both able to constitutively activate NF-κB canonical and non-canonical pathways. This constitutive activation sustains latent infection and favors viral persistence *in vivo* increasing cell survival and proliferation, and is even essential for progression of virus-associated lymphomas [8].

1.3 EPSTEIN-BARR VIRUS

EBV was discovered in 1964 by Epstein, Achong and Barr [9] and was the first virus to be associated with human cancer [10]. EBV is an enveloped linear double stranded DNA virus with a genome of 184 kb [7]. For the structure of EBV virions see Figure 1.

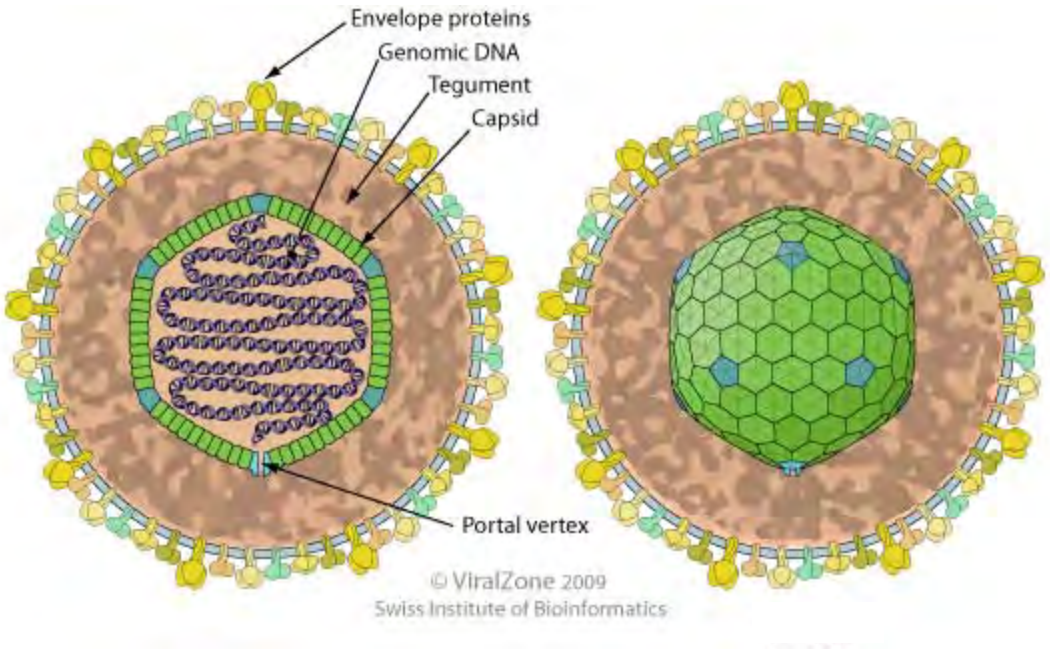

Figure 1: Structure of EBV virion EBV is composed of a genomic DNA core in a capsid of 162 capsomeres, an envelope with external glycoproteins, and a tegument between the capsid and the envelope. Taken from: http://expasy.org/viralzone/all_by_species/185.html.

EBV is a highly successful parasite infecting more than 95% of human adult population worldwide and is mainly spread by oral route through saliva. Primary infection is generally asymptomatic and occurs in oropharyngeal cavity. In developing countries most children are

infected before the age of three years and infection is mostly asymptomatic; whereas in developed countries primary infection is often delayed. Fifty percent of children are still seronegative at the age of 10 years and the acquisition of the virus occurs as adolescent or young adult. In that case 25% of the infections lead to infectious mononucleosis (IM) [7]. IM is a benign lymphoproliferative disorder, which symptoms are fever, sore throat, enlarged and painful lymph glands in the neck, severe and debilitating fatigue, and may last several months [11]. Among young males with X-linked lymphoproliferative disease (XLP), EBV infection is lethal and they succumb to fulminant IM with an excessive immune response. XLP is due to a single mutation in SAP (signaling lymphocytic-activation molecule [SLAM] associated protein) that is expressed in T and natural killer cells [11,12,13,14]. EBV is associated with malignances: nasopharyngeal carcinomas (NPC), Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL), B and T/NK cell lymphoma, post transplant lymphoproliferative disorder (PTLD), lymphoproliferative disease in immunosuppression, undifferentiated carcinomas of nasopharyngeal type, gastric carcinoma. More details about EBV associated diseases can be found in these book and reviews [7,11,15].

EBV is not cleared after primary infection, but establishes a life-long latent infection in human memory B cells as episome or rarely integrates host DNA. Under some circumstances the virus is reactivated and lytic cycle occurs with production of new EBV virions [7].

EBV can establish three different types of latencies in cells, which are associated with different disorders (Table 1). In type III latency EBV expresses the 9 latent proteins: 6 EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, LP), 3 latent membrane proteins (LMP1, 2A, 2B) and two small non-coding RNAs: EBER1 and EBER2 (Figure 2 and Table 1). This type of latency is found in lymphoblastoid cell lines (LCLs), infectious mononucleosis, X-linked disorder and lymphoproliferative disease in immunosuppression. In type II latency only EBNA1, LMP1 and LMP2 are expressed in cells. This type of latency is found in tumor cells

from NPC, HL and T/NK cell lymphoma. In type I latency, only EBNA1 and EBER RNAs are found. This panel of genes is only expressed in Burkitt's lymphoma and in some memory B cells [7,16,17].

Some of these proteins play an essential role: EBNA1 is essential during the cellular division by binding the viral DNA to the cellular chromosomes, LMP1 and LMP2 prevent apoptosis of EBV infected cells [7].

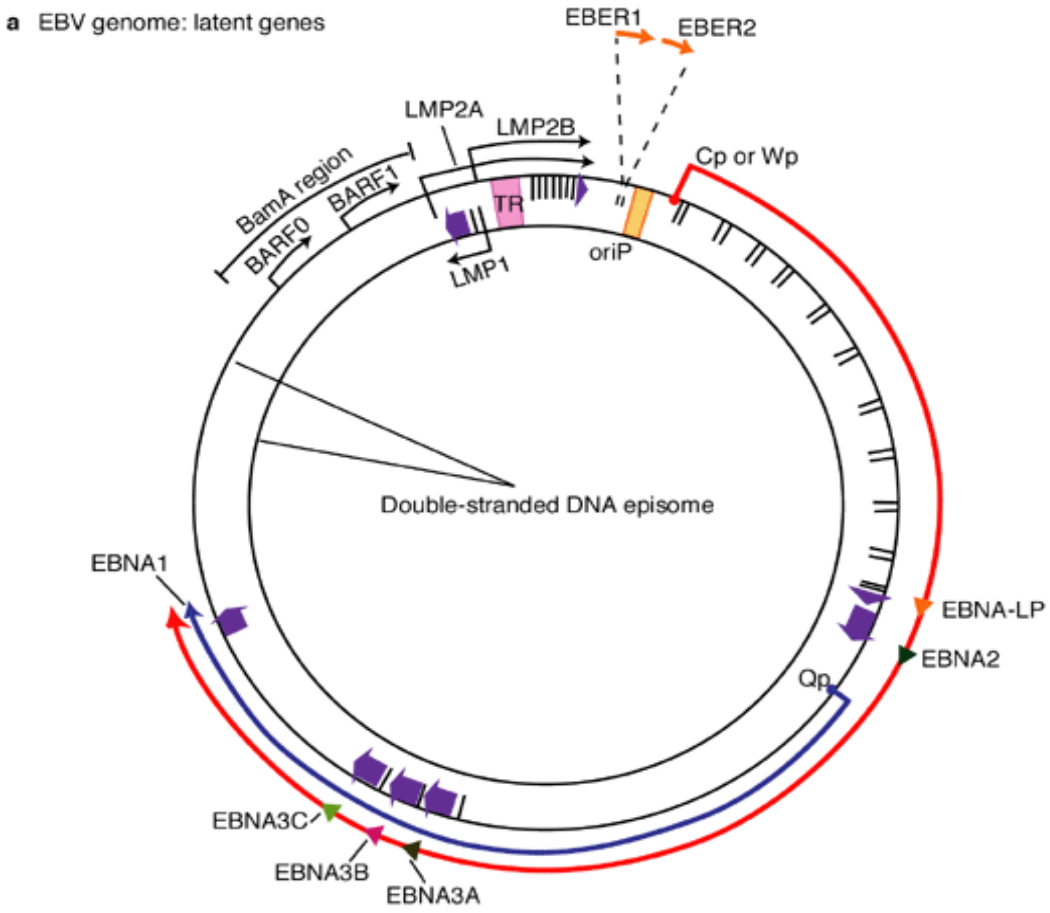

Figure 2: EBV genome and proteins expressed in la tencies. The six EBV nuclear antigens (EBNAs), the three latent membrane proteins (LMPs) and the two small non-coding RNAs (EBERs) are shown with arrows. Taken from [16].

Table 1: EBV types of latencies and related diseases.

Latency type	EBV proteins expressed	Occurrence/disorders
0		- Memory B cells
I	EBNA1 LMP2A	- Burkitt's lymphoma - Memory B cells
II	EBNA1 LMP1 LMP2A, 2B	- Classical Hodgkin lymphoma - Nasopharyngeal carcinoma - Nasal NK/T-cell lymphoma
III	EBNA1,2,3A,3B,3C,-LP LMP1 LMP2A, 2B	- Lymphoblastoid cell lines - Infectious mononucleosis - Post-transplant lymphomas - AIDS-related lymphomas

Two different types of EBV are described: type 1 EBV based on B95-8 strain and type 2 EBV based on AG876 strain. Type 1 and type 2 EBV differ in their nuclear protein genes and contain specific polymorphisms in EBNA-2, EBNA-3A, EBNA-3B, and EBNA-3C [18,19]. 80% to 90% of EBV isolated from Caucasian and Southeast Asian populations are of type 1 EBV. In African populations type 2 reaches an almost equal prevalence as type 1 EBV. Even if type 1 EBV transforms B cells to LCL more efficiently than type 2, no strain specific disease association has been observed [7]. Two or more EBV co-resident strains can be present in one individual, either of both type 1 and 2 or all of the same type. The question is still open whether the different strains are acquired at the same time or serially over time. It has been suggested that multiple co-resident strains are mainly found in immunocompromised patients, but with determination by heteroduplex tracking assay multiple strains have been found in immunocompetent patients too [20,21,22].

1.3.1 EBV AND HIV

EBV, as KSHV, is an important health problem for HIV infected patients, because of its high capacity to cause malignancies. In HIV-infected patients about 75% of non-Hodgkin lymphomas and the majority of HL are EBV positive [23,24]. In immunocompetent individual EBV reaches equilibrium with the immune system. When a person is infected by HIV, the tight equilibrium between EBV and the host immunity breaks. EBV viral load increases in HIV infected patients early after HIV seroconversion [25,26]. Primary HIV infection increases B-cell stimulation, an environment favorable for proliferating B-cells and thus reactivation of EBV leading to a higher EBV viral load [27]. Indeed, EBV viral load is high in HIV-infected patients it is not a predictive value for tumor formation, such as in transplant recipients [28,29,30,31]. Patients with higher EBV viral load are not those who develop cancer and the AIDS progressors are not those with higher EBV viral load, meaning that the degree of immunosuppression is not linked with EBV viral load but rather with B cell status [26].

EBV reactivation is not only linked with cancer in HIV-infected patients, but also with hairy leukoplakia that is characterized by the appearance of white irregular plaques on the tongue due to uncontrolled infection in the oropharynx of patients with severe immunosuppression [32].

Data from the Swiss HIV Cohort Study (SHCS) and cancer registries in Switzerland estimate excess cancer risk in persons infected with HIV and investigate the modifying effects of highly active antiviral therapy (HAART) use on cancer risk [33,34]. These analyses revealed that persons infected with HIV who are treated with HAART have a lower risk of developing Kaposi sarcoma and non-Hodgkin lymphoma than untreated patients; however, the incidence risk for Hodgkin lymphoma remains high in patients treated with HAART [33,34]. These

results suggest that additional factors than HIV-induced immunosuppression such as genetic viral variations are involved in the development of this type of lymphoma.

1.3.2 EBV AND INTERFERON-β

The host immunity is an efficacious way of defending cells against microbes. Viruses, as obligate pathogens, need to infect host cells to replicate and survive [7]. In this way, they evolved many different mechanisms to evade the host defenses, one of them the subversion of the innate immune response. This first line of defense, which first recognizes the invading agents and avoids their spread, leads to the induction of Type I interferon (IFNs) (IFNα/IFNβ) [35,36]. The IFNβ is the major player of the Type I interferon and will be the focus of this chapter.

The activation of the IFNβ pathway is a two steps process. During the first step, pathogens are sensed by the first defense line of the innate immune system, the pattern-recognition receptors (PRRs) among them Toll-like receptors (TLR) or RIG-like receptors (RLR) [37,38]. This sensing leads to the activation of the three transcription factors NF-κB, IRF3 and AP-1 (c-Jun/ATF-2), which translocate to the nucleus, bind to IFNβ-promoter and together induce the expression of IFNβ that is secreted [35,38]. During the second step, the secreted IFNβ bind to the Interferon-Alpha/Beta receptors (IFNAR) 1 and 2 of the same cell or of the neighboring cells to warn them of the danger. Thanks to this alert, cells mount antiviral response by inducing the activation of the Jak/STAT pathway leading to the induction of IFN-stimulated genes (ISGs). The simultaneous expression of several ISGs induces antiviral state [35,38].

Herpesviruses have to evade host immune defense at the infection and replication steps (lytic cycle), but also to stay hidden in the host cells during the persistent infection (latent cycle) [38,39]. EBV activates the IFN pathway during the first steps of infection [40,41]. This

activation is counteracted by the synthesis of *de novo* viral proteins, which inhibit both IFNα and IFNβ [38,39,40,41]. Whereas several strategies of immune escape during lytic infection have been identified, how EBV may circumvent the innate immune responses in the context of a latent infection is much less understood. During the latent infection, few viral latent proteins are expressed, their number differing according to the latency type, as well as small non coding nonpolyadenylated RNAs [42]. It has been shown that LMP2A and LMP2B limit the IFN signaling by degrading IFNAR 1 and 2 [43] and that LMP1 plays a determinant role in the block of Type I IFN by suppressing the phosphorylation of STAT1 and -2 through Tyk2 binding [44], but the observations do not fully explain how the virus counteract innate immune defenses.

1.4 LATENT MEMBRANE PROTEIN 1

1.4.1 GENERALITIES

Latent membrane protein 1 (LMP1), encoded by the *BNLF-1* gene, is the major transforming protein of EBV. LMP1 is an integral membrane protein composed by a short cytoplasmic N-terminal domain of 24 amino acids, 6 markedly hydrophobic transmembrane domains linked by short loops (aa 25-186), a long cytoplasmic C-terminal domain of about 200 amino acids (Figure 3) [45,46,47]. The gene encoding LMP1 contains two short introns. The N-terminal part of the protein is important for the correct insertion of the protein in the plasma membrane and is probably the attachment point for ubiquitin. LMP1 has a short half-life of two to five hours depending on the cell type [48,49,50] and is degraded via the proteasome [51]. The transmembrane segments are involved in self-aggregation and oligomerization of the protein. LMP1 localizes in large patches in the plasma membrane [52]. The C-terminal part possesses most of the signaling activity, e.g. NF-κB activation, through two C-terminal activating regions (CTAR) 1 (aa 351-386) and CTAR2 (aa 351-386) [53]. The two regions

have consensus sequences that have docking activities for cellular proteins permitting their signaling activities. CTAR1 and CTAR2 are both involved in NF-κB activation, but CTAR2 at a greater level [53].

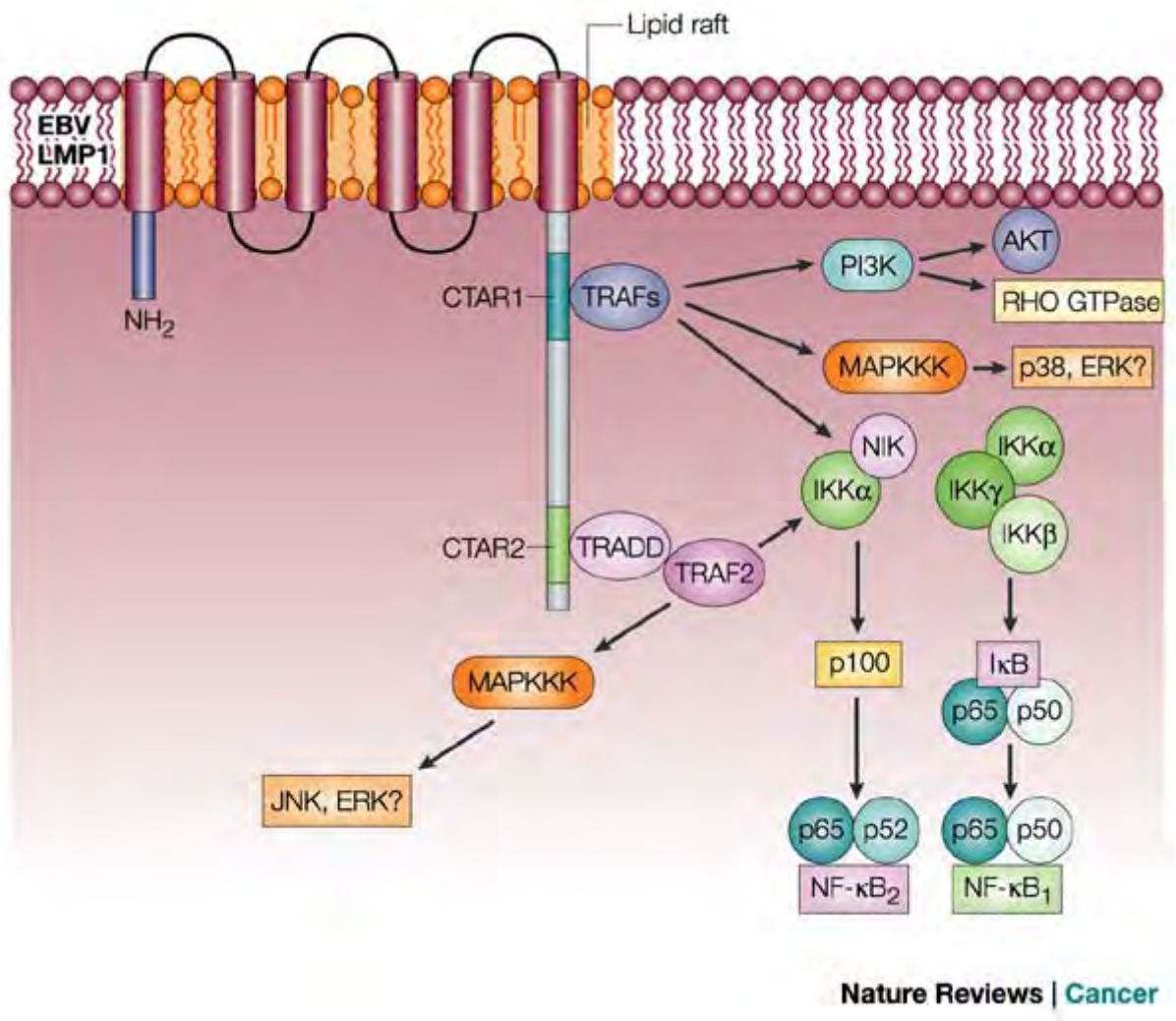

Figure 3: Schematic representation of LMP1. EBV-encoded LMP1 is an integral membrane protein composed of a short cytoplasmic N-terminal region (aa 1-24), six transmembrane spanning domains linked by extra- and intra-cellular loops of 4 to 12 amino acids (aa 25-186) and a long cytoplasmic C-terminal region (aa 187-386) containing two major regions for signaling, C-terminal activating region 1 (CTAR1) and CTAR2. LMP1 is involved in several signaling pathways and activates both NF-κB1 and NF-κB2 pathways. Taken from [42].

LMP1 acts as a constitutively active tumor necrosis factor receptor (TNFR) mimicking the CD40 receptor [54], binds to TRAFs [55] and thereby can engage several pathways, among them NF-κB [56,57,58], AP-1 [59], PI3K/Akt [60], c-Jun N terminal kinase (JNK) [10],

p38/ATF2 [61] or JAK/STAT [62] (Figure 3). NF-κB activation by LMP1 is critical for EBV-transformed lymphoblastoid cell survival [63]. Therefore LMP1 is essential for the conversion of primary B lymphocytes to lymphoblastoid cell lines (LCL) and protect them from apoptosis through the induction of Bcl-2 [64]. LMP1 has oncogenic effects in rodent fibroblast cell lines [65] and is able to create tumors in nude mice by inducing phenotypic changes in a non-tumorigenic human keratinocyte line [66] and causes lymphoma in transgenic mice [67].

1.4.2 LMP1 VARIANTS

LMP1 gene, *BNLF-1*, is highly polymorphic. Multiple EBV strains with different LMP1 variants are naturally present in the population. Interestingly, LMP1 variants diverge more within type 1 EBV than between type 1 and type 2 EBV [68].

The first and best characterized LMP1 is B95-8, known as the prototype LMP1, which was isolated from a cell line infected by an EBV from an American patient with IM [46,47,69,70].

CAO LMP1 isolated from a Chinese NPC is 88% similar to the prototype B95-8 LMP1 and differs by 27 amino acids, an insertion of three additional repeats (total of 28 aa) and a 30-bp deletion (10 aa: ^{343}GGGHSHDSGH^{352}) in the C-terminal end adjacent to the CTAR2 region. CAO has a higher molecular weight than B95-8 due to its protein length, 404 amino acids versus 386, respectively [71]. CAO LMP1 activates NF-κB and AP-1 pathways more potently than B95-8, is more strongly expressed upon transfection and more tumorigenic in a nude mice model [50,72].

AG876 LMP1, type 2 EBV, isolated from an African BL is 93% similar to the prototype and contains 12 amino acid changes and the same 30-bp deletion as CAO LMP1 [68]. It induces NF-κB at the same level as B95-8 LMP1 [72].

Raji LMP1 isolated from an African BL is 97.5% identical to the prototype and contains 19 aa changes [71,73].

Other LMP1 variants have been studied for their differences in amino acid sequences and classified according to these differences. Two different classifications have been proposed by Sandvej et *al.* [74] and Edwards et *al.* [75].

Despite the genetic diversity, EBV is a DNA virus that is stable over time. Brousset et *al.* demonstrated that LMP1 amplified from samples of two patients taken at first tumor episode and after relapse have the same sequence even after several years [76].

1.4.3 LMP1 INTERACTION AND SIGNALING

1.4.3.1 NF-κB

Nuclear factor-kappaB (NF-κB) comprises a family of eukaryotic inducible transcription factors, which are involved in inflammation, stress, immune response and malignant transformation. The proteins expressed by this family are conserved from *Drosophila melanogaster* to humans reflecting the importance of the NF-κB pathways [77].

In the absence of stimulus, NF-κB is tightly bound to the inhibitor of κB (IκB), which masks domains for nuclear localization and DNA binding. The NF-κB/IκB complex is therefore sequestered in the cytoplasm of the cell.

In stimulated cells, two different NF-κB pathways can be activated: the canonical or classical one and the noncanonical or alternative one. Canonical NF-κB pathway involves IκB kinase (IKK) complex composed of IKKα, IKKβ and IKKγ. IKK mediates the phosphorylation of IκB, which permits its ubiquitination by the ubiquitin ligase $SCF^{\beta\text{-}TrCP}$, followed by its degradation mediated by the 26S proteasome. This allows the release of NF-κB dimer, such as

p50-RelA or p50-cRel [77,78,79]. Noncanonical NF-κB pathway involves NF-κB inducing kinase (NIK) that in association with IKKα binds the C-terminal part of p100, which is the autoinhibitory part of the dimer, allowing its phosphorylation and subsequent recognition and ubiquitination by $SCF^{\beta\text{-}TrCP}$. This allows its recognition by the 26S proteasome, its proteolysis, its processing in p52 and the release of NF-κB dimer, such as p52-RelB. In both pathways uncovered dimers are able to enter the nucleus and activate target gene expression. The canonical pathway is self-regulated through the activation of the gene encoding IκBα, is transient and lasts 30-60 minutes. The duration of the noncanonical NF-κB pathway is longer [77,78,79].

NF-κB transcription factors bind to 9-10 base pairs DNA sites called κB sites as dimers [77]. They activate more than one hundred genes with key cellular function, e.g. Il-2 or Interferon gamma, which are involved in immunity, CD40 which is an immunoreceptor, IκBα which is a NF-κB inhibitor, as well as nfkb2 which is the NF-κB p100 precursor [77].

NF-κB plays a determinant role in cell transformation: tumor promotion is mediated by its anti-apoptotic functions and aberrant activation of NF-κB is associated with tumorigenesis [80,81].

Interestingly, LMP1 activates both NF-κB pathways: the classical mostly through CTAR2 and the alternative mostly through CTAR1.

1.4.3.2 β-TrCP

Beta transducing-repeat containing protein (β-TrCP) is the Fbox subunit of the S-phase-kinase-associated protein-1 (Skp-1)-Cullin1-Fbox protein (SCF) E3 Ubiquitin ligase [78,82]. $SCF^{\beta\text{-}TrCP}$ recognizes specifically phosphorylated substrates and confers their ubiquitination leading to their degradation by the 26S proteasome and plays a critical role in the regulation

of cell cycle progression, cell proliferation and survival. Among $SCF^{\beta\text{-TrCP}}$ substrates are β-catenin, IκB and caspase-3; HIV-1 encoded Vpu is a pseudosubstrate of the β-TrCP [78,82,83]. By controlling the substrate specificity, β-TrCP is a key subunit of the ubiquitin ligase complex. Via the N-terminal F-box domain β-TrCP binds to Skp-1 and via the C-terminal WD-40 domain to phosphorylated substrates [78]. The majority of β-TrCP substrates contain a canonical DSGxxS motif in which the phosphorylation of the two serine residues is required to allow recognition [84]. A similar DSG motif is found in the C-terminal part of LMP1, within the CTAR1 [82]. β-TrCP2 was shown to bind LMP1 motif [82].

β-TrCP is a highly conserved protein from Xenopus to humans. Human β-TrCP is present in cells in two homologs: β-TrCP1 (aka FWD1/Fbw1a) and β-TrCP2 (aka HOS/Fbw1b). β-TrCP1 was mainly found in the nucleus, whereas β-TrCP2 is predominantly localized in the cytoplasm [84]. They are divided in several spliced forms all containing the F-box motif at the N-terminus and seven WD40 repeats at the C-terminus [78].

1.4.3.3 TRAF3

Tumor necrosis factor receptor (TNFR) associated factors (TRAFs) are cytoplasmic adapter proteins. Members of the TRAF family share a similar TRAF domain in the C-terminal part of the molecule allowing the homo-/hetero-trimerization, interaction with receptor or downstream signaling molecules. They contain three other domains: a Zinc binding motif in their N-terminal part, a RING finger and an isoleucine zipper [79].

TRAFs play vital roles in inflammatory responses, lymphoid tissue development, and orchestration of adaptive immune responses via activation of both canonical and noncanonical NF-κB signaling pathways [79]. TRAF2, TRAF5 and TRAF6 have a role in activation of NF-κB canonical pathway, whereas the role of TRAF3 is much more complex because it has

been shown to be a negative regulator of the noncanonical pathway [85]. It was shown that a lack of TRAF3 leads to an increase of NIK level implying a constitutive p100 processing and so an activation of the noncanonical NF-κB pathway [79].

TRAF3 can associate with the CD40 receptor, which is mimicked by EBV LMP1 protein. TRAF3 can also bind to the LMP1 upon a PxQxT motif present in CTAR1 [86].

2 AIMS

2.1 AIM OF THE CHAPTER 1

A large part of the population is infected by EBV, but only few people develop EBV-associated malignancies. Factors contributing to the development of tumors are of environmental nature, genetic background of either host or virus. In our study we focus on the possible role of LMP1 strain variations in tumor pathogenesis. We were interested to study LMP1 because it was shown to be EBV's major oncogene and expressed in the majority of tumor types. The gene encoding LMP1 is polymorphic. CAO, a variant isolated from Chinese NPC was found to have increased potential to transform rodent fibroblasts and to induce tumors in nude mice [71], leading to the hypothesis that polymorphisms within LMP1 gene influence the susceptibility to develop EBV-associated diseases. Moreover, when compared to B95-8 LMP1, CAO induces higher levels of NF-κB activation, an effect that has been reported in several cell types [50,72]. Despite extensive studies, polymorphisms associated with functional differences such as an increased capacity to activate NF-κB have not yet been identified.

2.2 AIM OF THE CHAPTER 2

LMP1, a mimic of CD40 receptor, is an integral membrane protein with a short cytoplasmic N-terminus, six transmembrane-spanning segments (TMs) and a 195 amino acids long C-terminal domain [45,46,54]. The N- and C-terminal domains of the protein were widely analyzed for their role in LMP1 signaling properties [87,88,89]. The TMs were less studied and few are known about the relative contribution of each pair of TMs to the functional activity [90,91,92,93,94]. To analyze the importance of each TM pairs we used deletion mutants with four or two TMs to perform functional analyses.

3 CHAPTER 1 -

LMP1 IN THE SWISS HIV COHORT STUDY

3.1 CHAPTER 1 - ORIGINAL ARTICLE (MANUSCRIPT IN REVISION)

« Genetic diversity of EBV-encoded LMP1 and implication for NF-κB activation and Hodgkin's lymphoma susceptibility in the Swiss HIV Cohort Study »

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Manuscript in revision

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Wrote the paper: **E. Zuercher**, A. Telenti, S. Rothenberger

Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution:

I was the main person in charge of the project. I designed and analyzed the questionnaires about the patients enrolled in the SHCS, and collected the samples provided by the different cohort and pathology centers. I performed and analyzed the vast majority of the experiments presented in this study (Figure 1B-C, 2-4). Finally, I actively participated in the writing of the manuscript.

Comments on the manuscript:

It has been reported that LMP1 is highly polymorphic and several variants have been described. Among these LMP1 variants different abilities to activate NF-κB pathways were observed. The aim of this study was first to map polymorphisms involved in increased activation of NF-κB and then to determine whether these polymorphisms were enriched in samples from patients with EBV-associated cancer in a pilot study in the Swiss HIV Cohort Study (SHCS).

We found three polymorphisms determinant for increased NF-κB activity, two of them, linked with additional polymorphisms, defined two distinct LMP1 phylogenetic groups: I124V/I152L and F144I/D150A/L151I. They are predictors of high NF-κB activation levels, and were tested for their role in Hodgkin's lymphoma etiology. No association was found between these predictors of high NF-κB activation levels *in vitro* and the development of Hodgkin's lymphoma among HIV-infected individuals enrolled in the SHCS.

The data not shown contained in the manuscript are described at the end of the Chapter 1 under Supplementary figures and methods. The sequences of the LMP1 variants obtained in the study are reported in the Appendix 1.

ABSTRACT

Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1), a multifunctional oncoprotein, is a powerful activator of the transcription factor NF-κB, a property that is essential for EBV-transformed lymphoblastoid cell survival. Previous studies reported LMP1 sequence variations and induction of higher NF-κB activation levels compared to the prototype strain B95-8 LMP1 by some variants. Here we used blood of individuals included in the Swiss HIV Cohort Study (SHCS) to analyze LMP1 genetic diversity and LMP1-mediated NF-κB activation levels by gene reporter assay. We found that a number of variants mediate higher NF-κB activation levels when compared to B95-8 LMP1 and mapped several polymorphisms responsible for this phenotype, among them F144I and I124V. The sets of polymorphisms I124V/I152L and F144I/D150A/L151I were chosen as markers to assess whether the presence of LMP1 variants with increased NF-κB activation *in vitro* influences Hodgkin's lymphoma (HL) susceptibility in a pilot epidemiological study within the SHCS.

The analysis of LMP1 variants in blood of SHCS participants with or without HL diagnosis showed that I124V/I152L and F144I/D150A/L151I, when combined, were present in more than 50% of the samples from each group. Concordant with a high distribution in the two groups of patients, the two sets of polymorphisms are not identified as predictive factors associated with HIV-HL susceptibility when analyzed either together or individually by a logistic regression model. In order to determine to which extend EBV strains differ between blood and tumor, we further compared LMP1 sequences present in the blood and in the corresponding biopsy from HIV-HL. The good correspondence of LMP1 variants between blood and tumor biopsies in 10/15 cases indicates that larger future studies using blood samples would be relevant to the identification of viral polymorphisms influencing the development of EBV-associated malignancies.

INTRODUCTION

Epstein-Barr virus (EBV) infects more than 90% of human adults worldwide. EBV causes infectious mononucleosis and is associated with several human malignancies, among them nasopharyngeal carcinoma (NPC), Burkitt's lymphoma and Hodgkin's lymphoma (HL) [42]. EBV infection of B-lymphocytes is mostly non-lytic and results in the expression of a limited number of nuclear and membrane proteins. EBV-encoded latent membrane protein 1 (LMP1) is a multifunctional oncoprotein essential for EBV-induced B-cell proliferation and transformation *in vitro* [65,95,96,97]. LMP1 also has transforming effects on non-lymphoid cells such as rodent fibroblasts and keratinocytes [65,98,99]. LMP1 is a powerful inducer of nuclear factor-κB (NF-κB)-mediated transcription [56,57], a property that is essential for EBV-transformed lymphoblastoid cell survival [63]. NF-κB plays a determinant role in cell transformation: tumor promotion is mediated by its anti-apoptotic functions and aberrant activation of NF-κB is associated with tumorigenesis [80,81]. Moreover, this transcription factor is essential for the progression of EBV-associated lymphomas *in vivo* [100]. Up to date, the vast majority of functional studies on LMP1 has used as a prototype B95-8, an infectious mononucleosis derived isolate. CAO LMP1, a variant isolated from a NPC, was found to have increased potential to transform rodent fibroblasts and to induce tumors in nude mice, when compared to B95-8 LMP1 [71], leading to the hypothesis that polymorphisms within LMP1 gene influences the susceptibility to develop EBV-associated tumors. When compared to B95-8 LMP1, CAO LMP1 induces higher levels of NF-κB activation [50]. This observation has been extended to several cell types, such as HEK 293, Elijah-BL, Daudi BL, DG75 and Jurkat [72], demonstrating that this property is not cell-type specific. However, polymorphisms modulating NF-κB activation have not been mapped to date.

Patients infected with human immunodeficiency virus (HIV) are at high risk of developing EBV-associated lymphoproliferative disorders [101]. Earlier, a study of the Swiss HIV

Cohort demonstrated that immune responses contribute to the development of EBV-associated brain lymphoma [102]. However, the contribution of viral factors to the development of EBV-associated malignancies is not well understood. EBV has been detected in 80-100% of Hodgkin's lymphoma arising in the setting of HIV, supporting the notion that EBV plays a pivotal role in the pathogenesis of this type of tumor [24,103]. Since the introduction of highly active antiretroviral therapy (HAART) the number of cancer cases has decreased in HIV-infected individuals worldwide. In contrast, the frequency of HL among HAART treated HIV-infected patients increases with the prolonged life expectancy [24,34,104], suggesting that factors other than HIV-induced immunosuppression are involved in the etiology of HL. Of special importance is the detection of EBV type II latency program in HL, which is characterized by the expression of LMP1 and two other viral proteins [105]. The strong association between EBV and HL in HIV-infected individuals (HIV-HL) and the limited number of viral products expressed in the tumor cells provide ideal conditions to study the impact of LMP1 genetic variations on the etiology of EBV-associated tumors.

We mapped several single amino acid polymorphisms leading to significantly enhanced capacity for NF-κB activation compared to B95-8 LMP1 and used samples from the Swiss HIV Cohort Study (SHCS) to evaluate the importance of LMP1 polymorphisms relevant to NF-κB activation in the etiology of EBV-associated HL.

MATERIALS AND METHODS

Samples

Paraffin blocks of biopsies of B-cell and T-cell lymphomas were provided by the Institute of Pathology in Lausanne. One biopsy from a patient with a post-transplant lymphoproliferative disorder (PTLD) was obtained from Geneva University Hospital. Biopsies specimen of Hodgkin's lymphoma of HIV-infected individuals were obtained from the Institutes of Pathology of the universities of Bern, Geneva, Lausanne and Zurich. Blood samples of HIV-infected individual were provided by several centers of the SHCS: Bern, Basel, Geneva, Lausanne, Lugano and Zurich. This study was approved by the scientific review board of the Swiss HIV Cohort Study**.** All SHCS participants provided informed written consent and the SHCS has been approved for Lausanne by the commission cantonale d'éthique de la recherche sur l'être humain du canton de Vaud.

DNA extraction

DNA of tumor samples of B and T cell lymphomas and a case of PTLD was prepared according to standard procedures in pathology centers of Lausanne and Geneva. DNA of tumor samples from Zurich was extracted at the pathology center of Zurich according to standard procedures. Isolation of DNA from biopsies and cell samples of HIV-infected individual was performed with QIAamp DNA Mini Kit (QIAGEN, Basel, Switzerland) according to manufacturer's instructions. Frozen biopsies were crushed before DNA extraction. Paraffin from paraffin embedded biopsies was removed by dissolution with xylol 100% before DNA extraction.

Amplification by polymerase chain reaction and isolation of LMP1 genes

The region between positions 169.508 and 168.111 of the EBV genome was amplified using a specific primer pair based on the published prototype B95-8 LMP1 sequence [46]. PCRs were carried out either with Pwo DNA Polymerase (Roche Applied Science) on the four biopsies of EBV-associated tumors (A1, A2, A3 and P1) or with AmpliTaq Gold DNA Polymerase on samples from HIV-infected individuals. Primers and PCR programs are available on request. PCR products were purified with QIAquick Gel Extraction Kit (QIAGEN) or MSB Spin PCRapace (Invitek, Berlin, Germany) and sequenced directly using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The whole gene was subcloned into a eukaryotic expression vector, pCR3.1 (Invitrogen, Basel, Switzerland), using adapters with HindIII and XbaI restriction sites. At least two independent clones of each variant were sequenced on both strands. B95-8 and AG876 full length LMP1 were constructed by cloning LMP1 gene from the lymphoblastoid cell lines B95-8 [106] and AG876 (kindly provided by A. Rickinson, Birmingham, UK) into pCR3.1 vector. CAO variant [71] (kindly provided by F. Grässer, Homburg, Germany) was subcloned into the same background vector.

Construction of LMP1 mutants

Chimeras were built by enzymatic digestion of the gene encoding B95-8, A2 and P1 LMP1s using the internal DNA restriction sites NaeI or BglII. Mutations were introduced by PCR using Pwo polymerase. All constructs were cloned into the expression vector pCR3.1 and sequenced. Plasmids were amplified and purified using Qiagen plasmid MIDI kit (QIAGEN) and quantified using NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Cell culture

Human embryonic kidney 293 (HEK) [107] cells were cultured in Dulbecco's modified Eagle medium (DMEM - Gibco, Basel, Switzerland) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin and streptomycin at 37°C with 5% CO_2. Lymphoblastoid cell lines B95-8 and AG876 were cultured in Roswell Park Memorial Institute medium (RPMI - Gibco, Basel, Switzerland) supplemented with 10% (v/v) heat-inactivated FCS, penicillin and streptomycin at 37°C with 5% CO_2.

Gene reporter assay

Gene reporter assay system was used to measure NF-κB activation levels induced by LMP1. Expression vectors for either prototype B95-8 LMP1, variants or mutants were co-transfected with the reporter κB-conA-luc (kindly provided by F. Grässer) that comprises *Firefly* luciferase gene under the control of a conalbumin reporter with 3 integrated κB elements derived from the immunoglobulin κ chain enhancer. HEK cells were transfected in 24-well plates with 50 ng of NF-κB *Firefly* reporter construct κB-conA-luc and 50 ng of LMP1 expression vectors using FuGENE6 (Roche Applied Science). Twenty-four hours after transfection, cells were lysed in Cell Culture Lysis Reagent (Promega, Amriswil, Switzerland) and assayed for light emission in a tube luminometer Lumat LB 9507 or a microplate luminometer Tristar LB 941 (BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany) using Luciferase Assay System (Promega). Final quantifications were calculated on three separate experiments performed in triplicates. Statistical analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, http://www.graphpad.com).

Immunoblot

Samples from gene reporter assays were boiled at 95°C for 4 min in 2x sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 100 mM DTT) and separated through 10% SDS-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membrane, probed with the appropriate antibodies and revealed by enhanced chemiluminescence (ECL - LiteAblot, Euroclone, Italy). Primary antibodies were used at the following dilutions: LMP1 rat monoclonal 8G3 [108], 1:500 – 1:1.000 (provided by F. Grässer). Mouse anti-alpha-tubulin (Sigma), 1:10.000. Secondary antibodies were used at the following dilutions: Polyclonal rabbit anti-mouse HRP and polyclonal rabbit anti-rat HRP (Dako Cytomation, Glostrup, Denmark), 1:4.000 – 1:5.000.

Cell viability

Toxicity of the different LMP1s was measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to manufacturer's protocol. This method allows determining the amount of viable cells by measuring ATP levels in samples. Briefly, HEK cells in 24-well plates were transfected with 50 ng expression plasmids using FuGENE 6. Fourteen hours post transfection cells were seed in 96-well plates and twenty-four hours post transfection cells were lysed in CellTiter-Glo® Reagent and assessed for luminescent signal in a microplate luminometer Tristar LB 941.

Phylogenetic tree

For phylogenetic analysis, LMP1 nucleotide sequences from position 169474 to 168160 of EBV genome were aligned with Geneious (v5.1; A. J. Drummond et al., Biomatters Ltd., http://www.geneious.com) and controlled manually for repeats and deletion alignment. The alignment was used to build neighbor-joining tree (Jukes-Cantor model) using Geneious.

EBV typing

EBV type was determined on EBNA2 gene as described in Telenti *et al.*[25].

Analysis of samples from patients of the SHCS with or without HL

Among participants of the SHCS, 48 have had a confirmed diagnosis of HL at the date of our study. We included in our study 42 HIV-HL with available blood samples (37 men and 5 women; age at the date of blood sampling 44.2 ± 10.7 years) and 90 SHCS participants without any tumor history (72 men and 18 women; age at the date of blood sampling 41.9 ± 9.2 years). For 16 HIV-HL we obtained blood as well as tumor biopsy. Sequences of LMP1 gene encoding amino acids 96 to 202 were determined using PCR amplification on genomic DNA with AmpliTaq Gold DNA polymerase followed by sequencing with nested primers, using the same method as above. Sequences were obtained for 41/42 HIV-HL (36 men and 5 women, mean age 44.3 ± 10.8 years), for 15/16 biopsies and for 85/90 participants without tumor (67 men and 18 women, mean age 42 ± 9.4 years). The strength of the association between the two sets of polymorphisms I124V/I152L and F144I/I150A/L151I and the etiology of HL was tested with a simple logistic regression and then with a logistic regression model controlling for sex, age, CD4 levels and the number of years of antiretroviral therapy at the date of blood sampling, using Stata 11 software (StataCorp, http://www.stata.com).

RESULTS

Polymorphism F144I modulates NF-κB activation mediated by variant A2

Among a panel of LMP1 variants with different abilities to activate NF-κB we selected two variants, P1 and A2, on the basis of their activation profile to perform further studies (Figure 1A). The level of activation of variant P1 is close to that of prototype B95-8 LMP1, whereas that of variant A2 has a highly increased level of activation close to that of CAO LMP1. We observed that the prototype LMP1 and the two variants were all well expressed (Figure 1B) and non-toxic using a cell viability assay (Figure 1C). Furthermore enhanced NF-κB activation is not associated with a prolonged half-life, since half-lives of 3.6 ± 0.6 and 2.8 ± 0.4 hours were determined for P1 and A2, respectively (data not shown). An overview of amino acid changes identified in variants P1 and A2 with respect to reference B95-8 LMP1 [46] is shown in Figure 1D. Variant A2 has a very high overall similarity to CAO LMP1 [50,71,109].

We then mapped polymorphisms responsible for the differences in NF-κB activation. In order to identify the regions implicated we constructed chimeras between variants and B95-8 LMP1. In a first set of chimeras, we exchanged the first 231 amino acids of either variant with B95-8 LMP1 and constructed the reciprocal chimeras using the same strategy (Figure 2A). Functional analysis by gene reporter assay showed that B95-8^{231}/P1 and P1^{231} /B95-8 activate NF-κB at levels close to P1 (Figure 2B). In contrast, B95-8^{231}/A2 and A2^{231}/B95-8 show a striking difference in their signaling potential. Whereas B95-8^{231}/A2 induces NF-κB to level close to those of the prototype, A2^{231}/B95-8 induces very high level of NF-κB activation, close to that of A2. Our results showed that polymorphisms within the 231 N-terminal amino acids of A2 are responsible for the increased capacity to activate NF-κB, whilst polymorphisms between amino acids 232 and 386, such as the 10 amino acids deletion and variations in the direct repeats, do not contribute to the enhanced NF-κB activation

phenotype. To better refine regions associated with increased NF-κB activation, we exchanged the first 118 amino acids of either variant with B95-8 LMP1, and generated reciprocal constructs using the same strategy (Figure 2C). $P1^{118}$/B95-8 is slightly more effective than the reciprocal construct B95-8^{118}/P1 (Figure 2D). Neither B95-8^{118}/A2 nor $A2^{118}$/B95-8 displays the full A2 phenotype. Interestingly, $A2^{118}$/B95-8 induces levels comparable to $P1^{118}$/B95-8, suggesting that A2 and P1 share polymorphisms in the N-terminal part of the molecule (amino acids 1-118) associated with a modest increase in NF-κB activation levels with respect to the prototype. NF-κB activation level induced by B95-8^{118}/A2 is significantly higher than that of the prototype, indicating that one or a combination of polymorphisms localized between amino acids 119 and 231 are associated with enhanced NF-κB activation. The comparison of the amino acid sequences of variants P1 and A2 within residues 1 to 118 shows two common polymorphisms: I85L and F106Y (Figure 1D), which were introduced individually in the B95-8 sequence to evaluate their influence. Mutation F106Y, but not I85L, is associated with an increase in NF-κB activation level (Figure 2E). Interestingly, the single mutation F106Y in the context of B95-8 increases NF-κB activation level 3-fold, whereas in the context of chimeras $P1^{118}$/B95-8 and $A2^{118}$/B95-8 its effect is limited indicating that other polymorphisms located between amino acids 1 and 118 counteract this effect. We tested the influence of 8 additional polymorphisms present in variant A2 between residues 119 and 231 (Figure 1D). Functional analysis of LMP1 mutants shows that mutation F144I leads to a significant increase in NF-κB activation (Figure 2E). In contrast, other mutations are slightly deleterious (M129I, D150A or L151I) or neutral (Q189P, S192T and G212S) (Figure 2E and data not shown). In order to confirm the importance of F144I we mutated isoleucine 144 to phenylalanine in variant A2. NF-κB activation level induced by A2 I144F is close to that of B95-8 (Figure 2F). In contrast, mutation of tyrosine 106 to phenylalanine in variant A2 does not change NF-κB signaling

potential of the variant (Figure 2F). These results demonstrate that F144I is the unique polymorphism responsible for the high NF-κB activation level mediated by A2.

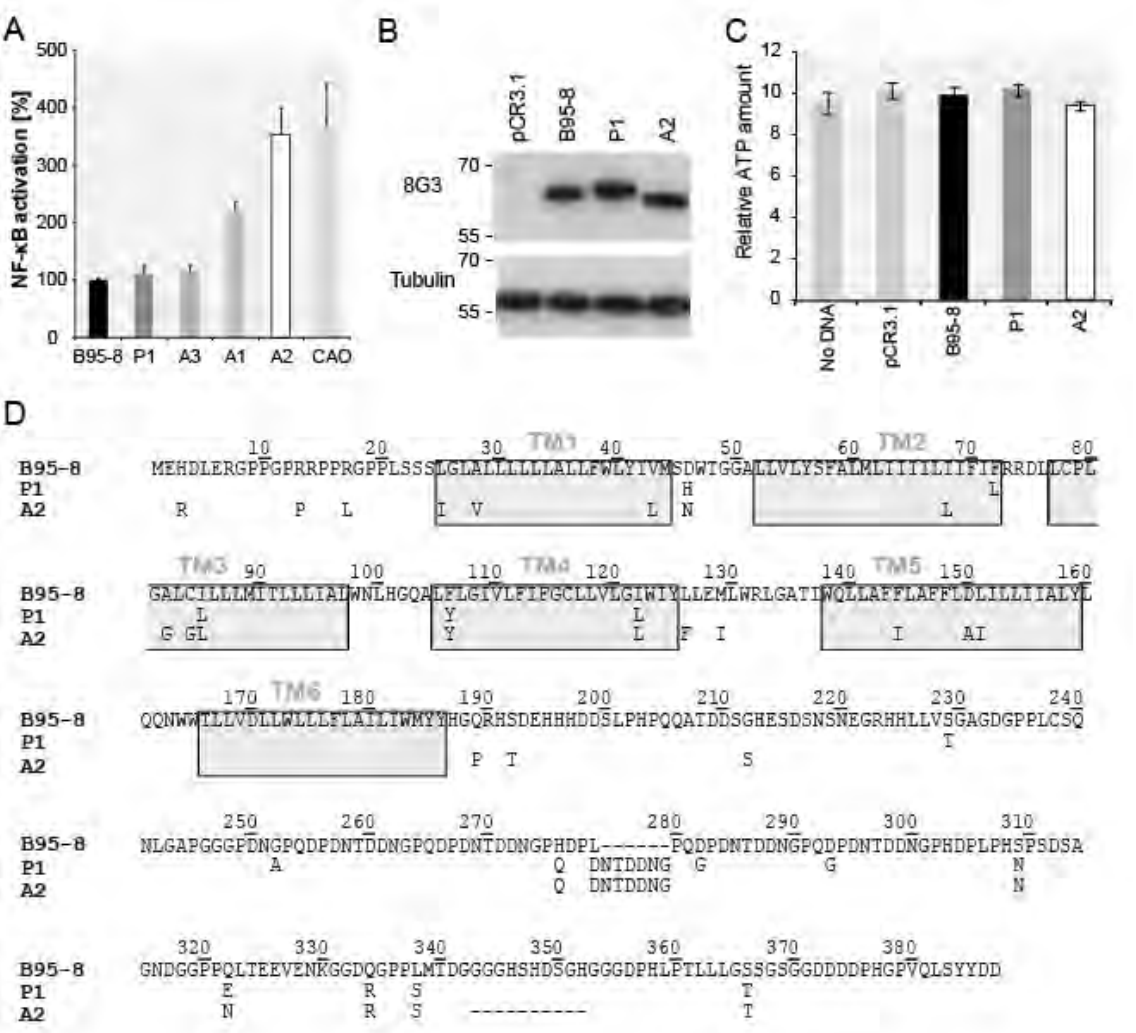

Figure 1. Analysis of LMP1 variants. (A) NF-κB activation by LMP1 variants. Cells were transfected with 50 ng of expression vector coding for LMP1 prototype (B95-8) and variants (P1, A3, A1, A2, CAO) and 50 ng of *Firefly* luciferase reporter plasmid. An analogous strategy of subcloning was applied to variants, prototype and CAO LMP1, to ensure appropriate comparisons. NF-κB activity was measured twenty hours after transfection using luciferase assay (Promega). Data are mean ± SD of triplicates and shown is a representative of three independent experiments with similar results. **(B)** Expression of LMP1 B95-8, A2 and P1 was visualized by SDS-PAGE and Western blotting with anti-LMP1 8G3 antibody. Detection against tubulin was used as internal control. **(C)** Measure of the toxicity of LMP1 variants. Cells were transfected with 50 ng of LMP1 B95-8, P1 or A2. Untransfected cells and cells transfected with empty vector were used as experimental controls. ATP amount was measured 24 hours after transfection using CellTiter-Glo Luminescent Cell Viability Assay (Promega). Shown is a representative experiment of three independent experiments with similar results. **(D)** Amino acid sequence alignment of B95-8, P1 and A2 LMP1. Only amino acids that differ from the sequence of prototype B95-8 LMP1 are indicated. Transmembrane segments are indicated by light gray boxes and deletions by dashes.

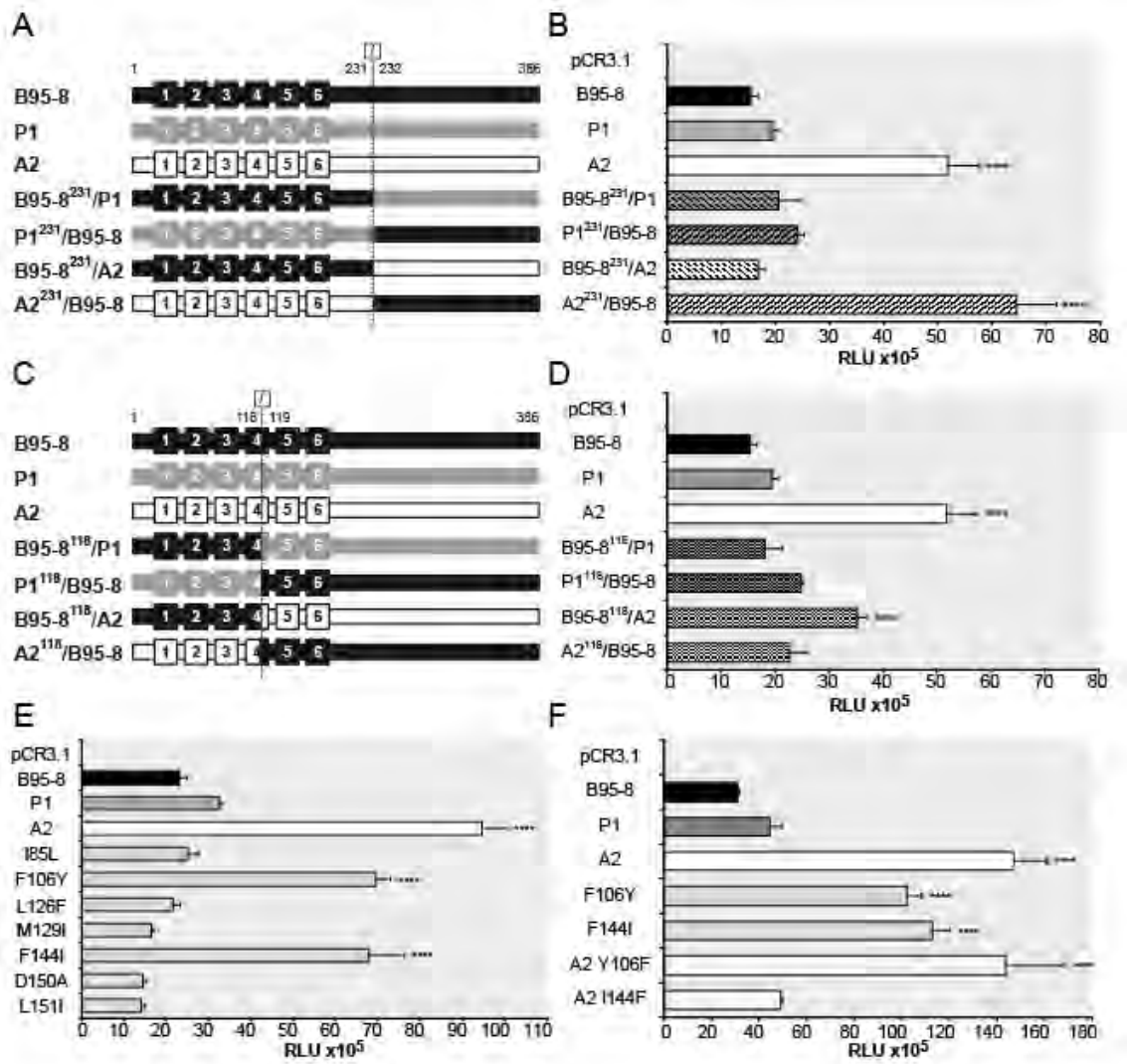

Figure 2. NF-κB activation levels by LMP1 chimeras and mutants. (A, C) Schematic representation of LMP1 chimeras split at amino acid 231 **(A)** and 118 **(C)**. The six transmembrane segments are represented by boxes. **(B, D-F)** NF-κB activation by LMP1 chimeras split at amino acid 231 **(B)** and 118 **(D)**, and LMP1 mutants on B95-8 background **(E)** and on A2 background **(F)**. HEK cells were transfected with 50 ng of LMP1 vector and 50 ng of NF-κB reporter plasmid. Empty vector was used as control. NF-κB activity was measured twenty-four hours after transfection using luciferase assay (Promega). Shown are representative of three independent experiments with similar results. Data are given as mean ± SD of triplicates. Statistical analysis was done using one-way ANOVA with Bonferroni posttest using GraphPad Prism, n=9 triplicates of three experiments. **** $P<0.0001$ relatively to the NF-κB activation of B95-8 LMP1. RLU: relative light units.

Enhanced LMP1-mediated NF-κB activation is linked to polymorphisms in the transmembrane region

To better understand the importance of polymorphism F144I in the context of LMP1 sequence variation in individuals without a diagnosis of EBV-associated tumor we characterized LMP1 from blood of HIV-infected individuals included in the SHCS. Among

randomly chosen genomic DNA, we obtained 31 full length LMP1 genes in a single amplification. The frequencies of amino acid changes compared to the B95-8 prototype are reported in Figure 3A. F106Y is present in 31/31 variants. The distribution of F144I is more restricted (5/31). 14 variants with a 10 amino acids deletion and 2 with a 23 amino acids deletion were isolated. A neighbor joining tree was built based on LMP1 nucleotide sequences from position 169474 to 168160 of EBV genome. Variants A1, A2, A3 and P1, the 31 variants isolated from HIV-infected individuals and the references LMP1 B95-8, CAO, Raji and AG876 were included in the analysis (Figure 3B). Three different phylogenetic groups emerged from the tree. The first group comprising 5 LMP1 variants – 7836, 7885, 7850, 7825 and 7939– is characterized by two polymorphisms I124V and I152L, which always segregate together. The second group includes A2, CAO and the five variants 7924, 7705, 7950, 7823 and 7910. All variants from the second group are characterized by F144I, D150A and L151I and are more closely affiliated to the China1 strain described by Edwards et *al.* than to any other LMP1 strain [109]. The other variants formed a more heterogeneous group. Functional analysis revealed that a number of LMP1 variants display enhanced NF-κB activation profiles when compared to B95-8 LMP1 (Figure 3C). Variants from the first and second groups have all significantly increased capability to activate NF-κB compared to B95-8 LMP1. A few variants from the third group also display this phenotype. By sequence comparisons and directed mutagenesis we identified I124V as the amino acid change responsible for enhanced NF-κB activation of a variant of the first group, 7825 (data not shown). As shown above, F144 I is the determinant of the second group of variants. Finally, we found that F106Y leads to increased NF-κB activation in the context of variants 7795, 7815, 7918, 7948 and 7821 of the third group (data not shown). Since we have previously shown that this polymorphism has no influence in the context of A2 LMP1 (Figure 2F) and is present in all LMP1 variants sequences obtained in this study (Figure 1D and 3A), the effect

of F106Y is modulated by sequence variations. To determine whether the phylogenetic groups are associated with an EBV subtype, we performed an amplification analysis based on the variability in EBNA-2 as described by Telenti and coworkers [25]. Twenty samples contained only EBV type 1 and had the same pattern as reference strains B95-8 and Raji; five samples contained only EBV type 2 as the reference strain AG876, and 9 samples harbored both EBV types (Figure 3D). The analysis showed that both EBV types are distributed among the phylogenetic groups and that some samples contained several EBV strains since both types were detected simultaneously. In summary, we found two phylogenetic groups of LMP1 variants characterized by polymorphisms I124V/I152L and F144I/D150A/L151I, respectively, and all have an increased ability to activate NF-κB when compared to B95-8 LMP1.

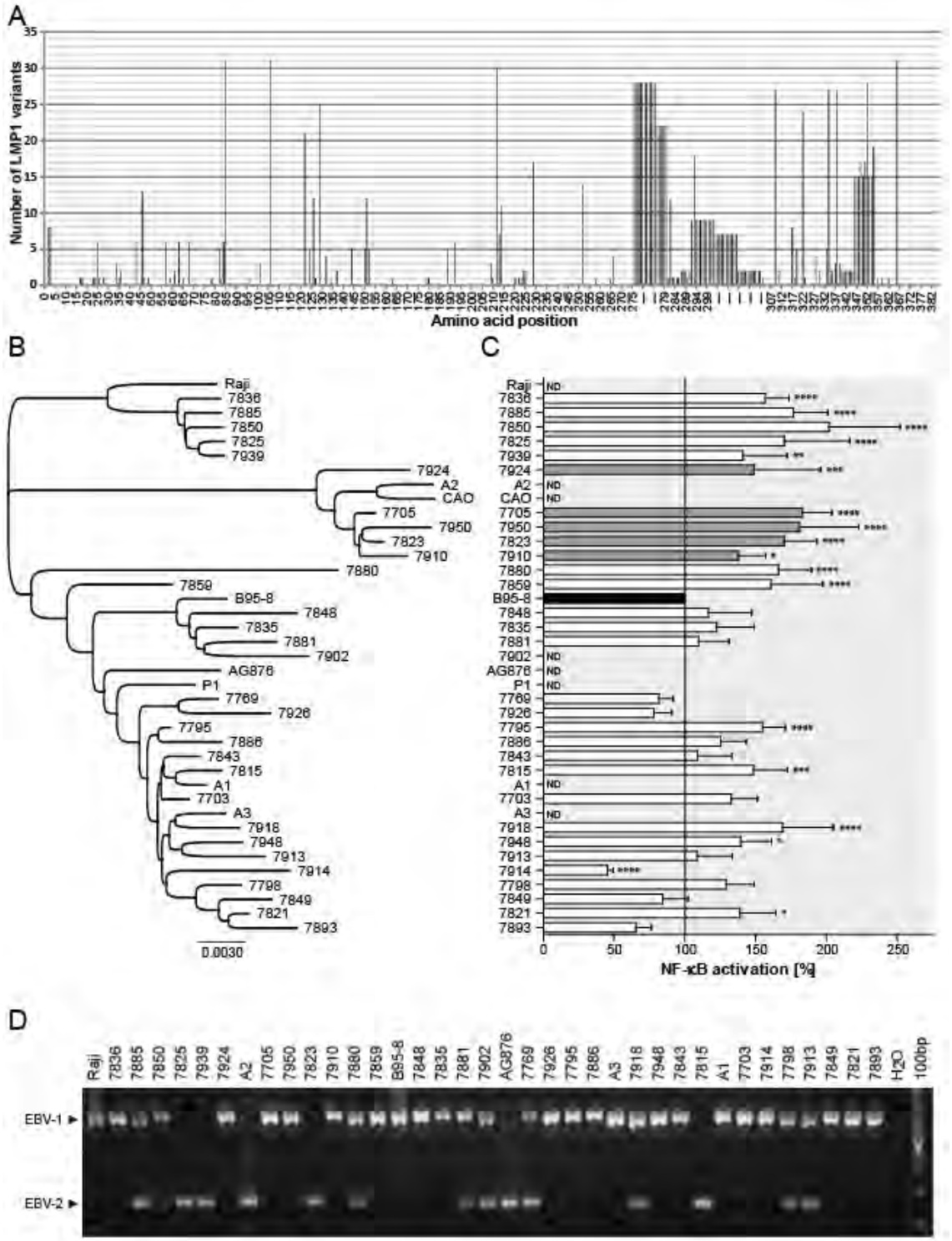

Figure 3. Analysis of LMP1 variants amplified from blood of HIV-infected individuals. **(A)** Frequency of polymorphisms from 31 LMP1 variants compared to the B95-8 prototype reference sequence. Dashes represent insertion of amino acids in direct repeats region compared to B95-8. Deletion of 10 (aa 343 to 352) or 23 (aa 332 to 354) amino acids are present in 13 and 2 variants, respectively. **(B)** Phylogenetic tree was built on LMP1 nucleotide sequences by employing neighbor-joining method using Geneious software. **(C)** Percentages of NF-κB activation induced by the 31 LMP1 variants. HEK cells were transfected with 50 ng of LMP1 vector and 50 ng of NF-κB reporter plasmid. NF-κB activity was measured twenty four hours after transfection using luciferase assay (Promega). Values were normalized to the B95-8 activation value fixed at 100%. Data are given as mean ± SD of percentages of triplicates of three independent experiments. Statistical analysis was done using one-way ANOVA with Bonferroni posttest using GraphPad Prism. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$

relatively to the NF-κB activation of B95-8 LMP1. RLU: relative light units. ND: not done. **(D)** Typing based on EBNA-2 gene was performed on genomic DNA according to Telenti and coworkers **[25]**. B95-8 and Raji are EBV type 1 references and AG876 is EBV type 2 reference.

Polymorphisms I124V/I152L and F144I/D150A/L151I are not associated with HIV-HL

Since LMP1 driven NF-κB activation is essential for EBV-transformed cells survival, we tested whether enhanced NF-κB activation potential favors the etiology of EBV-associated HL. We analyzed samples from HIV-infected individuals enrolled in the SHCS who have had a diagnosis of HL or not in a pilot epidemiological study (Figure 4A). At the time of our study forty-eight HL were diagnosed among participants of the SHCS and blood samples from 42 participants were available. Blood samples from 90 participants without tumor diagnosis were used as comparators. To determine the presence of the relevant polymorphisms we amplified and sequenced a region of LMP1 gene corresponding to amino acids 96 to 202 (Figure 4A and Materials and Methods). Among the 41 HIV-HL with LMP1 amplification and sequence we detected single sequences in samples from 31 participants and two sequences in samples from 10 participants. Among the 85 participants without tumor diagnosis with LMP1 amplification and sequence we detected single sequences in samples from 36 participants, two sequences in samples from 46 participants and three sequences in samples from 3 participants.

We assessed the presence of polymorphisms I124V/I152L and F144I/D150A/L150I for each participant's sample (Table 1). I124V/I152L was found in 12.2% of HIV-HL and in 24.7% of participants without tumor. F144I/D150A/L150I was found in 41.5% of HIV-HL and in 31.8% of participants without tumor. The simultaneous presence of both sets of polymorphisms was found in blood from one HIV-HL and from four participants without tumor. The strength of the association between the polymorphisms of interest and the etiology of HL was tested with a simple logistic regression and then with a logistic regression model adjusted for sex, age, CD4 levels and the number of years of antiretroviral therapy at the date

of blood sampling (Table 2). Our results showed that I124V/I152L and F144I/D150A/L151I are not associated with HL development neither in the simple and nor in the multivariable analyses.

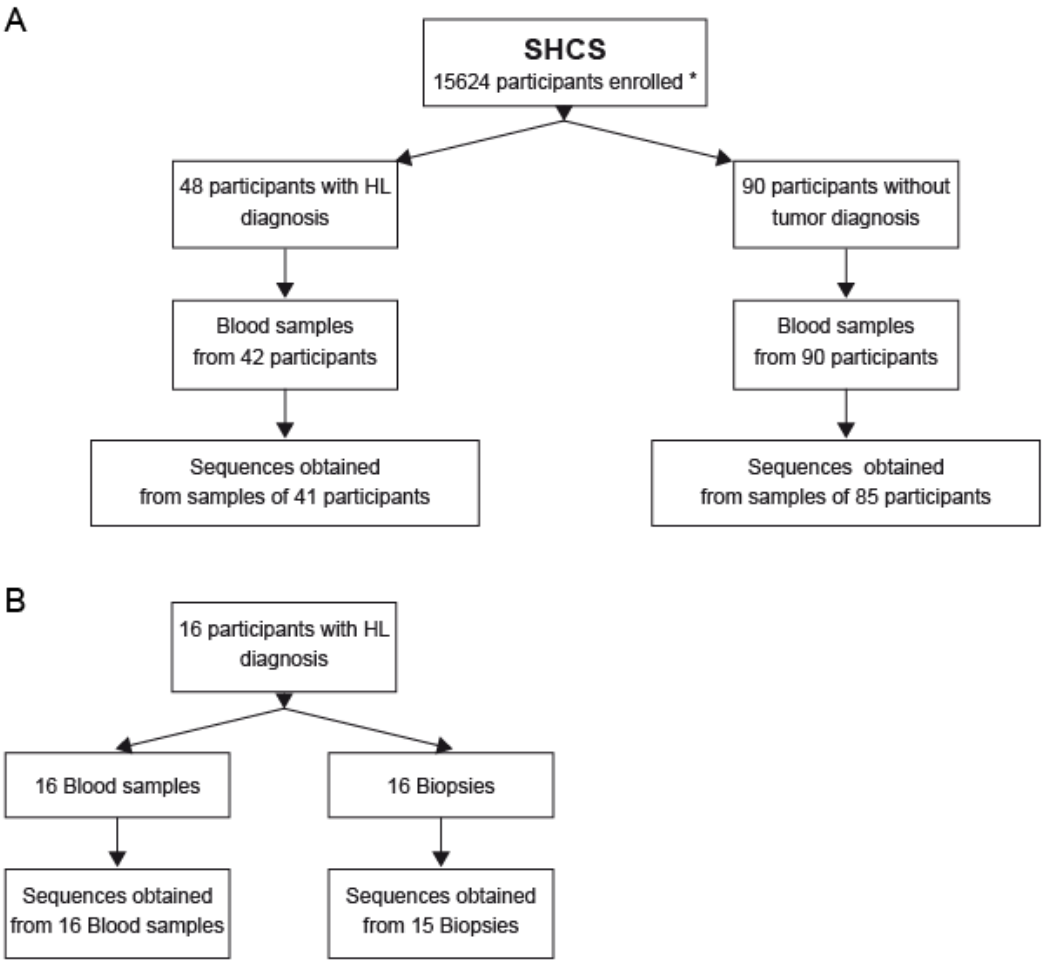

Figure 4. Flow chart of the study. **(A)** Comparison of LMP1 polymorphisms in blood samples from HIV-infected individuals with or without HL. *patients enrolled in the SHCS at the beginning of the study by March 2009 **[110]**. **(B)** Comparison of LMP1 polymorphisms in blood and corresponding biopsy from HIV-HL.

Table 1. Comparison of LMP1 polymorphisms in blood from HIV-HL and from HIV-infected individuals without tumor

Polymorphisms	Frequency	%†
Sequences obtained from samples of 41 HIV-HL*		
I124V/I152L	5	12.2
F144I/D150A/L151I	17	41.5
Other	20	48.8
Sequences obtained from samples of 85 participants without tumor§		
I124V/I152L	21	24.7
F144I/D150A/L151I	27	31.8
Other	41	48.2

* Single sequences were obtained from 31 participants and 2 sequences from 10 participants.

§ Single sequences were obtained from 36 participants, 2 sequences from 46 participants and 3 sequences from 3 participants.

† The total is more than 100% due to the simultaneous presence of both sets of polymorphisms in 1 HIV-HL and in 4 participants without tumor.

Table 2. Association between the presence of polymorphisms I124V/I152L or 144I/D150A/L151I and HIV-HL susceptibility measured by logistic regression

Polymorphisms	OR	95% CI	OR*	95% CI*
I124V/I152L	0.42	[0.15 / 1.22]	0.44	[0.14 / 1.39]
F144I/D150A/L151I	1.52	[0.70 / 3.29]	1.25	[0.52 / 3.00]
I124V/I152L and 144I/D150A/L151I	0.98	[0.46 / 2.06]	0.78	[0.33 / 1.82]

* Adjusted for sex, age, number of years of antiretroviral therapy, CD4 levels, all at the date of blood sampling.

We further analyzed the distribution of the relevant polymorphisms in the corresponding biopsies from 16 HIV-HL (Figure 4B). LMP1 sequences were obtained from 15/16 biopsies, among them single sequences were obtained in biopsies from 10 participants and two sequences in biopsies from 5 participants. We found a good concordance between LMP1 sequences from HIV-HL biopsies and corresponding blood in 10/15 cases (Table 3). As a second marker we used EBV subtype based on EBNA2 differences. We were able to type EBV from the blood of 15 HIV-HL and from 12 biopsies and the data overall confirm the good concordance between blood and biopsy.

Table 3. Comparison of LMP1 polymorphisms and EBNA2 subtype in blood and in the corresponding biopsy of HIV-HL

Cases		Sequences obtained			EBV subtype*
#	Sample	# LMP1	Polymorphisms	Identical sequences	
1	Blood	2	Others		1
	Biopsy	1	Other	Yes	NA
2	Blood	1	V124		1+2
	Biopsy	1	Other	No	1
3	Blood	1	I144		1
	Biopsy	1	I144	Yes	NA
4	Blood	1	Other		2
	Biopsy	1	Other	Yes	2
5	Blood	1	Other		1
	Biopsy	1	Other	Yes	1
6	Blood	1	I144		2
	Biopsy	2	I144 - Other	Yes	2
7	Blood	1	I144		2
	Biopsy	2	I144 - Other	Yes	1+2
8	Blood	1	Other		1+2
	Biopsy	1	Other	No	1+2
9	Blood	1	Other		1+2
	Biopsy	1	Other	Yes	1+2
10	Blood	2	I144		1+2
	Biopsy	1	I144	Yes	1
11	Blood	1	Other		1+2
	Biopsy	1	Other	No	1+2
12	Blood	1	Other		1+2
	Biopsy	2	V124 - I144	No	2
13	Blood	1	Other		NA
	Biopsy	0	NA	NA	NA
14	Blood	1	I144		1
	Biopsy	1	I144	Yes	1
15	Blood	1	I144		1
	Biopsy	2	Others	No	NA
16	Blood	1	Other		1
	Biopsy	1	Other	Yes	1

NA: not amplified

* based on EBNA2 differences

DISCUSSION

In the present study polymorphisms leading to enhanced LMP1-mediated NF-κB activation were identified and their importance in the etiology of EBV-associated HIV-HL was assessed in a pilot epidemiological study. Overall, the results presented here improve the understanding of the landscape of LMP1 genetic variation associated with NF-κB activation and show that markers of increased NF-κB activation levels *in vitro* are not predictive factors for EBV-associated HIV-HL susceptibility in the SHCS.

Since the initial description of LMP1 variant CAO isolated from a NPC [71], it has been hypothesized that LMP1 polymorphisms influence the development of EBV-associated diseases. Previous studies reported LMP1 sequence variations [74,75,109,111] and differences in the extent of NF-κB activation for some variants [50,72,112]. Induction of higher NF-κB activation levels by CAO LMP1 compared to B95-8 LMP1 was consistently observed in several cell lines of B cell and epithelial origin when measured by reporter assays [50,72,111,112]. In addition, CAO LMP1 was more oncogenic than B95-8 LMP1 in a nude mice model by inducing phenotypic changes in a non-tumorigenic human keratinocyte line [66]. This led us to test whether polymorphisms responsible for increased LMP1-mediated NF-κB activation levels *in vitro* were associated with the etiology of HIV-HL.

The risk of HL in HIV-infected individuals is significantly higher than in the general population [34]. The use of HAART that improved the immunity status of HIV-infected individuals is associated with reduced incidence of Kaposis's sarcoma and high grade non-Hodgkin's lymphoma, but paradoxically with an increased risk of HIV-HL [33]. Moreover, HL risk could not be associated with absolute $CD4^+$ cell counts [104]. Hodgkin's lymphoma arising in HIV-infected individuals show distinctive features compared to the HL cases in the general population, among them the association of HIV-HL with EBV in almost all cases [24,103,113]. The high frequency of EBV association with HIV-HL and the detection of a

strong expression of LMP1 in tumor tissue in the context of a type II latency pattern [24,114,115] indicate that LMP1 is a relevant factor involved in the pathogenesis of this disease. Additional evidences highlight the central importance of the NF-κB signaling pathway for pathogenesis: NF-κB activation by LMP1 is critical for B cell transformation *in vitro* and *in vivo* [97,100,116,117,118], NF-κB hyperactivity was shown to be associated with tumorigenesis [119] and mutations in NF-κB regulatory pathways leading to increased activity have been identified in EBV-negative HL [120,121,122].

In this study LMP1 variants isolated from HIV-infected individuals living in Switzerland were characterized with respect to their sequence variations and their NF-κB activation potential measured by gene reporter assay. The increased EBV load in blood samples from HIV infected individuals [25,26] allowed the amplification of the LMP1 gene as a single fragment, an essential step to avoid PCR-driven recombination between EBV strains in samples with multiple EBV infection. Overall our analysis gives a new insight into LMP1 sequence variation in individuals without a diagnosis of EBV-associated tumor. Concomitantly we found that a number of variants mediate higher NF-κB activation levels when compared to the prototype B95-8 LMP1 and mapped several polymorphisms that have an effect on signaling. Single amino acid change F144I is present in variants that cluster with CAO in a phylogenetic analysis and that have the ability to activate NF-κB at high levels. This group, characterized by amino acid changes F144I/D150A/L151I, is more closely related to China1 than to the other LMP1 strains with F144I described by Edwards et al. [109,112]. Another amino acid change associated with enhanced NF-κB activation is I124V, present in group of variants characterized by polymorphisms I124V/I152L that have not been previously described. The last amino acid change we found associated with enhanced NF-κB activation is F106Y. This polymorphism is present in all LMP1 variants isolated in our study and its effect is counteracted in most variants by one or several polymorphisms that have not yet

been mapped. Thus F106Y could not be used to predict NF-κB activation levels. Taken together our results demonstrate that several groups of LMP1 variants, through distinct mutational paths, mediate enhanced NF-κB activation levels compared to B95-8 LMP1 and point out the transmembrane segments 4 and 5 for their importance on NF-κB modulation.

Polymorphisms I124V/I152L and F144I/D150A/L151I were chosen as markers of enhanced NF-κB activation in a pilot epidemiological study within the SHCS. By comparing LMP1 variants present in blood of SHCS participants with or without HL diagnosis, we observed that I124V/I152L and F144I/D150A/L151I, combined, were found in more than 50% of the participants from each group. Concordant with a high distribution in the two groups of patients, the two sets of polymorphisms are not identified as predictive factors associated with HL etiology when analyzed by a logistic regression model, either together or individually. However, a lower percentage of I124V/I152L in samples from HIV-HL compared to samples from individuals without tumors (12.2% versus 24.7%) was observed, and in parallel, a higher percentage of variants with F144I/D150A/L151I (41.5% versus 31.8%). More than one variant was detected in some samples, as demonstrated by EBV typing based on EBNA-2 gene and analysis of LMP1 sequences. The percentage of samples presenting a co-infection by EBV type 1 and type 2 was concordant with what was previously reported in the literature [123]. The presence of distinct EBV strains in the blood and tumor site has been previously reported [124]. In contrast, another study showed a good concordance with respect to EBNA-2 region and LMP1 gene in normal and neoplastic cells [125]. In order to determine to which extend EBV strains differ between blood and tumor biopsy samples on a larger group of patients, we further compared LMP1 sequences present in blood from HIV-HL and in its corresponding biopsy. EBV typing based on EBNA-2 was chosen as an additional marker. Using these two criteria we found a good correspondence between blood and tumor samples in 10/15 cases. The major limit of this study is the low number of HL cases in the SHCS and

the low number of available biopsies. The relatively good correspondence of LMP1 variants between blood and tumor biopsies indicates that larger future studies using blood samples would be relevant to the identification of viral polymorphisms influencing the development of EBV-associated malignancies.

Further studies will be needed to find out the complex effects of LMP1 variants in tumor cells in the context of EBV latency type II program. Our results extend the previous observations made on CAO LMP1 and demonstrate that several groups of LMP1 variants through distinct mutational paths mediate increased NF-κB activation levels *in vitro*. Moreover, naturally occurring polymorphisms that lead to enhanced NF-κB activation *in vitro* are not identified as risk factors associated with HIV-HL susceptibility.

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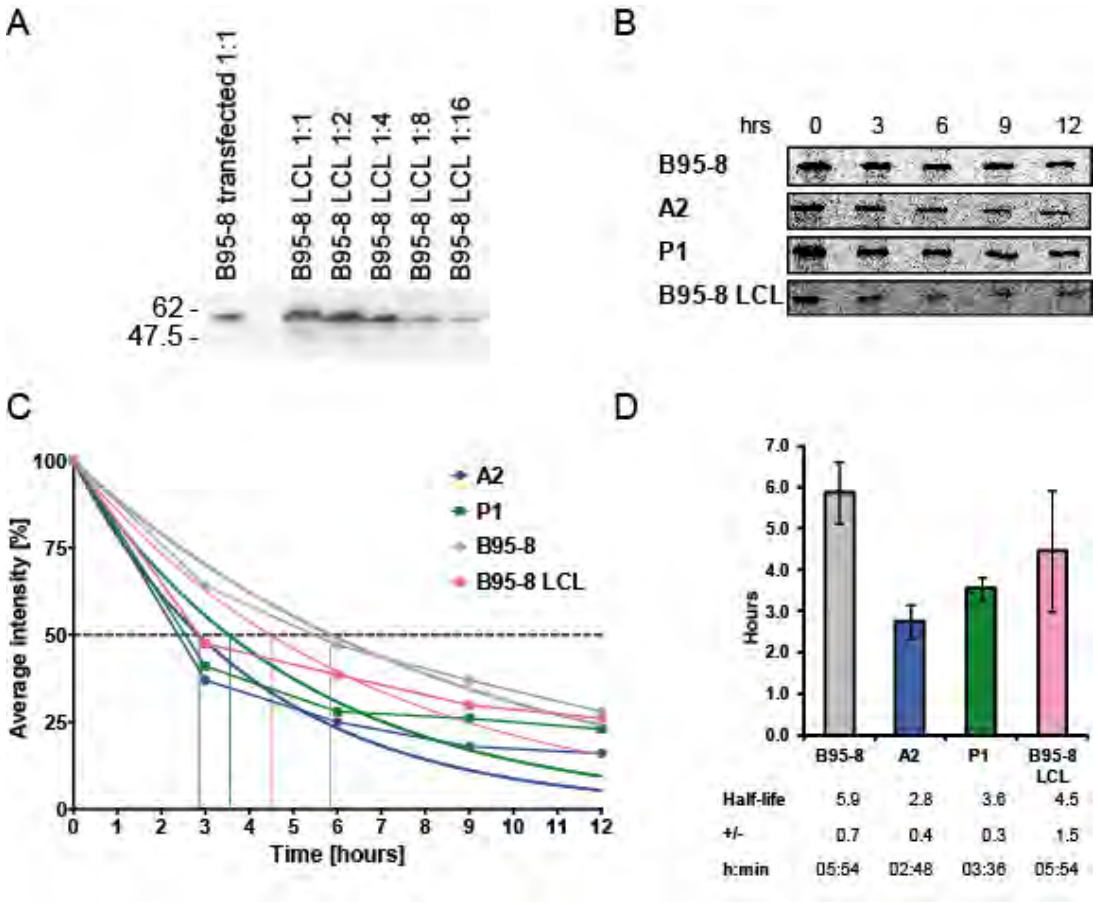
S, Rickenbach M (Head of Data Center), Rudin C (Chairman of the Mother & Child Substudy), Schmid P, Schultze D, Schöni-Affolter F, Schüpbach J, Speck R, Taffé P, Tarr P, Telenti A, Trkola A, Vernazza P, Weber R, Yerly S.

3.2 CHAPTER 1 - SUPPLEMENTARY FIGURES AND METHODS

3.2.1 DETERMINATION OF PROTEIN HALF-LIFE BY PULSE CHASE ANALYSIS

HEK cells were transfected in 100-mm plates with 2 μg of the expression plasmids using Fugene 6. 24 hours post-transfection cells pooled from two 100-mm plates were seeded in six 60-mm plates coated with poly-L-Lysine and grown for 14 hours. Cells were starved at 37°C for 30 minutes in methionine and cysteine-free DMEM supplemented with 2% FCS and pulse-labeled with 100 μCi [^{35}S] labeled cysteine/methionine per ml (Hartman) at 37°C for 45 minutes. Cells were washed with complete medium and then replaced in the incubator with complete DMEM for incubation from 0 to 12 hours chase times. Cells were rinsed with chilled PBS and lysed in RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 8, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) supplemented with inhibitors of proteases (minicomplete, Roche Applied Science) and inhibitors of phosphatases (20 mM NaF, 25 mM glycerophosphate, and 1 mM orthovanadate). Lysates were cleared by centrifugation at 16,000g for 10 minutes and deep frozen in nitrogen at each time point. Samples were immunoprecipitated with mouse anti-LMP1 S12 monoclonal antibody bound to protein-A sepharose for 2 hours at 4°C. Precipitates were washed three times with RIPA buffer and twice with phosphate buffered saline and boiled for 4 min in 50 μl of 2x sample buffer and separated by 10 % SDS-PAGE. Gels were dried, exposed on a phosphor screen cassette and then the signal was read with a Typhoon (Amersham Biosciences) and quantification was done using ImageQuantTM TL2005 software (Amersham Biosciences).



Supplementary Figure 1: Determination of the half-life of B95-8, A2 and P1 LMP1. (A) Comparison of the expression of B95-8 LMP1 upon plasmid transfection in 293T cells and upon natural expression in B95-8 LCL. For the transient transfection 293T cells were transfected with 50 ng of B95-8 LMP1 vector. B95-8 LCL cells were grown in RPMI. Both kinds of samples were lysed and the protein amount measure with BCA (Pierce). B95-8 LCL sample was serially diluted with H2O. Samples were then separated on a 10% SDS PAGE and immunobloted with S12 anti-LMP1 antibody. **(B)** Scan of the radioactive signals. **(C)** Plotted quantifications of the signals detected in A and determination of the half-life with a non-linear regression model using GraphPad Prism version 5.03 for Windows. **(D)** Histogram of the half-life and values.

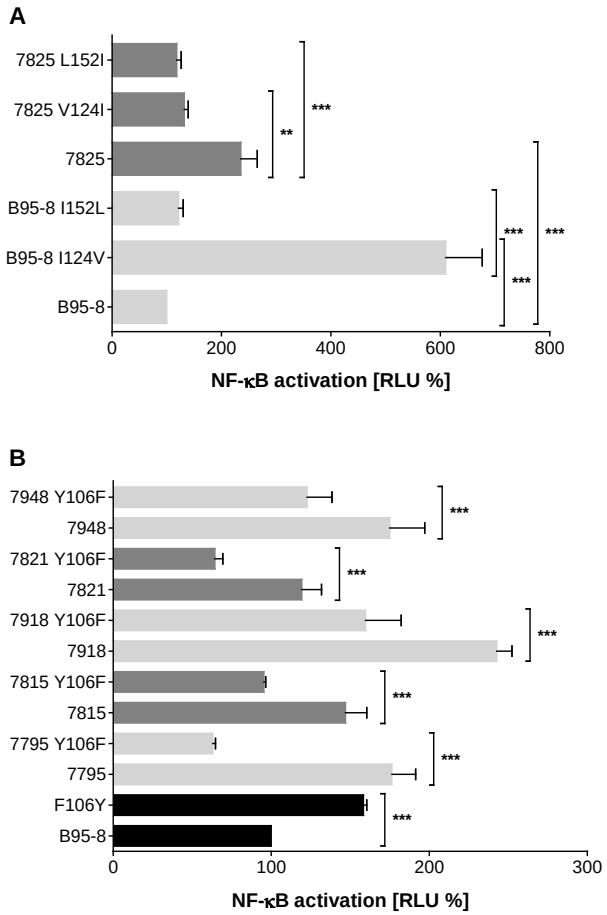
3.2.2 NF-κB ACTIVATIONS LEVELS OF LMP1 PROTOTYPE AND MUTANTS

Supplementary Table 1:
NF-κB activation levels of LMP1 prototype and mutants.

LMP1		%	SD %	p-value
Prototype	B95-8	100	0	
Mutants	B95-8 I85L	101	48	ns
	B95-8 F106Y	296	114	***
	B95-8 L126F	93	35	ns
	B95-8 M129I	76	24	ns
	B95-8 F144I	308	108	***
	B95-8 D150A	83	12	ns
	B95-8 L151I	64	22	ns
	B95-8 Q189P	87	15	ns
	B95-8 S192T	115	23	ns
	B95-8 G212S	87	41	ns
	B95-8 F106Y	289	38	***
	B95-8 F144I	334	30	***
	A2 Y106F	448	60	***
	A2 I144F	167	10	ns

NF-κB activities were measured 24 hours after transfection. Values are percentages ± SD of means of 9 independent transfections normalized to the activity of B95-8 LMP1 fixed at 100%. Significant p-values compared to the activation of B95-8 LMP1 are indicated with asterisks ($p<0.05$, one-way ANOVA with Bonferroni posttest performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

3.2.3 *INVOLVEMENT OF POLYMORPHISMS 124-152 AND 106 FOR NF-κB ACTIVATION*



Supplementary Figure 4: NF-κB activation. (A-B) NF-κB activation by LMP1 mutants on B95-8 background and on variants background. HEK cells were transfected with 50 ng of LMP1 vector and 50 ng of NF-κB reporter plasmid. Empty vector was used as control. NF-κB activity was measured twenty-four hours after transfection using luciferase assay (Promega). Shown are representative of three independent experiments with similar results. Data are given as mean ± SD of triplicates. Statistical analysis was done using one-way ANOVA with Bonferroni posttest using GraphPad Prism. ** $P<0.01$, *** $P<0.001$ relatively to the NF-κB activation of B95-8 LMP1. RLU: relative light units. **(A)** LMP1 mutants with positions 124 and 152 mutated in B95-8 and variant in 7825, a member of the first group of variants. **(B)** LMP1 mutants with position 106 mutated in B95-8 and in LMP1 variants of the third group.

3.2.4 *PRIMERS AND PCR CONDITIONS FOR LMP1 AMPLIFICATION FROM GENOMIC DNA*

The region between positions 169.508 and 168.111 of EBV genome was amplified using a pair of primers based on the published prototype B95-8 LMP1 sequence [46]:

Fwd: 5’-TCAACTGCCTTGCTCCTGACAC-3’

Rev: 5’-AGGCAAGCCTATGACATGGTAATGC-3’.

PCRs were carried out either with Pwo DNA Polymerase (Roche Applied Science): [98°C 5 min, 5 cycles (94°C 1 min, 65°C 1 min, 72°C 1.5 min), 5 cycles (94°C 1 min, 63°C 1 min, 72°C 1.5 min), 25 cycles (94°C 1 min, 60°C 1 min, 72°C 1.5 min), 5 cycles (94°C 1 min, 57°C 1 min, 72°C 1.5 min), 72°C 10 min] or with AmpliTaq Gold DNA Polymerase (Applied Biosystem): [95°C 10 min, 5 cycles (95°C 1 min, 65°C 1min, 72°C 1.5 min), 5 cycles (94°C 1 min, 63°C 1min, 72°C 1.5 min), 25 cycles (95°C 1 min, 60°C 1min, 72°C 2 min), 5 cycles (95°C 1 min, 57°C 1 min, 72°C 1.5min), 72°C 10 min] on the four biopsy samples used for the mapping of the polymorphisms and samples from HIV-infected individuals, respectively.

3.2.5 EBV TYPING

EBV type was determined on EBNA2 gene by performing PCR and nested PCR on this gene with AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Rotkreuz, Switzerland). Primers and PCR program used in our study are based on the publication of Telenti et al. [25]:

Common forward primer:	5’-AGGGATGCCTGGACACAAGA-3’
Common reverse primer:	5’-TGGTGCTGCTGGTGGTGGCAAT-3’
EBV-1 forward nested primer:	5’-TCTTGATAGGGATCCGCTAGGATA-3’
EBV-1 reverse nested primer:	5’-ACCGTGGTTCTGGACTATCTGGATC-3’
EBV-2 forward nested primer:	5’-CATGGTAGCCTTAGGACATA-3’
EBV-2 reverse nested primer:	5’-AGACTTAGTTGATGCCCTAG-3’

PCR were carried out with this program: [94°C 10 min, 30 cycles (94°C 1.5 min, 60°C 1 min, 72°C 2 min), 72°C 10 min].

4 CHAPTER 2 -

LMP1: IMPORTANCE OF TRANSMEMBRANE SEGMENTS

4.1 CHAPTER 2 - INTRODUCTION

Epstein-Barr virus (EBV), the etiological agent of infectious mononucleosis, is an important human oncogenic virus being implicated in the pathogenesis of several malignancies including nasopharyngeal carcinoma, Burkitt's lymphoma, nasal NK/T cell lymphoma, Hodgkin's lymphoma and post-transplant lymphoproliferative disorders [126]. EBV is a ubiquitous gammaherpesvirus that can infect multiple cell types including B cells, which provide a reservoir for the virus [127,128]. EBV infection of B-lymphocytes is mostly latent and results in the expression of a limited number of nuclear and membrane proteins. Latent membrane protein 1 (LMP1), which is detected in tumor cells of human malignancies associated with EBV, is a multifunctional oncogene with potent transforming effects in cell culture and animal models. LMP1 has transforming effects on non-lymphoid cells such as rodent fibroblasts and keratinocytes [65,98,99], is essential for EBV-induced B-cell transformation and *in vitro* transformation [95,96,97] and causes lymphoma in transgenic mice [67]. LMP1 is a powerful inducer of nuclear factor-κB (NF-κB)-mediated transcription [56,57], a property that is indispensable for EBV-transformed lymphoid cell survival *in vitro* and *in vivo* [63,100,116].

LMP1 is an integral membrane protein with a short cytoplasmic N-terminus, six transmembrane-spanning segments (TMs) and a 195 amino acids long C-terminal domain [45,46]. Two motifs within the cytoplasmic C-terminal domain of the protein are essential for NF-κB activation and cell transformation. The first motif, designated as C-terminal activating region 1 (CTAR1), spans residues 199-231, interacts with TRAF1-6 [53,55,129,130,131,132,133,134]. The second motif, CTAR2, is located between residues 352-386 [130,133]. This region of LMP1 is thought to mediate NF-κB activation through direct binding of the TNF-associated death domain protein (TRADD), which interacts with TRAF2, thus mimicking TNF-receptor 1 (TNFR1)-mediated NF-κB activation [135,136].

This region is also linked to the activation of the AP-1 family of transcription factors through the c-Jun N-terminal kinase (JNK) pathway [59,137,138].

LMP1 acts as a constitutively active receptor-like molecule that mimics CD40 and alters cell growth [54,95,139]. However, unlike members of the tumor necrosis factor receptor (TNFR) family, LMP1 signals in a ligand-independent fashion, a feature that is largely due to the properties of its TMs. Indeed chimeric proteins with LMP1's amino terminus and membrane-spanning segments fused to the carboxy-terminal signaling domain of TNFR-1, TNFR-2 and CD40 signal in the absence of ligand, whereas chimeric proteins with extracellular ligand binding domain of cell surface receptors fused to LMP1's carboxy terminus are activated in a ligand-dependent manner [54,140,141,142]. However, the relative contribution of the six TMs to the many functional properties of LMP1 is not well understood. In the present study we have constructed series of LMP1 mutants with either four or two TMs to evaluate the relative importance of the three pairs of TMs for membrane association, self-association, binding to cellular factors and modulation of NF-κB activation. Our results show that LMP1 TMs are implicated in a number of functions, some of which have not been described before. Notably, the three pairs of TMs and the two intracellular loops that link them are not functionally equivalent and interchangeable. Overall, the transmembrane domain of LMP1 is a key player in protein-protein interactions and signaling.

4.2 CHAPTER 2 - RESULTS

4.2.1 LMP1 TMS MUTANTS

LMP1 is an integral membrane protein with six transmembrane spanning domains (TMs) (Figure 1A) [46]. In contrast to the N- and C-terminal domains of LMP1, which were the focus of many studies, much less is known about TMs. It has been demonstrated that LMP1 acts as a constitutively active receptor by means of aggregation mediated by the TMs. However the question of the relative importance of the six segments has not been addressed before in comparative experiments.

To better understand the role of each pair of TMs we constructed LMP1 mutants with either four or two TMs (Figure 1B-C). These TMs mutants allowed us to analyze the contribution of the three pairs of TMs to self-association, binding to the cellular partners TRAF3 and β-TrCP, and NF-κB activation. Finally, the naturally occurring polymorphisms identified in LMP1 TMs will be presented in the perspective of these biochemical analyses.

LMP1 mutants with four TMs are schematically represented in Figure 1B. TM3-4-5-6 comprises the LMP1 N-terminus fused to TMs 3 to 6 and the C-terminal domain. The two constructs TM1-2+5-6 (L2) and TM1-2+5-6 (L4) differ only in the intracellular loops L2 or L4 that link the TM pairs and comprise LMP1 N-terminus fused to the TMs 1-2 and TMs 5-6, followed by the C-terminus. Finally, TM1-2-3-4 encodes LMP1 N-terminus fused to the TMs 1 to 4 and the C-terminal domain. LMP1 mutants with two TMs harbor a single pair of TMs fused to the N- and C-terminal domains (Figure 1C). Importantly, all TMs mutants contain identical N- and C-terminus for optimal comparisons.

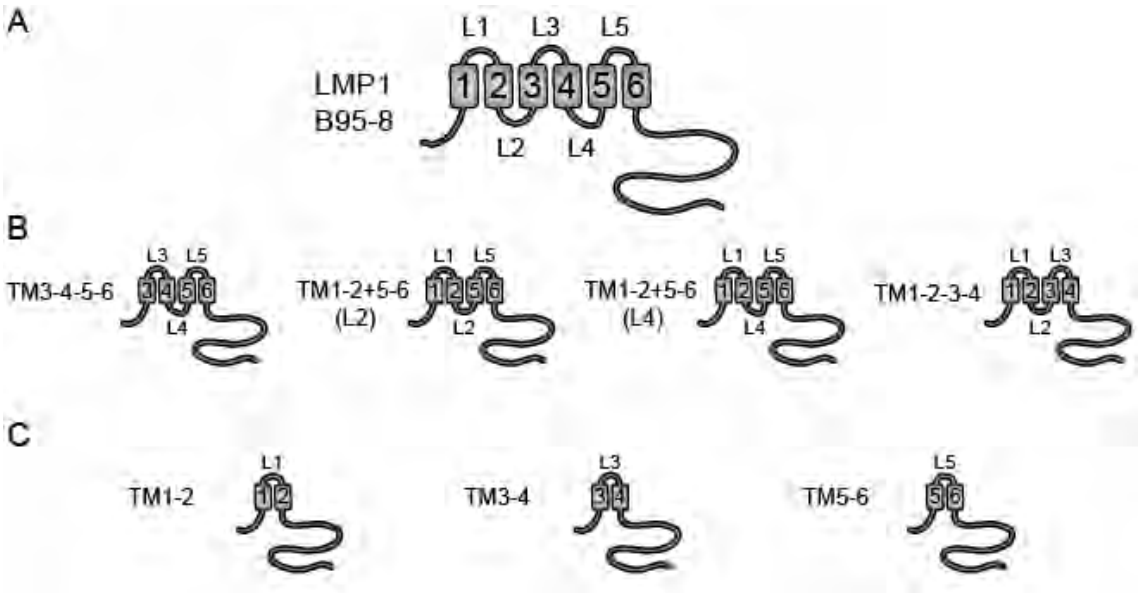


Figure 1: Schematic representation of LMP1 B95-8 prototype and transmembrane mutants. The TMs are indicated by small boxes and numbered 1 to 6. The loops that link the TMs are numbered L1 to L5. **(A)** B95-8 LMP1 prototype contains 6 TMs and 5 loops. **(B-C)** LMP1 TMs mutants were constructed by PCR-driven mutagenesis. All TMs mutants have identical N- and C-terminus. **(B)** TMs mutants with four TMs. The only difference between TM1-2+5-6(L2) and TM1-2+5-6(L4) is the intracellular loop 2 or 4. **(C)** TMs mutants with two TMs.

4.2.2 LMP1 MUTANTS WITH 4 TMS ASSOCIATE WITH THE PLASMA MEMBRANE

LMP1 is inserted in the membrane thanks to the first TM, which plays the role of leader sequence, and to the cytoplasmic N-terminus that provides the structural information whereby proper membrane orientation is achieved [87]. To confirm the membrane association of the different LMP1 constructs, we performed a subcellular fractionation of cells transfected either with LMP1 B95-8, TMs mutants or C-GFP (Figure 2 and 5G for C-GFP scheme). The transmembrane transferrin receptor and caspase-3 were chosen as membrane and cytoplasmic markers, respectively. The transferrin receptor was recovered in the pellet containing membrane proteins, whereas the caspase-3 was detected in the supernatant only, confirming the quality of the fractionation. C-GFP consists of the C-terminal part of LMP1 fused to EGFP. As expected this construct, which lacks TMs, was exclusively found in the cytoplasmic fraction. All TMs mutants with 4 TMs were fully associated with the membrane. In contrast, mutants TM3-4 and TM5-6 were found both in the membrane and in the

cytoplasmic fractions. This analysis showed that the three pairs of TMs were not equivalent with respect to membrane insertion and that multiple TMs were required for optimal membrane association.

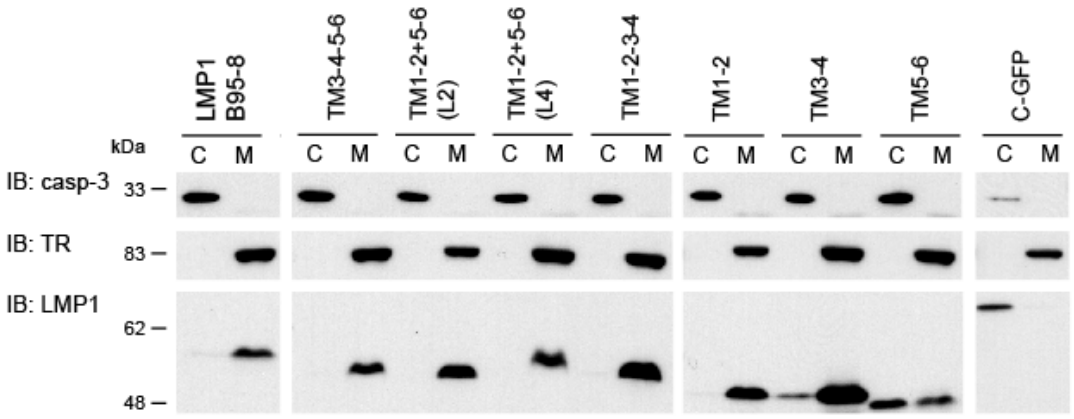


Figure 2: Multiple TMs ensure membrane association. 293T cells transfected with expression vectors coding for B95-8 LMP1 or LMP1 transmembrane mutants were homogenized and fractionated as described in Materials and Methods. The proteins present in the membrane (M) and the cytoplasmic (C) fractions were analyzed on 10% SDS-PAGE. LMP1, transferrin receptor (TR) and caspase-3 (casp-3) were detected using the appropriate antibodies. IB: immunoblot.

4.2.3 EACH PAIR OF LMP1 TMS CONTRIBUTED TO SELF-ASSOCIATION

LMP1 is a functional mimic of the cellular receptor CD40 [54,95,139]. However, unlike members of the tumor necrosis factor (TNFR) family, LMP1 signals in a ligand-independent fashion, a feature that is attributed to the properties of its six TMs. TM 1 is thought to play a special role in this process due to the presence of a leucine heptad [143] and residues FWLY [94].

We tested the relative ability of the three TMs pairs to mediate interactions in co-immunoprecipitation (co-IP) experiments. Flag-tagged and HA-tagged versions of B95-8 LMP1 (Figure 3A) and mutants with one pair of TMs were constructed and co-expressed in HEK cells. After cell lysis, HA-tagged LMP1 was immunoprecipitated and the associated Flag-tagged molecules subsequently revealed by immunoblotting.

B95-8 LMP1 strongly associated with itself in co-IP experiments even using a stringent buffer for cell lysis, binding and washing (Figure 3B lane 4). The analysis of LMP1 mutants with a single pair of TMs showed that each mutant was able to associate with itself (Figure 3B lane 5-7), the strongest association being between mutants containing TMs 3 and 4. Interestingly the three TMs mutants were also able to associate with each other (Figure 3B lane 8-10), the weakest association was seen between TM1-2 and TM5-6, whereas TM3-4 and TM5-6 associated more strongly.

In summary, these results demonstrated that TMs 1 and 2 did not play a unique role in self-association. Indeed, the comparative analysis showed that TMs 3 and 4 probably played a key role in association.

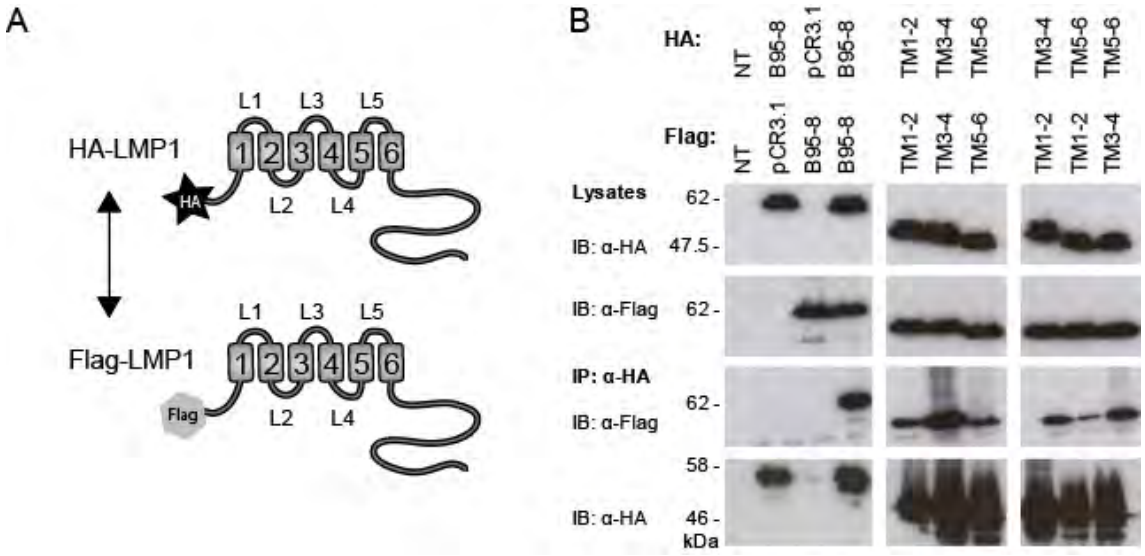


Figure 3: LMP1 association occurs through its TMs. (A) Schematic representation of HA- and Flag-tagged B95-8 LMP1. (B) Association of HA- and Flag-tagged LMP1. HEK cells were transfected with HA- and Flag-tagged LMP1 constructs. Cells were lysed 38h later and extracts were immunoprecipitated with anti-HA antibody bound to protein A Sepharose. LMP1 immunoprecipitates (IP) and cells lysates were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using the indicated antibodies. NT: non-transfected; pCR3.1: empty vector.

4.2.4 TRAF3 BINDING REQUIRED THE 6 TMS OF LMP1

TNF receptor-associated factor 3 (TRAF3) was first described as a CD40-interacting molecule [144,145,146] and simultaneously as a cellular interacting factor of LMP1 [55].

TRAF3 is the best characterized cellular partner of LMP1. The exact role of TRAF3 in CD40 and LMP1 signaling remained elusive for many years. Recent studies reveal major functions played by TRAF3 in the alternative NF-κB signaling and the control of type I interferon production [147].

LMP1 binds TRAF3 through the PxQxT motif of CTAR1 (Figure 4A) [131,148]. As shown in the control experiment, CTAR1- and CTAR1-/2- mutants harboring a mutated TRAF binding site lost the ability to bind endogenous TRAF3 in co-IP experiments (Figure 4A-B). We further tested the impact of the deletion of one or two pairs of TMs on TRAF3 association. Co-IP experiments showed that the deletion of a single pair of TMs had a major impact on TRAF3 binding (Figure 4C-D). These results extended a previous observation showing that LMP1 mutant containing only TMs 1 and 2 does not interact detectably with TRAF3 [148]. Interestingly, these data revealed that the deletion of any pair of TMs had a similar effect on TRAF3 binding. Since the PxQxT motif was conserved in all TMs mutants the loss of TRAF3 binding was likely the result of conformational changes and loss of aggregation.

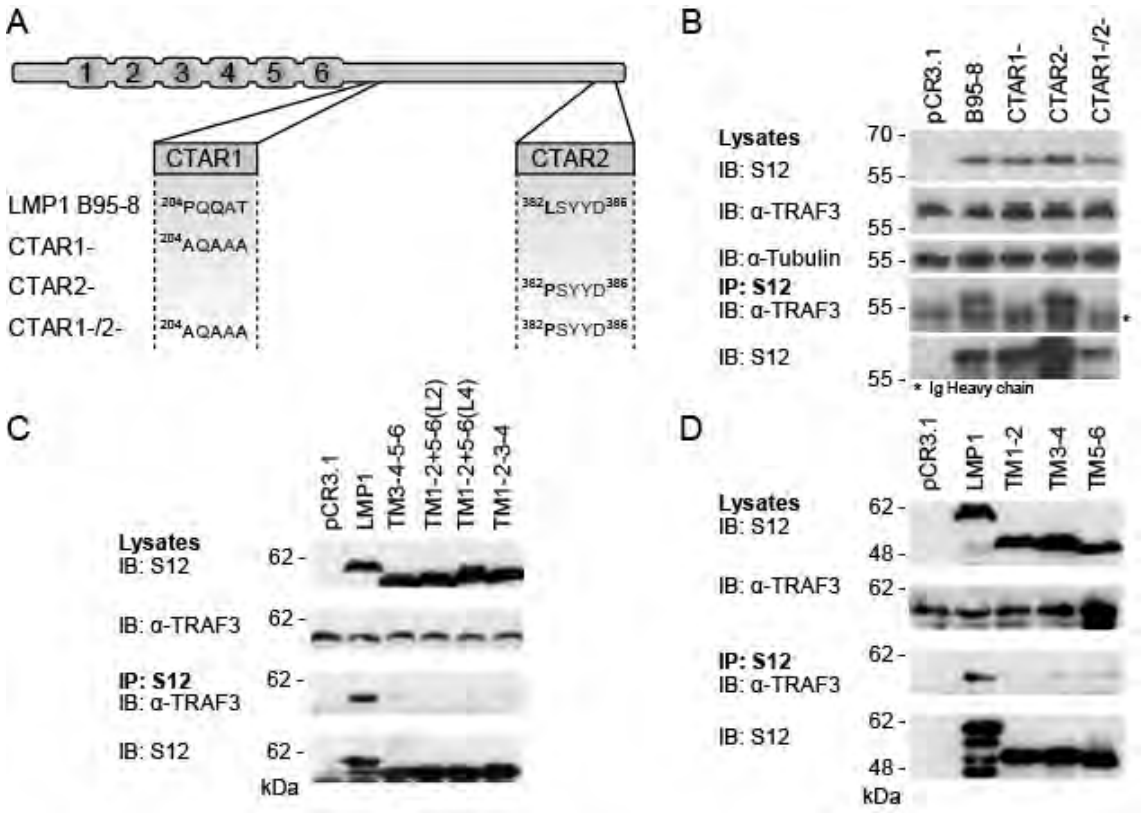


Figure 4: TRAF3 binding requires LMP1 six TMs. (A) Schematic representation of LMP1 B95-8 and CTAR mutants. Mutations in the CTAR1 and CTAR2 sites are indicated. **(B-C-D)** Co-IP of endogenous TRAF3 with B95-8 LMP1 and mutants. 293T cells were transfected with LMP1 constructs, lysed 38h later and extracts were immunoprecipitated with S12 anti-LMP1 antibodies bound to protein A Sepharose. LMP1 immunoprecipitates (IP) and cells lysates were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using the indicated antibodies. Endogenous TRAF3 binding to **(B)** CTAR mutants, **(C)** TMs mutants with 4 TMs and **(D)** TMs mutants with 2 TMs. pCR3.1: empty vector.

4.2.5 β–TrCP BINDING REQUIRED LMP1 TMS 3 AND 4

In constrast to TRAF3, which was the focus of many studies, little is known about the interaction between LMP1 and the cellular β-transducing-repeat containing protein (β-TrCP). LMP1 was shown to associate with β-TrCP2 [82]. This binding was proposed to occur through a canonical DSG binding site close to the TRAF binding site (Figure 5A) and a cryptic site comprising S350 and S366 [82]. Mainou et *al*. confirmed a binding between natural LMP1 variants and β-TrCP2, but showed that this interaction is more complex than previously described by Tang et *al*. [112] and probably involved alternative binding sites.

To better understand this interaction, we constructed DSG mutants by mutating the canonical and cryptic β-TrCP2 recognition site in LMP1 to create DSGm1 (G212S/Δ346-355/S366T), DSGm2 (mutation of the two serines S211A/S215A of the DSGxxS site) and DSGm3 (D210N/S211A/E214Q/S215A/D216N) (Figure 5A). DSGm1 resembled the triple mutant used by Tang et *al.* [82]. Co-IP experiments of DSG mutants with β-TrCP2 showed that all DSG mutants interacted with β-TrCP2 as B95-8 LMP1 (Figure 5B). These results showed that the DSG binding motif was not the major β-TrCP2 binding site of LMP1. We further tested whether the loss of TRAFs binding interfered with β-TrCP2 binding. Both CTAR1- and CTAR1-/2- bound β-TrCP2 as B95-8 LMP1. It is to note that in all the co-IP experiments we observed a weaker detection of β-TrCP2 in cell lysates when co-transfected with the empty vector, than when co-transfected with the different LMP1 constructs (Figure 5).

By controlling the substrate specificity, β-TrCP2 is a key subunit of the SCF $E3^{\beta\text{-TrCP2}}$ ubiquitin ligase complex. Via the N-terminal F-box domain β-TrCP2 binds to Skp-1 and via the C-terminal WD40 domains to the phosphorylated substrates [78]. To test the direct interaction of β-TrCP2 with LMP1 we used a construct encoding the WD40 domains of β-TrCP2 and lacking the F-box domain in co-IP experiments. The results showed that LMP1 is able to co-immunoprecipitate both full length β-TrCP2 and its WD40 domains (Figure 5C-D). A similar result was found in the reciprocal experiment (Figure 5E). These findings demonstrated that the interaction between LMP1 and β-TrCP2 was mediated by the WD40 domains and did not involve other subunits of the SCF complex.

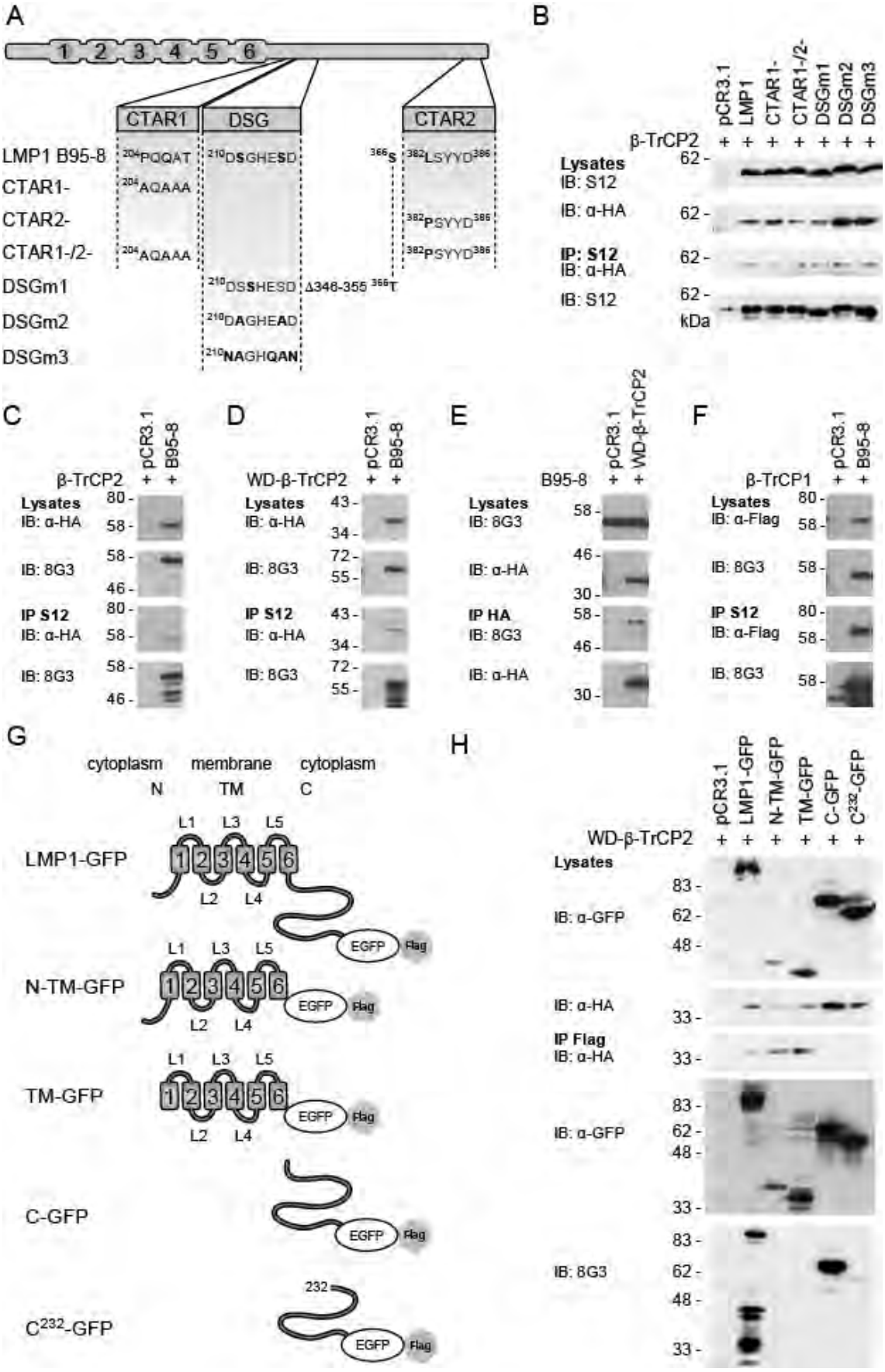
In humans, β-TrCP family includes two homologous proteins, β-TrCP1, encoded by *BTRCP*, and β-TrCP2, encoded by *FBXW11*, also known as HOS [78,149,150]. The WD40 domains of β-TrCP1 and β-TrCP2 are highly homologous and show similar electrostatic surface properties with a conservation of the central groove covered by positively charged amino acids [84]. Consistently, HIV-encoded Vpu protein was shown to interact with both β-TrCP1

and β-TrCP2 [84]. We showed that β-TrCP1 bound B95-8 LMP1 (Figure 5F), a result providing an additional example of a functional redundancy of the two homologous proteins.

In a first approach to define the LMP1 regions involved in the interaction, we used LMP1 mutants with deletions of the entire N- or C-terminal domains and of the TM domain (Figure 5G). Mutants were tagged with EGFP and Flag to allow expression and detection. Mutants N-TM-GFP and TM-GFP bind to WD-β-TrCP2, whereas C-GFP and C^{232}-GFP lost this ability (Figure 5H). A mutant containing the TM-C-Flag also had the ability to bind β-TrCP2 and even stronger than B95-8 LMP1 (data not shown). These results showed that the deletions of both N- and C-terminal cytoplasmic domains did not impair β-TrCP2 binding and thus highlighted a determinant role of the transmembrane domain for the interaction.

To dissect the involvement of each pair of TMs in β-TrCP2 binding we performed co-IP experiments with TMs mutants. An association with β-TrCP2 was observed for LMP1 mutants containing TMs 3 and 4 (Figure 5I lanes 3 and 6; Figure 5J lane 4), but was lost for the mutants lacking these two TMs (Figure 5I lanes 4 and 5; Figure 5J lanes 3 and 5). These results revealed a unique role of the central pair of TMs for LMP1-β-TrCP2 binding.

In summary, these results show that LMP1 not only binds to β-TrCP2, but also to its homolog β-TrCP1. We showed that the interaction was mediated by β-TrCP2-WD40 domains and depended on the presence of LMP1 TMs 3 and 4.


A
1 2 3 4 5 6
CTAR1
DSG
CTAR2
LMP1 B95-8
CTAR1-
CTAR2-
CTAR1-/2-
DSGm1
DSGm2
DSGm3
B
pCR3.1
LMP1
CTAR1-
CTAR1-/2-
DSGm1
DSGm2
DSGm3
β-TrCP2
Lysates
IB: S12
IB: α-HA
IP: S12
IB: α-HA
IB: S12
kDa
C
β-TrCP2
Lysates
IB: α-HA
IB: 8G3
IP S12
IB: α-HA
IB: 8G3
D
WD-β-TrCP2
Lysates
IB: α-HA
IB: 8G3
IP S12
IB: α-HA
IB: 8G3
E
B95-8
WD-β-TrCP2
Lysates
IB: 8G3
IB: α-HA
IP HA
IB: 8G3
IB: α-HA
F
β-TrCP1
Lysates
IB: α-Flag
IB: 8G3
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IB: α-Flag
IB: 8G3
G
cytoplasm
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TM
C
LMP1-GFP
N-TM-GFP
TM-GFP
C-GFP
C232-GFP
EGFP
Flag
H
pCR3.1
LMP1-GFP
N-TM-GFP
TM-GFP
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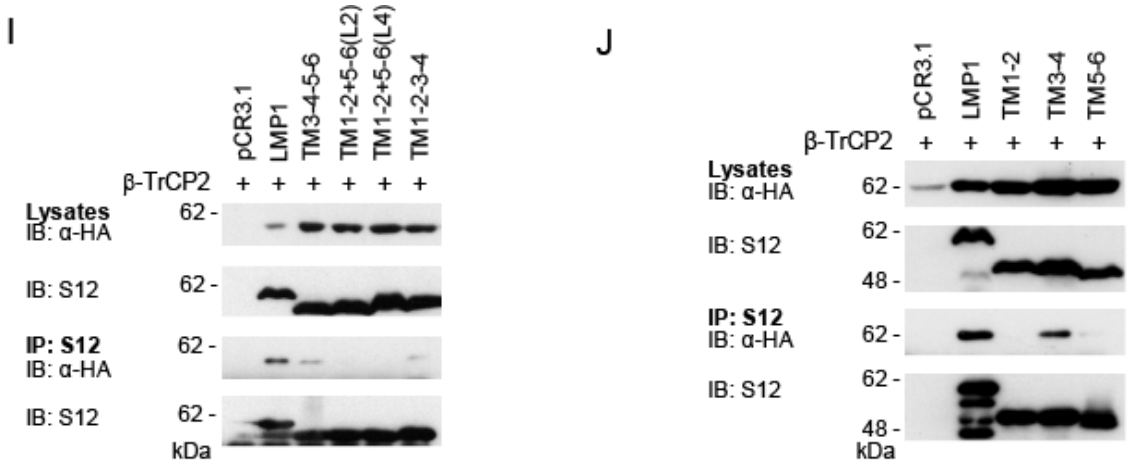


Figure 5: The presence of TMs 3 and 4 is essential for β-TrCP2 binding. (A) Schematic representation of LMP1 and localization of the putative β-TrCP2 binding site. The DSG binding motif lies close to the TRAF binding site. Mutations in the CTAR and DSG sites are indicated. **(B-F, H-J)** Association of β-TrCP with LMP1. 293T or HEK cells were transfected with the indicated β-TrCP and LMP1 constructs and lysed 38h later. Extracts were immunoprecipitated with S12 anti-LMP1 or HA antibodies bound to protein A Sepharose. LMP1 immunoprecipitates (IP) and cells lysates were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using the indicated antibodies. **(B)** HA-tagged β-TrCP2 binding to CTAR and DSG mutants in 293T cells with LMP1 immunoprecipitation. **(C)** HA-tagged β-TrCP2 binding to B95-8 LMP1 in HEK cells with LMP1 immunoprecipitation. **(D-E)** HA-tagged β-TrCP2-WD domains binding to B95-8 LMP1 in HEK cells with LMP1 **(D)** and HA **(E)** immunoprecipitation. **(F)** Flag-tagged β-TrCP1 binding to B95-8 LMP1 in HEK cells with LMP1 immunoprecipitation. **(G)** Schematic representation of LMP1-GFP mutants. **(H)** HA-tagged WD-β-TrCP2 binding to LMP1-GFP mutants in 293T cells with LMP1 immunoprecipitation. 8G3 epitope is located in the C-terminal region explaining the loss of detection of mutants lacking the C-terminal part. **(I-J)** HA-tagged β-TrCP2 binding to **(I)** TMs mutants with 4 TMs and **(J)** with 2 TMs in 293T cells with LMP1 immunoprecipitation. pCR3.1: empty vector.

4.2.6 LMP1 TMs AND INTRACELLULAR LOOPS L2 AND L4 CONTRIBUTED DIFFERENTIALLY TO NF-κB ACTIVATION

Two regions CTAR1 and CTAR2 within LMP1 C-terminal cytoplasmic domain have been shown to be critical for NF-κB activation (Figure 4A and 5A) [53]. As shown in the control experiment, mutation of the PxQxT motif of CTAR1 diminished the capability of LMP1 to activate NF-κB to 34% ± 12% of B95-8 LMP1 when measured by gene reporter assay (Figure 6A and Table 1). Mutation of CTAR2 diminished the activation to 32% ± 7%, whereas the combined mutations of CTAR1 and CTAR2 abolished NF-κB activation completely. DSG mutants, containing mutations close the PxQxT motif of CTAR1, activated NF-κB at levels similar to the B95-8 LMP1 (Figure 6B and Table 1).

Multiple observations support a role for LMP1 TMs in signaling [50,57,93]. However, the lack of consistency among LMP1 mutants does not allow to draw conclusions about the relative importance of each pair of TMs. We first analyzed the signaling properties of LMP1 TMs mutants with two pairs of TMs. As shown in Figure 6C, mutant TM3-4-5-6 had the lowest capability to activate NF-κB (45%) compared to the full length LMP1 (Table 1). Surprisingly, mutants TM1-2-3-4 and TM1-2+5-6(L2) activated NF-κB to the level of full-length LMP1, despite the fact that they displayed impaired TRAF3 binding (Figure 4C). Mutant TM1-2+5-6(L4) has an intermediate level of activation of 75%. The further comparison of TM1-2+5-6(L2) and TM1-2+5-6(L4) revealed that the intracellular loops L2 and L4 had a different impact on the signaling properties of the molecule. This observation suggests that L2 favored NF-κB activation or, alternatively, that L4 was associated with lower NF-κB activation levels.

We then analyzed the ability of mutants carrying a single pair of TMs to activate NF-κB. The three mutants are able to activate the transcription factor, but at different levels (Figure 6D and Table 1). The 70% of NF-κB activation displayed by mutant TM1-2 confirmed the major importance of TMs 1 and 2 for signaling [94,143]. The lower NF-κB activation levels induced by TM3-4 and TM5-6 (55% and 27%, respectively) was consistent with a less efficient membrane insertion (Figure 2). It is interesting to note that despite the loss of TRAF3 binding and suboptimal membrane insertion, LMP1 mutants with a single pair of TMs mediate substantial NF-κB activation compared to CTAR1-/2-.

Mutant TM1-2+5-6(L4) activated NF-κB less than TM1-2+5-6(L2). To better understand the function of the intracellular loop L4, we mutated residues that were conserved in B95-8 LMP1 and Rhesus LMP1 (Figure 6E and 8A). Both LMP1 and RhLMP1 contain a di-leucine motif within L4. To assess the importance of this motif, we mutated L126 and L127 to alanines (mutant L4m2) (Figure 6F and Table 1). Similarly, we mutated to alanines two pairs

of residues located near or present in the loop 4: W123, Y125 (mutant L4m1) and G134, W138 (mutant L4m3).

L4 mutants induced higher levels of NF-κB activation than B95-8 LMP1 (Figure 6G). L4m2, in which the di-leucine motif within L4 was replaced by two alanines, induced maximal NF-κB activation levels. The finding of several independent mutations near or in L4 with a positive impact on NF-κB signaling indicated a possible participation of the intracellular loop L4 in a negative regulation.

In summary, these results highlighted the differential behavior of the TMs as well as the two loops L2 and L4. A possible negative regulation by L4 was shown by mutating into alanines residues that are conserved between human and rhesus LMP1.

Table 1:
NF-κB activation levels of LMP1 B95-8 prototype and CTAR, DSG, TMs and L4 mutants.

LMP1	%	SD	p-value
B95-8	100	-	
CTAR1-	34	12	****
CTAR2-	32	7	****
CTAR1-/2-	1	0	****
DSGm1	102	6	ns
DSGm2	96	8	ns
DSGm3	86	9	ns
TM3-4-5-6	45	7	****
TM1-2+5-6(L2)	106	9	ns
TM1-2+5-6(L4)	75	8	****
TM1-2-3-4	107	10	ns
TM1-2	69	7	****
TM3-4	55	9	****
TM5-6	27	5	****
L4m1	156	18	****
L4m2	183	39	****
L4m3	157	23	****

NF-κB activities were measured 24 hours after transfection. Values are percentages ± SD of means of minimum 9 independent transfections normalized to the activity of B95-8 LMP1 fixed at 100%. Statistical analysis was done using one-way ANOVA with Bonferroni posttest using GraphPad Prism. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ relatively to the NF-κB activation of B95-8 LMP1. RLU: relative light units.

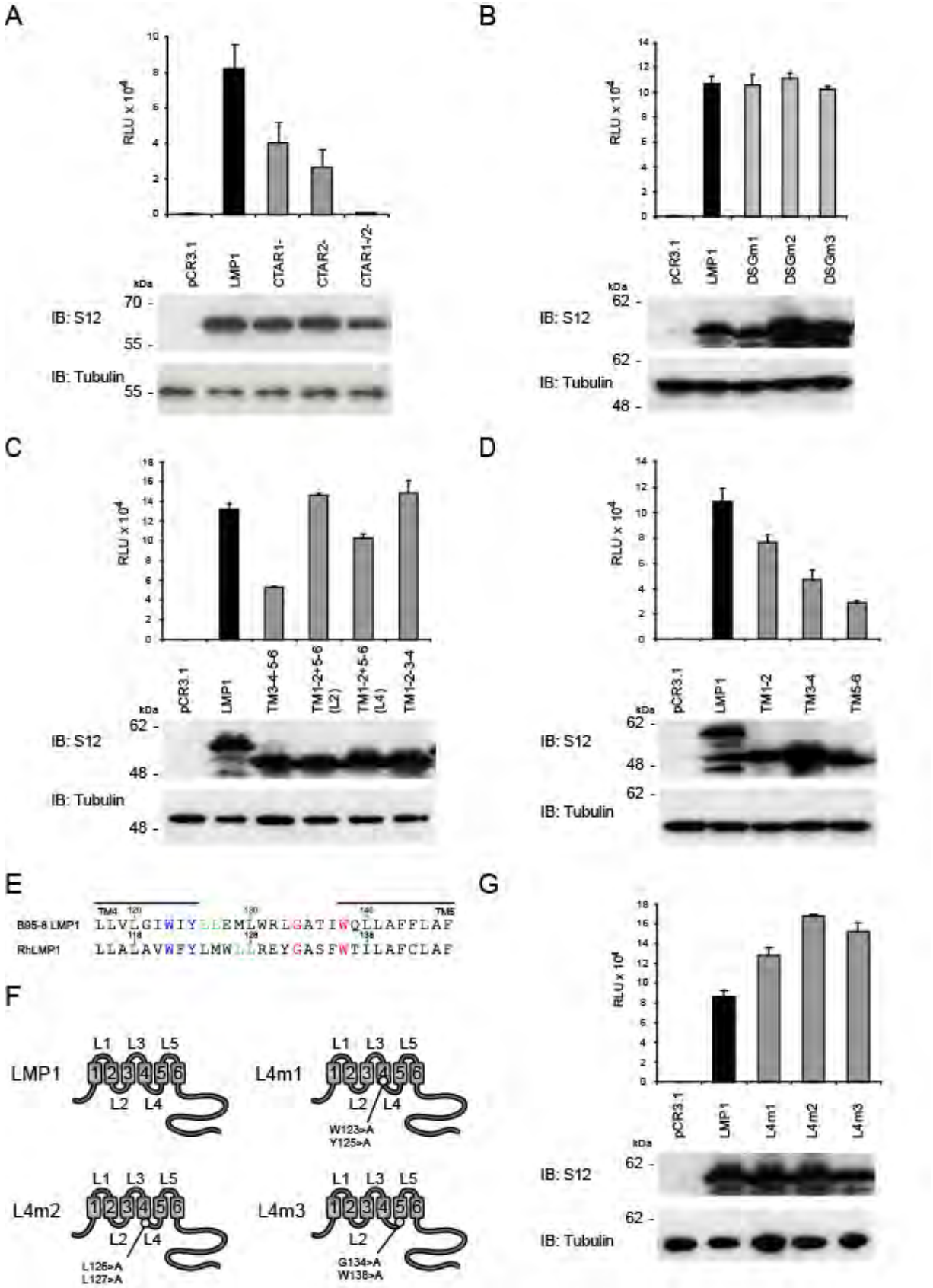


Figure 6: NF-κB activation by LMP1 CTAR and TMs mutants. (A-D, G) 293T cells were transfected with a NF-κB *Firefly* luciferase reporter, a *Renilla* luciferase reporter and the indicated LMP1 constructs. Cells were lysed 24h later and assayed for light emission using a dual-luciferase assay. Results are reported as mean + SD of the ratio of Firefly versus Renilla luciferase activity from triplicate wells. Shown is a representative of three

separate experiments. RLU: relative light units. Extracts were assayed for LMP1 expression by Western blot (lower panel). The amount of LMP1 detected in each lane is representative of triplicate transfection in a single experiment. pCR3.1: empty vector. LMP1: B95-8 LMP1. NF-κB activation by **(A)** CTAR mutants, **(B)** DSG mutants, **(C)** TMs mutants with 4 TMs and **(D)** with 2 TMs, **(G)** L4 mutants. **(E)** Alignment of B95-8 and RhLMP1 in the region of the loop L4. **(F)** Schematic representation of L4 mutants.

4.2.7 LMP1 INHIBITS MAVS-MEDIATED INTERFERONB ACTIVATION

As mentioned before, TRAF3 was the first LMP1 interacting partner described [55]. LMP1 recruits endogenous cytoplasmic TRAF3 to lipid rafts [106] and TRAF3 sequestration by LMP1 is the key event leading to the activation of the alternative NF-κB pathway [151,152,153]. Recent work showed that TRAF3 is also an essential activator of type I interferon production and thus is a critical link between NF-κB and IRF3 signaling pathways [154,155,156,157].

To investigate the impact of LMP1 on the IFNβ signaling pathway and test a possible correlation with LMP1's ability to bind TRAF3 or activate NF-κB we used reporter genes for NF-κB, ISRE and IFNβ-promoter (described in Materials and Methods). We observed that LMP1 B95-8 was able to activate the NF-κB reporter plasmid but not the ISRE and IFNβ-promoter reporter plasmids (Figure 7A). As previously observed in Figure 6A, the CTAR mutants had decreased abilities to activate NF-κB. None of the LMP1 proteins had the ability to activate ISRE and IFNβ-promoter reporter genes. In contrast, strong activations of ISRE and IFNβ-promoter reporter genes were measured upon overexpression of MAVS. Transcriptional activation of the IFNβ-promoter requires assembly of a multiprotein complex, which consists of activating transcription factor (ATF)2, c-Jun, IRF3 and NF-κB [36,158]. Our experiments showed that despite being a strong activator of c-Jun/AP-1 [159] and NF-κB, LMP1 did not activate the IFNβ-promoter.

A physical interaction between TRAF3 and MAVS has been recently demonstrated [157]. According to a model in which TRAF3 positively regulates MAVS the sequestration of

TRAF3 by LMP1 should inhibit MAVS-mediated ISRE activation. We tested this hypothesis by overexpressing RIG-I, MAVS, TBK-1 and IKKε, which all activated the ISRE reporter plasmid in presence of a 1:1 ratio of LMP1 or the control protein EGFP (Figure 7B). Whereas ISRE activation by TBK-1 and IKKε was not impaired by the co-expression of LMP1 B95-8, its activation by RIG-I and MAVS was specifically affected (Figure 7B). This result indicated that LMP1 blocked MAVS-mediated ISRE activation upstream of TBK-1 and IKKε.

We further measured the influence of the expression of LMP1 mutants on MAVS-mediated activation of ISRE and IFNβ-promoter reporter genes. As shown previously, CTAR1- and CTAR1-/2- did not bind TRAF3 (Figure 4B). Although CTAR1- and CTAR1-/2- were not as effective as B95-8 LMP1 in blocking MAVS-mediated ISRE and IFNβ-promoter activation, both mutants clearly displayed inhibitory activities. The data showed that TRAF3 binding leads to a mild inhibition and indicated that TRAF3 was not the unique mechanism by which LMP1 blocked MAVS-mediated ISRE activation. Moreover, the lack of inhibition by CTAR2- suggested that complex regulatory processes may be involved.

Inhibition of the IRF3 signaling pathway is a novel function of LMP1 that has not been described before. We further tested whether it required the integrity of the transmembrane region. Most interestingly, TM1-2+5-6(L4) totally lost the ability to inhibit ISRE and IFNβ-promoter reporter genes, whereas TM3-4-5-6, TM1-2+5-6(L2) and TM1-2-3-4 kept residual inhibitory activities (Figure 7D). These results again highlighted the differential properties of the two intracellular loops L2 and L4. LMP1 mutants with a single pair of TMs did not inhibit ISRE and IFNβ-promoter reporter genes effectively (Figure 7E), the highest inhibitory effect was displayed by TM5-6 with about a 10% of inhibition level.

In summary, we described a novel property of the LMP1 oncogene, the inhibition of MAVS-mediated ISRE and IFNβ-promoter activation. We showed that the binding of TRAF3 by

LMP1 played a minor role in this process. Finally, the integrity of the transmembrane domain of LMP1 was critical to this inhibitory activity.

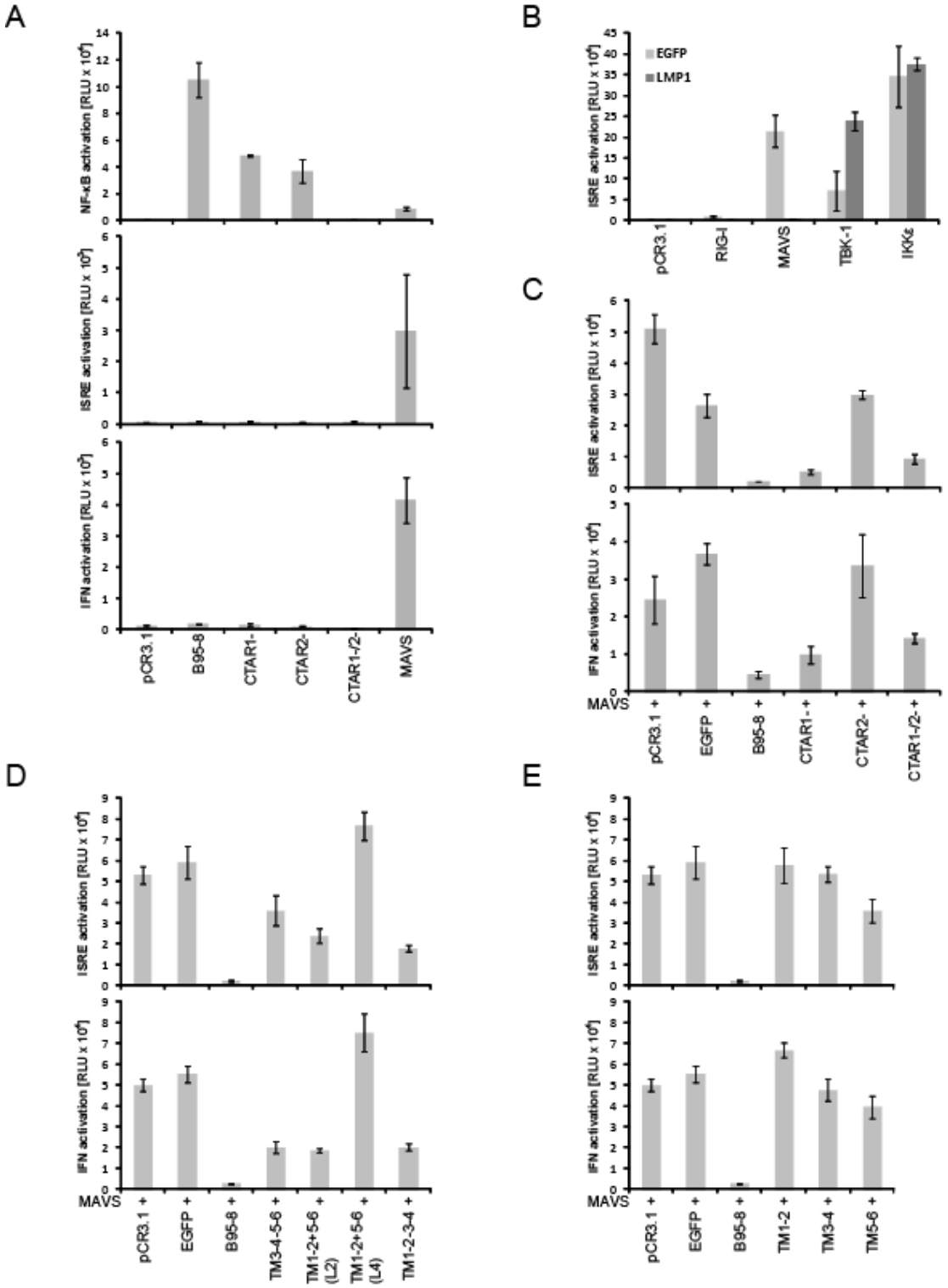


Figure 7: LMP1 inhibited MAVS-mediated activation of IFNβ pathway. (A) Activation of NF-κB, ISRE and IFNβ-promoter reporter plasmids by different LMP1 and MAVS. HEK cells were co-transfected with 50 ng of one reporter plasmid and 50 ng of expression vectors encoding for LMP1 or MAVS. **(B-E)** HEK cells were

co-transfected with 50 ng of either ISRE-luc or IFNβ-luc and 25 ng of expression vector for MAVS and 25 ng of expression vector for LMP1. Transfection with the empty vector pCR3.1 was used as basal level. Transfection with EGFP was used as control for the effect of the expression of unrelated protein. **(B)** Activation of ISRE reporter gene by different activators of the pathway and effect of LMP1 B95-8. EGFP was used as negative control. **(C)** Inhibition of MAVS-mediated ISRE and IFNβ pathways by LMP1. **(D-E)** Inhibition of MAVS-mediated activation of ISRE and IFNβ reporter genes by TMs mutants **(D)** with four TMs and **(E)** with two TMs.

4.2.8 RhLMP1 AND EBV LMP1 SHARE SIMILAR FUNCTIONAL PROPERTIES.

EBV shares many molecular and biologic features with simian lymphocriptoviruses naturally infecting primates [160,161]. Franken and co-workers isolated and characterized the LMP1 homolog RhLMP1 from EBV-related rhesus lymphocryptovirus (cercopithicine Herpesvirus 15) [162]. LMP1 and RhLMP1 shared an overall homology of 27.2% (Figure 8A). Interestingly, a good conservation was observed within the six TMs with 54.9% homology among residues 25 to 186. In contrast, the two C-terminal domains were strikingly divergent, except for the last 22 amino acids comprising CTAR2.

RhLMP1 C-terminal domain contains five putative TRAF binding sites PxQxT/S but no putative β-TrCP binding motif DSG. We extended previous observations [162] by showing that RhLMP1 bound endogenous TRAF3 (Figure 8B) and activated NF-κB very effectively as assessed by gene reporter assay (Figure 8C). Moreover, we demonstrated by co-IP experiments that RhLMP1 was able to interact with β-TrCP2 and the WD40 domains of β-TrCP2 (Figure 8D). RhLMP1 also shared with LMP1 the ability to inhibit MAVS-mediated ISRE and IFNβ-promoter activation (Figure 8E). The lower efficacy of RhLMP1 ompared to B95-8 is likely due to its lower expression level. Our observations indicated that the different features were common to human LMP1 and RhLMP1, despite major sequence divergences in the C-terminal domains.

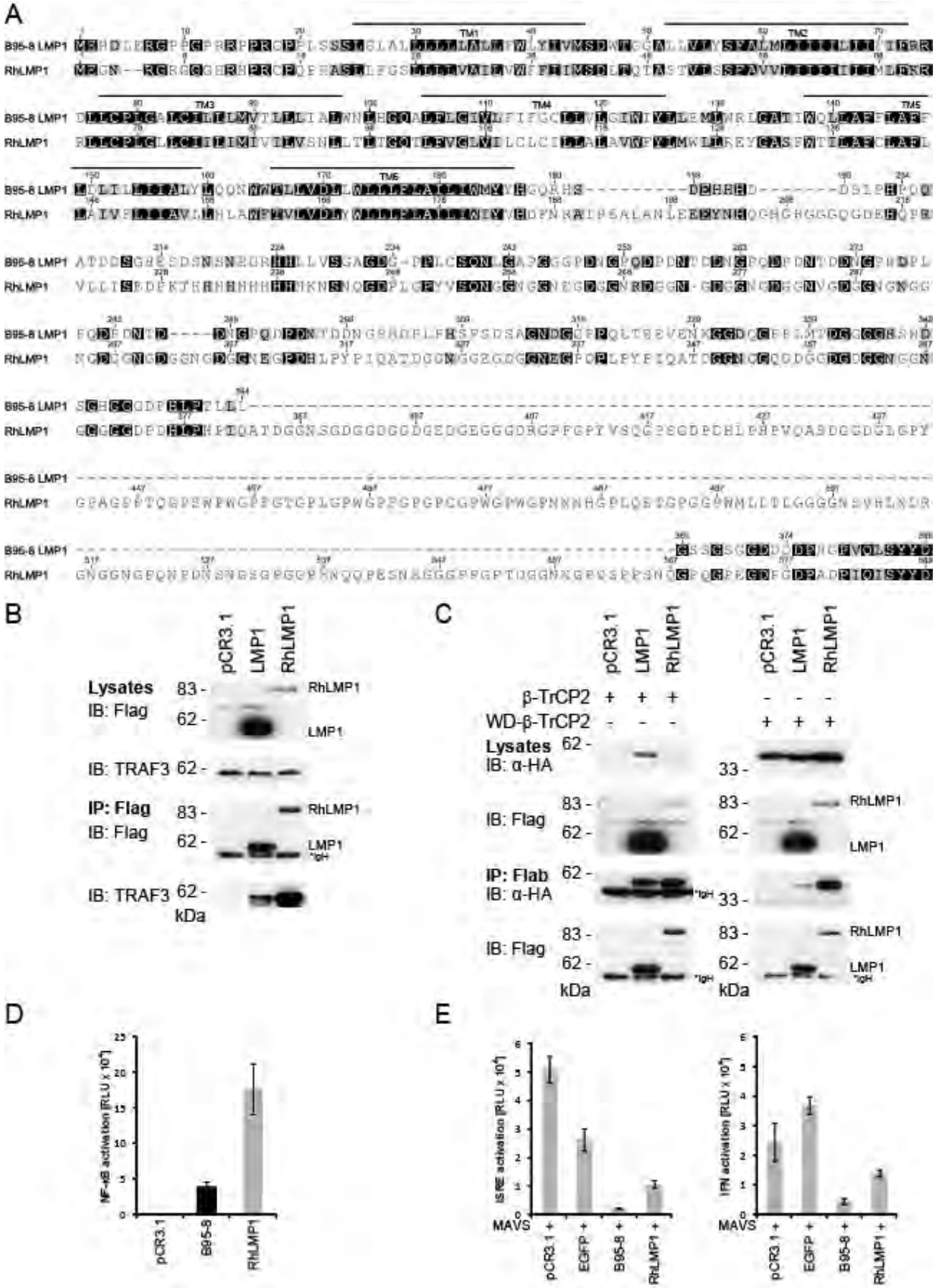


Figure 8: Rhesus LMP1 shared common features with human LMP1. (A) Alignment of B95-8 LMP1 and RhLMP1. Alignment and measure of homology was performed using Geneious software. The 6 TMs are highlighted by black lines. The strength of the homology was depicted with a black-white gradation. **(B-C)** Association of endogenous TRAF3 **(B)** or HA-tagged β-TrCP2 **(C)** with B95-8 LMP1 and RhLMP1. 293T cells

were transfected with Flag-tagged LMP1 constructs and **(C)** HA-tagged β-TrCP2, lysed 38h later. Extracts were immunoprecipitated with anti-FlagM2 beads. LMP1 immunoprecipitates (IP) and cells lysates were resolved by SDS-PAGE and analyzed by immunoblotting (IB) using the indicated antibodies. **(D)** NF-κB activation by B95-8 LMP1 and RhLMP1. HEK cells were transfected with 50 ng of LMP1 vector and 50 ng of NF-κB reporter plasmid. Empty vector was used as control. NF-κB activity was measured 24h after transfection using luciferase assay (Promega). Shown are representative of three independent experiments with similar results. Data are given as mean ± SD of triplicates. **(E)** Inhibition of MAVS-mediated activation of ISRE and IFNβ reporter genes by LMP1 and RhLMP1. HEK cells were co-transfected with 50 ng of either ISRE-luc or IFNβ-luc and 25 ng of expression vector for MAVS and 25 ng of expression vector for LMP1 or RhLMP1. Transfection with the empty vector pCR3.1 was used as basal level. Transfection with EGFP was used as control for the effect of the expression of unrelated protein.

4.2.9 B95-8 LMP1 AND VARIANT A2 SHARED FUNCTIONAL PROPERTIES

We have seen in the Chapter 1 the great diversity of natural LMP1 variants and their ability to display different levels of NF-κB activation. We have taken B95-8 and A2 LMP1 as examples to show the good conservation among variants of LMP1 functional properties.

We already knew that A2 displayed increased levels of NF-κB activation (Figure 9A and Chapter 1). Similarly to B95-8 LMP1, variant A2 interacted with TRAF3 (Figure 9 B) and β-TrCP2-WD40 domains (Figure 9C). As B95-8 LMP1, A2 did not activate ISRE and IFNβ-promoter by itself (Figure 9A) and inhibited MAVS-mediated ISRE and IFNβ-promoter activation (Figure 9D).

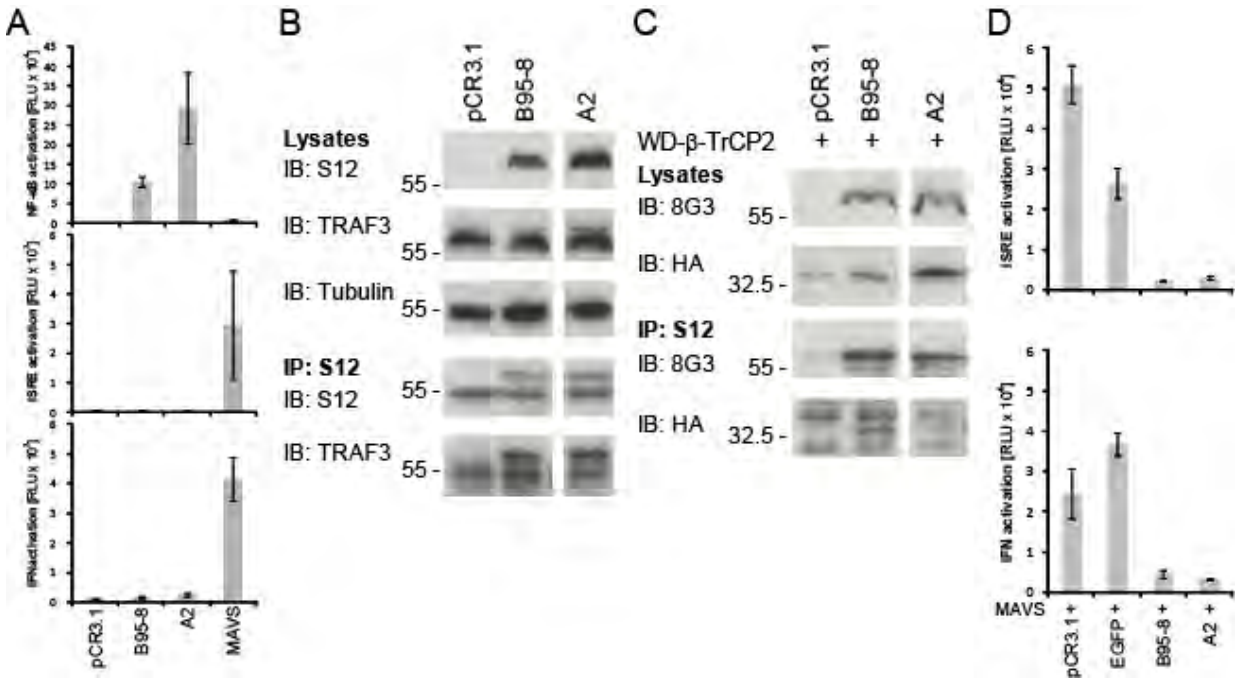


Figure 9: B95-8 LMP1 and variant A2 shared functional properties. (A) Activation of NF-κB, ISRE and IFNβ-promoter reporter plasmids by LMP1 and MAVS. HEK cells were co-transfected with 50 ng of one reporter plasmid and 50 ng of expression vectors encoding for LMP1 or MAVS. **(B-C)** Association of TRAF3 and β-TrCP2 with A2 LMP1. Cells were transfected with the indicated constructs, lysed 38h later and extracts were immunoprecipitated with S12 anti-LMP1 antibodies bound to protein A Sepharose. LMP1 immunoprecipitates (IP) and cells lysates were resolves by SDS-PAGE and analyzed by immunoblotting (IB) using the indicated antibodies. **(B)** HEK cells were transfected with LMP1 constructs. Binding of the endogenous TRAF3 with LMP1 was analyzed. **(C)** 293T cells were co-transfected with HA-tagged β-TrCP2-WD domains and LMP1 constructs. pCR3.1: empty vector. **(D)** Inhibition of MAVS-mediated ISRE and IFNβ pathways. HEK cells were co-transfected with 50 ng of either ISRE-luc or IFNβ-luc and 25 ng of expression vector for MAVS and 25 ng of expression vector for LMP1. Transfection with the empty vector pCR3.1 was used as basal level. Transfection with EGFP was used as control for the effect of the expression of unrelated protein.

4.2.10 ANALYSIS OF NATURALLY OCCURRING POLYMORPHISMS IN TMS

In view of the importance of TMs for LMP1's functions, genetic variations in the transmembrane region of the protein merit significant attention.

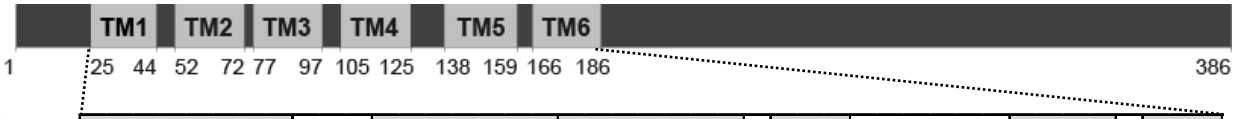
We reported all polymorphisms identified between aa 25 to 186 in LMP1 variants characterized in Chapter 1 (Figure 10, Appendix 1 for full length sequences).

When compared to the B95-8 LMP1 prototype sequence, the number of amino acid changes in this region ranged from two (variant 7835) to 15 (variant A2). It is to note that these numbers included two polymorphisms I85L and F106Y that were identified in all LMP1 isolates except B95-8.

Variations were identified in all six transmembrane segments. However, in most cases the amino acid changes were highly conservative. Most interestingly, we did not identify any polymorphism in the intracellular loop L2.

The phylogenetic groups described in the Figure 3 of Chapter 1 could be defined from the polymorphisms in the transmembrane region. In particular, variants from group 1 and 2 each display a very distinctive set of linked polymorphisms and few rarer variants.

Finally, some key residues identified in B95-8 LMP1 were not conserved in all variants. These include leucine 29 and leucine 36 in the Leucin heptad [143], cystein 78, which was shown to be palmitoylated [163], and methionine 129, which was described as the initiation methionine of lytic LMP1 [48,164,165] .



	TM1								L1			TM2							TM3								L3	TM4			L4						TM5				L5	TM6		
	25	26	28	29	34	35	36	43	46	47	49	57	59	61	63	65	68	72	78	79	82	83	84	85	96	101	106	122	124	126	127	129	132	134	137	144	150	151	152	163	178	179	180
Raji	I	.	.	.	.	.	.	I	N	.	.	A	.	.	V	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	I	.	.	.	.	.
7836	I	.	.	.	.	.	.	I	N	.	.	A	.	.	L	.	.	.	.	.	.	.	.	L	.	Q	Y	.	V	F	.	I	.	.	.	.	.	I	L	.	.	.	.
7885	I	.	.	.	.	.	.	I	N	.	.	A	.	.	V	.	.	.	.	.	.	.	.	L	.	.	Y	.	V	F	.	I	.	.	.	.	.	I	L	.	.	.	.
7850	I	.	.	.	.	.	.	I	N	.	.	A	.	.	V	.	.	.	.	.	.	.	.	L	.	.	Y	.	V	F	.	I	.	.	.	.	.	I	L	.	.	.	.
7825	I	.	.	.	.	.	.	I	N	.	.	A	.	.	V	.	.	.	.	.	.	.	.	L	.	.	Y	.	V	F	.	I	.	.	.	.	.	I	L	.	.	.	.
7939	I	.	.	.	.	.	.	I	N	.	.	A	.	.	V	.	.	.	.	.	.	.	.	L	.	.	Y	.	V	F	.	I	.	.	.	.	.	I	L	.	.	V	.
7924	.	.	.	.	.	.	I	.	N	.	.	.	.	.	.	.	.	.	Y	.	G	.	G	L	.	.	Y	L	.	F	.	I	.	.	.	I	A	I	.	.	.	.
A2	I	.	.	V	.	.	.	L	N	.	.	.	.	.	.	.	L	.	.	.	G	.	G	L	.	.	Y	L	.	F	.	I	.	.	.	I	A	I	.	.	.	.
CAO	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	L	.	.	Y	L	.	F	.	I	.	.	L	I	A	I	.	.	M	.
7705	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	G	.	G	L	.	.	Y	L	.	F	.	I	G	.	.	I	A	I	.	.	.	.
7950	.	.	.	.	.	.	.	.	N	L	A	.	.	.	.	.	.	.	.	.	G	.	G	L	.	.	Y	L	.	F	.	I	G	.	.	I	A	I	.	.	.	.
7823	.	.	.	.	F	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	G	.	G	L	.	.	Y	L	.	F	.	I	G	.	.	I	A	I	.	.	.	.
7910	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	G	.	G	L	.	.	Y	L	.	F	.	I	G	.	.	I	A	I	.	.	.	.
7880	I	T	.	.	V	.	.	I	.	.	.	A	.	I	.	.	.	.	.	T	.	I	A	L	.	.	Y	L	.	.	M	.	.	.	.	.	.	I	.	.	.	L
7859	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	R	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B95-8	**L**	**G**	**A**	**L**	**L**	**A**	**L**	**V**	**D**	**W**	**G**	**S**	**A**	**M**	**I**	**I**	**I**	**F**	**C**	**P**	**A**	**L**	**C**	**I**	**A**	**H**	**F**	**I**	**I**	**L**	**L**	**M**	**R**	**G**	**I**	**F**	**D**	**L**	**I**	**N**	**L**	**A**	**I**
7848	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	L	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7835	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7881	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7902	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	V	.	.	.	.	.	D	V	.	.	.	.	.	.	.	.
AG876	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
P1	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	L	.	.	.	.	.	L	.	.	Y	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7769	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7926	.	.	.	.	.	.	.	.	N	.	.	.	.	.	L	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7795	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7886	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	N	Y	I	.	.	.	I	.	.	.	.	.	I	.	H	.	.	.
A3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7918	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7948	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	L	.	.	Y	L	.	F	.	I	.	.	L	.	.	.	.	.	.	.	.
7843	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7815	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	F	.	I	.	.	.	.	.	.	.	.	.	.	.
A1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7703	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7914	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7798	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7913	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7849	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	L	V	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7821	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
7893	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	L	.	.	Y	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.

Figure 10: Schematic representation of LMP1 protein and table of polymorphisms present in the TMs and loops of the variants analyzed in Chapter 1. The sequence of reference is the B95-8 LMP1 prototype. The different variants were listed according to the phylogenetic tree constructed in Chapter 1 Figure 3. Only the positions changing were described. For the full sequences alignment see Appendix 1.

4.3 CHAPTER 2 – MATERIALS AND METHODS

4.3.1 PLASMIDS

The expression vectors for EBV B95-8 strain full length LMP1 was constructed by cloning the LMP1 gene from the lymphoblastoid cell line B95-8 [106] into pCR3.1 (Invitrogen). The following deletions were introduced by PCR: **TMs mutants**: TM3-4-5-6 (deletion of amino acids 25 to 76), TM1-2+5-6 (L2) (deletion of amino acids 77 to 138), TM1-2+5-6 (L4) (deletion of amino acids 73 to 125), TM1-2-3-4 (deletion of amino acids 126 to 186), TM1-2 (deletion of amino acids 73 to 186), TM3-4 (deletion of amino acids 25 to 76 and 126 to 186), TM5-6 (deletion of amino acids 25 to 138). **GFP mutants**: LMP1-GFP contains full-length LMP1 fused in C-terminal to GFP and Flag tag. N-TM-GFP contains the N-terminal domain and the six TMs fused to GFP and Flag tag. TM-GFP contains the six TMs fused to GFP and Flag tag. C-GFP contains the whole C-terminal domain fused to GFP and Flag tag. C^{232}-GFP contains the 154 last amino acids of the C-terminal part of LMP1 fused to GFP and Flag tag. Flag-tagged LMP1 was constructed by fusing the C-terminal part of LMP1 to Flag tag. Point mutations were introduced by PCR using the full length LMP1 as template. **CTAR mutants**: CTAR1- has the PxQxT TRAF binding motif mutated into alanines, CTAR2- contains the mutation L382P and CTAR1-/2- has both CTAR sites mutated. **DSG mutants**: DSGm1 contains mutations of the canonical and cryptic recognition sites described in [82]. DSGm2 has the two serines of the DSGxxS motif mutated. DSGm3 has, besides the mutations of the two serines, changes in the acidic residues present in the DSG region. All LMP1 constructs were cloned into pCR3.1 and verified by sequencing.

The expression vector for RhLMP1 was a gift of F. Wang (Boston, USA). It encodes an N-terminally Flag-tagged LMP1 homologue from simian EBV infecting rhesus monkeys (cercopithicine herpes virus 15) [162].

The expression vectors for Flag-tagged β-TrCP1 [166] and HA-tagged β-TrCP2 [167] were the gift of Y. Ben Neriah (Jerusalem, Israel) and S. Fuchs (Philadelphia, USA), respectively. The construct encoding the WD40 domain of β-TrCP2 (amino acids 215 to 542) was built by PCR.

The expression vector for EGFP was given by S. Kunz (Lausanne, Switzerland).

The reporter construct κB-conA-luc, provided by F. Grässer (Homburg, Germany), comprises the *Firefly* luciferase gene under the control of a conalbumine promoter with three integrated κB elements derived from the immunoglobuline κ chain enhancer (Figure 11).

pISRE-luc reporter vector (Stratagene), provided by M. Thome (Lausanne, Switzerland), expresses the *Firefly* luciferase gene under the control of a synthetic promoter containing five enhancer elements $(TAGTTTCACTTTCCC)_5$ of the transcription recognition sequences for the interferon-stimulated response element (ISRE). pISRE-luc is stimulated by IFNα and IFNβ and regulated by IRF3.

IFNβ-luc reporter vector, provided by M. Thome, expresses the *Firefly* luciferase gene under the control of the human IFNβ promoter that harbors responsive elements for AP1, $(ISRE)_2$ and NF-κB [168].

The *Renilla* luciferase construct pRL-RSV, provided by E. Buetti (Lausanne, Switzerland), was constructed by inserting the BglII-HindIII fragment containing the RSV LTR from pRc/RSV (Invitrogen) into the plasmid pRL-null (Promega).

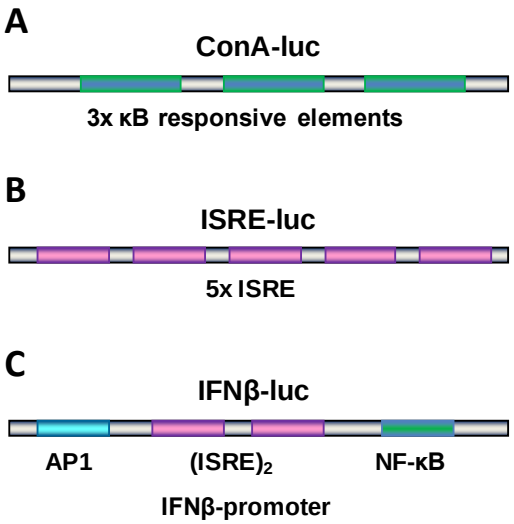


Figure 11: Schematic representation of the promoters of *Firefly* luciferase gene reporters. For the description see the Plasmids section of Materials and Methods.

4.3.2 ANTIBODIES

4.3.2.1 Primary antibodies

The following antibodies rat monoclonal against HA tag – 1:3,000 - 1:4,000 (Roche Applied Science), mouse IgG1 monoclonal Flag tag – 1:10,000 (M2, Sigma), mouse IgG1 monoclonal transferrin receptor – 1:3,000 (Zymed), mouse IgG2a monoclonal caspase-3 – 1:3,000 (Signal transduction), mouse antibody against tubulin – 1:10,000 (Sigma), as well as rabbit polyclonal antibodies against TRAF3 – 1:1,000 (Santa Cruz) were purchased. S12 mouse IgG2a monoclonal antibody – 1:24,000 - 1:48,000 [45,169] and 8G3 rat monoclonal antibody – 1:500 - 1:1,000 [108] against LMP1 were provided by F. Meggetto (Toulouse, France) and F. Grässer, (Hombourg, Germany), respectively. Both anti-LMP1 antibodies recognize epitopes located in the C-terminal domain of the protein.

4.3.2.2 Secondary antibodies

Secondary antibodies used after primary incubation were all purchased: polyclonal rabbit anti-mouse HRP and polyclonal rabbit anti-rat HRP – 1:3,000 - 1:5,000 (Dako Cytomation,

Glostrup, Denmark), anti-rabbit HRP – 1:3,000 (Santa Cruz), goat anti-mouse IgG2a HRP – 1:10,000 and IgG1 HRP – 1:6,000 (SouthernBiothech, Birmingham, UK).

4.3.3 CELL CULTURE

Human embryonic kidney 293 T (293T) cells (ATCC CRL-1126) and Human embryonic kidney 293 (HEK) cells (ATCC CRL-1573) [107] were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin and streptomycin at 37^{o}C with 5% CO2.

4.3.4 SUBCELLULAR FRACTIONATION

293T cells from 100-mm plates were washed once in cold PBS and resuspended in 1 ml homogenization buffer (20 mM Tris HCl pH7.4, 10 mM EDTA, 5 mM EGTA) supplemented with inhibitors of proteases (minicomplete, Roche Applied Biosytems). Cells were broken with 100 strokes of a tight-fitting pestle in a dounce homogenizer. The nuclei and unbroken cells were removed by a low speed centrifugation (500 x*g* for 5 min at 4°C). The homogenates were centrifuged at 100,000 x*g* for 1 h at 4°C. The high speed supernatant (soluble fraction) was removed and pellets including membranes were resuspended in an equal volume of homogenization buffer containing 1 % Triton X-100. Both fractions were analyzed by SDS-PAGE.

4.3.5 CELL LYSIS, IMMUNOPRECIPITATION AND IMMUNOBLOTTING

293T cells were transfected in 100-mm plates with 2 μg of the expression plasmids using Fugene 6 (Roche Applied Science). All transfections were equalized using the empty vector

pCR3.1**.** Thirty-eight hours post-transfection cells were lysed in RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 8, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) supplemented with inhibitors of proteases (minicomplete, Roche Applied Science) and inhibitors of phosphatases (20 mM NaF, 25 mM glycerophosphate, 1 mM orthovanadate). Lysates were cleared by centrifugation at 16,000 x*g* for 10 min at 4°C. Fifty µl of lysate were saved and used to detect the level of each protein. Proteins were immunoprecipitated with either M2-agarose beads or S12-bound protein-A Sepharose for 2 h at 4°C. Precipitates were washed three times with RIPA buffer and twice with phosphate buffered saline. Samples were boiled for 4 min in 50 µl of 2x sample buffer (62 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 100 mM DTT). Samples were separated through SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with the appropriate antibodies and revealed by chemiluminescence.

4.3.6 REPORTER GENE ASSAYS

293T cells were transfected in 24-well plates with 50 ng of the NF-κB *Firefly* reporter construct κB-conA-luc, 2.5 ng of the *Renilla* reporter construct pRL-RSV and 50 ng of LMP1 expression vectors using Fugene 6 (Roche Applied Science). Twenty-four hours after transfection, cells were lysed in Passive lysis buffer (Promega) and assayed for light emission on a Bertold Lumat LB 9507 luminometer using the Dual-Luciferase Reporter Assay System (Promega, Amriswil, Switzerland). All transfection efficiencies were normalized to the levels of *Renilla* luciferase. Final quantifications were performed on three separate experiments executed in triplicates.

HEK cells were transfected in 24-well plates with the indicated amount of the different reporter constructs κB-conA-luc, ISRE-luc or IFNβ -luc and with the indicated amount of

expression vectors encoding for LMP1 constructs, pCR3.1 empty vector, EGFP or MAVS using FuGENE6. Twenty-four hours after transfection, cells were lysed in Cell Culture Lysis Reagent (Promega) and assayed for light emission in a Bertold microplate luminometer Tristar LB 941 using Luciferase Assay System (Promega).

4.4 CHAPTER 2 – DISCUSSION

In the present study we found that diverse biological properties of LMP1 crucially depend on its six transmembrane-spanning segments. TMs ensured membrane insertion, self-aggregation, contributed to the recruitment of cellular proteins and modulated signaling. In particular we showed that the three pairs of TMs and the two intracellular loops that link them are not functionally equivalent and interchangeable.

Previous observations supported a key role for LMP1's TMs in signaling: a derivative of EBV with a LMP1 gene lacking the entire transmembrane region transformed B cells with less than 1% of the efficiency of the full length form [96] and studies using chimeric proteins between LMP1 and CD40 demonstrated that LMP1 signals in a ligand-independent fashion due to its six TMs (24, 28, 32, 39). However the relative importance of TMs has not been evaluated before. To address this question we generated LMP1 mutants with deletions of either one or two pairs of TMs. Importantly, all constructs had identical N- and C-terminus and were equally well expressed in cells upon transfection. Moreover, all TMs mutants including mutants with a single pair of TMs induced substantial NF-κB activation compared to CTAR1-/2-, demonstrating that C-terminus were accessible to cytoplasmic factors and thus oriented correctly.

LMP1 TMs are particularly rich in leucine and isoleucine residues. Previous studies focusing on TM 1 demonstrated by alanine mutagenesis that the leucin heptad and residues FWLY contributed to intermolecular interactions and signaling [93,94,143]. We performed cellular fractionation to assess the membrane association of the LMP1 mutants. We found that TM3-4 and TM5-6 drove a less efficient membrane insertion compared to TM1-2. Concordantly, TM3-4 or TM5-6 activated NF-κB to lower levels compared to TM1-2 in a gene reporter assay. NF-κB activation levels were correlated with the Grand average of hydropathicity

(GRAVY) index of the TMs: the combined GRAVY of TMs1-2 is the highest, that of TMs 3-4 intermediate, and that of TMs 5-6 the lowest.

We evaluated self-aggregation of LMP1 mutants with a single pair of TMs in co-immunoprecipitation experiments and found that all three pairs of TMs participated in self-association and association with neighboring LMP1 molecules. However in the absence of a crystal structure and the lack of homology to proteins with known crystallographic data, it is impossible to draw conclusions about the exact spatial organization of LMP1 TMs. Co-immunoprecipitation analysis indicated that the three pairs of TMs engaged intramolecular and intermolecular interactions and that the central pair of TMs seemed to play a key part in LMP1 aggregation as TM3-4 led to the strongest associations.

We used LMP1 mutants to assess the binding to endogenous TRAF3 and found that the deletion of a single pair of TMs strongly impaired TRAF3 binding. Despite impaired TRAF3 binding, mutants with two pairs of TMs induced higher activation levels than CTAR1-. Surprisingly TM1-2+5-6(L2) and TM1-2-3-4 even induced levels similar to full length B95-8 LMP1. These results, together with the differential activation by TM1-2+5-6(L2) and TM1-2+5-6(L4), are compatible with a negative regulation of NF-κB signaling in the transmembrane region. The finding that mutations within the intracellular loop L4 led to enhanced NF-κB activation compared to B95-8 LMP1 further support this hypothesis.

Little is known about the interaction between LMP1 and β-TrCP. The F-box protein β-TrCP serves as the substrate recognition subunit in the $SCF^{\beta\text{-TrCP}}$ E3 ubiquitin ligase complex that plays a pivotal role in cell cycle progression, cell survival and signaling. The majority of β-TrCP substrates contain a canonical DSGxxS motif in which the phosphorylation of the two serine residues is required to allow recognition. Although LMP1 contains a canonical DSGxxS motif in the C-terminus, this motif was dispensable for β-TrCP2 binding. We found that LMP1 with mutations S211A /S215A either alone, or in combination with the mutation

of the adjacent residues D210N/E214Q/D216N bound β-TrCP2 as efficiently as B95-8 LMP1. Moreover, RhLMP1 bound β-TrCP2 despite the absence of a DSGxxS motif in its sequence. Instead we found that the interaction was driven by LMP1 TMs and did not require the F-box domain of β-TrCP2. The biological relevance of this association remains largely unknown. Since IκB is a target of β-TrCP, the interaction may modulate LMP1-mediated NF-κB activation. Initial studies showed that overexpression of a dominant negative β-TrCP2 did not change LMP1's half-life [82], suggesting that the E3 ubiquitin ligase activity of $SCF^{\beta\text{-TrCP}}$ does not target LMP1 for degradation. Most interestingly, alternative binding sites were described for hnRNP-U [170], growth hormone receptor [171] and USP47 [172], indicating that other modes of interaction with β-TrCP2 exist. It is to note that these interactions were similarly not linked to the degradation of the target protein.

Evidences of a direct interaction between TRAF3 and MAVS [173] suggested to us that TRAF3 sequestration by LMP1 may impair MAVS function. To test this hypothesis we took advantage of the observation that MAVS, when overexpressed in cells, induced efficiently ISRE and IFNβ-promoter reporter genes [174]. Using this assay we discovered that LMP1 was a strong inhibitor of MAVS-mediated ISRE and IFNβ-promoter activation. The biological advantage of such an inhibition may be multiple for EBV in the context of viral persistence in memory B-cells and tumors. In cells latently infected with EBV, EBER1 and EBER2 are the most abundant viral transcripts and EBERs are considered reliable markers for the detection of EBV in tumors [175]. EBER1 and EBER 2, which are small uncapped, polyA-, non-coding, non-translated RNA form extensive secondary structures that could induce the expression of type I INFs through direct activation of RIG-I in Burkitt's lymphoma derived cells [175,176]. Interestingly, the transcriptome analysis of EBV-transformed lymphoblastoid cell lines which express LMP1 revealed a two- to four-fold suppression of INFα and IFNβ gene expression [177].

We found that TRAF3 binding to LMP1 only modestly contributed to the inhibition of a MAVS-mediated ISRE and IFNβ-promoter activation. Instead, LMP1 TMs and intracellular loop L2 played a key role. Our results also indicated that LMP1 targets either MAVS or a positive regulator of MAVS and not the downstream kinases TBK-1 and IKKε. Interestingly, LMP1 did not induce MAVS degradation (data not shown), suggesting that molecular mechanisms involved are different from those described for hepatitis C virus [168]. LMP1 may block MAVS-induced signaling cascade at the interface between the endoplasmic reticulum and the mitochondria. LMP1 was shown to signal principally from intracellular compartments [178] and to interact through the transmembrane domain with prenylated Rab acceptor 1 (PRA1, also designated as PRA1 domain family member 2, PRAF2) [179], a multispan protein implicated in vesicular traffic and lipid metabolism. It will be of greatest interest to test whether LMP1 interacts through the transmembrane region with the MAVS regulator stimulator of interferon genes (STING, also known as MITA, ERIS, MPYS), a multispan protein localized to the endoplasmic reticulum membrane.

In conclusion, the present study showed that TMs were implicated in broader biological functions than previously reported by providing a platform where association with the membrane, intramolecular and intermolecular interactions and binding to cellular proteins occurred. Therefore transmembrane segments could be considered as essential modulators of LMP1's activities.

5 CONCLUSIONS AND PERSPECTIVES

In Chapter 1, we determined which polymorphisms were involved in the increased NF-κB activation phenotype displayed by some LMP1 variants. Surprisingly, we found single polymorphisms as responsible for the major difference in NF-κB activation levels. F106Y and F144I were defined as important determinants for higher NF-κB activation levels; whereas I124V was described for the first time in this study.

F106Y is present in all variants we cloned and moreover at a high frequency in LMP1 described in the literature. It is present in CAO LMP1 [71,72], AG876 LMP1 [18] and in different LMP1 subgroups: A, B, C and D described by Fielding et *al.* [111] and Ch1, Ch2, AL and NC described by Edwards et *al.* [109]. Our study showed that the effect of polymorphism F106Y is modulated according to the variants. In the majority of the variants F106Y is not main feature associated with enhanced NF-κB activation, as shown with A2 variant or in CAO [50]; whereas in variants 7795, 7815, 7918, 7948 and 7821 it is the major determinant leading to high NF-κB activation levels. Further work will be needed to better understand how other polymorphisms modulate the effect of F106Y.

F144I has a more restricted distribution. It is present in CAO LMP1, AG876 LMP1 and in subgroups C [111], Ch2 and AL [109]. Moreover F144I is present at a high frequency in published sequences of LMP1 from EBV-associated NPC: 5/5 variants isolated from Vietnamese patients [180], 21/21 variants isolated from patients in Southern China [181], 5/5 variants isolated from patients in Hong Kong [182]. These observations suggest either a more aggressive potential of these LMP1 strains or as markers of geographical origin of the strain.

With the clinical pilot study on the potential association of LMP1 polymorphisms with HL, we demonstrated the feasibility and interest of such a study even if the statistical power was too low to draw definitive conclusions on the lack of association found. The further step of

this work would be the extension to a larger cohort and inclusion of a higher number of HL cases to increase the statistical power of the study in order to have more reliable results (Appendix 2).

In Chapter 2, a more biochemical aspect of the LMP1 field was approached. In this study, we improved the knowledge about the relative contribution of each pair of LMP1 TMs regarding membrane association, self-aggregation, binding to LMP1 cellular partners TRAF3 and β-TrCP, and NF-κB activation. We described an unknown function of LMP1, which consists in the repression of MAVS-mediated ISRE and IFNβ-promoter reporter genes. It would be relevant to further investigate the molecular mechanisms involved in this new function. Furthermore, since we cloned from SHCS samples a wide collection of LMP1 variants, it would be of great interest to test the different features with some variants representative of the different phylogenetic groups.

Overall we have shown in this work the importance of the transmembrane domain of LMP1. All critical polymorphisms reside in LMP1 TMs 4 and 5. Finally, we have demonstrated the importance of the TMs for the regulation of LMP1 signaling.

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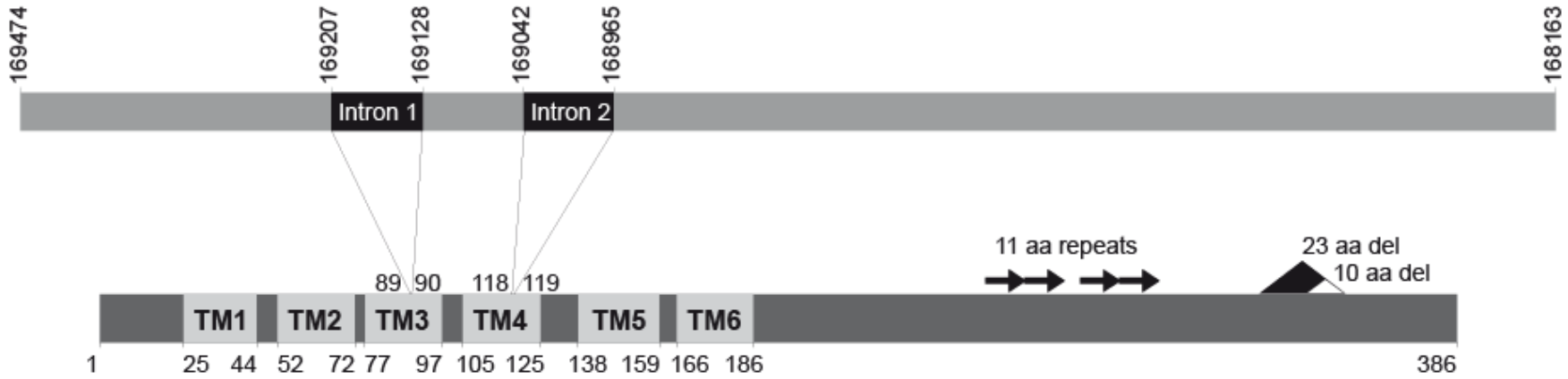
7 APPENDIX

7.1 AMINO ACID SEQUENCE OF LMP1 VARIANTS



Schematic representation of LMP1 gene (upper) with the position of the two introns and protein (lower) with the six transmembrane domains represented by grey boxes, the region of the direct repeats and the position of the two deletions.

Alignment of LMP1 variants used in the study and specific GenBank accession number (BankIt1498710) for each new LMP1 sequence:

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                10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Raji    .DL.....................I.................I..N..........A.....V.....................L..............
7836    .DL.....................I.................I..N..........A.....L.....................L..............
7885    .DL.....................I.................I..N..........A.....V.....................L..............
7850    .DL.....................I.................I..N..........A.....V.....................L..............
7825    .DL.....................I.................I..N..........A.....V.....................L..............
7939    .DL.....................I.................I..N..........A.....V.....................L..............
7924    .--.................................I.........N.................................Y...G.GL..............
A2      ..R.........P...L.......I...V.............L..N.....................L.............G.GL..............
CAO     ..R.........P...L.......I.....................................................G.GL..............
7705    ..............................................N.....................................G.GL..............
7950    ......................F.....................NL.A...................................G.GL..............
7823    ................................F............N.....................................G.GL..............
7910    ..................Q.............................N.....................................G.GL..............
7880    .DR.....................IT.......V........I.............A...I.................T...IAL..............
7859    .DL.....................................................................................L..............
B95-8   MEHDLERGPPGPRRPPRGPPLSSSLGLALLLLLLALLFWLYIVMSDWTGGALLVLYSFALMLIIIILIIFIFRRDLLCPLGALCILLLMITLLLIALWNL
7848    ........................F............I.............................L...................L..............
7835    ..........................................................................................L..............
7881    ..............................................N.....................................L..............
7902    ............................V.....................................................L..............
AG876   ..................................................I.....................L..............
P1      ..............................................H.....................L.............L..............
7769    ..............................................N.....................................L..............
7926    ..............................................N.................L...................L..............
7795    ..................................................G.....................L..............
7886    ..........................................................................L..............
A3      ..................................................................L.....L..............
7918    ................................V.....................................L..............
7948    ..................................................................L.....L..............
7843    ..........................................................................L..............
7815    ..................................................I.....................L..............
A1      ..................................................L.....................L..............
7703    ..........................................................................L..............
7914    ..................R.....................................................L..............
7798    ..................................................................L.....L..............
7913    ..................................................................L.....L..............
7849    ..................................................................L.....L..........V....
7821    ..................................................................L.....L..............
7893    ................................S.................................L.....L..............
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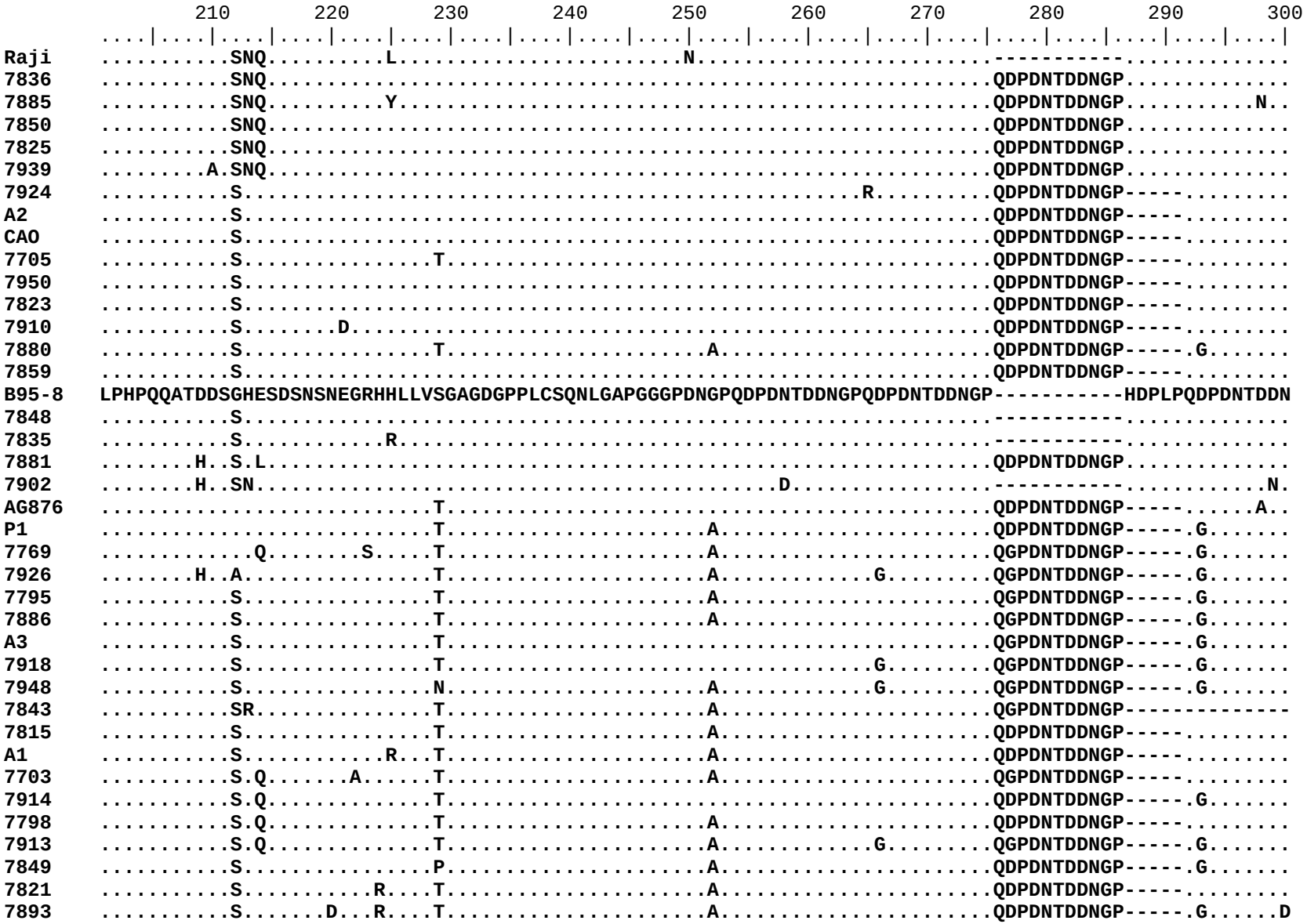
                110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Raji      .....Y..............L......I.....................I.....................................................
7836      Q....Y..............V.F..I.....................IL....................................................
7885      .....Y..............V.F..I.....................IL....................................................
7850      .....Y..............V.F..I.....................IL....................................................
7825      .....Y..............V.F..I.....................IL....................................................
7939      .....Y..............V.F..I.....................IL.....................V..............................
7924      .....Y..............L...F..I.............I.....AI.....................................P..T........
A2        .....Y..............L...F..I.............I.....AI.....................................P..T........
CAO       .....Y..............L...F..I.......L.....I.....AI.....................M...............P..T........
7705      .....Y..............L...F..I..G...........I.....AI.....................................P..T........
7950      .....Y..............L...F..I..G...........I.....AI.....................................P..T........
7823      .....Y..............L...F..I..G...........I.....AI.....................................P..T........
7910      .....Y..............L...F..I..G...........I.....AI.....................................P..T........
7880      .....Y..............L....M.I.....................I.....................L.....................
7859      R....Y.....................................................................................
B95-8     HGQALFLGIVLFIFGCLLVLGIWIYLLEMLWRLGATIWQLLAFFLAFFLDLILLIIALYLQQNWWTLLVDLLWLLLFLAILIWMYYHGQRHSDEHHHDDS
7848      .....Y.....................................................................................
7835      .....Y.....................................................................................
7881      .....Y.....................................................................................
7902      .....Y..............V...........D..V.....................................................
AG876     .....Y..............L......I.....................................................
P1        .....Y..............L.....................................................
7769      .....Y..............L......I.....................................................
7926      .....Y..............L......I.....................................................
7795      .....Y..............L......I.....................................................
7886      N....Y.....................I.....................I...........H.....................T........
A3        .....Y..............L......I.....................................................
7918      .....Y..............L......I.....................................................
7948      .....Y..............L...F..I.......L.....................................................
7843      .....Y..............L......I.....................................................
7815      .....Y..............L...F..I.....................................................
A1        .....Y..............L......I.....................................................
7703      .....Y..............L......I.....................................................
7914      .....Y..............L......I.....................................................
7798      .....Y..............L.....................................................
7913      .....Y..............L......I.....................................................
7849      .....Y..............L......I.....................................................
7821      .....Y..............L......I.....................................................
7893      .....Y..............L......I.....................................................
```


210 220 230 240 250 260 270 280 290 300
Raji .....SNQ.....L.....N.....----------.....
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7885 .....SNQ.....Y.....QDPDNTDDNGP.....N..
7850 .....SNQ.....QDPDNTDDNGP.....
7825 .....SNQ.....QDPDNTDDNGP.....
7939 .....A.SNQ.....QDPDNTDDNGP.....
7924 .....S.....R.....QDPDNTDDNGP----------
A2 .....S.....QDPDNTDDNGP----------
CAO .....S.....QDPDNTDDNGP----------
7705 .....S.....T.....QDPDNTDDNGP----------
7950 .....S.....QDPDNTDDNGP----------
7823 .....S.....QDPDNTDDNGP----------
7910 .....S.....D.....QDPDNTDDNGP----------
7880 .....S.....T.....A.....QDPDNTDDNGP-----G.....
7859 .....S.....QDPDNTDDNGP----------
B95-8 LPHPQQATDDSGHESDSNSNEGRHHLLVSGAGDGPPLCSQNLGAPGGGPDNGPQDPDNTDDNGPQDPDNTDDNGP----------HDPLPQDPDNTDDN
7848 .....S.....----------.....
7835 .....S.....R.....----------.....
7881 .....H..S.L.....QDPDNTDDNGP.....
7902 .....H..SN.....D.....----------.....N.
AG876 .....T.....QDPDNTDDNGP----------A..
P1 .....T.....A.....QDPDNTDDNGP-----G.....
7769 .....Q.....S.....T.....A.....QGPDNTDDNGP-----G.....
7926 .....H..A.....T.....A.....G.....QGPDNTDDNGP-----G.....
7795 .....S.....T.....A.....QGPDNTDDNGP-----G.....
7886 .....S.....T.....A.....QGPDNTDDNGP-----G.....
A3 .....S.....T.....QGPDNTDDNGP-----G.....
7918 .....S.....T.....G.....QGPDNTDDNGP-----G.....
7948 .....S.....N.....A.....G.....QGPDNTDDNGP-----G.....
7843 .....SR.....T.....A.....QGPDNTDDNGP----------
7815 .....S.....T.....A.....QDPDNTDDNGP----------
A1 .....S.....R...T.....A.....QDPDNTDDNGP----------
7703 .....S.Q.....A.....T.....A.....QGPDNTDDNGP----------
7914 .....S.Q.....T.....QDPDNTDDNGP-----G.....
7798 .....S.Q.....T.....A.....QDPDNTDDNGP----------
7913 .....S.Q.....T.....A.....G.....QGPDNTDDNGP-----G.....
7849 .....S.....P.....A.....QDPDNTDDNGP-----G.....
7821 .....S.....R....T.....A.....QDPDNTDDNGP----------
7893 .....S.....D...R....T.....A.....QDPDNTDDNGP-----G.....D

```
                310       320       330       340       350       360       370       380       390       400
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Raji      ..........----------.....N.....................................D.I.........T.
7836      .S........----------.....N...........N..........R...S.....---------.........T.
7885      ..........----------.....N...........N..........R...S.....---------.........T.
7850      ..........----------.....N...........N..........R...S.....---------.........T.
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7939      ..........----------.....N...........N..........R...S.....---------.........T.
7924      ..........----------.....N...........N..........R...S.....---------.........T.
A2        ..........----------.....N...........N..........R...S.....---------.........T.
CAO       ..........QDPDNTDDNGPQDPDNTDDNGP.....N...........N.....A.....R...S.....---------.........T.
7705      ..----------.....N...........NF.........R...S.....---------.........T.
7950      ..----------.....N...........N.......T...R...S.....---------.........T.
7823      ..........----------Q.....N...........N..........R...S.....---------.........T.
7910      ..........QDPDNTDDNGP----------.....N...........N.........HR...SV.....---------.........T.
7880      ..----------.....N...........E..........R...S.....------------R.....T.
7859      ..........----------.....N...........N..........R...S.....---------.........T.
B95-8     GPQDPDNTDDNGP----------HDPLPHSPSDSAGNDGGPPQLTEEVENKGGDQGPPLMTDGGGGHSHDSGHGGGDPHLPTLLLGSS
7848      ..........----------.....N.....................................................I.....T.
7835      ..........----------.....Q.....................N....S.....T.
7881      ..........----------.....Q.....................S.....T.
7902      ..........----------.....Q.....T.
AG876     ..----------.....N...........N..........R...S.....---------.........T.
P1        ...G.....----------.....N...........E..........R...S.....T.
7769      ...G.....QGPDNTDDNGP----------.....N.......E....E.....----------.....M...A.
7926      ...G.....----------.....N.......E.R..E.....----------.....A.
7795      ..----------.....N...........E..........R...S.....------------R.....T.
7886      ..----------.....N...........E..........R...S.....------------R.....T.
A3        ..----------.....N...........E..........R.....------------R.....T.
7918      ...G.....----------.....D...........E..........R...S.....------------R.....T.
7948      ...G.....QGPDNTDDNGPQGPDNTDDNGP.....N...........E.....Q.....R...S.....RR.....T.
7843      ----------.....N...........D..........R...S.....---------.........T.
7815      ..----------.....N...........E..........R...S.....A..R.....T.
A1        ..........QGPDNTDDNGP----------.....N...........E..........R...S.....------------R.....T.
7703      ...G.....QGPDNTDDNGP----------.....N...........E..........R...S.....------------R.....T.
7914      ...G.....----------.....N.......N....E..........R...S.....G..R.....T.
7798      ...G.....QGPDNTDDNGPQGPDNTDDNGP.....N.......Y.H..E..........R...S.....------------R..C.....T.
7913      ..----------.....K.G.....Y....E.......T...R...S.....---------.........T.
7849      ...G.....----------.....N.......N.D.....------------AR...S.....------------R..C.....T.
7821      ...G.....QGPDNTDDNGP----------.....N.......N.D.....------------R...S.....------------R..C.....T.
7893      ..----------.....N.......N.D.....------------HR..SS.....------------R..C.....T.
```

```
                410       420
          ....|....|....|....|
Raji      ...................*
7836      ...................* 7836 JQ240434
7885      ...................* 7885 JQ240435
7850      ...................* 7850 JQ240436
7825      ...................* 7825 JQ240437
7939      ...................* 7939 JQ240438
7924      ...................* 7924 JQ240439
A2        ...................* A2   JQ240440
CAO       ...................*
7705      ...................* 7705 JQ240441
7950      ...................* 7950 JQ240442
7823      ...................* 7823 JQ240443
7910      ...................* 7910 JQ240444
7880      ...................* 7880 JQ240445
7859      ...................* 7859 JQ240446
B95-8     GSGGDDDDPHGPVQLSYYD*
7848      ...................* 7848 JQ240447
7835      ...................* 7835 JQ240448
7881      ...................* 7881 JQ240449
7902      ...................* 7902 JQ240450
AG876     ...................*
P1        ...................* P1   JQ240451
7769      ...................* 7769 JQ240452
7926      ...................* 7926 JQ240453
7795      ...................* 7795 JQ240454
7886      ...................* 7886 JQ240455
A3        ...................* A3   JQ240456
7918      ...................* 7918 JQ240457
7948      ...................* 7948 JQ240458
7843      ...................* 7843 JQ240459
7815      ...................* 7815 JQ240460
A1        ...................* A1   JQ240461
7703      ...................* 7703 JQ240462
7914      ...................* 7914 JQ240463
7798      ...................* 7798 JQ240464
7913      ...................* 7913 JQ240465
7849      ...................* 7849 JQ240466
7821      ...................* 7821 JQ240467
7893      ...................* 7893 JQ240468
```

7.2 EXERCISE OF FICTIVE PROTOCOL

This fictive protocol was written in the context of a certificate in clinical research done the last year of my PhD studies. This protocol will be a possible continuation of the clinical study developed in the Chapter 1. Since our study has a low power due to few patients HIV-HL in the SHCS, increasing the sample size will increase the power of the study and thus confirm or infirm the results obtained in the pilot study.

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Institut de Microbiologie, CHUV, Lausanne

Relation chez les personnes VIH+ entre la présence de modifications dans la protéine de latence 1 du virus d'Epstein-Barr et le développement de lymphomes de Hodgkin

[LMP1POL]

13.09.2011 et version 6

N° d'identification LMP1POL-2011

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1 Informations générales

1.1 Date de l'envoi du protocole à la commission d'éthique : 15.09.2011

1.2 Date prévue pour le début de l'étude : Dès acceptation par la C.E.

1.3 Lieu d'étude : Institut de microbiologie (IMUL) - CHUV

1.4 Coordonnées du promoteur et des investigateurs

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Collaborations :

Cohorte EuroSIDA

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2 Table des matières

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3 Résumé

3.1 Introduction

Le virus d'Epstein-Barr (EBV) infecte plus de 90% de la population adulte mondiale et est lié à différents types de tumeurs, dont le lymphome de Hodgkin (LH). La protéine membranaire de latence 1 (LMP1), l'oncogène majeur du virus, active différentes voies de signalisation, dont celle du facteur de transcription NF-κB. L'activation de NF-κB par LMP1 est une étape essentielle de la transformation cellulaire par EBV. L'activation accrue de NF-κB peut être liée avec le développement de tumeurs. Certains variants naturels de LMP1 sont capables d'activer plus fortement NF-κB que le LMP1 prototype B95-8. Nous avons précédemment déterminé deux groupes de polymorphismes permettant une activation accrue de NF-κB: I124V/I152L et F144I/D150A/L151I.

L'avènement des trithérapies a permis aux personnes infectées par VIH (VIH+) d'avoir une durée de vie prolongée et moins de maladies liées à l'immunosuppression. Au contraire, la proportion de LH a augmenté. Chez les personnes VIH+, les LH ont une forme plus agressive que dans le reste de la population et sont associés à EBV dans la majorité des cas. En comparaison, dans le reste de la population ce taux environne les 50%.

Nous avons mené une étude pilote prospective cas-témoins nichée dans l'étude suisse de cohorte VIH (SHCS) afin d'observer si les groupes de polymorphismes susmentionnés pouvaient être des facteurs prédictifs pour le développement de LH. Dû au faible nombre de cas dans la SHCS, nous souhaitons mener une étude similaire dans une cohorte de plus grande taille afin de recenser plus de cas et pouvoir confirmer ou infirmer les résultats obtenus lors de l'étude pilote. Celle-ci nous a permis de démontrer la faisabilité du design expérimental.

3.2 Déroulement de l'étude

Nous voulons mener un projet d'étude observationnelle, cas-témoins nichée dans le cadre d'une cohorte – EuroSIDA, incluant des patients VIH+ ayant eu un diagnostique de LH versus des patients VIH+ n'ayant jamais eu de diagnostique de tumeur associée à EBV. Le diagnostique de LH devra avoir été confirmé par histologie.

Les échantillons de sang seront obtenus de la biobanque de la cohorte. Pour les cas, les échantillons seront choisis le plus proche de la date du diagnostique de LH (±1 an). Les témoins seront idéalement appariés aux cas pour le sexe, l'âge, le niveau de CD4 dans le sang, la durée d'infection par le VIH et la durée du traitement antirétroviral au moment du prélèvement sanguin, avec un ratio cas-témoin 1 :2. L'ADN génomique sera extrait des cellules sanguines et la région du gène codant pour les acides aminés 96 à 202 de LMP1 sera amplifiée et séquencée. La présence des groupes de polymorphismes I124V/I152L et F144I/D150A/L151I de LMP1 sera ensuite déterminée et son association avec l'étiologie de LH sera analysée par régression logistique pour chaque groupe individuellement ainsi que combiné.

3.3 Intérêt du présent travail de recherche dans le domaine

Si les groupes de polymorphismes sont associés au développement de LH chez les personnes VIH+, un screen de ces polymorphismes permettrait aux personnes positives d'avoir un dépistage de LH plus fréquent résultant en un traitement précoce de la maladie.

3.4 Mots-clé

EBV, LMP1, polymorphisme, VIH, lymphome de Hodgkin

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4 Glossaire et abréviations

CHUV	Centre hospitalier universitaire vaudois
EBV	Virus d'Epstein-Barr (Epstein-Barr virus)
LH	Lymphome de Hodgkin
IMUL	Institut de microbiologie de l'université de Lausanne
LMP1	Protéine membranaire de latence 1 (latent membrane protein 1)
NF-κB	Facteur nucléaire kappaB (nuclear factor kappaB)
OR	Rapport de cote (odds ratio)
PCR	Réaction en chaîne par la polymérase (polymerase chain reaction)
SHCS	Cohorte Suisse pour le VIH (Swiss HIV Cohort Study)
VIH	Virus de l'immunodéficience humaine
VIH+	Infecté par le virus de l'immunodéficience humaine
95%CI	Intervalle de confiance à 95%

5 Mise en perspective de l'étude

5.1 Etat des connaissances

Le virus d'Epstein-Barr (EBV), de la famille des Herpesvirus, infecte plus de 90% de la population adulte mondiale. En plus d'être l'agent causatif de la mononucléose infectieuse, EBV est lié à différents types de tumeurs, dont le lymphome de Hodgkin (LH), le lymphome de Burkitt, ou encore le carcinome nasopharyngé[1]. La protéine membranaire de latence 1 (LMP1), l'oncogène majeur du virus, active différentes voies de signalisation de la cellule hôte, dont celle du facteur de transcription NF-κB[2,3]. LMP1 est une protéine polymorphique et divers variants naturels de LMP1 ont été décrits[4-6]. Certains de ces variants ont montré une capacité accrue à activer NF-κB comparé au prototype B95-8 LMP1[7-9].

L'activation de NF-κB par LMP1 est une étape essentielle de la transformation cellulaire par EBV[1]. De plus, NF-κB a été montré comme étant essentiel pour la progression de lymphomes associés à EBV *in vivo*[10]. CAO LMP1, un variant isolé d'un carcinome nasopharyngé[5], a montré une activation de NF-κB accrue[8] ainsi qu'un pouvoir oncogène plus élevé que le prototype B95-8 LMP1 dans un modèle de souris nues[11]. Une activation accrue de NF-κB a été reliée au développement de certaines tumeurs[12-14]. Nous avons donc posé comme hypothèse que certains variants de LMP1 en activant NF-κB à de plus haut niveaux pourraient être davantage oncogènes et ceci dû à la polymorphicité de la protéine. Nous avons déterminé deux groupes de polymorphismes de LMP1 - F144I/D150A/L151I et I124V/I152L - permettant une activation accrue de NF-κB (E. Zuercher et *al.*, soumis).

Chez les individus infectés par le virus de l'immunodéficience humaine (VIH+), la charge virale pour EBV est plus élevée que dans le reste de la population et ce dès la séroconversion à VIH[15,16]. Dû à l'avènement des trithérapies, les personnes VIH+ ont une durée de vie augmentée. Ces traitements ont également permis une réduction des maladies liées à l'immunosuppression dans cette population. Au contraire, la proportion de LH a augmenté en même temps que la durée de vie[17-19]. De plus, chez les personnes VIH+, les LH ont une forme plus agressive que dans le reste de la population et sont associés à EBV dans la majorité des cas. En comparaison, dans le reste de la population ce taux environne les 50%[19,20].

Nous avons mené une étude pilote prospective cas-témoins nichée dans l'étude suisse de cohorte VIH (SHCS) afin d'observer s'il existe un lien entre la présence des groupes de polymorphismes I124V/I152L et F144I/D150A/L151I de LMP1 et le développement de LH (E. Zuercher et *al.*, soumis). 42 cas ont pu être inclus dans notre étude, ce qui n'est pas suffisant pour que l'étude soit assez puissante et permette de tirer des conclusions solides. Cette étude pilote nous a permis de démontrer la faisabilité de notre design expérimental. Nous avons également pu observer une tendance en faveur de notre hypothèse de départ, c'est-à-dire que les groupes de polymorphismes sont des facteurs prédictifs de l'étiologie de LH. C'est pourquoi, nous souhaitons mener une étude similaire dans une cohorte de plus grande taille afin de recenser plus de cas et pouvoir confirmer ou infirmer les résultats obtenus lors de l'étude pilote.

5.2 But général de l'étude

Dans la présente étude, nous souhaitons définir si deux groupes de polymorphismes de LMP1, I124V/I152L et F144I/D150A/L151I, sont des facteurs prédictifs de l'étiologie de LH dans une population de patients VIH+.

5.3 Question de recherche

Est-ce que les personnes infectées par le virus de l'immunodéficience humaine (VIH) sont plus à risque de développer un lymphome de Hodgkin (LH) s'ils sont porteurs d'un virus

d'Epstein-Barr avec une protéine membranaire de latence 1 (LMP1) ayant une capacité accrue à activer le facteur de transcription NF-κB?

5.4 Hypothèse(s) de recherche

Les polymorphismes I124V/I152L et F144I/D150A/L151I sont des facteurs prédictifs du développement de LH.

5.5 Justification

Si ces groupes de polymorphismes s'avèrent liés au développement de LH dans cette population, leur présence pourrait être déterminée à partir d'échantillons sanguins et les personnes positives auraient un dépistage de LH plus fréquent permettant un traitement à un stade plus précoce de la maladie.

6 Plan général

6.1 Critères de jugement (Outcomes)

La présence des groupes de polymorphismes I124V/I152L et F144I/D150A/L151I comme potentiels facteurs prédictifs de l'étiologie de LH sera analysée par amplification par PCR et séquençage d'un fragment du gène de LMP1.

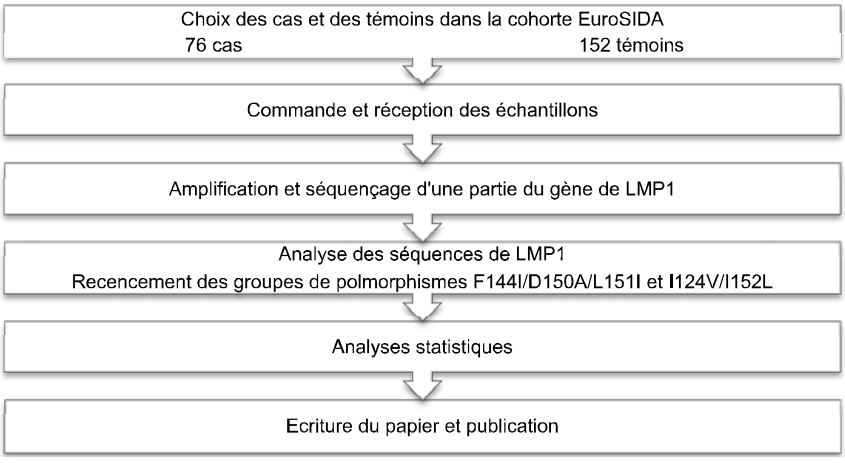
6.2 Population cible

Nous nous intéressons à des individus infectés par le VIH ayant développé un lymphome de Hodgkin pour la population de cas et n'ayant pas développé de tumeur pour la population témoin.

6.3 Type / design d'étude

La présente étude est prospective, observationnelle, cas-témoin nichée dans une cohorte (EuroSIDA). Les témoins proviendront de la même cohorte que les cas est seront appariés pour le sexe, l'âge, le niveau de CD4 dans le sang, la durée d'infection par le VIH et la durée du traitement antirétroviral au moment du prélèvement sanguin, avec un ratio cas-témoin de 1:2.

Figure 1: Schéma du design de l'étude



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6.4 Méthodes d'investigation

Les échantillons sanguins seront obtenus de la biobanque de la cohorte EuroSIDA. Pour les cas, ils seront choisis le plus proche de la date du diagnostique de LH (±1 an). Pour les témoins, ils seront appariés selon le sexe, l'âge, le niveau de CD4 dans le sang, la durée d'infection par le VIH et la durée du traitement antirétroviral au moment du prélèvement sanguin. L'ADN sera extrait des cellules sanguines et la région du gène codant pour les acides aminés 96 à 202 de LMP1 sera amplifiée et séquencée; cette étape sera effectuée par le service de diagnostique de l'IMUL. La présence des groupes de polymorphismes I124V/I152L et F144I/D150A/L151I sera ensuite déterminée. L'association entre la présence des groupes de polymorphismes et l'étiologie de LH sera analysée par régression logistique pour chaque groupe individuellement, ainsi que combiné. Si l'appariement n'est pas parfait un modèle ajustant pour les facteurs confondants sera construit.

6.5 Planification de l'étude

Tableau 1: Timetable de l'étude

Phase 1		Phase 2		Phase 3		Phase 4	
2 mois		1-2 mois		1 mois		3 mois	
Début	Fin	Début	Fin	Début	Fin	Début	Fin
01.10.2011	01.12.2011	01.12.2011	15.01.2012	15.01.2012	15.02.2012	15.02.2012	15.05.2012

Phase 1: Recherche des cas au sein de la cohorte et appariement des témoins

Phase 2: Réception et préparation des échantillons

Phase 3: PCR et séquençage

Phase 4: Analyse des données, écriture du manuscrit et premier envoi pour publication

7 Sélection des sujets

7.1 Définition de la population cible

Dans la présente étude, nous nous intéresserons à une population composée de personnes VIH+ inclues dans la cohorte EuroSIDA. De plus, la population de cas sera composée de personnes ayant eu un diagnostique de LH confirmé par histologie, alors que la population de témoins sera composée de personnes n'ayant jamais eu de diagnostique de tumeur associée à EBV.

7.2 Critères d'inclusions

Tous les patients inclus dans la cohorte EuroSIDA sont éligibles depuis la date de commencement de celle-ci. Ce qui signifie que tous les sujets inclus dans notre étude ont eu un diagnostique positif pour VIH. Le consentement pour l'utilisation des échantillons sanguins à but de recherche est donné dans le cadre de la cohorte EuroSIDA.

Critères d'inclusion spécifiques pour les cas: avoir eu un diagnostique avéré de LH.

Critères d'inclusion spécifiques pour les témoins: ne jamais avoir eu de diagnostique de tumeur associée à EBV. Les témoins seront appariés pour le sexe, l'âge, le niveau de CD4 dans le sang, la durée d'infection par le VIH et la durée du traitement antirétroviral au moment du prélèvement sanguin selon un ratio cas-témoins 1:2.

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7.3 Critères d'exclusion

S'il s'avère qu'aucun échantillon sanguin n'est disponible: pour un témoin apparié, celui-ci ne pourra pas être inclus dans l'étude; pour un cas, celui-ci ne pourra pas être inclus dans l'étude, ainsi que ses témoins appariés.

7.4 Procédure de retrait d'un sujet en cours d'étude

S'il s'avère qu'aucune amplification et/ou séquence de LMP1 ne sont obtenues à partir du matériel génomique d'un cas ou d'un témoin, celui-ci sera retiré de l'étude et si possible remplacé par un sujet similaire dont un échantillon sanguin est disponible et dont l'amplification et la séquence de LMP1 sont obtenus. Si aucun cas avec des caractéristiques similaires n'est trouvé, les témoins appariés du cas retiré devront également être exclus.

8 Plan statistique

8.1 Méthodes statistiques

Les analyses statistiques seront effectuées à la fin de l'étude, lorsque toutes les séquences auront été analysées. Les données seront analysées par régression logistique et le résultat donné sous forme d'odds ratio (OR) avec intervalle de confiance 95% (95%CI). Un modèle incluant les facteurs utilisés pour l'appariement sera construit (sexe, âge, niveau de CD4 dans le sang, durée d'infection par le VIH, durée du traitement antirétroviral à la date du prélèvement sanguin).

Hypothèses statistiques:

H0: La présence des groupes de polymorphismes I124V/I152L ou F144I/D150A/L151I n'est pas liée à l'étiologie de LH.

H1: La présence des groupes de polymorphismes I124V/I152L ou F144I/D150A/L151I est liée à l'étiologie de LH.

8.2 Taille de l'échantillon

Il faudra inclure dans l'étude 76 cas et 152 témoins pour avoir une puissance de 80%, unα à 0.05, une proportion attendue dans le groupe cas de 0.4 et dans le groupe témoin de 0.2, ainsi qu'un ratio de 2 entre les deux groupes.

8.3 Degré de signification

Pour être considéré comme significatif l'OR devra être supérieur à 2 et le 95% CI ne devra pas inclure la valeur 1.

8.4 Données récoltées

Les séquences du gène codant pour LMP1 entre les acides aminés 96 et 202 seront obtenues pour les cas et les témoins. La présence des deux groupes de polymorphismes I124V/I152L ou F144I/D150A/L151I sera ensuite déterminée. Ces groupes de polymorphismes sont mutuellement exclusifs et ne peuvent donc pas être présents sur la même séquence.

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8.5 Critères de traitement des données

Seront inclus dans l'analyse tous les sujets pour lesquels au moins une séquence aura été obtenue.

8.6 Traitement des données manquantes

Les sujets pour lesquels aucune séquence n'aura été obtenue seront exclus de l'analyse. Si pour plusieurs sujets les valeurs pour un facteur confondant ne sont pas disponibles, il faudra exclure du modèle cette variable. Si ce n'est le cas que pour un sujet, ce sujet sera exclu de l'analyse et le facteur confondant sera conservé dans le modèle. S'il s'avère que ce facteur confondant n'est pas essentiel au modèle, l'exclusion du sujet de l'analyse ne sera pas nécessaire.

8.7 Critère de fin d'étude

L'étude sera terminée lorsque tous les échantillons auront été récoltés, amplifiés et séquencés, et les séquences analysées.

8.8 Limites de l'étude

L'étude sera menée sur des échantillons de patients HIV+, la population étudiée est de ce fait très restreinte. Il ne sera donc pas possible d'extrapoler les résultats à la population générale. De plus, chez les patients VIH+ la charge virale pour EBV est plus élevée que dans la population générale, ce qui permet une amplification plus aisée du gène de LMP1. La même étude menée dans une population plus globale ne donnerait probablement pas les mêmes résultats.

Afin d'éviter un biais de sélection des témoins, ceux-ci seront appariés aux cas pour les principaux facteurs influençant le développement de tumeurs. En ce qui concerne le biais de mesure, l'amplification par PCR et le séquençage sont des techniques de biologie moléculaire fiables et reproductibles. La limitation majeure se trouve dans la possibilité d'infection multiple à EBV. Dans ce cas, plusieurs séquences différentes de LMP1 peuvent être obtenues pour un même patient; ceci est grandement lié à la prévalence des souches. Pour éviter tout problème lié à ce facteur, nous n'ajusterons pas pour le nombre de séquences trouvées, mais nous regarderons uniquement si chez un certain patient nous observons une LMP1 avec les groupes de polymorphismes I124V/I152L ou F144I/D150A/L151I. La lecture sera donc de type présence / absence et non quantitative.

8.9 Stockage des données

Les échantillons d'ADN génomique seront stockés pour une durée de 15 ans dans les congélateurs -80°C se trouvant au 4ème étage de l'institut de microbiologie, local 406. Les données informatiques seront stockées dans le serveur du CHUV pour la même durée.

9 Considérations éthiques

Cette étude observationnelle cas-témoins nichée dans la cohorte EuroSIDA n'implique pas d'enrôlement direct de patients. Les échantillons utilisés pour cette étude sont des prélèvements sanguins effectués lors du suivi régulier des patients de la cohorte, ce qui n'implique aucun désagrément pour les patients. L'utilisation des ces échantillons, somme toute précieux, ne se fait pas dans une optique de recherche fondamentale, mais dans le but de définir de potentiels facteurs prédictifs de l'étiologie de LH. Nous pensons que cette étude peut apporter un outil permettant de définir les personnes plus à risque de développer

un LH dans une population VIH+, qui a déjà plus de risques de développer un LH. Ce qui nous permettrait de mettre sur pied une meilleure surveillance de cette population. Nous considérons que cette étude peut apporter un bénéfice à la population ciblée.

10 Assurance

L'étude étant observationnelle, il ne sera pas nécessaire de contracter une assurance.

11 Budget et source de financement:

Budget de l'étude

Charges salariales directement liées à l'étude				
Poste (ex : supervision, encadrement, chercheur, etc.)	Mois	%	Salaire annuel sur 13 mois à 100% [CHF]	Montant tot. (y c. charges sociales)
Médecin chef	2	5	140'399.-	1348.90.-
Chercheur responsable	7	10	79'402.-	4861.30.-
Chercheur associé	7	30	65'065.-	11'954.70.-

Autres charges			
	Montants total [CHF]	Facturé par	Payé par
Prestations cohorte			
Charges administratives cohorte	500.-	Cohorte EuroSIDA	Fond du projet
Prix échantillons (~230)	2300.-	Cohorte EuroSIDA	Fond du projet
Prix envois	500.-	Cohorte EuroSIDA	Fond du projet
Prestations laboratoires (CHUV ou externes)			
Frais projet	500.-	Diagnostique IMUL	Fond du projet
Prix PCR et séquençage	5750.-	Diagnostique IMUL	Fond du projet
Traitement des données			
Expertise statistique	500.-	Statisticien	Fond du projet
Congrès / formation			
Déplacement	700.-	Agence de voyage	Fond du projet
Inscription	450.-	Congrès	Fond du projet
Logement (forfait journalier)	1000.-	Hôtel	Fond du projet
Repas (forfait journalier)	300.-	Divers	Fond du projet

Source(s) de financement externe(s)

Provenance du financement (y c. adresse et personne de contact)	**Receveur**	**Montant par tranche**	**Dates prévues pour versements**
SNF	Barr Epstein	50'000	Octobre 2011 Mars 2012
Cohorte EuroSIDA	Barr Epstein	25'000	Octobre 2011
Ligue Suisse contre le Cancer	Zürcher Emilie	65'000	Début 2012

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Source de financement interne

Dénomination du fond	Montant total
CGRB00000	14'800

12 Conflits d'intérêt potentiels

Les investigateurs de cette étude déclarent n'avoir aucun conflit d'intérêt que ce soit au niveau politique, financier ou intellectuel.

13 Collaborations

13.1 Internes à l'institution

L'extraction de l'ADN génomique, ainsi que l'amplification et le séquençage de LMP1 seront effectués par la plateforme diagnostique de l'IMUL.

13.2 Externes à l'institution

Pour la détermination des cas et des témoins, ainsi que pour l'obtention des échantillons nous allons collaborer avec la Cohorte EuroSIDA.

EuroSIDA website : http://www.cphiv.dk/EuroSIDA/tabid/59/Default.aspx

Coordinateur de l'étude: Ole Kirk

Chef de projet: Jens D. Lundgren

14 Plan de publication

Les résultats obtenus lors de cette étude ne pourront être publiés qu'avec l'accord de Barr Epstein, promoteur de l'étude. Le premier envoi pour publication devra se faire au plus tard deux mois après la fin de l'analyse des données.

Barr Epstein en tant que promoteur et investigateur responsable sera dernier auteur des publications. Zürcher Emilie en tant qu'investigateur principal sera premier auteur et Human Viriou en tant que co-investigateur sera second auteur. Ces trois personnes se chargeront de la rédaction du(des) manuscript(s). Les autres investigateurs du projet seront listés selon l'importance de leur travail. Si plusieurs auteurs ont effectué un travail similaire, leurs noms seront listés selon l'ordre alphabétique.

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