Comparative analysis of oncogenic properties and nuclear factor-κB activity of latent membrane protein 1 natural variants from Hodgkin's lymphoma's Reed-Sternberg cells and normal B-lymphocytes

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

In Epstein-Barr virus-associated Hodgkin's lymphomas, neoplastic Reed-Sternberg cells and surrounding non-tumor B-cells contain different variants of the *LMP1-BNLF1* oncogene. In this study, we raised the question of functional properties of latent membrane protein 1 (LMP1) natural variants from both Reed-Sternberg and non-tumor B-cells.

Design and Methods

Twelve LMP1 natural variants from Reed-Sternberg cells, non-tumor B-cells of Hodgkin's lymphomas and from B-cells of benign reactive lymph nodes were cloned, sequenced and stably transfected in murine recombinant interleukin-3-dependent Ba/F3 cells to search for relationships between LMP1 cellular origin and oncogenic properties as well as nuclear factor-κB activation, and apoptosis protection.

Results

LMP1 variants of Reed-Sternberg cell origin were often associated with increased mutation rate and with recurrent genetic events, such as del15bp associated with S to N replacement at codon 309, and four substitutions I85L, F106Y, I122L, and M129I. Oncogenic potential (growth factor-independence plus clonogenicity) was consistently associated with LMP1 variants from Reed-Sternberg cells, but inconstantly for LMP1-variants from non-tumor B-cells. Analysis of LMP1 variants from both normal B-cells and Reed-Sternberg cells indicates that protection against apoptosis through activation of nuclear factor-κB - whatever the cellular origin of LMP1 - was maintained intact, regardless of the mutational pattern.

Conclusions

Taken together, our results demonstrate that preserved nuclear factor-κB activity and protection against apoptosis would be the minimal prerequisites for all LMP1 natural variants from both normal and tumor cells in Hodgkin's lymphomas, and that oncogenic potential would constitute an additional feature for LMP1 natural variants in Reed-Sternberg cells.

Key words: Epstein-Barr virus, LMP1, Hodgkin's lymphoma, NF-κB, oncogenic potential.

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Introduction

Epstein-Barr virus (EBV), an ubiquitous gamma herpes virus, has been implicated in the pathogenesis of several human malignancies of both lymphocyte and epithelial cell lineages, including nasopharyngeal carcinomas, Burkitt's lymphomas, immunodeficiency-related lymphomas, some T-cell lymphomas, and Hodgkin's lymphomas.[1] EBV infection is mainly characterized by the expression of latent genes. In nasopharyngeal carcinomas and Hodgkin's lymphomas, viral expression is restricted to EBNA1, LMP1, LMP2 and the EBER and BART RNA.[2] Latent membrane protein 1 (LMP1) was the first EBV latent protein found to be able to transform cell lines, and further the development of lymphomas in LMP1 transgenic mice due to its oncogenic potential.[3,4]

LMP1, which mimics a constitutively active tumor necrosis factor receptor,[5] is an integral membrane protein which can be divided into three segments: (i) a short cytosolic N-terminus (codon 1-24) which tethers and orients LMP1 to the cell surface,[6] (ii) six membrane-spanning hydrophobic domains (codon 25-186) which are involved in spontaneous aggregation and oligomerization,[5] and (iii) a large cytoplasmic C-terminus (codon 187-386) which possesses most of LMP1 functions.[7] Two functional domains have been identified within the C-terminal region, *i.e.* C-terminal-activating region 1 and 2 (CTAR1 and CTAR2), which trigger various cellular signaling pathways including IKK/NF-kB, p38MAPK/ATF2, JNK/AP1 and PI3k/Akt.[7-10] Activation of these signaling pathways leads to cell proliferation and inhibition of apoptosis via activation of the expression of several genes such as *c-myc*, *A20*, *ICAM1*, *Bcl2*, *BclXL*, *TRAF1*, and *cIAPs*.[2]

The EBV genome exhibits considerable genetic diversity, and different EBV strains or genotypes have been described. The first source of diversity comes from the circularization of the EBV DNA double-stranded molecule via the terminal repeats during virion production in the saliva.[11] As a second source of diversity, allelic polymorphisms of *EBNAs* genes define type 1 and type 2 EBV isolates.[12] In addition, *LMP1-BNLF1* gene polymorphisms, the most discriminating genetic locus, reflect intrastrain diversity.[13,14] EBV primary B-cell infection is polyclonal, always corresponding to an infection by a mixture of different EBV strains regarding the polymorphism of *EBNAs* and/or *LMP1-BNLF1* genes.[15] Hodgkin's lymphoma shows a monoclonal pattern of infection which persists throughout the course of the disease,[16] suggesting that a selection pressure is exerted on the virus during the oncogenic process and raising the question of its role in the pathogenesis of Hodgkin's lymphoma.

Hodgkin's lymphoma, the most frequent B-cell lymphoma in young adults, is a model for *in vivo* LMP1 diversity between the tumor and the non-tumor compartment.[17] We previously showed that LMP1 variants isolated from both Reed-Sternberg cells (*i.e.* tumor cells) and non-tumor B-cells are different but phylogenetically related, suggesting that they could be derived from the same ancestral EBV strain by accumulating mutations.[18] In order to understand the relation between the cellular origin of LMP1 natural variants and functional properties, we present a comprehensive analysis of nuclear factor (NF)-κB activation, apoptosis protection, and oncogenic properties, by LMP1 natural variants from Reed-Sternberg cells, non-tumor B-cells, as well as from B-cells extracted from non-neoplastic reactive lymph nodes.

Design and Methods

Patients

Biological material from biopsies was obtained according to institutional regulations and after approval from the scientific committees of the tumor banks of the university hospitals of Toulouse. Lymph nodes from six cases of Hodgkin's lymphomas (HL1 to HL6) and four benign reactive lymph nodes samples from patients with non-neoplastic lymphoproliferative disorders (N1 to N4) were the same as those investigated in a previous study.[17,18] HL1 to HL5 were EBV-positive-Hodgkin's lymphoma cases with EBV-positive Reed-Sternberg cells associated with few EBER-positive non-tumor B-lymphocytes. HL6 was a Hodgkin's lymphoma case with EBV-negative Reed-Sternberg cells associated with EBER-positive non-tumor B-cells. In benign reactive lymph nodes samples (N1 to N4), EBV was detected in rare EBER-positive normal B-lymphocytes.

Cell culture

LCL.B95.8 is a marmoset lymphoblastoid cell line.[19] EBV-transformed lymphoblastoid cell lines LCL.4 and LCL.6, described in the previous reports,[17,18] were established by spontaneous outgrowth from patients HL4 and HL6, respectively. These cell lines appeared to be monoclonal[20] and thus corresponded to one LMP1-variant from one original blood circulating EBV-B-lymphocyte. Ba/F3 is a murine pro-B lymphoid cell line that depends on 2 ng/mL of murine recombinant interleukin 3 (mrIL-3) (R&D Systems, DPC Bierman GmbH, Wiesbaden, Germany) for survival and proliferation.[21] Cell lines were grown in standard culture conditions.

LMP1 natural variants cloning, sequencing and transfection

We previously published the amino acid sequence of LMP1-variants from cases of this series after two separate polymerase chain reaction (PCR) amplifications of the 3' and 5' regions of the *LMP1-BNLF1* gene,[18] a method inadequate for LMP1 functional studies. Thus, using the AccuPrime Taq DNA polymerase high fidelity (Invitrogen), the 1315 bp of the complete *LMP1-BNLF1* gene were nested PCR- amplified from DNA using two sets of primers (5'CCAAGAAACACGCGTTACTCTGACGTAGCC3', 5'GCCTGGTAGTTGTGTTGTCCAGAGGTC3'; nested PCR: 5'CCTGACACACTGCCCTCGAGG3', 5'GGCGGAGTCTGGCAACGCCCGGGTCCTTG3'). Purified PCR products from nucleotide 167.909 to 169.496 (B95.8 coordinates) were cloned into the pCR3.1-Uni vector (Invitrogen): pCR3.1-LMP1. As a DNA source, we used the combination of whole tissue, LCL and/or single Reed-Sternberg cells, as

described previously.[18] In some rare cases, such as HL1, PCR amplification of the *LMP1-BNLF1* gene from whole lymph node DNA extract yielded two fragments as previously described.[17] *Single cell PCR* analysis of eight isolated Reed-Sternberg cells of HL1 (mean efficiency = 20%) clearly showed that one fragment corresponds to the LMP1-variant of Reed-Sternberg cells (RS1-LMP1-variant). Based upon this result, we cloned this RS1-LMP1-variant and the non-tumor B-cell LMP1-variant (NT1-LMP1-variant) from both PCR fragments amplified from the whole DNA extracted from HL1 lymph nodes. By contrast, we found in a previous study that in most EBV-positive-Hodgkin's lymphomas (HL2 to HL5) EBV-infected non-tumor B-lymphocytes were too rare to allow *LMP1-BNLF1* gene amplification.[17] Indeed, the frequency of infected non-tumor B-cells was $1/10^6$, whereas Reed-Sternberg tumor cells reached 5% of the total population. So the fragment obtained from whole DNA extracted from Hodgkin's lymphoma lymph nodes with EBV-infected Reed-Sternberg and non-tumor B-cells corresponds to the *LMP1-BNLF1* gene of the main cellular population, *i.e.* Reed-Sternberg cells.[17] Thus for HL2 to HL5 cases, we took advantage of the fact that the unique LMP1-BNLF1-PCR fragment amplified from whole DNA isolated from Hodgkin's lymphoma lymph nodes was correlated to the LMP1-variant of Reed-Sternberg cells, *i.e.* the most represented population.[17,18] Therefore, RS2, RS3, RS4, and RS5-LMP1-variants corresponded to EBV strains infecting Reed-Sternberg cells of HL2, HL3, HL4, and HL5, respectively. In addition, we cloned LMP1-BNLF1-PCR fragments corresponding to NT4 and NT6-LMP1-variants from non-tumor B-lymphocytes of cases HL4 and HL6 using LCL.4 and LCL.6, respectively. Finally, we cloned reactive lymph node B-cell LMP1-variants (RN-LMP1-variants) from DNA extracted from N1 to N4 reactive lymph nodes, *i.e.* RN1 to RN4-LMP1-variants.

The pCR3.1-neo (empty control vector) and pCR3.1-LMP1 vectors, were stably transfected into 10^7 Ba/F3 cells by electroporation with the Gene pulser II Electroporation system (Bio-Rad, California, USA) at 250V and 950 μF with 30 μg of DNA. Then, neo- and LMP1-Ba/F3 clones were obtained by limiting dilution in the presence of 1 mg/mL of G418 (Invitrogen). Entire nucleotide sequences of the 12 cloned *LMP1-BNLF1* genes were obtained both from the *LMP1-BNLF1* gene cloned into the pCR3.1 expression vector and from the *LMP1-BNLF1* gene amplified from the genomic DNA extracted from each of the corresponding stable LMP1-Ba/F3 clones. All these pairs of sequences were exactly identical (*Online Supplementary Figure S1*).

Immunoblotting analysis

Cells (10^7 cells) were lysed in 200 μL of Passive Lysis Buffer (Promega, Madison, USA). Western blots were performed with 50 μg of proteins. LMP1 and β-actin were detected with the S12 and the AC-15 (Sigma, Steinheim, Germany) monoclonal antibodies, respectively. LMP1 expression was then normalized according to β-actin expression using Gene Tools Software (GTS, SynGene).

Proliferation assay in liquid culture and soft agar clonogenic assay

Neo- and LMP1-Ba/F3 clones were plated in 96-well plates (10^3 /well), in 10% fetal calf serum (FCS) medium containing or not 2 ng/mL of mrIL-3. Over 6 days, proliferation rates were measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). For clonogenicity assays, 5×10^2 cells were suspended in 10% FCS medium and 2 ng/mL of mrIL-3 with 0.3% agar (Sigma) and plated onto 0.5% agar in each 3.5-cm well of a 6-well dish. After 3 weeks, colonies were stained by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT (Sigma). Colonies were counted using GTS.

Nuclear factor-κB-dependent dual-luciferase reporter assay

After 17 h of mrIL-3 starvation, 5×10^6 neo- and LMP1-Ba/F3 clones were transfected with 100 ng of pRL-TK Renilla luciferase expression vector (Promega) and 5 μg of 3X-κB-L with three copies of the major histocompatibility complex (MHC) class I κB element or its mutated counterpart 3X-mutkB-L (m*)[22] using the Amaxa G-016 program (AMAXA Biosystems, Cologne, Germany). Six hours after transfection, cells were lysed and luciferase activities were measured using the Dual-Luciferase Reporter Assay System and the Turner Designs TD-20/20 Luminometer (Promega).

Apoptosis analysis by flow cytometry

Neo- and LMP1-Ba/F3 clones were grown with or without mrIL-3 for 24, 48, and 72 h and double stained with annexin V-fluorescein isothiocyanate (BD Pharmingen™, San Diego, USA) and propidium iodide (Sigma) in cold phosphate-buffered saline-$CaCl_2$-$MgCl_2$ (Invitrogen). The DNA content of cells was assessed with the DNA Prep Kit (Beckman Coulter Immunotech, Marseille, France), according to standard procedures recommended by the manufacturer. Cells were analyzed using a FACS Calibur cytometer (BD Pharmingen).

Results

LMP1 natural variants from Reed-Sternberg cells are highly polymorphic with recurrent genetic events

We raised the question of the role of LMP1 genetic diversity in the emergence of tumor cells in Hodgkin's lymphomas. Our strategy was to amplify the whole *LMP1-BNLF1* genes of various, previously described LMP1 natural variants,[17,18] with their starting and stop codons and to clone them in order to functionally analyze their oncogenic capacity. The three different origins of LMP1 were Reed-Sternberg cells (RS-LMP1-variants), non-tumor B-cells (NT-LMP1-variants) of Hodgkin's lymphomas and B-cells of non-neoplastic reactive lymph nodes (RN-LMP1-variants).

Nucleotide sequences were aligned against the *B95.8-LMP1-BNLF1* gene sequence, taken as a reference (*Online Supplementary Figure S1*). All natural LMP1-variants were mutated, but to various degrees. Mutations were found predominantly in the C-termi-

nal region. The mean percentage of nucleotide sequence homology to the *B95.8-LMP1-BNLF1* gene C-terminus was 90.3% (±4.26 s.e.m) for RS-LMP1-variants, 96.4% (±2.90 s.e.m) for NT-LMP1-variants, and 99.7% (±0.18 s.e.m) for RN-LMP1-variants. The RS-LMP1-variants were significantly more polymorphic than RN-LMP1-variants ($p<0.05$, Student's t test)[18]. Of note, most nucleotide substitutions in RS-LMP1-variants were found in the coding sequence (frequency=1.34±0.35 s.e.m), and the nucleotide substitution frequency of the intronic regions was 0.52 (±0.24 s.e.m), a clear indication of a non-random mutational selection process. The main recurrent features consisted of: (i) the absence of significant changes in the N-terminal domain; (ii) the occurrence of four *hot spot mutations* in the transmembrane-spanning hydrophobic domains at codons 85 (I to L), 106 (F to Y), 122 (I to L) and 129 (M to I), as previously described[18,24]. This association, called I85L, F106Y, I122L, and M129I, was observed in RS1, RS2, RS4, and RS5-LMP1-variants; and (iii) the occurrence of two recurrent genetic events in the cytoplasmic C-terminal region encompassing the CTAR1 and CTAR2 domains. In this region, the first sequence variation corresponds to the number of 11 amino acid repeat units with the presence or deletion of one half-repeat unit in the middle of the third repeat, called del15bp (codon 276-280). As in the B95.8-LMP1 prototype, three of five RS-LMP1-variants, three of three NT-LMP1-variants and four of four RN-LMP1-variants have the four perfect repeats. The other variants have five (RS1 and RS5-LMP1-variants) or seven (RS2-LMP1-variants) perfect repeats. Changes in the number of repeat units were always associated with the del15bp. Interestingly, the del15bp was systematically associated with a replacement at codon 309 (S to N) as recently reported in NK/T cell lymphomas.[23] In the CTAR2 domain, the second genetic event corresponds to the deletion of codons 346-355, called del30bp. This deletion was found in only one of five RS-LMP1-variants (RS5-LMP1-variants), and in one of three NT-LMP1-variants (NT1-LMP1-variants); it was not found in RN-LMP1-variants. The del30bp was always associated with the del15bp. Altogether, these results show that RS and NT-LMP1 natural variants from Hodgkin's lymphomas presented considerable amino acid alterations with the same recurrent genetic events, such as del15bp associated with S to N replacement at codon 309 and del30bp, which suggests a selection pressure.

LMP1 variants from Reed-Sternberg cells have enhanced proliferating and clonogenic potential

To analyze the role of LMP1 natural variants in B-cell oncogenesis, we established stably transfected Ba/F3 clones with the different LMP1-variants. The Ba/F3 lymphoid cell line is an mrIL-3-dependent murine B-cell model that has been proven to be useful in oncogenicity analysis.[25,26] In this experimental model, constitutive LMP1 protein expression levels were heterogeneous although in the same order of magnitude (Figure 1). Thus, the LMP1-Ba/F3 clones were not compared between them but to the neo-Ba/F3 control clone for their ability to induce mrIL-3-independent growth and to form macroscopic colonies in soft agar, so-called clonogenicity, properties usually associated with oncogenicity.[25,27]

In optimal culture conditions (*i.e.* with mrIL-3), all LMP1-Ba/F3 clones proliferated at the same rate as the neo-Ba/F3 clone, with a 17 h doubling time (*data not shown*). This observation suggests, on the one hand, that *LMP1-BNLF1* gene genomic insertion did not alter Ba/F3 cell growth and, on the other hand, that expression of LMP1-variants did not have a significant effect on mrIL-3-dependent proliferation. In the absence of mrIL-3, proliferation markedly decreased in the neo-Ba/F3 clone (Figure 2, left axis). When compared to the neo-Ba/F3 clone, a significant increase of growth rate was found for five of five RS-LMP1-Ba/F3 clones, one of three NT-LMP1-Ba/F3 clones and two of four RN-LMP1-Ba/F3 clones ($p<0.05$, Student's t test). However, this LMP1-transforming potential did not appear to be related to the recurrent genetic events observed in the various LMP1-variants. Thus, the whole amino acid sequence had to be considered in its totality. Indeed, the B95.8, RS3, NT4, RN3 and RN4-LMP1-variants, which do not have the del15bp, del30bp, nor the I85L, F106Y, I122L, and M129I substitutions, were able to sustain Ba/F3 mrIL-3 independent growth. Moreover, the fact that NT1, NT6, RN1 and RN2-LMP1-Ba/F3 clones were not transformed suggests that, in normal B-cells, *in vivo* acquisition and selection of the amino acid signature,

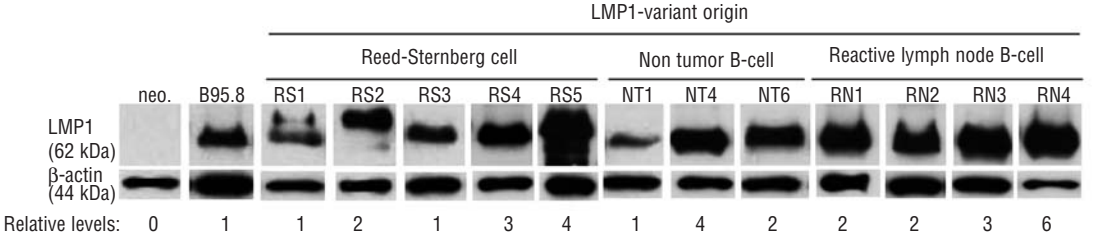

Figure 1. LMP1 expression in LMP1-Ba/F3 clones. Murine Ba/F3 cells were stably transfected with pCR3.1 vectors leading to the expression of RS, NT and RN-LMP1-variants. The vectors pCR3.1-B95.8 and -neo were used as controls. LMP1 expression was tested by an immunoblotting method using anti-LMP1 and anti-β-actin antibodies. The relative level of LMP1 was normalized to β-actin. The B95.8-LMP1-Ba/F3 clone was used as the reference control and set at 1. To maintain the order of the LMP1-Ba/F3 clones, different parts of the filter were used, and dividing lines were inserted.

specific to each LMP1 natural variant, could be associated with the loss of its transforming properties.

To asses LMP1 clonogenicity, we analyzed the capacity of neo- and LMP1-Ba/F3 clones to proliferate in semi-solid agar medium (Figure 2, right axis). Colony-forming efficiency was increased for five of five RS-LMP1-Ba/F3 cell clones, with a significant difference when compared to neo-Ba/F3 control cells ($p<0.05$, Student's t test). A significant increase of clonogenicity was also found for one of three NT-LMP1-Ba/F3 clones, and for three of four RN-LMP1-Ba/F3 clones. Two independant LMP1-Ba/F3 clones were tested for RS1, NT6, RN1, and RN3-LMP1-variants, giving similar results (*data not shown*). This suggests that the genomic integration sites do not have a significant influence on the functional results observed. Reed-Sternberg cells were the only cell-type of origin consistently associated with LMP1 variants with enhanced oncogenic potential (defined as growth factor-independent proliferation plus clonogenicity). This property did not seem to be related to LMP1 protein expression levels. For example, on the one hand, RS1 and RS4-LMP1-Ba/F3 clones had the same oncogenic potential (Figure 2) although the levels of LMP1 protein expression were different (Figure 1). On the other hand, RS1 and NT1-LMP1-Ba/F3 clones had similar LMP1 expression levels but different oncogenic potential.

Oncogenic potential was, therefore, consistently associated with Reed-Sternberg cell origin of LMP1 variants, suggesting that this functional property could be a criterion for selection of LMP1 variants in Reed-Sternberg cells but not in non-tumor cells.

Nuclear factor-κB activation and apoptosis protection are constant functional properties of LMP1-variants from Reed-Sternberg cells and normal B-cells in Hodgkin's lymphomas

In a next step, since strong constitutive activation of NF-κB is a hallmark of Hodgkin's lymphomas, we looked at NF-κB activation properties of LMP1 natural variants.[28] To perform this analysis, NF-κB transcriptional activity assays were done on mrIL-3-deprived LMP1-Ba/F3 clones to avoid interference with mrIL-3-induced NF-κB.[29] A significant but weak increase of NF-κB transcriptional activity was found in B95.8, RS2, RS3, RS5, NT6, and RN4-LMP1-Ba/F3 clones compared to that of the neo-Ba/F3 clone ($p<0.05$, Student's t test) (Figure 3). In comparison to the B95.8-LMP1-Ba/F3 clone, we observed a significant increase of NF-κB activity in RS1, RS4, NT1, NT4, RN1, RN2, and RN3-LMP1-Ba/F3 clones ($p<0.05$, Student's t test). Of note, NF-κB activities did not seem to be related to LMP1 protein expression levels, as shown, for example by comparing the LMP1 protein level of RS2 and RS4-LMP1-Ba/F3 clone or NT1 and NT6-LMP1-Ba/F3 clone in Figure 1 with their respective NF-κB activity in Figure 3. The results indicate that while all LMP1 natural variants could activate NF-κB signaling, some of them may exhibit enhanced NF-κB activation capacities. This characteristic did not appear to be related either to the cellular origin of LMP1-variants or to the transformation of Ba/F3 cells.

Since NF-κB is known to protect against cell death,[30] we studied the induction of apoptosis by mrIL-3 starvation on LMP1-Ba/F3 clones (Figure 4). The data show that all the LMP1-Ba/F3 clones were protected against

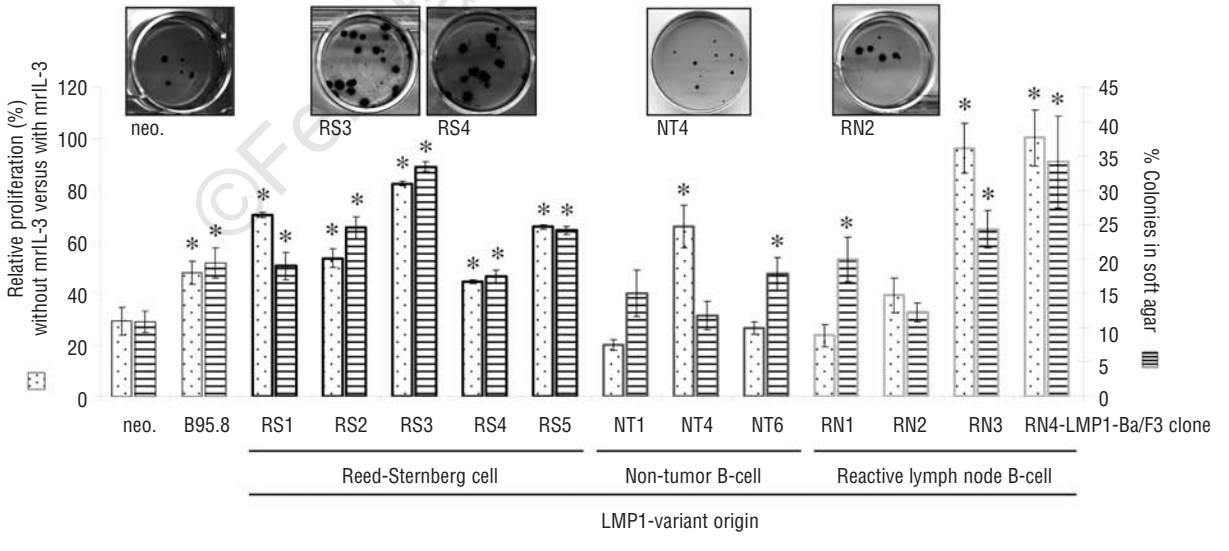

Figure 2. Oncogenicity of LMP1 natural variants on the Ba/F3 lymphoid cellular model. Proliferation in mrIL-3 starved medium and formation of macroscopic colonies in soft agar, *i.e.* clonogenicity of LMP1-Ba/F3 clones. Left axis, cells were cultured in mrIL-3-free medium for 6 days. At day 3, residual proliferation of neo- or LMP1-Ba/F3 clone was normalized to its control in the presence of mrIL-3, *i.e.* relative proliferation rate without mrIL-3 versus with mrIL-3. Right axis, cells were suspended in 10% FCS medium and 2 ng/mL of mrIL-3 with 0.3% agar. After 3 weeks, colonies were stained by MTT and counted. The percentage of colonies is the number of colonies divided by the number of seeded cells. Error bars indicate the standard error from the mean from three independent sets of triplicate experiments. A significant difference ($p<0.05$) compared to the control neo-Ba/F3 clone using Student's t test is indicated by an asterisk (*). Two independent LMP1-Ba/F3 clones were tested for RS1, NT6, RN1, and RN3-LMP1-variants, giving similar results (*data not shown*).

apoptosis when compared to the neo-Ba/F3 clone, although to various degrees (Figure 4). Levels of apoptosis were first quantified by the sub-G_1 peak and were divided into three categories; low, intermediate and high (Figure 4B). All LMP1-Ba/F3 clones with high NF-κB activity showed low (4/7, RS1, RS4, RN1, and RN3-LMP1-Ba/F3 clones) or intermediate (3/7, NT1, NT4, and RN2-LMP1-Ba/F3 clones) levels of apoptosis after mrIL-3 starvation (Figure 4A and B). All LMP1-Ba/F3 clones with low NF-κB activity exhibited high (4/6, B95.8, RS2, NT6, and RN4-LMP1-Ba/F3 clones) or intermediate (2/6, RS3, and RS5-LMP1-Ba/F3 clones) levels of apoptosis (Figure 4A and B). In this regard, results of sub-G_1 peak and annexin V analyses were consistent, showing nearly identical distribution of the different Ba/F3 clones between these three different categories of apoptosis levels (Figure 4C). This is a clear indication that protection against apoptosis is related to the intensity of NF-κB transcriptional activity induced by LMP1 variants. It is interesting to note that all LMP1 natural variants seemed to protect the Ba/F3 cells against mrIL-3 starvation better than the prototypic B95.8-LMP1-variant.

Altogether, our results suggest that NF-κB activation potential associated with protection against apoptosis is a functional property of LMP1 natural variants and is maintained through the process of accumulation of genetic events, a very strong indication that activation of NF-κB is absolutely necessary for LMP1 function.

Discussion

LMP1 polymorphism participates in EBV genetic diversity. The nasopharyngeal carcinoma CAO-LMP1-variant was the first described.[31] This variant has multiple single-base mutations, as well as seven copies of the 11 amino acid repeated sequence and two deletions of 15 bp (del15bp) and 30 bp (del30bp) in the C-terminal part. In the human keratinocyte line Rhek1, CAO-LMP1 was found to be more oncogenic than LMP1 protein from the prototypic B95.8 strain[32] and less immunogenic.[33] Subsequent to this initial report, numerous LMP1-variants from both nasopharyngeal carcinomas and Hodgkin's lymphomas have been described by various groups.[34-36] However, to our knowledge, results published in the literature fail to demonstrate direct associations between any single LMP1-variant and any specific EBV-associated disease.[34-36] Based on phylogenetic analysis, Nancy Raab-Traub's group proposed classifing LMP1 natural variants into seven distinct strains, and showed a bias regarding the frequency of some LMP1 strains and nasopharyngeal carcinomas, suggesting a potential selection pressure on these variants during epithelial cell oncogenesis.[24] Our previous analysis of the LMP1 polymorphism in both Reed-Sternberg cells and normal B-lymphocytes from Hodgkin's lymphomas clearly suggested an intra-host diversity for the two viral strains from a common viral ancestor and showed that LMP1-variants in tumor cells are highly mutated when compared to LMP1-variants from healthy donors.[18] This raises the question of the functional properties of LMP1-variants from both Reed-Sternberg cells and non-tumor B-cells.

Infection with EBV causes naïve B-cells to transform into proliferating blasts, which can then differentiate *in vivo* into long-lived resting memory EBV-infected B-cells.[2] Our data indicate that LMP1-variants from normal B-cells could exert or not an oncogenic potential on Ba/F3 cells, which suggests that *in vivo* LMP1 selection in normal B-cells is not based on their oncogenic properties. In contrast, all the LMP1-variants from Reed-Sternberg cells in Hodgkin's lymphomas were able both to transform the Ba/F3 cell line into mrIL-3-independent cells and to allow clonogenic growth of Ba/F3 cells, an indication that their oncogenic potential[25,27] was associated with their origin from Reed-Sternberg cells. These observations suggest that a selection step based on increased oncogenic properties (*i.e.* growth factor-independence plus clonogenicity) of LMP1-variants would take place for these LMP1-variants in the context of the transformation of Reed-Sternberg cell precursors.

It has recently been shown that inhibition of NF-κB impairs establishment of gamma herpes virus latency.[37] However, very few functional studies on NF-κB activation have been performed with LMP1 natural variants. A study by Rothenberger *et al.* on the C-terminal region of

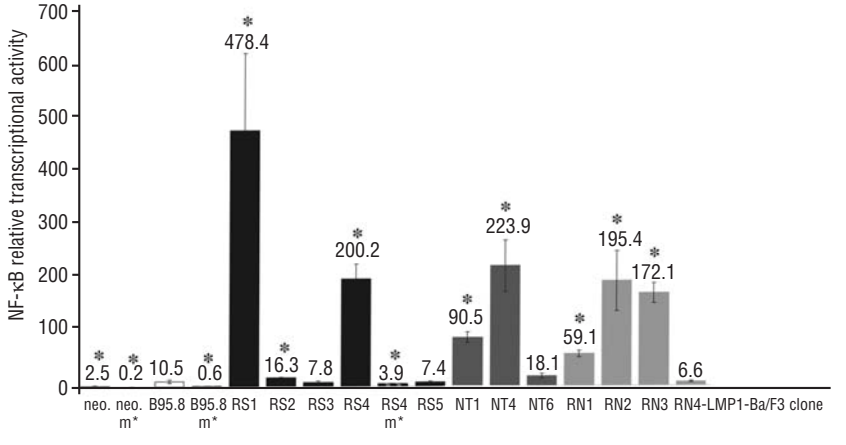

Figure 3. NF-κB transcriptional activity of LMP1 natural variants. After 17 h of mrIL-3 starvation, neo- and LMP1-Ba/F3 clones were co-transfected with a Renilla luciferase vector (pRL-TK) and 3X-κB-L vector, with three copies of the MHC class I κB site upstream of a Firefly luciferase reporter gene, or its mutated counterpart 3X-mutκB-L plasmid (indicated by m*). Results are expressed as NF-κB relative transcriptional activity which is the ratio between Firefly and Renilla luciferase activities relative to NF-κB transcriptional activity of parental Ba/F3 cells. Each bar corresponds to the mean of at least four independent experiments. Error bars represent the corresponding standard error from the mean. A significant difference ($p<0.05$) compared to the reference B95.8-LMP1-Ba/F3 clone using Student's *t* test is indicated by an asterisk (*).

a series of seven LMP1 natural variants isolated from both Hodgkin's lymphomas and EBV-associated lymphoproliferative disorders suggested that NF-κB activation potential would be conserved whatever the mutational status was [22]. A recent study showed that the signature amino acid changes of LMP1 variants from nasopharyngeal carcinomas do not hinder or enhance their *in vitro* oncogenic potentials on Rat-1 fibroblasts despite an increase of their NF-κB activity in 293T epithelial cells.[38] Our data indicate that selection of LMP1-variants in normal B-cells and Reed-Sternberg cells maintained intact NF-κB activation potential at sufficient levels to protect cells against apoptosis. Several aberrantly activated signaling pathways contribute to the survival of Reed-Sternberg cells, but NF-κB is perhaps the most important one. Activation of the NF-κB pathway is critical for LMP1-induced immortalization of human primary B-lymphocytes and oncogenic transformation of some rodent cell lines.[39,40] From our results, none of the individual sequence variations was clearly

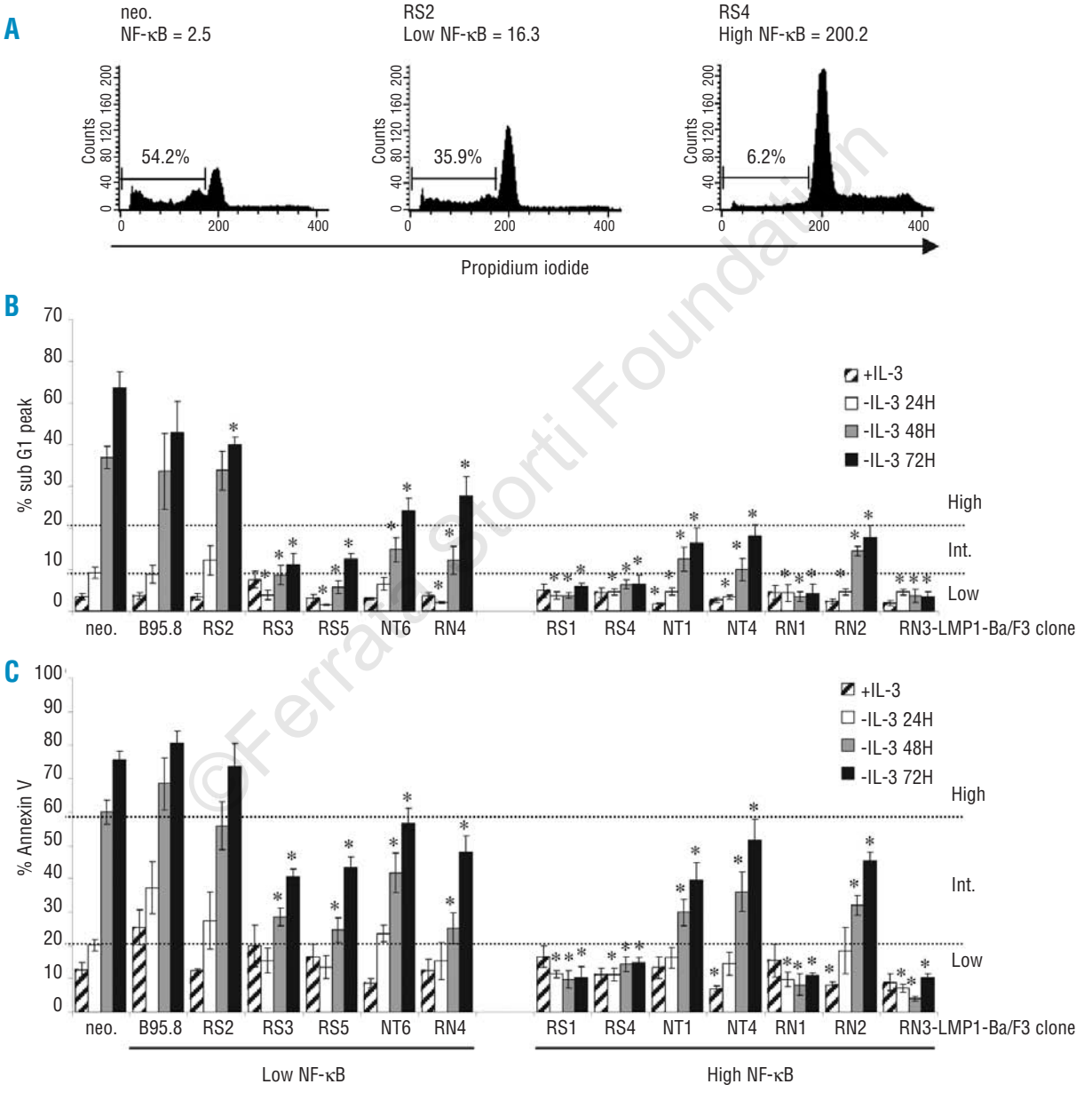

Figure 4. Effect of mrIL-3 starvation on the induction of apoptosis of LMP1-Ba/F3 clones. (A) Typical histograms of sub-G_1 peak of neo., RS2 and RS4-LMP1-Ba/F3 clones after 72 h of mrIL-3 starvation. On each histogram, the percentage of cells with a decreased DNA content (sub G_1), and the corresponding NF-κB relative transcriptional activity (Figure 3) are indicated. (B and C) Percentages of apoptotic cells after mrIL-3 starvation of neo- and LMP1-Ba/F3 clones. Apoptosis was assessed by flow cytometry measuring the sub-G_1 peak (B) and annexin V-positive cells (C). The time course of mrIL-3 starvation was from 0-72 h. The dotted lines represent the cut-offs for defining the three categories of apoptosis level: low, intermediate (Int.) and high indicated on the right. Each bar corresponds to the mean of at least three independent experiments. Error bars represent the corresponding standard error from the mean. A significant difference ($p<0.05$) compared to the neo-Ba/F3 clone using Student's *t* test is indicated by an asterisk (*).

associated with differences in LMP1 activation of NF-κB. Several studies postulate that the efficiency of NF-κB activation can be influenced by more than one type of naturally occurring amino acid substitution in the N-terminus and/or the transmembrane domains of LMP1.[41,42] Our results support these observations, since the only amino acid changes in RS4 and NT4-LMP1-variants, with a strong NF-κB activation, were localized in the transmembrane domains.

As demonstrated by Küppers's group, EBV infection of the Reed-Sternberg cell precursor (*i.e.* a germinal center B-cell with B-cell receptor-destructive mutations) and LMP1 expression are important events to rescue this precursor from apoptosis.[43] This supports an early role of EBV in the pathogenesis of Hodgkin's lymphomas. Levels of NF-κB activity were indeed roughly correlated to apoptosis protection. Some LMP1-Ba/F3 clones with high NF-κB activation potential exhibited intermediate levels of apoptosis after mrIL-3 starvation. In this regard, at least two hypotheses could be proposed. The equilibrium between RelA/p50 and RelB/p52 NF-κB complexes could be determined by the mutational pattern of LMP1 and thus affect the level of protection against apoptosis. Alternatively, LMP1 natural variants could co-activate alternative activation pathways, such as AP1, this co-activation depending also on the mutational pattern of LMP1. Nevertheless, all LMP1 natural variants were able to induce both activation of NF-κB and protection against apoptosis. This indicates that, whatever the mutational status of LMP1-variants is, a selection pressure would exist to keep intact the capability to activate NF-κB and protect cells against apoptosis.

The rate of somatic mutations was increased for LMP1-variants from Reed-Sternberg cells. The well-established propensity of Reed-Sternberg cells to carry numerous, often crippling, mutations in immunoglobulin light and heavy chain genes and others genes would suggest that LMP1 could be a target of such genetic instability.[44] In this context, our results clearly indicate that not only preservation of NF-κB activation potential but also oncogenic potential would be the minimal evolutionary constraint for LMP1 genetic diversity in these tumor cells.

Authorship and Disclosures

NF: designed research, performed research, analyzed data and wrote the paper; AC, AB, NC: performed research; GD: designed research; JF: designed research, analyzed data and wrote the paper; FM: designed research, analyzed data and wrote the paper.

The authors reported no potential conflicts of interest.

References

1. Thorley-Lawson DA. EBV the prototypical human tumor virus-just how bad is it? J Allergy Clin Immunol 2005;116:251-61.
2. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757-68.
3. Kaye KM, Izumi KM, Kieff E. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc Natl Acad Sci USA 1993;90:9150-4.
4. Kulwichit W, Edwards RH, Davenport EM, Baskar JF, Godfrey V, Raab-Traub N. Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. Proc Natl Acad Sci USA 1998;95:11963-8.
5. Gires O, Zimber-Strobl U, Gonnella R, Ueffing M, Marschall G, Zeidler R, et al. Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. Embo J 1997;16:6131-40.
6. Coffin WF 3rd, Erickson KD, Hoedt-Miller M, Martin JM. The cytoplasmic amino-terminus of the latent membrane protein-1 of Epstein-Barr virus: relationship between transmembrane orientation and effector functions of the carboxy-terminus and transmembrane domain. Oncogene 2001;20:5313-30.
7. Huen DS, Henderson SA, Croom-Carter D, Rowe M. The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. Oncogene 1995;10:549-60.
8. Dawson CW, Tramountanis G, Eliopoulos AG, Young LS. Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. J Biol Chem 2003;278:3694-704.
9. Eliopoulos AG, Gallagher NJ, Blake SM, Dawson CW, Young LS. Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. J Biol Chem 1999;274:16085-96.
10. Kieser A, Kilger E, Gires O, Ueffing M, Kolch W, Hammerschmidt W. Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade. Embo J 1997;16:6478-85.
11. Moody CA, Scott RS, Su T, Sixbey JW. Length of Epstein-Barr virus termini as a determinant of epithelial cell clonal emergence. J Virol 2003;77:8555-61.
12. Rowe M, Young LS, Cadwallader K, Petti L, Kieff E, Rickinson AB. Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. J Virol 1989;63:1031-9.
13. Miller WE, Edwards RH, Walling DM, Raab-Traub N. Sequence variation in the Epstein-Barr virus latent membrane protein 1. J Gen Virol 1994;75:2729-40.
14. Walling DM, Shebib N, Weaver SC, Nichols CM, Flaitz CM, Webster-Cyriaque J. The molecular epidemiology and evolution of Epstein-Barr virus: sequence variation and genetic recombination in the latent membrane protein-1 gene. J Infect Dis 1999;179:763-74.
15. Srivastava G, Wong KY, Chiang AK, Lam KY, Tao Q. Coinfection of multiple strains of Epstein-Barr virus in immunocompetent normal individuals: reassessment of the viral carrier state. Blood 2000;95:2443-5.
16. Brousset P, Schlaifer D, Meggetto F, Bachmann E, Rothenberger S, Pris J, et al. Persistence of the same viral strain in early and late relapses of Epstein-Barr virus-associated Hodgkin's disease. Blood 1994;84:2447-51.
17. Faumont N, Al Saati T, Brousset P, Offer C, Delsol G, Meggetto F. Demonstration by single-cell PCR that Reed–Sternberg cells and bystander B lymphocytes are infected by different Epstein–Barr virus strains in Hodgkin's disease. J Gen Virol 2001;82:1169-74.
18. Faumont N, Trempat P, Brousset P, Delsol G, Meggetto F. In Hodgkin's disease Reed-Sternberg cells and normal B-lymphocytes are infected by related Epstein-Barr virus strains. Virus Res 2004;101:163-73.
19. Miller G, Shope T, Lisco H, Stitt D, Lipman M. Epstein-Barr virus: transformation, cytopathic changes, and

viral antigens in squirrel monkey and marmoset leukocytes. Proc Natl Acad Sci USA 1972;69:383-7.
20. Meggetto F, Muller C, Henry S, Selves J, Mariame B, Brousset P, et al. Epstein-Barr virus (EBV)-associated lymphoproliferations in severe combined immunodeficient mice transplanted with Hodgkin's disease lymph nodes: implications of EBV-positive bystander B lymphocytes rather than EBV-infected Reed-Sternberg cells. Blood 1996;87:2435-42.
21. Palacios R, Steinmetz M. Il-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell 1985;41:727-34.
22. Rothenberger S, Bachmann E, Berger C, McQuain C, Odermatt BF, Knecht H. Natural 30 base pair and 69 base pair deletion variants of the LMP1 oncogene do stimulate NF-kappaB-mediated transcription. Oncogene 1997;14:2123-6.
23. Nagamine M, Takahara M, Kishibe K, Nagato T, Ishii H, Bandoh N, et al. Sequence variations of Epstein-Barr virus LMP1 gene in nasal NK/T-cell lymphoma. Virus Genes 2007; 34:47-54.
24. Edwards RH, Sitki-Green D, Moore DT, Raab-Traub N. Potential selection of LMP1 variants in nasopharyngeal carcinoma. J Virol 2004; 78: 868-81.
25. Adam M, Pogacic V, Bendit M, Chappuis R, Nawijn MC, Duyster J, et al. Targeting PIM kinases impairs survival of hematopoietic cells transformed by kinase inhibitor-sensitive and kinase inhibitor-resistant forms of Fms-like tyrosine kinase 3 and BCR/ABL. Cancer Res 2006;66: 3828-35.
26. Schwaller J, Frantsve J, Aster J, Williams IR, Tomasson MH, Ross TS, et al. Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. EMBO J 1998;17:5321-33.
27. Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci USA 1997;94:6658-63.
28. Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, et al. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 1997;100:2961-9.
29. Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia 2004;18:189-218.
30. Shishodia S, Aggarwal BB. Nuclear factor-kappaB: a friend or a foe in cancer? Biochem Pharmacol 2004; 68:1071-80.
31. Hu LF, Zabarovsky ER, Chen F, Cao SL, Ernberg I, Klein G, et al. Isolation and sequencing of the Epstein-Barr virus BNLF-1 gene (LMP1) from a Chinese nasopharyngeal carcinoma. J Gen Virol 1991;72:2399-409.
32. Hu LF, Chen F, Zheng X, Ernberg I, Cao SL, Christensson B, et al. Clonability and tumorigenicity of human epithelial cells expressing the EBV encoded membrane protein LMP1. Oncogene 1993;8:1575-83.
33. Trivedi P, Hu LF, Chen F, Christensson B, Masucci MG, Klein G, et al. Epstein-Barr virus (EBV)-encoded membrane protein LMP1 from a nasopharyngeal carcinoma is non-immunogenic in a murine model system, in contrast to a B cell-derived homologue. Eur J Cancer 1994;30A:84-8.
34. Knecht H, Bachmann E, Brousset P, Sandvej K, Nadal D, Bachmann F, et al. Deletions within the LMP1 oncogene of Epstein-Barr virus are clustered in Hodgkin's disease and identical to those observed in nasopharyngeal carcinoma. Blood 1993;82: 2937-42.
35. Sandvej K, Peh SC, Andresen BS, Pallesen G. Identification of potential hot spots in the carboxy-terminal part of the Epstein-Barr virus (EBV) BNLF-1 gene in both malignant and benign EBV-associated diseases: high frequency of a 30-bp deletion in Malaysian and Danish peripheral T-cell lymphomas. Blood 1994;84:4053-60.
36. Zhou XG, Sandvej K, Li PJ, Ji XL, Yan QH, Zhang XP, et al. Epstein-Barr virus gene polymorphisms in Chinese Hodgkin's disease cases and healthy donors: identification of three distinct virus variants. J Gen Virol 2001;82(Pt 5):1157-67.
37. Krug LT, Moser JM, Dickerson SM, Speck SH. Inhibition of NF-kappaB activation in vivo impairs establishment of gammaherpesvirus latency. PLoS Pathogens 2007;3:e11.
38. Mainou BA, Raab-Traub N. LMP1 strain variants: biological and molecular properties. J Virol 2006;80:6458-68.
39. Cahir McFarland ED, Izumi KM, Mosialos G. Epstein-Barr virus transformation: involvement of latent membrane protein 1-mediated activation of NF-kappaB. Oncogene 1999;18:6959-64.
40. He Z, Xin B, Yang X, Chan C, Cao L. Nuclear factor-kappaB activation is involved in LMP1-mediated transformation and tumorigenesis of rat-1 fibroblasts. Cancer Res 2000;60: 1845-8.
41. Blake SM, Eliopoulos AG, Dawson CW, Young LS. The transmembrane domains of the EBV-encoded latent membrane protein 1 (LMP1) variant CAO regulate enhanced signalling activity. Virology 2001;282:278-87.
42. Soni V, Yasui T, Cahir-McFarland E, Kieff E. LMP1 transmembrane domain 1 and 2 (TM1-2) FWLY mediates intermolecular interactions with TM3-6 to activate NF-kappaB. J Virol 2006;80:10787-93.
43. Brauninger A, Schmitz R, Bechtel D, Renne C, Hansmann ML, Kuppers R. Molecular biology of Hodgkin's and Reed/Sternberg cells in Hodgkin's lymphoma. Int J Cancer 2006;118:1853-61.
44. Liso A, Capello D, Marafioti T, Tiacci E, Cerri M, Distler V, et al. Aberrant somatic hypermutation in tumor cells of nodular-lymphocyte-predominant and classic Hodgkin lymphoma. Blood 2006;108:1013-20.