

# The HIV-1 matrix protein p17 activates the transcription factors c-Myc and CREB in human B cells

Song Li, Luisa Bozzo, Zhibin Wu, Wuyuan Lu, Fabio Romerio

*Institute of Human Virology, University of Maryland, School of Medicine, Baltimore*

## SUMMARY

The human immunodeficiency virus matrix protein p17 plays a critical role in many steps of the virus life cycle. In addition, p17 displays biological activities outside infected cells. Indeed, virus-neutralizing antibodies against p17 in plasma of infected patients correlate with slower disease progression, and p17 has been shown to interact with an as yet unidentified cell surface receptor expressed on peripheral blood B cells. The present study investigated intracellular signaling pathways triggered following this interaction. Using protein/DNA arrays, we show that p17 increases phosphorylation and the DNA-binding activity of CREB and c-Myc through the time- and dose-dependent activation of the cAMP/PKA and MEK/ERK signaling pathways. Interestingly, we found that both signaling pathways are synergistically activated upon co-stimulation through the CD19 receptor. As both CREB and c-Myc are involved in the regulation of cell proliferation, differentiation, and survival, our findings might suggest a potential mechanism of B cell lymphomagenesis during HIV-1 infection.

**KEY WORDS:** HIV-1 p17, c-Myc, CREB, AIDS-associated lymphoma

Received July 29, 2009

Accepted August 28, 2009

## INTRODUCTION

The pathogenicity of the human immunodeficiency virus type 1 (HIV-1) is determined by host factors as well as by viral proteins. The HIV-1 proteins Tat and Nef enhance viral replication, infectivity and immune dysregulation (Nekhai *et al.*, 2006; Zagury *et al.*, 1998; Alimonti *et al.*, 2003), and the envelope glycoprotein gp120 induces apoptosis of uninfected T cells (Jekle *et al.*, 2003; Herbein *et al.*, 1998; Holm *et al.*, 2004). There is an increasing amount of evidence suggesting that HIV-1 p17 is not only an integral component of the HIV-1 viral particle, but might also be a regulatory protein involved in enhancing HIV-1 pathogenesis.

The HIV-1 matrix protein, p17 is generated after cleavage of the precursor Gag polyprotein by the viral protease, and is situated just beneath the viral envelope, where it forms a matrix layer (Freed, 1998). Several studies have shown that p17 is important in many steps of the virus life cycle. In the early stage of a new infection p17 dissociates from the viral membrane and - via its nuclear localization signal - targets the core-derived pre-integration complex (PIC) to the nucleus of host cells (Bukrinsky *et al.*, 1993; Gasllay *et al.*, 1995). Newly synthesized p17 migrates and accumulates into the cell nucleus, where it binds viral RNA promoting its transfer to the plasma membrane (Bukrinskaya *et al.*, 1992; Yuan *et al.*, 1993). Finally, p17 plays a role in the assembly and release of mature viral particle (Spearman *et al.*, 1994; Facke *et al.*, 1993).

The role of p17 in the HIV-1 life cycle is underlined by evidence that p17 increases HIV-1 replication (De Francesco *et al.*, 1998), while neutralizing antibodies against p17 suppress it. Furthermore, p17 is the target of neutralizing an-

*Corresponding author*

Fabio Romerio, PhD

Institute of Human Virology

725 West Lombard Street

Baltimore, MD 21201

E-mail: fromerio@ihv.umaryland.edu

tibodies against HIV-1 (Papsidero *et al.*, 1989; Naylor *et al.*, 1987; Buratti *et al.*, 1997), and high serum levels of anti-p17 antibodies correlate with slower disease progression (Chargelegue *et al.*, 1995; Lange *et al.*, 1987). As p17 is located in the interior surface of the viral membrane, the findings of a protective role for antibodies against p17 are surprising and suggest a possible activity of p17 externally from the virus particle in supporting virus replication and promoting disease progression. Indeed, *in vitro* studies demonstrated that recombinant p17 binds an unidentified cell surface receptor expressed on various cell types, leading to enhanced expression of TNF- $\alpha$  and IFN $\gamma$  in IL-2 stimulated PBMC (De Francesco *et al.*, 1998; Vitale *et al.*, 2003; Marini *et al.*, 2008).

In the present report, we have used chemically synthesized p17 to study the intracellular signaling pathways triggered in the B cell line, Raji. We show that p17 up-regulated the PKA and MEK/ERK pathways, leading to phosphorylation and activation of the transcription factors, CREB and c-Myc. We also demonstrate synergistic activation of the PKA and MEK/ERK pathways following co-stimulation with p17 and monoclonal antibodies to CD19, a cell surface receptor involved in B cell activation and proliferation.

## MATERIALS AND METHODS

### *Chemical synthesis of p17*

The HIV-1 matrix protein, p17 and N-myristoylated p17 (myr-p17) were obtained by total chemical synthesis as described previously (Wu *et al.*, 2008). Protein folding was carried out by dissolving purified and lyophilized polypeptides at 1 mg/ml in 0.2 M phosphate buffer containing 6 M guanidinium hydrochloride and 10 mg/ml DTT (pH 7.5), followed by extensive dialysis against acidified water. Folded proteins were quantified by UV absorbance measurements at 280 nm with a molar extinction coefficient of 16,960, determined as described previously (Pace *et al.*, 1995). All analytical RP-HPLC runs were performed on a Waters Alliance system (Milford, MA) or an Agilent 1100 series (Palo Alto, CA). Solvent A was water containing 0.1% trifluoroacetic acid, and solvent B was acetonitrile containing 0.1% trifluoroacetic acid.

### *Cell culture and treatments*

Raji cells (ATCC, Manassas, VA) were cultured in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin sulfate, and 2 mM L-glutamine; all from Invitrogen Corporation, Carlsbad, CA) at 37°C in 5% CO<sub>2</sub>. For treatment with synthetic p17 proteins, cells were centrifuged, re-suspended at 2x10<sup>6</sup> cells/ml in complete RPMI medium, and incubated with 1  $\mu$ g/ml p17 for the times indicated in each experiment. Dose-dependent experiments were performed with 0.05–1  $\mu$ g/ml p17.

### *Flow cytometry*

For cell surface detection of the p17 receptor by flow cytometry, p17 and myr-p17 were first labeled with biotin using the EZ-Link NHS-LC-Biotin reagent following the manufacturer's instructions (Pierce, Rockford, IL). At the end of the reaction, excess reagent was neutralized with glycine Li *et al.* HIV-1 p17 upregulates c-Myc and CREB and removed by dialysis. Then, 2x10<sup>5</sup> Raji cells were suspended in 200  $\mu$ l of PBS containing 2% FCS, and incubated for 30 minutes on ice in the presence of 100–200 ng of the biotinylated proteins. Cells were then centrifuged, washed in PBS plus 2% FCS, suspended in 200  $\mu$ l of the same buffer, and incubated for 30 minutes on ice with 100 ng of streptavidin-PE-Cy5 (BD Biosciences, San Jose, CA). Finally cells were analyzed by flow cytometry using FACScalibur instrument (BD Biosciences). Data were analyzed with FlowJo (Tree Star, Ashland, OR, USA).

### *Preparation of whole-cell lysate*

Cells were washed once in ice-cold PBS and subsequently lysed in ice-cold lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerolphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml Leupeptin and 1 mM PMSF) for 20 min. Lysates were centrifuged for 20 min at 14,000 x g. The supernatants were collected as whole-cell lysates.

### *Preparation of nuclear extracts*

Cells were first lysed in isotonic sucrose buffer containing 0.32 M sucrose, 3 mM CaCl<sub>2</sub>, 2 mM magnesium acetate, 0.1 mM Tris-Cl (pH 7.5),

0.5% NP-40, 0.1 mM EDTA, 1 mM PMSF and protease inhibitors for 15 minutes on ice. Lysates were centrifuged at 3,000 x g, 4°C for 3 minutes. Pellets were then incubated in Dignam buffer [20 mM Tris (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 25% glycerol, 0.2 mM EDTA (pH 8), 1 mM PMSF and protease inhibitors] for 20 minutes on ice and centrifuged at 14,000 x g, 4°C for 20 minutes. The supernatants were collected as nuclear extracts.

#### *Protein/DNA binding assays*

The effect of p17 and myr-p17 on the activation of multiple transcription factors was assessed using the TransSignal Protein/DNA Array from Panomics (Fremont, CA).

The results obtained by protein/DNA array were then validated in DNA binding assays for individual transcription factors by assaying nuclear extracts from p17-treated or control untreated cells using the Gel-Shift Kit from Panomics. All kits were used following the instructions of the manufacturer.

#### *Western blotting*

Equal amounts of whole cell lysates were separated on a 4-20% SDS polyacrylamide gel and then transferred to PVDF filters in a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. The blotted membranes were first blocked in 5% skim milk, 0.9% NaCl, 10 mM Tris pH 7.5 and 0.1% Tween 20. Next, the membranes were incubated with the primary antibodies and then HRP-conjugated secondary antibodies. Both steps were performed in blocking buffer, and were followed by extensive washes in blocking buffer. The blots were finally developed using the ECL-Plus kit from GE Healthcare (Piscataway, NJ). To detect total protein levels after incubation with phospho-specific antibodies, the blots were stripped for 30 minutes in stripping buffer (100 mM b-mercaptoethanol, 2% SDS and 62.5 mM Tris pH 6.7) at 50°C. Thereafter, blots were washed three times, and subsequently blocked and probed with primary antibody as described above. Anti-phospho-MEK1/2, anti-phospho-ERK1/2 and anti-phospho-PKA were obtained from Cell Signaling Technology (Danvers, MA). Anti-MEK1/2, anti-ERK1/2 and anti-PKA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

## RESULTS

### *Chemically synthesized HIV-1 p17 and N-myristoylated p17 proteins interact with a cell surface molecule expressed on human lymphoma B cell lines*

Previous studies showed that HIV-1 p17 expressed in bacteria specifically interacts with a cell surface receptor on human B cells (De Francesco *et al.*, 2002). In an attempt to understand whether this event has biological function, we sought to identify intracellular signaling pathways triggered following this interaction. For our studies, we used chemically synthesized HIV-1 p17, both in its unmodified (p17) and its N-myristoylated (myr-p17) form. Figure 1 shows the mass spectrometry profiles of the two synthetic products. Detailed structure/function analyses of these two molecules were published elsewhere (Wu *et al.*, 2004). These proteins represent a reliable and highly pure source of p17 in the correct folding and three-dimensional structure.

Next, we tested whether synthetic p17 proteins interact with a cell surface marker on B cells, as previously demonstrated with the recombinant form of the protein. Flow cytometry assays with p17 and myr-p17 showed that both synthetic proteins recognized a cell surface marker in the Burkitt's lymphoma B cell line, Raji (Fig. 2A and 2B), thus demonstrating the same B cell surface-binding properties identified with the recombinant polypeptide. These two proteins have the clear advantage of being available in pure form, free of any contaminating bacterial protein and - most importantly - free of lipopolysaccharide (LPS), which may affect results from cell signaling studies.

### *Synthetic p17 and myr-p17 increase DNA binding activity of c-Myc and CREB*

The approach we adopted in the screening of intracellular signaling pathways activated by synthetic p17 was to analyze changes in the DNA-binding capacity of cellular transcription factors by means of Protein/DNA Arrays. We prepared nuclear extracts from p17-treated and untreated Raji cells, and then we compared the DNA-binding capacity of several transcription factors in the two nuclear extract samples. We found modulation of the binding activity for several transcription factors. However, the most consistent and

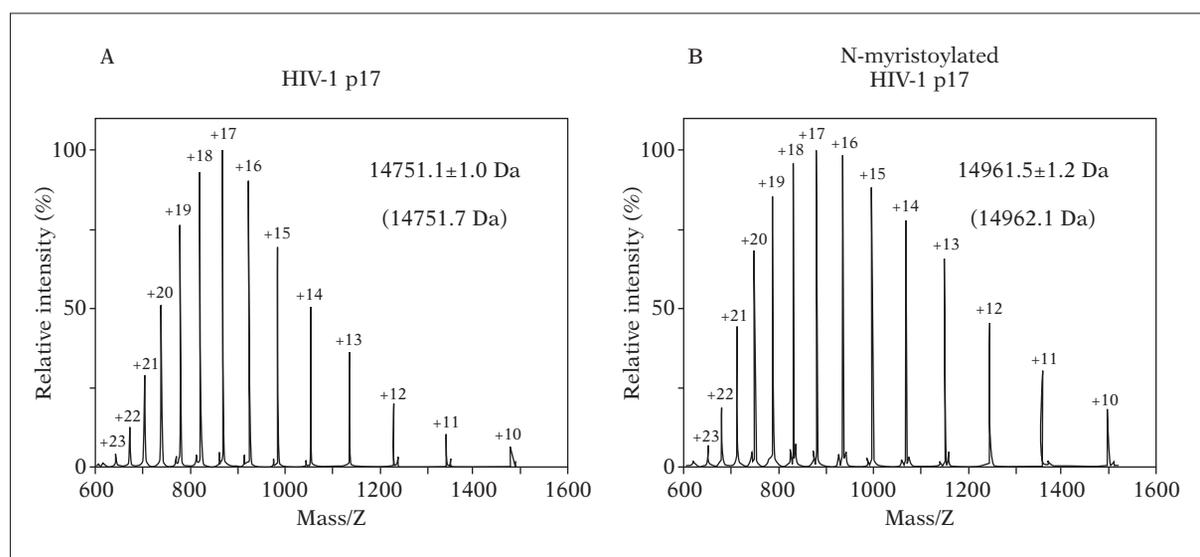


FIGURE 1 - Mass spectrometry profiles of unmodified (panel A) and N-myristoylated (panel B) p17 produced by total chemical synthesis. The two proteins were obtained as described in Materials and Methods. The figure shows the results of the electrospray ionization mass spectrometry for the two proteins. The molecular masses determined by this method were in agreement with the expected values calculated based on the average isotopic composition of the peptides (in parentheses).

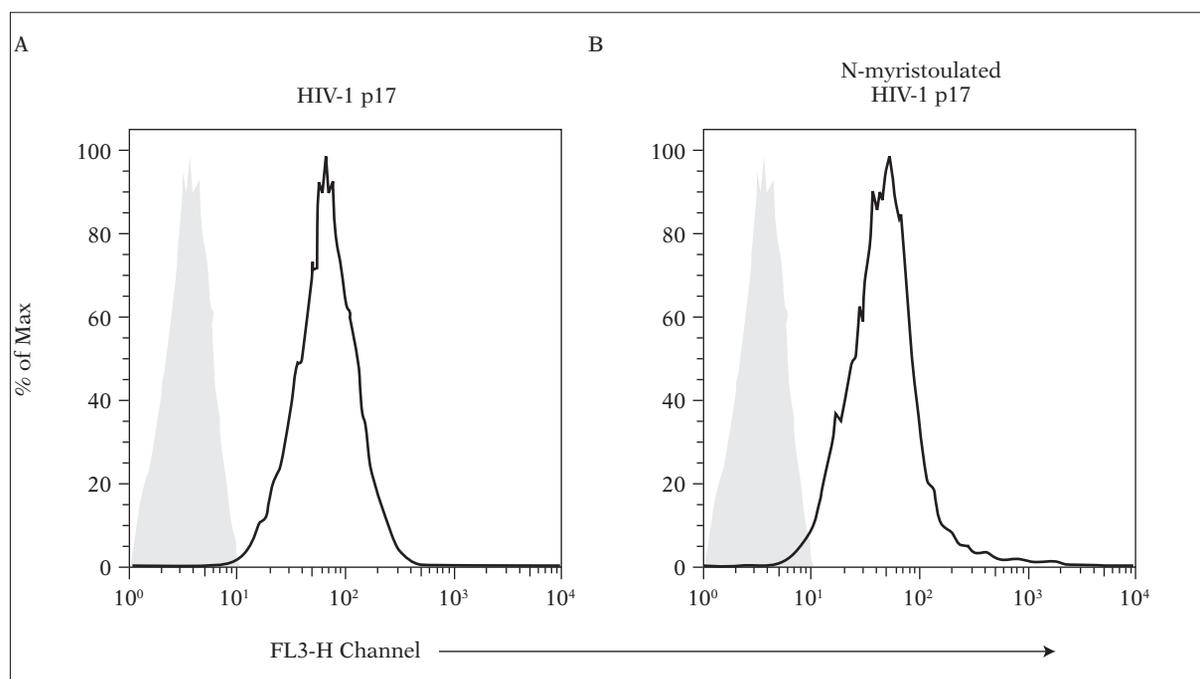
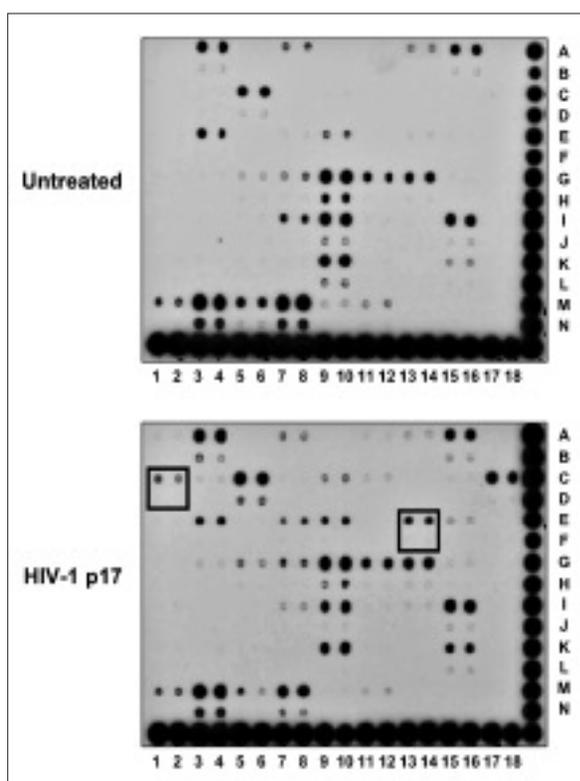


FIGURE 2 - Flow cytometry detection of the binding between unmodified (panel A) or N-myristoylated (panel B) p17 to its receptor expressed on Raji cells. Cells were incubated with p17 and myr-p17 (both biotinylated as described in Materials and Methods), and interaction with its receptor was detected with streptavidin-PE-Cy5. The bold black line shows staining with biotinylated p17 plus streptavidin-PE-Cy5, whereas the solid gray histograms show staining with streptavidin-PE-Cy5 alone. The plots show results obtained with at least 100,000 events collected in the live-cell population as determined by forward and side light scatter profiles, and are representative of at least five independent experiments.

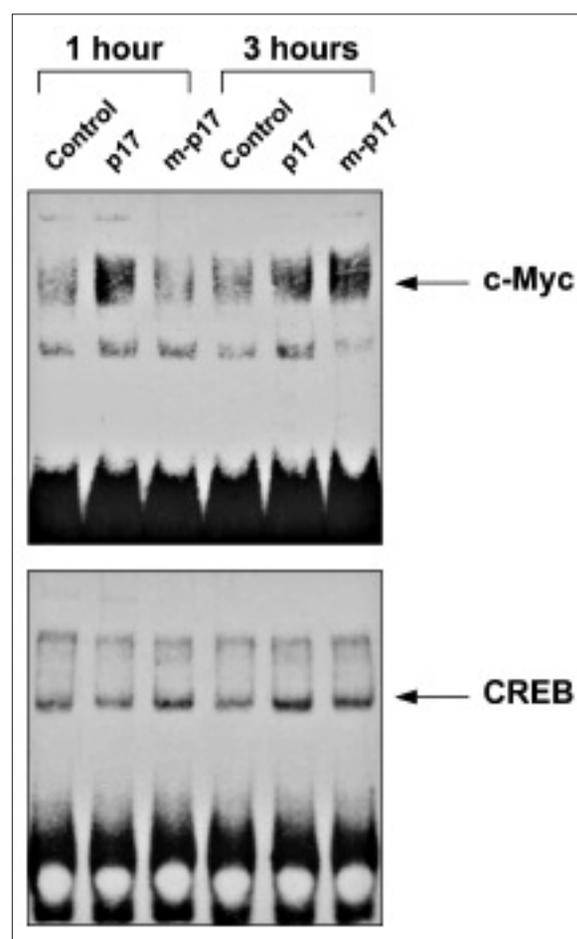
notable results were the up-regulation of DNA binding activity for CREB and c-Myc following treatment with p17 (compare dots at position C1-C2 and at position E13-E14 between upper and lower panels of Figure 3).

Identical results were obtained with unmodified and N-myristoylated p17. In some experiments we also found up-regulation of AP-2 (C17-C18), IRF-1 (E7-E8), MEF-1 (E9-E10) and c-Myb (A15-A16) following p17 treatment. However, because of their relevance in B cell differentiation and proliferation, and because other transcription factors did not behave consistently in response to p17 treatment, we focused our efforts on CREB and c-Myc. First, we performed gel mobility shift assays to confirm the up-regulation of CREB and c-Myc following treatment with p17. Thus, Raji cells were treated with p17 and myr-p17 for 1 and

3 hours. Nuclear extracts obtained from these and from untreated control cells were tested by EMSA using oligonucleotide probes specific for CREB and c-Myc. As shown in Figure 4, both forms of p17 up-regulated CREB and c-Myc in Raji cells. In addition, similar results were obtained when nuclear extracts from p17-treated and untreated Raji cells were tested in ELISA-based DNA binding assays with probes specific for c-Myc and CREB (not shown). The lower



**FIGURE 3** - Analysis of modulation of transcription factor DNA binding activity upon treatment with p17. Nuclear extracts prepared from control and p17-treated Raji cells were used to assess modulation in transcription factor binding activity using the TranSignal Protein/DNA array from Panomics (see Materials and Methods for details). The results shown are representative of at least three independent experiments.



**FIGURE 4** - Effect of p17 on the DNA binding activity of c-Myc and CREB. Raji cells were treated with synthetic p17 proteins (unmodified and myristoylated) for the indicated times. Nuclear extracts were prepared and assayed in EMSA assays using the Gel-Shift kits from Panomics (see Materials and Methods for details). Results shown are representative of at least three independent experiments. Control: nuclear extract from untreated Raji cells; p17: nuclear extract from Raji cells treated with synthetic, unmodified p17 protein; m-p17: nuclear extract from Raji cells treated with synthetic, myristoylated p17 protein.

baseline DNA binding activity for CREB and c-Myc. HIV-1 p17 upregulates c-Myc and CREB. Myc in untreated Raji cells as detected by Protein/DNA array compared to EMSA is likely due to several factors: different DNA probe sequence and concentration, different amount of non-specific DNA competitor, different buffer conditions. Nonetheless, all three techniques employed indicated that p17 up-regulates CREB and c-Myc in Raji cells.

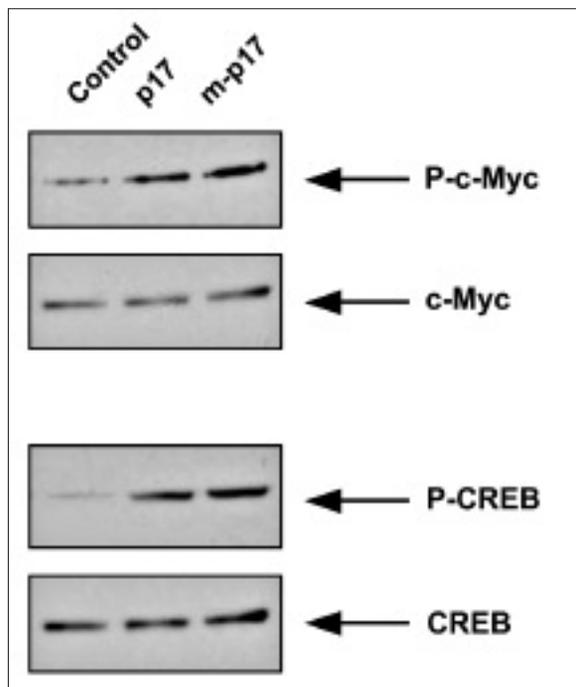
Next, we assessed whether this phenomenon was due to increased expression of these two transcription factors, or rather to an up-regulation of their DNA binding activities. Whole-cell lysates were prepared from control Raji cells and from

cells treated for 1 hour with synthetic p17 and myr-p17. Equal amounts of total protein were assayed by western blot with antibodies specific for the phosphorylated form of CREB and c-Myc, as well as for the total proteins. As shown in Figure 5 (upper panels), treatment with both forms of p17 augmented phosphorylation of c-Myc at position Thr58 and Ser62, which are required for the transactivating function of c-Myc. However, p17 did not affect the total level of c-Myc protein in Raji cells. Similarly, treatment with p17 strongly induced phosphorylation of CREB at position Ser133 - which is known to correlate with increased transcriptional activity - but did not augment the total level of CREB protein within the cells (Fig. 5, lower panels).

Altogether, these results demonstrate that stimulation of B cells with the HIV-1 matrix protein, p17 results in augmented DNA binding activity of the transcription factors CREB and c-Myc through increased phosphorylation.

#### *Synthetic p17 and myr-p17 up-regulate the MAPK and PKA signaling pathways*

Next, we investigated whether p17 increases the DNA binding activity of CREB and c-Myc via up-regulation of specific intracellular signaling pathways. To this end, we used western blot to test the phosphorylation of ERK1/2 and PKA, the kinases that regulate c-Myc and CREB function, respectively, through direct phosphorylation of serine/threonine residues. Increased phosphorylation of ERK1/2 and PKA is a direct index of their kinase activity, and reflects activation of signaling pathways downstream cell surface receptors. Whole-cell lysates were prepared from Raji cells treated with p17 and myr-p17, and from control cells, and probed with specific antibodies that detect ERK1/2 phosphorylated at residues Thr202/Tyr204. As shown in Figure 6A, treatment with both p17 and myr-p17 led to an increase in phosphorylation of ERK1/2, detectable as early as 15 minutes after stimulation. Consistently with this result, the phosphorylation of MEK1 at residues Ser217 and Ser221 - which regulates ERK1/2 function in the MAPK signaling cascade - was also increased soon after p17 treatment (Fig. 6A). However, the total levels of ERK1/2 and MEK1/2 remained unchanged during the course of the experiments. Next, we used antibodies that specifically detect PKA phosphorylated at Thr197

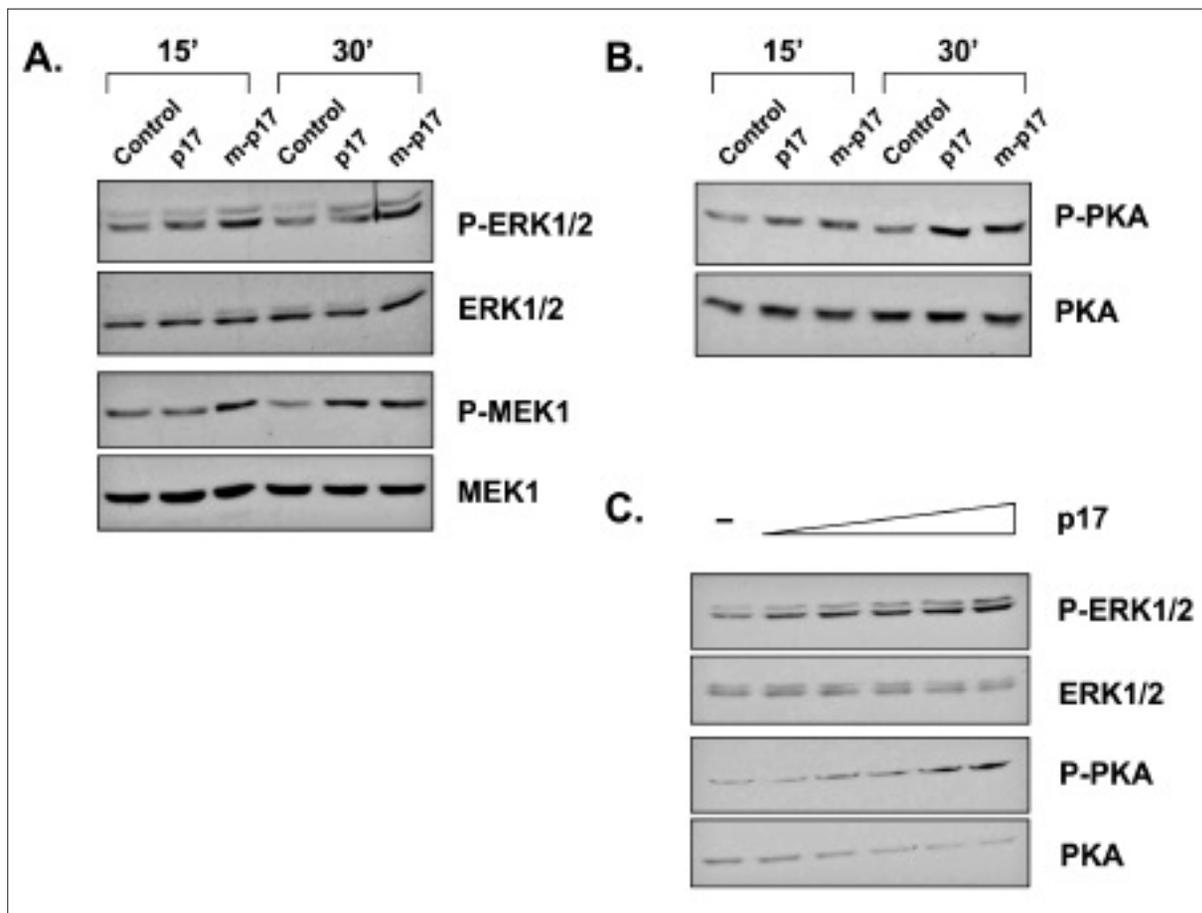


**FIGURE 5 - Effect of p17 on the phosphorylation levels of c-Myc and CREB.** Raji cells were treated with synthetic p17 proteins (unmodified and myristoylated) for 1 hour. Whole-cell lysates were then analyzed by western blot with polyclonal antibodies detecting phosphorylated c-Myc (Thr58 and Ser62) and CREB (Ser133). After stripping, the blots were re-probed with antibodies detecting the total protein levels of c-Myc and CREB. Results are representative of at least three independent experiments. Control: whole-cell lysate from untreated Raji cells; p17: whole-cell lysate from Raji cells treated with synthetic, unmodified p17 protein; m-p17: whole-cell lysate from Raji cells treated with synthetic, myristoylated p17 protein; P-c-Myc, P-CREB: phosphorylated c-Myc, or CREB.

to analyze PKA activation following treatment of Raji cells with synthetic p17 and myr-p17. Our results indicate that p17 promotes an increase in PKA function 30 minutes following treatment, but it does not alter total levels of the protein (Figure 6B). In addition, the activation of ERK1/2 and PKA phosphorylation by unmodified, synthetic p17 was dose-dependent (Fig. 6C).

The PKA and MEK/ERK pathways are critically involved in B cell activation and proliferation following stimulation of the B cell receptor (BCR). CD19 is cell surface molecules associated with the B cell receptor (BCR), which provides co-

stimulatory signals leading to an amplification of the intracellular activation pathways. Thus, we sought to investigate whether stimulation through CD19 amplifies PKA and MEK/ERK activation by p17. To this end, we treated Raji cells with sub-optimal amounts of p17 and anti-CD19 monoclonal antibodies, either alone or in combination. Then we analyzed by western blot ERK1/2 and PKA phosphorylation in response to the three modes of stimulation. We observed that co-stimulation with p17 and anti-CD19 antibodies had a synergistic effect on the phosphorylation levels of both ERK1/2 and PKA (Fig. 7). We



**FIGURE 6 - Effect of treatment with p17 on phosphorylation of upstream regulators of c-Myc and CREB.** Raji cells were treated with synthetic 1  $\mu\text{g/ml}$  p17 proteins (unmodified and myristoylated) for 15 and 30 minutes. Dose-dependent experiments were performed with concentrations of p17 ranging from 0.05 to 1  $\mu\text{g/ml}$ . Whole-cell lysates were prepared as described in Material and Methods, and then analyzed by western blot with polyclonal antibodies detecting phosphorylated ERK1/2 (Thr202 and Tyr204), MEK1 (Ser217 and Ser221), and PKA (Thr197). After stripping, the blots were re-probed with antibodies detecting the total protein levels of ERK1/2, MEK1 and PKA. Results are representative of at least three independent experiments. Control: whole-cell lysate from untreated Raji cells; p17: whole-cell lysate from Raji cells treated with synthetic, unmodified p17 protein; m-p17: whole-cell lysate from Raji cells treated with synthetic, myristoylated p17 protein; P-ERK1/2, P-MEK1, P-PKA: phosphorylated ERK1/2, MEK1 or PKA.

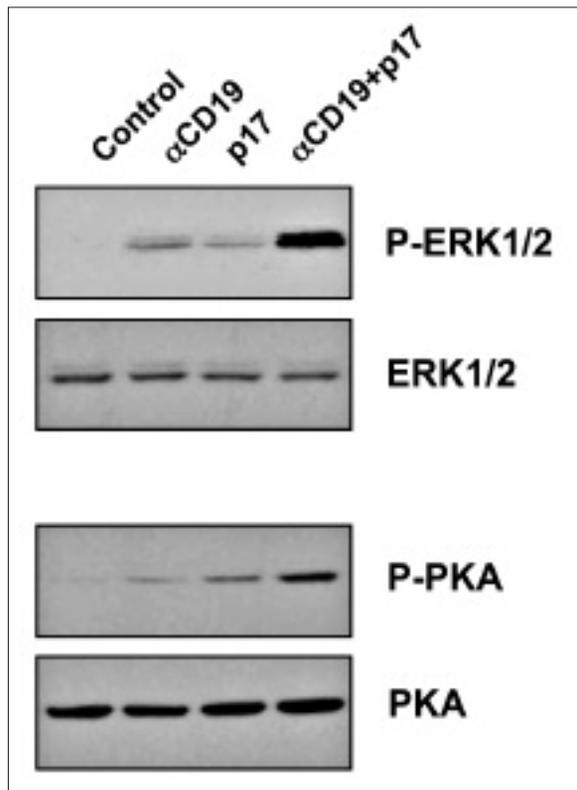


FIGURE 7 - Synergistic effect of CD19 and p17 receptor co-stimulation on ERK1/2 and PKA activation. Raji cells were left untreated (control) or exposed to monoclonal antibodies to CD19 ( $\alpha$ CD19), to Li *et al.* HIV-1 p17 up-regulates c-Myc and CREB synthetic unmodified p17, or both. Whole-cell lysates were then subjected to western blot as described in Materials and Methods using antibodies detecting phosphorylated ERK1/2 (Thr202/Ty204; P-ERK1/2), phosphorylated PKA (Thr197; P-PKA) or total ERK1/2 and PKA levels. The results shown are representative of at least three independent experiments.

conclude that the signaling pathways downstream the putative p17 receptor and CD19 engage in crosstalk upon co-stimulation.

## DISCUSSION

The HIV-1 protein p17 is a structural component of the viral particle, forming a matrix layer immediately underneath the viral membrane (Freed 1998). Moreover, p17 promotes infectivity by targeting the pre-integration complex to the cell nucleus after viral entry, and is involved in viral assembly by chaperoning viral RNA to the plasma membrane (Bukrinsky *et al.*, 1993; Gally *et al.*,

1995; Bukinskaya *et al.*, 1992; Yuan *et al.*, 1993; Sperman *et al.*, 1994; Fache *et al.*, 1993). However, p17 may play important roles outside the cell, as a free soluble Li *et al.* HIV-1 p17 upregulates c-Myc and CREB molecule. This hypothesis arises from the evidence that antibodies against p17 correlate with better prognosis (Papsidero *et al.*, 1989; Naylor *et al.*, 1987; Buratti *et al.*, 1997; Cargelegue *et al.*, 1995; Lange *et al.*, 1987). Indeed, HIV-1 p17 can be released from infected cells and is detectable in serum of HIV-1 patients (Marini *et al.*, 2008; Wu *et al.*, 2004, Pace *et al.*, 1995; Fiorentini *et al.*, 2006). In addition, recombinant p17 has been shown to interact with a cell surface receptor expressed on several cell types (De Francesco *et al.*, 1998; De francesco *et al.*, 2002; Vitale *et al.*, 2003; Marini *et al.*, 2008). Experiments employing the protein synthesis inhibitor cycloheximide, or the proteolytic enzyme trypsin demonstrated that the p17 receptor is a cell surface protein (Marini *et al.*, 2008). Interaction between p17 and its cell surface receptor was found to increase proliferation of IL-2- and phytohemagglutinin-stimulated CD4+ and CD8+ T cells, and NK cells (De Francesco *et al.*, 1998; Vitale *et al.*, 2003). Moreover, p17 increased the release of such pro-inflammatory cytokines as IFN $\gamma$  and TNF- $\alpha$  from T and NK cells (De Francesco *et al.*, 2002; Vitale *et al.*, 2003). A more recent report investigated the effects of p17 on primary human monocytes, which constitutively express the p17 receptor on their surface, and found that p17 promotes expression of MCP-1 through activation of the transcription factor AP-1 (Vitale *et al.*, 2003).

Human primary B cells present constitutive expression of the p17 receptor (De Francesco *et al.*, 2002). In the present study

we sought to investigate some of the intracellular events triggered by p17 in B cells, using the Burkitt's lymphoma cell line Raji as a cellular model. First, we demonstrate that synthetic p17 - both unmodified and myristoylated - interacts with a cell surface receptor expressed on Raji cells. This result strengthens evidence obtained previously with recombinant p17, in that it shows that the interaction between p17 and its putative receptor is direct, and not mediated by possible bacterial-derived contaminants in the p17 preparations. More importantly, we have assessed the biological relevance and the intracellular conse-

quences of the interaction between p17 and its cell surface receptor. We employed protein/DNA arrays to perform a broad-spectrum survey of the modulation in the function of key cellular transcription factors induced by p17, and we found that engagement of the p17 receptor augments the DNA binding capacity of CREB and c-Myc. Gel shift assays with probes specific for CREB and c-Myc - but different than the ones included in the protein/DNA array kit - confirmed these results. In addition to CREB and c-Myc, we have found upregulated DNA binding activity for other transcription factors in response to p17 treatment, such as IRF-1, MEF-1, c-Myb, and AP-2. However, the increase in DNA binding activity for these transcription factors was not consistent in all the experiments that we performed, and in some cases was very moderate. Therefore, in the present study we focused our attention on c-Myc and CREB because of their established role in B cell proliferation and lymphomagenesis. Our results also demonstrate that the increased DNA binding activity of CREB and c-Myc is the consequence of increased phosphorylation at Thr58 and Ser62 (for c-Myc) and Ser133 (for CREB), which are known to be phosphorylation sites that play a critical role in regulating the transcriptional activity of these two factors. On the other hand we did not observe any change in the total protein level of either transcription factor. We concluded that the increased DNA binding activity of CREB and c-Myc observed following stimulation with p17 is due to triggering of signaling pathways downstream of the p17 receptor. Indeed, our results show that p17 induces rapid phosphorylation of MEK1 and ERK1/2, and PKA, which are upstream regulators of c-Myc and CREB phosphorylation and transcriptional activity, respectively.

The transcription factor c-Myc forms a heterodimer with Max (Gu *et al.*, 1993; Grandori *et al.*, 1996) and serves as a potent positive regulator of B cell proliferation through activation of key molecules involved in cell cycle progression (Karn *et al.*, 1989; Marcu *et al.*, 1992). In addition, c-Myc promotes B cell growth by increasing the cell metabolism and promoting survival in anaerobic conditions (Shim *et al.*, 1997; Mai *et al.*, 1994). Because of these and other potent effects on B cell proliferation and survival, increased expression and transcriptional activity of

c-Myc is an important event in the multi-step process that leads to some forms of B cell lymphoma, particularly Burkitt's lymphoma (Hecht *et al.*, 2000; Sanchez-Beato *et al.*, 2003). CREB is an additional important factor in B cell proliferation and survival. Indeed, CREB has been shown to induce expression of the cell cycle promoting cyclin D2, and the anti-apoptotic molecule Bcl-2 (White *et al.*, 2006; Wilson *et al.*, 1996). In addition, transgenic mice expressing a dominant-negative mutant of CREB (with an alanine substitution at position 133) display markedly impaired proliferation of B cells (Zhang *et al.*, 2002). Therefore, both c-Myc and CREB are key positive regulators of B cell proliferation and survival. Non-Hodgkin's lymphomas (NHL) represent a major proportion of AIDS-associated malignancies (Carbone *et al.*, 2005). Indeed, NHL is the first manifestation of AIDS in about 3% of HIV-1 infected patients, and serves as an AIDS-defining condition (Frisch *et al.*, 2001). Most AIDS-NHL derive from B cells, and are characterized by clinical aggressiveness (Little *et al.*, 2001). About 80% of all AIDS-related NHL are systemic B cell lymphomas, such as Burkitt's lymphoma, and diffuse large B-cell lymphomas (Aoki *et al.*, 2004). All cases of HIV-associated Burkitt's Li *et al.* HIV-1 p17 upregulates c-Myc and CREB lymphoma show activation of c-Myc with or without concurrent inactivation of p53, although EBV infection is detectable in only ~30% cases, suggesting that other factors are at play (Aoki *et al.*, 2004). Indeed, while it was generally assumed that HIV-1 infection has only an indirect role in cancer development through impairment of immune surveillance and increased risk of oncogenic virus infection, recent evidence suggests a more direct and active role through viral-encoded proteins. For example, the HIV-1 transactivator protein, Tat promotes Kaposi sarcoma (KS) tumor development through interaction with the vascular endothelial growth factor receptor 2 and induction of pro-angiogenic cytokines, adhesion molecules and matrix metalloproteinases (Albini *et al.*, 1995; Albini *et al.*, 1996; Aoki *et al.*, 2003; Lafreine *et al.*, 1997; Ensoli *et al.*, 1990; Barillari *et al.*, 1993). Moreover, Tat facilitates the replication of the KS-associated herpes virus (KSHV) and its transmission to endothelial cells (Aoki *et al.*, 2004). The direct and active role of Tat in promoting the aggressive nature and high incidence of KS suggests

that the ability of highly active anti-retroviral therapy (HAART) to reduce occurrence and severity of AIDS-associated KS is due in part to decreased levels of Tat (Aoki *et al.*, 2004). Whereas HAART has also reduced the incidence of B cell lymphomas of the central nervous system, its impact on Burkitt's and other forms of systemic B cell lymphomas has not been as successful (Little *et al.*, 2001). However, this evidence should not suggest that HIV-1 proteins are not co-factors in promoting these forms of malignancy. Indeed, a recent study has reported the persistent presence of HIV-1 structural proteins (p17, p24 and gp120) in the germinal centers of lymph nodes from HIV-1 patients undergoing successful HAART for more than 6 months, suggesting that germinal center B cells may remain exposed to HIV-1 structural proteins for prolonged periods of time, even in condition of suppressed viral replication (Popovic *et al.*, 2005). Prolonged B cell stimulation in the context of immune suppression is a major risk factor for the development of AIDS-associated NHL, including Burkitt's lymphoma, which derives from germinal center B cells and involves c-Myc activation (Gaidano *et al.*, 2000). Thus, the results we presented here suggest that interaction of p17 with its cells surface receptor leads to increased c-Myc and CREB function and may represent a cofactor in the insurgence of B cell lymphomas during HIV-1 infection.

#### ACKNOWLEDGEMENTS

*This work was supported by a research grant from the Fondazione della Cassa di Risparmio di Perugia (Italy). Luisa Bozzo was supported in part by a fellowship from Medestea Internazionale, SpA (Italy). The authors would like to thank Robert C. Gallo for continued support and helpful discussions.*

#### REFERENCES

- ALIMONTI J.B., BALL T.B., FOWKE K.R. (2003). Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *J. Gen. Virol.* **84**, 1649-1661.
- ALBINI A., BARILLARI G., BENELLI R., GALLO R.C., ENSOLI B. (1995). Angiogenic properties of human immunodeficiency virus type 1 Tat protein. *Proc. Natl. Acad. Sci. USA.* **92**, 4838-4842.
- ALBINI A., SOLDI R., GIUNCIUGLIO D., GIRAUDO E., BENELLI R., PRIMO L., NOONAN D., SALIO M., CAMUSSI G., ROCKL W., BUSSOLINO F. (1996). The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nat. Med.* **2**, 1371-1375.
- AOKI Y., TOSATO G. (2003). Targeted inhibition of angiogenic factors in AIDS-related disorders. *Curr. Drug. Targets. Infect. Disord.* **3**, 115-128.
- AOKI Y., TOSATO G. (2004). HIV-1 Tat enhances Kaposi sarcoma-associated herpesvirus (KSHV) infectivity. *Blood.* **104**, 810-814.
- AOKI Y., TOSATO G. (2004). Neoplastic conditions in the context of HIV-1 infection. *Curr. HIV. Res.* **2**, 343-349.
- BARILLARI G., GENDELMAN R., GALLO R.C., ENSOLI B. (1993). The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. *Proc. Natl. Acad. Sci. USA.* **90**, 7941-7945.
- BUKRINSKAYA A.G., VORKUNOVA G.K., TENTSOV Y.Y. (1992). HIV-1 matrix protein p17 resides in cell nuclei in association with genomic RNA. *AIDS Res. Hum. Retroviruses.* **8**, 1795-1801.
- BUKRINSKY M.I., HAGGERTY S., DEMPSEY M.P., SHAROYA N., ADZHUBEL A., SPITZ L., LEWIS P., GOLDFARB D., EMERMAN M., STEVENSON M. (1993). A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature.* **365**, 666-669.
- BURATTI E., TISMINETZKY S.G., D'AGARO P., BARALLE F.E. (1997). A neutralizing monoclonal antibody previously mapped exclusively on human immunodeficiency virus type 1 gp41 recognizes an epitope in p17 sharing the core sequence IEEE. *J. Virol.* **71**, 2457-2462.
- CARBONE A., GLOGHINI A. (2005). AIDS-related lymphomas: from pathogenesis to pathology. *Br. J. Haematol.* **130**, 662-670.
- CHARGELEGUE D., STANLEY C.M., O'TOOLE C.M., COLVIN B.T., STEWARD M.W. (1995). The affinity of IgG antibodies to gag p24 and p17 in HIV-1-infected patients correlates with disease progression. *Clin. Exp. Immunol.* **99**, 175-181.
- DE FRANCESCO M.A., BARONIO M., FIORENTINI S., SIGNORINI C., BONFANTI C., POIESI C., POPOVIC M., GRASSI M., GARRAFA E., BOZZO L., LEWIS G.K., LICENZIATI S., GALLO R.C., CARUSO A. (2002). HIV-1 matrix protein p17 increases the production of proinflammatory cytokines and counteracts IL-4 activity by binding to a cellular receptor. *Proc. Natl. Acad. Sci. USA.* **99**, 9972-9977.
- DE FRANCESCO M.A., CARUSO A., FALLACARA F., CANARIS A.D., DIMA F., POIESI C., LICENZIATI S., CORULLI M., MARTINELLI F., FIORENTINI S., TURANO A. (1998). HIV p17 enhances lymphocyte proliferation and HIV-1 replication after binding to a human serum factor. *AIDS.* **12**, 245-252.

- ENSOLI B., BARILLARI G., SALAHUDDIN S.Z., GALLO R.C., WONG-STAAI F. (1990). Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature*. **345**, 84-86.
- FÄCKE M., JANETZKO A., SHOEMAN R.L., KRÄUSSLICH H.G. (1993). A large deletion in the matrix domain of the human immunodeficiency virus gag gene redirects virus particle assembly from the plasma membrane to the endoplasmic reticulum. *J. Virol.* **67**, 4972-4980.
- FIorentini S., MARINI E., CARACCIOLo S., CARUSO A. (2006). Functions of the HIV-1 matrix protein p17. *New Microbiol.* **29**, 1-10.
- FREED E.O. (1998). HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology*. **251**, 1-15.
- FRISCH M., BIGGAR R.J., ENGELS E.A., GOEDERT J.J., AIDS-CANCER MATCH REGISTRY STUDY GROUP. (2001). Association of cancer with AIDS-related immunosuppression in adults. *JAMA*. **285**, 1736-1745.
- GAIDANO G., CAPELLO D., CARBONE A. (2000). The molecular basis of acquired immunodeficiency syndrome-related lymphomagenesis. *Semin. Oncol.* **27**, 431-441.
- GALLAY P., SWINGLER S., SONG J., BUSHMAN F., TRONO D. (1995). HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell*. **83**, 569-576.
- GRANDORI C., MAC J., SIÉBELT F., AYER D.E., EISENMAN R.N. (1996). Myc-Max heterodimers activate a DEAD box gene and interact with multiple E box-related sites in vivo. *EMBO J.* **15**, 4344-4357.
- GU W., CECHOVA K., TASSI V., DALLA-FAVERA R. (1993). Opposite regulation of gene transcription and cell proliferation by c-Myc and Max. *Proc. Natl. Acad. Sci. USA*. **90**, 2935-2939.
- HECHT J.L., ASTER J.C. (2000). Molecular biology of Burkitt's lymphoma. *J. Clin. Oncol.* **18**, 3707-3721.
- HERBEIN G., MAHLKNECHT U., BATLIWALLA F., GREGERSEN P., PAPPAS T., BUTLER J., O'BRIEN W.A., VERDIN E. (1998). Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. *Nature*. **395**, 189-194.
- HOLM G.H., ZHANG C., GORRY P.R., PEDEN K., SCHOLS D., DE CLERCO E., GABUZDA D. (2004). Apoptosis of bystander T cells induced by human immunodeficiency virus type 1 with increased envelope/receptor affinity and coreceptor binding site exposure. *J. Virol.* **78**, 4541-4551.
- JEKLE A., KEPPLER O.T., DE CLERCO E., SCHOLS D., WEINSTEIN M., GOLDSMITH M.A. (2003). In vivo evolution of human immunodeficiency virus type 1 toward increased pathogenicity through CXCR4-mediated killing of uninfected CD4 T cells. *J. Virol.* **77**, 5846-5854.
- KARN J., WATSON J.V., LOWE A.D., GREEN S.M., VEDECKI W. (1989). Regulation of cell cycle duration by c-myc levels. *Oncogene*. **4**, 773-787.
- LAFRENIE R.M., WAHL L.M., EPSTEIN J.S., YAMADA K.M., DHAWAN S. (1997). Activation of monocytes by HIV-Tat treatment is mediated by cytokine expression. *J. Immunol.* **159**, 4077-4083.
- LANG E.J., DE WOLF F., KRONE W.J., DANNER S.A., COUTINHO R.A., GOUDSMIT J. (1987). Decline of antibody reactivity to outer viral core protein p17 is an earlier serological marker of disease progression in human immunodeficiency virus infection than anti-p24 decline. *AIDS*. **1**, 155-159.
- LITTLE R.F., GUTIERREZ M., JAFFE E.S., PAU A., HORNE M., WILSON W. (2001). HIV-associated non-Hodgkin lymphoma: incidence, presentation, and prognosis. *JAMA*. **285**, 1880-1885.
- MAI S., JALAVA A. (1994). c-Myc binds to 5' flanking sequence motifs of the dihydrofolate reductase gene in cellular extracts: role in proliferation. *Nucleic Acids Res.* **22**, 2264-2273.
- MARCU K.B., BOSSONE S.A., PATEL A.J. (1992). Myc function and regulation. *Annu. Rev. Biochem.* **61**, 809-860.
- MARINI E., TIBERIO L., CARACCIOLo S., TOSTI G., GUZMAN C.A., SCHIAFFONATI L., FIorentini S., CARUSO A. (2008). HIV-1 matrix protein p17 binds to monocytes and selectively stimulates MCP-1 secretion: role of transcriptional factor AP-1. *Cell. Microbiol.* **10**, 655-666.
- NAYLOR P.H., NAYLOR C.W., BADAMCHIAN M., WADA S., GOLDSTEIN A.L., WANG S.S., SUN D.K., THORNTON A.H., SARIN P.S. (1987). Human immunodeficiency virus contains an epitope immunoreactive with thymosin alpha 1 and the 30-amino acid synthetic p17 group-specific antigen peptide HGP-30. *Proc. Natl. Acad. Sci. USA*. **84**, 2951-2955.
- NEKHAI S., JEANG K.T. (2006). Transcriptional and post-transcriptional regulation of HIV-1 gene expression: role of cellular factors for Tat and Rev. *Future Microbiol.* **1**, 417-426.
- PACE C.N., VAJDOS F., FEE L., GRIMSLEY G., GRAY T. (1995). How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**, 2411-2423.
- PAPSIDERO L.D., SHEU M., RUSCETTI F.W. (1989). Human immunodeficiency virus type 1-neutralizing monoclonal antibodies which react with p17 core protein: characterization and epitope mapping. *J. Virol.* **63**, 267-272.
- POPOVIC M., TENNER-RACZ K., PELSNER C., STELLBRINK H.J., VAN LUNZEN J., LEWIS G., KALYANARAMAN V.S., GALLO R.C., RACZ P. (2005). Persistence of HIV-1 structural proteins and glycoproteins in lymph nodes of patients under highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. USA*. **102**, 14807-14812.
- SÁNCHEZ-BEATO M., SÁNCHEZ-AGUILERA A., PIRIS M.A. (2003). Cell cycle deregulation in B-cell lymphomas. *Blood*. **101**, 1220-1235.
- SHIM H., DOLDE C., LEWIS B.C., WU C.S., DANG G., JUNGMANN R.A., DALLA-FAVERA R., DANG C.V. (1997). c-Myc transactivation of LDH-A: implications for

- tumor metabolism and growth. *Proc. Natl. Acad. Sci. USA*. **94**, 6658-6663.
- SPEARMAN P., WANG J.J., VANDER HEYDEN N., RATNER L. (1994). Identification of human immunodeficiency virus type 1 Gag protein domains essential to membrane binding and particle assembly. *J. Virol.* **68**, 3232-3242.
- VITALE M., CARUSO A., DE FRANCESCO M.A., RODELLA L., BOZZO L., GARRAFA E., GRASSI M., GOBBI G., CACCHIOLI A., FIORENTINI S. (2003). HIV-1 matrix protein p17 enhances the proliferative activity of natural killer cells and increases their ability to secrete proinflammatory cytokines. *Br. J. Haematol.* **120**, 337-343.
- WHITE P.C., SHORE A.M., CLEMENT M., McLAREN J., SOEIRO I., LAM, E.W., BRENNAN P. (2006). Regulation of cyclin D2 and the cyclin D2 promoter by protein kinase A and CREB in lymphocytes. *Oncogene*. **25**, 2170-2180.
- WILSON B.E., MOCHON E., BOXER L.M. (1996). Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Mol. Cell. Biol.* **16**, 5546-5556.
- WU Z., ALEXANDRATOS J., ERICKSEN B., LUBKOWSKI J., GALLO R.C., LU W. (2004). Total chemical synthesis of N-myristoylated HIV-1 matrix protein p17: structural and mechanistic implications of p17 myristoylation. *Proc. Natl. Acad. Sci. USA*. **101**, 11587-11592.
- YUAN X., YU X., LEE T.H., ESSEX M. (1993). Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. *J. Virol.* **67**, 6387-6394.
- ZAGURY D., LACHGAR A., CHAMS V., FALL L.S., BERNARD J., ZAGURY J.F., BIZZINI B., GRINGERI A., SANTAGOSTINO E., RAPPAPORT J., FELDMAN M., BURNY A., GALLO R.C. (1998). Interferon alpha and Tat involvement in the immunosuppression of uninfected T cells and C-C chemokine decline in AIDS. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3851-3856.
- ZHANG C.Y., WU Y.L., BOXER L.M. (2002). Impaired proliferation and survival of activated B cells in transgenic mice that express a dominant-negative cAMP-response element-binding protein transcription factor in B cells. *J. Biol. Chem.* **277**, 48359-48365.