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c-Myc and EBV–LMP1: two opposing regulators of the HLA class I antigen presentation machinery in epithelial cells

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BACKGROUND: Epstein–Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) up-regulates the human leukocyte antigen (HLA) class I antigen presentation machinery (APM). This appears counterintuitive with immune evasion in EBV-associated tumours like nasopharyngeal carcinoma (NPC).

METHODS: Latent membrane protein 1-transfected epithelial cell lines were used as a model system to study the impact of LMP1 and c-Myc on HLA class I components. The expression of components of the HLA class I APM, c-Myc and Ki-67 was analysed in LMP1 + and LMP1 − NPC by immunohistochemistry.

RESULTS: In epithelial cells, LMP1 up-regulated HLA class I APM. This effect could be counteracted by c-Myc, which itself was up-regulated by LMP1 apparently through IL6 induction and Jak3/STAT3 activation. Studies of NPC biopsies revealed down-regulation of HLA class I APM expression. No difference was observed between LMP1 + and LMP1 − NPC. However, expression of Ki-67 and c-Myc were up-regulated in LMP1 + tumours.

CONCLUSION: These findings raise the possibility that c-Myc activation in NPC might antagonise the effect of LMP1 on HLA class I expression thus contributing to immune escape of tumour cells.

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More than 90% of the world population are Epstein–Barr virus (EBV) carriers. Although EBV leads mostly to an asymptomatic infection in childhood, there is sufficient evidence for its carcinogenicity in the causation of lymphomas and epithelial cancers, such as gastric and nasopharyngeal carcinoma (NPC). In addition to tumour antigens, EBV-associated tumours express viral antigens that are potential targets for an anti-tumoural immunity (Young and Rickinson, 2004). However, the fact that they are not eliminated by the immune system suggests that EBV-positive tumour cells establish mechanisms to evade immune attack. One potential immune evasion mechanism is the down-regulation of components of the human leukocyte antigen (HLA) class I antigen presentation machinery (APM), which may allow tumour cells to evade cytotoxic T lymphocyte (CTL) attack (Seliger *et al*, 2002; Chang *et al*, 2003). Even partial HLA class I APM down-regulation promoted disease progression in several malignancies including NPC (Ogino *et al*, 2003, 2007; Demanet *et al*, 2004; Vitale *et al*, 2005). Interestingly, however, it has also been reported that the down-regulation of HLA class I makes tumour cells more sensible to natural killer (NK) cell attack (Ravetch and Lanier, 2000), so that the loss of HLA class I might be a disadvantage for the tumour.

Antigen presentation through HLA class I proteins involves antigen processing into peptides in the proteasome and transport into the endoplasmic reticulum (ER) via a transmembrane-transporter associated with antigen processing (TAP). In the ER, the peptides are loaded onto a complex of the HLA class I heavy chain (HC) and β_2-microglobuline (β_2-m) with the assistance of chaperones including tapasin. Thereafter, the complex is translocated via the *trans*-Golgi to the cell surface for antigen presentation (Flutter and Gao, 2004).

The EBV-encoded latent membrane protein (LMP)1, however, has been shown to induce the expression of HLA class I, TAP1 and TAP2 in B cells (Rowe *et al*, 1995) and of HLA class I in epithelial cells (Murray *et al*, 1998) inducing an immunogenic phenotype. This appears counterintuitive with immune evasion with regard to CTL attack. Latent membrane protein 1 acts as a constitutively active member of the tumour necrosis factor receptor family (CD40) (Uchida *et al*, 1999), activating a multitude of intracellular signalling pathways in a ligand-independent manner. Particularly, the STAT3 pathway can be strongly activated by LMP1 via JAK3 kinase (Eliopoulos *et al*, 1999; Gires *et al*, 1999; Chen *et al*, 2003) or an increase of IL6 secretion (Eliopoulos *et al*, 1997; Hirano *et al*, 2000). Signal transducer and activator of transcription 3, in turn, up-regulates c-myc (Bromberg *et al*, 1999; Bowman *et al*, 2001), which

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has been shown to down-regulate HLA class I APM expression in human melanoma cell lines (Versteeg *et al*, 1988; Blom *et al*, 1997), several carcinoma cell lines (Ottesen *et al*, 1990; Belldegrun *et al*, 1993) and in B cells (Staege *et al*, 2002). Therefore, one could hypothesise an auto-regulatory loop involving LMP1, STAT3 and c-myc to compensate the immunogenicity of LMP1.

The expression of LMP1 in epithelial tumours is both variable and heterogenous. Whereas LMP1 can be detected at the RNA level in the vast majority of NPC cases (Lin *et al*, 2001), and LMP1 specific antibodies are found in sera of >70% of patients (Xu *et al*, 2000), immunohistochemical staining of LMP1 protein is more variable, ranging between 20% and 60% of cases (Fahraeus *et al*, 1988; Niedobitek *et al*, 1992). Previous investigations on the expression of HLA class I APM components in NPC yielded conflicting results (Lai *et al*, 1990; Khanna *et al*, 1998; Sengupta *et al*, 2006; Ogino *et al*, 2007). Interestingly, no differences were observed when comparing LMP1+ and LMP1− NPC cases (Ogino *et al*, 2007). The present study was designed to investigate the interplay of LMP1, STAT3 and c-Myc in the regulation of HLA class I APM in epithelial cells and to test whether those mechanisms could be operational in an EBV-associated carcinoma using the example of NPC.

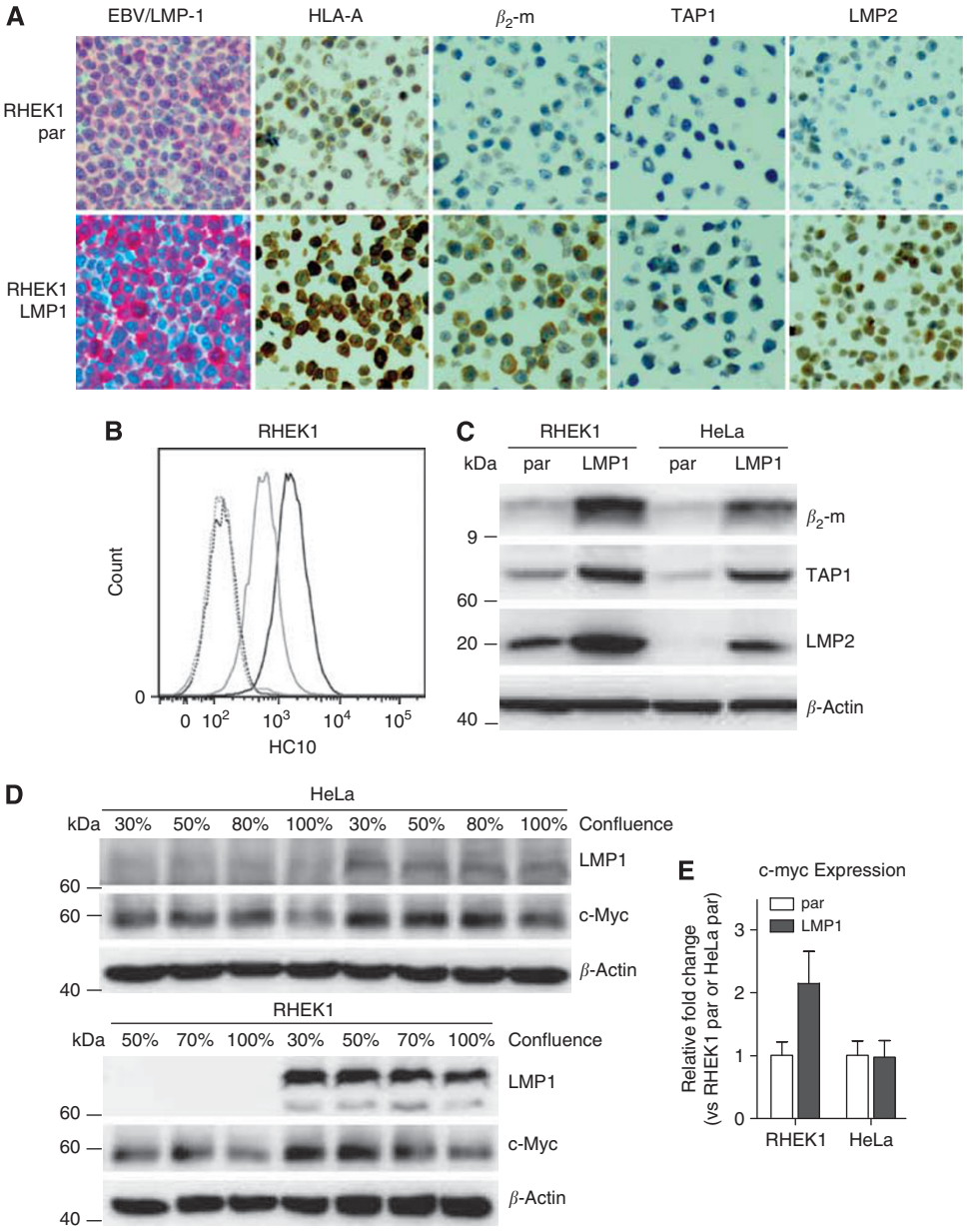

Figure 1 Induction of HLA class I APM components and of c-Myc in stably LMP1-transfected epithelial cell lines. (**A**) Comparative immunocytochemistry of RHEK1 and RHEK1/LMP1 cell lines. HLA-A, β_2-m, TAP1 and LMP2 were visibly up-regulated in response to LMP1 expression. One representative experiment of 3. (**B**) Flow cytometric analysis of RHEK1 and RHEK1/LMP1 showing the induction of the HLA class I HC (HC10) in LMP1 expressing cells. Dotted/solid light and dark lines represent the control staining without primary antibody/HC10 expression in RHEK1 and RHEK1/LMP1, respectively. One representative experiment of 3. (**C**) Western blot analysis of RHEK1 and HeLa cells. Up-regulation of β_2-m, TAP1 and LMP2 in stable LMP1-transfected cell lines. One representative experiment of 3. (**D**) Western blot analysis of c-Myc expression depending on the confluence of the cell culture. The amount of c-Myc protein decreases with increasing confluence. When comparing RHEK1 and HeLa parental with RHEK1/LMP1 and HeLa/LMP1 at 50% confluence, an increase of c-Myc is seen in the LMP1-transfected cell line. One representative experiment of 3. (**E**) qRT–PCR analysis of RHEK1, RHEK1/LMP1, HeLa and HeLa/LMP1 cells for c-myc expression. Latent membrane protein 1 induced c-myc expression in RHEK1 cells, whereas no effect in HeLa cells on RNA level was observed.

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METHODS

Tissues and cell lines

RHEK1 and HeLa and the stably LMP1 (type B95.8) transfected RHEK1/LMP1 and HeLa/LMP1 cell lines (Siegler *et al*, 2004; Dawson *et al*, 2008) were maintained at 37 °C and 5% CO_2 in RPMI-1640 + GlutaMAX supplemented with 10% fetal bovine serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Invitrogen, Darmstadt, Germany). Paraffin blocks of RHEK1 and RHEK1/LMP1 cells were generated using a commercially available Cell Block Preparation System (Shandon Cytoblock, Thermo Scientific, Astmoor, UK). The cell pellets were embedded in paraffin and transferred into tissue microarrays (TMA). Formalin-fixed and paraffin-embedded specimens of columnar epithelium ($n = 17$) and metaplastic squamous epithelium ($n = 10$) of the nasopharynx were retrieved from the files of the Institute of Pathology, University Erlangen and served as control tissue. In all, 16 biopsies of EBV-associated NPC have been described earlier (Heussinger *et al*, 2004; Buettner *et al*, 2006) or were retrieved from the files of the Institute of Pathology, University Erlangen and characterised by EBER *in situ* hybridisation and LMP1 immunohistochemistry as described earlier (Niedobitek and Herbst, 2001; Buettner *et al*, 2006). The control tissues and 12 of the NPC were investigated in TMA with cores of 2 mm diameter as described (Kasper *et al*, 2005; van Oers *et al*, 2007). Four NPC complete sections instead of TMA sections were investigated, in which the markers of the HLA class I APM, c-Myc and Ki-67 showed a diffuse staining pattern. Tissue biopsies were used in accordance with a statement of our local ethical board.

Immunohistochemistry

Sections (2 μm) were deparaffinised in xylene and rehydrated in graded ethanol (100–70%). For the detection of components of the HLA class I APM HLA-A and HC detected by HC10 mouse monoclonal antibodies (mAb) were applied, which were formerly described (Lampson *et al*, 1983; Stam *et al*, 1986; Sernee *et al*, 1998; Ogino *et al*, 2003; Perosa *et al*, 2003; Bandoh *et al*, 2005; Wang *et al*, 2005) (Supplementary Table S1). Antigen retrieval was performed as indicated in Supplementary Table S1. Endogenous peroxidase was blocked with Peroxidase-Block (Dako Cytomation, Hamburg, Germany). Primary antibodies were incubated on the slides for 30 min at room temperature (RT). An HRP-based EnVision + Kit (Dako Cytomation) was used for detection and DAB (Dako Cytomation) served as a chromogen. For the detection of c-Myc, Ki-67, HLA-B and EBV/LMP1 antigen retrieval was performed as indicated in Supplementary Table S1. Primary antibodies were added overnight at RT. Subsequently, a biotin-labelled polyclonal goat anti-rabbit secondary antibody (1 : 100, Dako Cytomation) was added for 30 min at RT followed by an

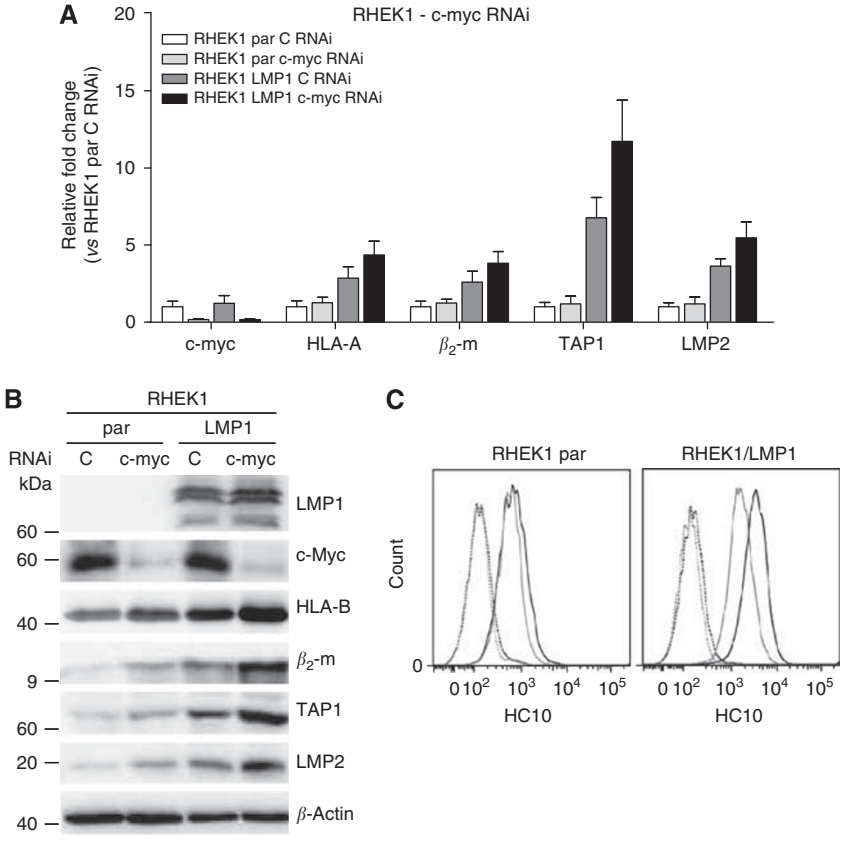

Figure 2 Inhibitory effect of c-myc on the expression of HLA class I APM components in epithelial cells. (**A**) qRT–PCR analysis ($\Delta\Delta C_T$ method with efficiency correction) of RHEK1 cells for HLA class I APM components after treatment with c-myc siRNA. c-myc knockdown stimulated gene expression of HLA-A, β_2-m, TAP1 and LMP2 in both RHEK1 parental and RHEK1/LMP1 cells. Latent membrane protein 1 expression enhanced the c-myc knockdown effect in RHEK1 cells. Data of three independent experiments are shown with standard deviations. (**B**) Western blot analysis of RHEK1 parental and RHEK1/LMP1 after knockdown of c-myc with siRNA. The expression of c-Myc protein is almost totally abolished. After c-myc knockdown, an up-regulation of HLA-B, β_2-m, TAP1 and LMP2 is observed in RHEK1 parental and RHEK1/LMP1. One representative experiment of 2. (**C**) Flow cytometric analysis of RHEK1 parental and RHEK1/LMP1 after knockdown of c-myc with siRNA. An up-regulation of HLA class I HC (HC10) is seen in parental cells and RHEK1/LMP1. The effect of LMP1 on the expression of HC10 is enhanced after c-myc knockdown. Dotted/solid light and dark lines represent the control staining without primary antibody/HC10 expression for control and c-myc knocked down cells, respectively. One representative experiment of 3.

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incubation with streptavidin-biotinylated horseradish peroxidase complex (Dako Cytomation). Then tyramide signal amplification was performed as described previously (Greiner *et al*, 2005) or an alkaline phosphatase-labelled polymer kit was added (Zytochem-Plus AP-PolymerKit, Zytomed Systems, Berlin, Germany). Aminoethyl carbazole (Zymed Laboratories, Berlin, Germany) or Fast Red (Sigma-Aldrich, Deisenhof, Germany) served as chromogens. Sections were counterstained with Mayer's Hemalaun solution (Merck, Darmstadt, Germany).

Semi-quantitative evaluation of immunohistochemical and immunocytochemical stainings

A similar scoring system has been used for the HLA class I APM-specific antibodies before (Seliger *et al*, 2010). Briefly, the percentage of positive tumour cells was scored as negative (0), $<25\%$ positive (1), 25–50% positive (2) or $>50\%$ positive (3) and the staining intensity was scored (0 = negative, 1 = weak, 2 = moderate, 3 = strong). The combined score was obtained by adding both values. For the quantification of the c-Myc and Ki-67 expression, the percentage of tumour or epithelial cells with positive nuclear signal was evaluated.

Statistical analysis

For comparisons of the semi-quantitative immunohistochemical stainings, Mann–Whitney tests were used for ordinal data and for data with a skewed distribution. Comparisons were made between NPC and controls, and between LMP1+ and LMP1− samples using SPSS17.0 bioinformatics software (SPSS Inc., Chicago, IL, USA). *P*-values <0.05 were accepted as statistically significant. When directional hypotheses were tested, one-tailed *P*-values were used.

Western blotting

For western blot analysis, monoclonal mouse anti-LMP1 (1 : 200, CS.1-4, Dako Cytomation), polyclonal rabbit anti-TAP1 (1 : 500, Stressgen, Victoria, Canada), monoclonal rabbit anti-c-Myc (1 : 1000, clone Y69, Abcam, Cambridge, UK), polyclonal rabbit anti-β_2-m (1 : 500, Dako Cytomation), polyclonal rabbit anti-LMP2 (1 : 1000, Abcam) and monoclonal rabbit anti-HLA-B (1 : 1000, Abcam) were used. In all, 50% confluent cells were lysed in RIPA buffer and sonicated. For cytoplasmic and nuclear fractionation, cells were incubated in cytoplasmic extraction buffer (10 mM Tris, pH 7.5; 40 mM KCl; 2 mM $MgCl_2$; 10% glycerol; 0.125% NP40; 1 mM PMSF; protease inhibitor cocktail (Roche, Mannheim, Germany)) for 5 min on ice and centrifuged. Pellets were resuspended in 0.25 M sucrose buffer, layered on a 0.35-M sucrose fraction and centrifuged. The pellet was resuspended in nuclear lysis buffer (10 mM Hepes; pH 7.5; 500 mM NaCl; 1% Triton X-100; 10% glycerol; 1 mM PMSF; protease inhibitor cocktail (Roche)) and sonicated. After heat-denaturation proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

qRT–PCR

Total RNA was extracted using an RNeasy kit (RNeasy kit, Qiagen, Hilden, Germany). Complementary DNA was generated by using random hexamers and RevertAid reverse transcriptase (Fermentas, Leon-Rot, Germany), applying 500 ng RNA. Primers used to quantify gene transcripts by qRT–PCR are summarised in Supplementary Table S2 (Dahl *et al*, 2007; Diosdado *et al*, 2009; Respa *et al*, 2011). Glyceraldehyde 3-phosphate dehydrogenase was stably expressed in control and treated samples and therefore used as housekeeping gene. RHEK1 par and RHEK1 par C RNAi, respectively, were used to normalise the data. The nucleic acid intercalating property of the fluorescent dye SYBR-Green (Applied Biosystems, Weiterstadt, Germany) was utilised to measure the extent of gene replication in line with $\Delta\Delta C_T$ qRT–PCR method with efficiency correction (Fink *et al*, 1998).

Knockdown of c-myc by siRNA and c-myc/LMP1 transfection

To knockdown c-myc, cells were reverse transfected with ON-TARGET plus SMART pool (Thermo Scientific, Dharmacon, Bonn, Germany). The following oligos were synthesised by Dharmacon: 5′UUACGCACAAGAGUUCCGU3′; 5′UCCAAGACGU UGUGUGUUC3′; 5′UGUUGGUGAAGCUAACGUU3′; and 5′UUCC ACAGAAACAACAUCG3′. The ON-TARGET plus Non-targeting Pool of at least four mismatches to any human gene served as a negative control. siRNAs were transfected using DharmaFECT1 Transfection Reagent (Thermo Scientific). For the (co)transfection of RHEK1 with pLNSX-LMP1 and pcDNA3-c-myc (Ricci *et al*, 2004) Lipofectamine LTX and PLUS Reagent (Invitrogen) was used. Empty vector controls were included.

Flow cytometric analysis

RHEK1 cells were stained with HC10 antibody (1 : 100; Stam *et al*, 1986; Perosa *et al*, 2003) for 30 min at RT. Flow cytometric analysis was performed on a FACSCanto II flow cytometer using the FACSDiva software v6.1.3 (BD, Heidelberg, Germany).

IL6 ELISA

In all, 96-well plates were coated with an IL6 antibody (MAB206, R&D, Minneapolis, MN, USA). After blocking with 1% BSA, standards and cell supernatants were added for 2 h. After incubation with the detection antibody (BAF206, R&D), streptavidin–HRP complex (R&D) and the substrate were added. To stop the staining reaction 0.18 M sulphur dioxide was used. Absorbance was measured at 450 and 590 nm as reference.

RESULTS

LMP1 induced up-regulation of HLA class I APM and c-Myc in epithelial cells

In order to analyse the regulation of the HLA class I APM components by LMP1, the expression of proteasome subunits, the transporter system, chaperones, HLA class I HC (HLA-A and HC10) and β_2-m (Supplementary Table S1) was investigated by immunocytochemistry in epithelial cells. Human leukocyte antigen-A, β_2-m, LMP2 and TAP1 were visibly up-regulated in RHEK1/LMP1 compared with RHEK1 cells (Figure 1A). No difference was seen in other components. In flow cytometric analysis, HC10 positivity was increased in RHEK1/LMP1 compared with RHEK1 (Figure 1B). For further experiments, HC10, HLA-A, β_2-m, LMP2 and TAP1 were chosen as LMP1− targets. The effect of LMP1 was confirmed in RHEK1 and HeLa cells in immunoblotting (Figure 1C) and qRT–PCR (data not shown). c-Myc expression decreased strongly with increasing cell confluence (Figure 1D) with highest c-Myc levels around 50% confluence (used for further experiments). At protein level, LMP1 induced c-Myc expression in RHEK1 and HeLa cells (Figure 1D). At RNA level, significant c-myc induction was only observed in RHEK1/LMP1 cells (Figure 1E).

Opposing effect of c-myc on LMP1− induced up-regulation of HLA class I APM

c-myc was knocked down in RHEK1 and HeLa (data not shown) cells using c-myc-specific siRNA and resulted in an increase in HLA class I HC, β_2-m, TAP1 and LMP2 expression at RNA

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(Figure 2A) and protein level (Figure 2B). The effect was more pronounced in RHEK1/LMP1 with higher baseline levels of c-Myc expression. An increase of superficial HLA class I HC10 was found in RHEK1 cells. This effect was even enhanced in RHEK1/LMP1 compared with RHEK1 (Figure 2C). Thereafter, RHEK1 cells were transiently reverse cotransfected with LMP1, c-myc or both. β_2-m, TAP1 and LMP2 expression was increased when LMP1 was transfected alone. This effect was diminished when cells were cotransfected with c-myc (Figure 3A). Latent membrane protein 1 expression levels under conditions of sole and cotransfection were similar in qRT–PCR (Figure 3B). In flow cytometric analysis, cotransfection with c-myc mildly decreased the effect of LMP1 on the surface expression of the HLA class I HC (HC10) (Figure 3C).

Induction of STAT3 pathway and IL6 by LMP1

Western blotting of fractionated cell lysates revealed increased STAT3-pY705 phosphorylation in both the cytosolic and the nuclear fractions of LMP1 expressing cells (Figure 4A). ELISA revealed a strong inductive effect of LMP1 on IL6 secretion (Figure 4B). Moreover, IL6 stimulation induced STAT3 phosphorylation and c-Myc expression (Figure 4C). Finally, treatment of RHEK1/LMP1 cells with the JAK3 inhibitor CP690550 almost completely abolished STAT3-pY705 phosphorylation accompanied by a dose-dependent decrease in c-Myc expression (Figure 4D).

Down-regulation of HLA-A, LMP7, TAP2 and tapasin in NPC and up-regulation of c-Myc and Ki-67 in LMP1+ NPC in immunohistochemistry

Nasopharyngeal epithelium was compared with NPC cases (LMP1−, $n=9$; LMP1+, $n=7$) using the same set of antibodies investigated in cell culture (Supplementary Table S1; Figure 5A). No significant differences were observed when comparing columnar or squamous epithelium. When comparing NPC and control tissues, a significant down-regulation of LMP7 ($P<0.001$), TAP1 ($P<0.001$), tapasin ($P=0.019$) and HLA-A ($P<0.001$) was observed in NPC. Only weak expression of LMP2 and TAP1 was detected in NPC and control tissue. No difference in the expression of APM components including HLA-A, HLA-B and HC10-detected HCs was found when comparing LMP1− and LMP1+ NPC cases. When evaluating the expression of the oncoprotein c-Myc and the proliferation associated antigen Ki-67, a significant increase of both markers was observed in NPC compared with control tissue (c-Myc, $P<0.001$; Ki-67, $P<0.001$) (Figure 5B and C). Moreover, c-Myc ($P=0.027$) and Ki-67 ($P=0.027$) were up-regulated in LMP1+ NPC compared with LMP1− NPC cases. To exclude that the lack of difference between LMP1− and LMP1+ NPC cases was a consequence of localised or focal expression of LMP1, serial sections of three LMP1+ NPC were stained for LMP1 and three APM components regulated by LMP1. The expression of LMP1 was patchy or focal in only few scattered cells in all three cases, whereas the expression of β_2-m, HLA-A and LMP2 showed a diffuse and homogenous distribution (Figure 5D).

DISCUSSION

Down-regulation of the HLA class I APM is a mechanism used by malignancies to elicit immune evasion (Chang *et al*, 2003). Therefore, it appears paradoxical that the EBV oncogene LMP1 induces HLA class I APM components. Here, we found that stable transfection of epithelial cells with LMP1 lead to a strong up-regulation of the HLA class I APM as has been reported for B cells (Rowe *et al*, 1995). At the same time, LMP1 induced c-Myc, which has been shown to down-regulate HLA class I APM expression (Versteeg *et al*, 1988; Ottesen *et al*, 1990; Belldegrun *et al*, 1993; Staege *et al*, 2002) inducing a non-immunogenic phenotype.

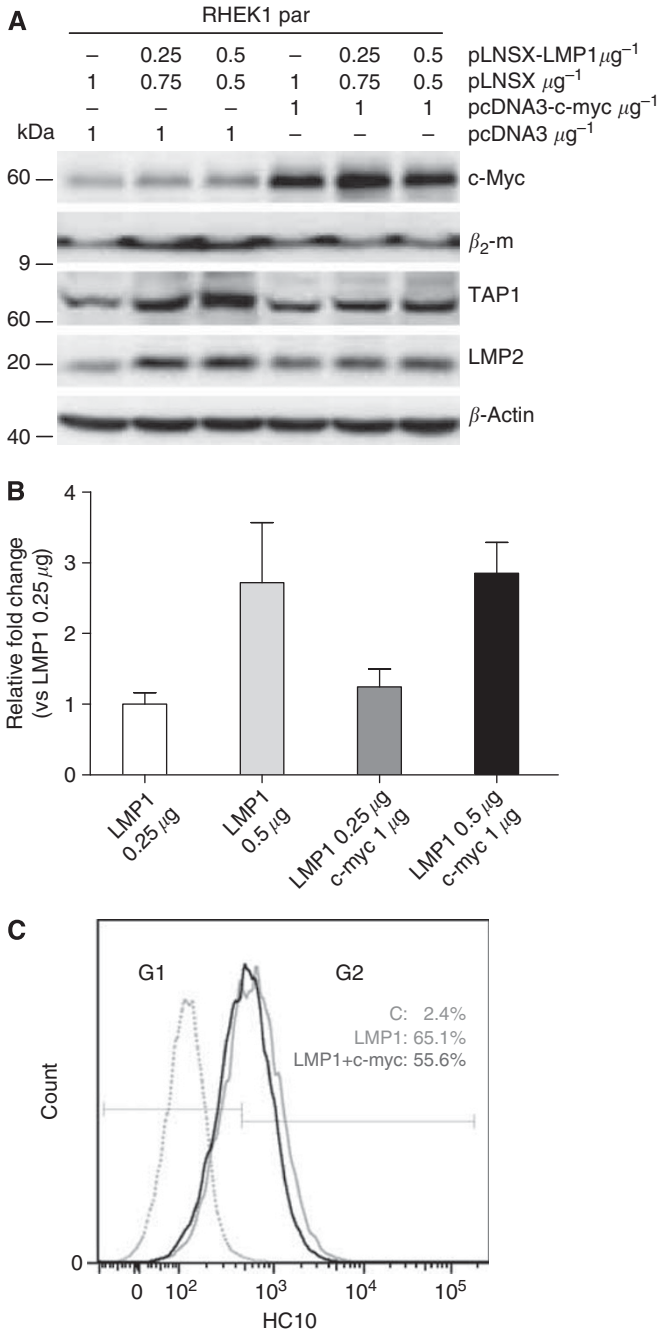

Figure 3 Inhibitory effect of c-myc on LMP1-mediated induction of HLA class I APM components in cotransfection experiments. (**A**) Western blot analysis showing expression of β_2-m, TAP1 and LMP2 after transient transfection with LMP1 or c-myc alone or after cotransfection with LMP1 and c-myc. As shown in the stably transfected cell lines, transient transfection with LMP1 leads to an induction of β_2-m, TAP1 and LMP2. The inductive effect of LMP1 is reduced when cotransfected with c-myc. One representative experiment of 3. (**B**) Comparison of LMP1 levels after transfection with LMP1 alone or after cotransfection with c-myc by qRT–PCR. Comparative levels of LMP1 were seen in the single transfection and cotransfection. One representative experiment of 3. (**C**) Flow cytometric anaylsis of RHEK1 parental comparing the transient transfection of LMP1 (0.5 μg) alone and the cotransfection of LMP1 (0.5 μg) and c-myc (1 μg). A significant inhibitory effect ($P=0.049$; $n=5$) of c-myc on the inductive effect of LMP1 on HLA class I HC (HC10) is seen. The dotted line represents the control staining without primary antibody. The solid light/dark line shows HC10 expression in transiently LMP1/c-myc and LMP1 cotransfected RHEK1 cells. The percentage of positive cells in gate G2 is indicated in the control (C), in LMP1-transfected cells (LMP1) and in LMP1 and c-myc cotransfected cells (LMP1 + c-myc) for the representative experiment of 5.

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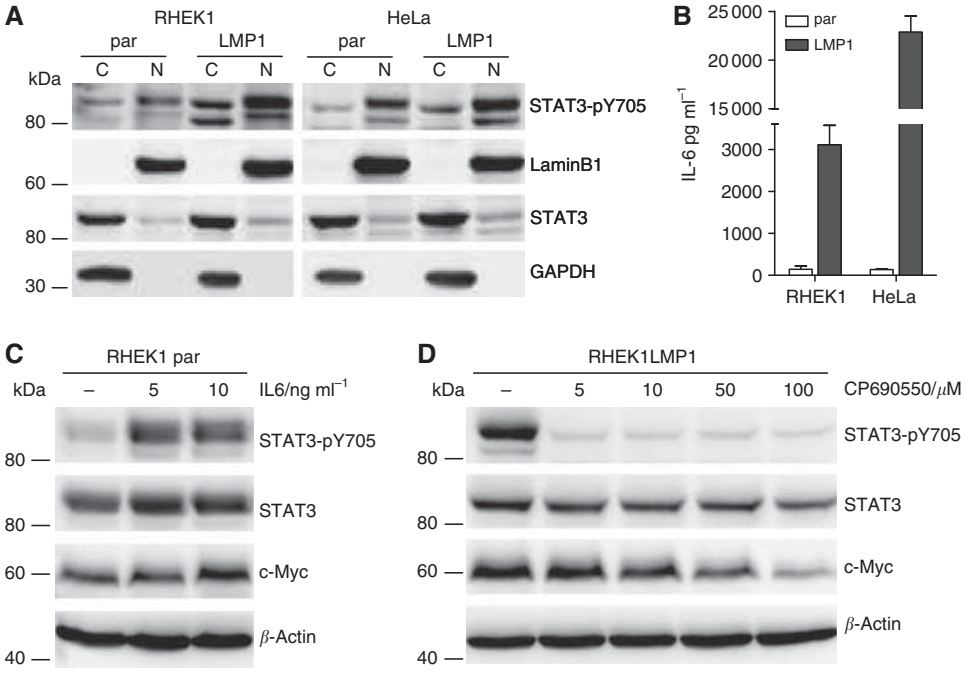

Figure 4 Interleukin-6 and STAT3 as mediators of the c-Myc regulation in epithelial cells. (**A**) Western blot analysis of the expression of STAT3 and STAT3-pY705 phosphorylation status in nuclear and cytoplasmic fractions of RHEK1 and HeLa cells depending on the presence of LMP1. Stable transfection with LMP1 leads to a mild induction of cytoplasmic STAT3 and to a pronounced induction of cytosolic and nuclear STAT3-pY705 phosporylation. One representative experiment of 3. (**B**) ELISA analysis of the effect of stable LMP1 transfection on the expression of IL6. A strong induction of secreted IL6 is observed in both RHEK1 and HeLa cells. Mean values and standard deviations of three independent experiments. (**C**) Western blot analysis of the effect of recombinant human IL6 on STAT3, STAT3-pY705 phosphorylation and c-Myc in RHEK parental. IL6 leads to a mild up-regulation of STAT3, c-ymc and a strong induction of STAT3-pY705 phosphorylation. One representative experiment of 3. (**D**) Western blot analysis showing the effect of the JAK3 inhibitor CP690550 on STAT3, STAT3-pY705 phosphorylation and c-Myc. Incubation of RHEK1/LMP1 with CP690550 for 48 h leads to strong reduction of STAT3-pY705 phosphorylation and to a reduction in STAT3 and c-Myc expression in a dose-dependent manner. One representative experiment of 3.

Therefore, c-Myc was a candidate to counteract the immunogenicity of LMP1. Indeed, c-Myc had an opposing effect on HLA class I APM expression seen as an up-regulation of the HLA class I APM after c-myc knockdown and an inhibition of the inductive effect of LMP1, when LMP1 and c-myc were coexpressed. Additionally, we found that STAT3 might mediate this effect, as LMP1 strongly up-regulated STAT3 phosphorylation. STAT3 activation can be achieved directly via JAK3 activation (Gires *et al*, 1999) or via secreted mediators in an autocrine or paracrine manner. Interleukin-6 is thought to be involved in a positive feedback loop of LMP1 expression and STAT3 activation (Chen *et al*, 2003). Moreover, IL6 is found in a subset of EBV-associated NPC cases in tumour cells and/or the infiltrate (Okamoto *et al*, 1997; Hirano *et al*, 2000; Beck *et al*, 2001; Lesina *et al*, 2011). We showed that LMP1 induced IL6 secretion in agreement with earlier reports (Eliopoulos *et al*, 1997) and that IL6 induced c-Myc expression and STAT3 activation. Whereas JAK2 inhibitors only weakly inhibited STAT3 phosphorylation (data not shown), the JAK3 inhibitor CP690550 had a strong negative effect on STAT3 phosphorylation and, in a dose-dependent manner, on c-Myc expression, indicating an important role of JAK3/STAT3 in c-myc regulation in this context. This is supported by an earlier observation showing that JAK3 is directly activated by LMP1 leading to STAT3 phosphorylation (Gires *et al*, 1999).

Taken together, we propose a model in which LMP1 induces the HLA class I APM in epithelial cells and at the same time opposes this effect by activating c-Myc via JAK3 and STAT3, which in turn down-regulates HLA class I APM. In this scenario, LMP1 could counteract its own immunogenicity.

To test the relevance of these findings *in vivo*, we investigated the expression of HLA class I APM components and of c-Myc in a small number of NPC biopsies. Previous studies examining the expression of HLA class I APM in NPC have produced conflicting results. While normal levels of LMP2, LMP7, TAP1, TAP2 and HLA class I alleles on NPC tumour cells have been described in one study (Khanna *et al*, 1998), others have shown heterogeneous expression with a partial loss of HLA-ABC in almost 50% of NPC cases (Lai *et al*, 1990). A genome-wide expression profiling study identified a positive correlation between EBV status and reduced MHC class I expression in NPC (Sengupta *et al*, 2006). Ogino *et al* (2007) found a down-regulation of HLA class I HC, tapasin, LMP2 and TAP1 in NPC, although no association was observed with respect to LMP1 status. Consistently, we detected down-regulation of tapasin in NPC cases. Additionally, we found a selective down-regulation of HLA-A and no evidence of HLA-B reduction as has been observed in Burkitt lymphoma (Masucci *et al*, 1987). Demanet *et al* (2004) reported a selective down-regulation of HLA-A and HLA-Bw6, but not of HLA-Bw4 and suggested that this effect might help to evade CTL and NK cells simultaneously, as HLA-Bw4 can inhibit NK cell function. Hypothetically, a similar effect using divergent functions of different HLA class I alleles in immune escape might be operational in NPC explaining the observed differential regulation of HLA class I subtypes.

The TAP2 and LMP7 were found to be down-regulated in NPC, which were either not investigated (Ogino *et al*, 2007) or unaltered in earlier studies (Khanna *et al*, 1998). In the latter study, lymphoblastoid cell lines served as controls compared with nasopharyngeal epithelium in our study, which might explain some of the difference. In contrast to previous findings (Ogino *et al*, 2007), TAP1 and LMP2 were not down-regulated but showed a low expression level in tumours and control tissues. The concomitant low levels of TAP1 and LMP2 could be explained by the regulation of both genes by the same bidirectional promoter (Wright *et al*, 1995). In agreement with Ogino *et al*, we observed no association between LMP1 and HLA class I APM expression, which contrasts with the findings in EBV-positive Hodgkin's

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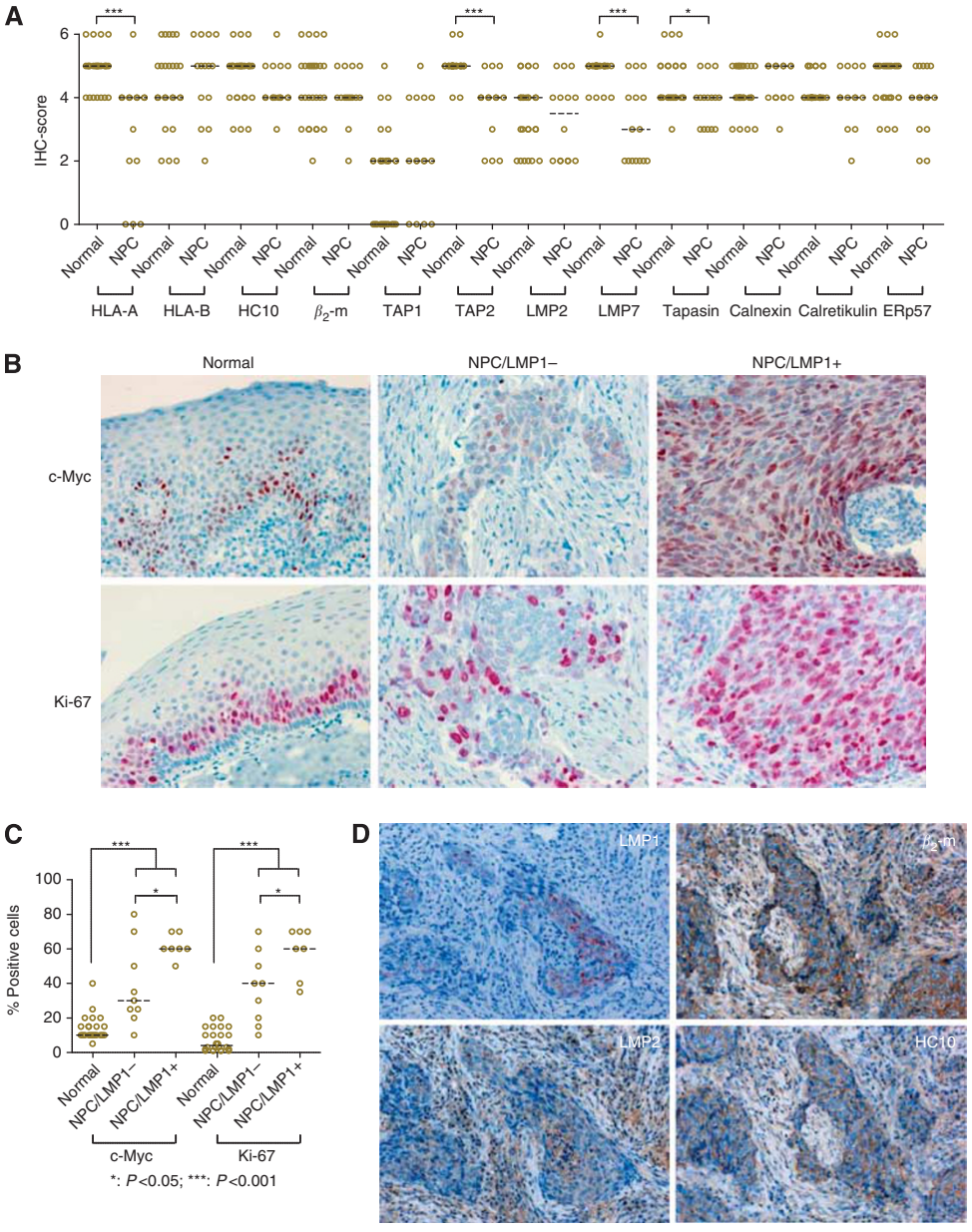

Figure 5 Down-regulation of HLA class I APM components in NPC and up-regulation of c-Myc in LMP1 + NPC. (**A**) Immunohistochemical scores of HLA class I APM components comparing normal tissue (columnar and squamous epithelium) with all NPC cases. HLA-A, TAP2, LMP7 and tapasin were down-regulated in NPC compared with control tissues. Expression of TAP1 and LMP2 was low but comparable in both NPC and controls. The median is indicated with a black dotted line. (**B**) c-Myc and Ki-67 immunohistochemistry of normal nasopharyngeal epithelium and one LMP1 − NPC showing a low expression of both markers compared with one LMP1 + NPC with high expression of c-Myc and Ki-67. Magnification × 400. (**C**) Immunohistochemical comparison of the nuclear expression of Ki-67 and c-Myc shown as the percentage of positive nuclei. All normal tissues are compared with LMP1 + and LMP1 − NPC. Ki-67 and c-Myc are up-regulated in all NPC compared with controls and in LMP1 + compared with LMP1 − NPC. The median is indicated with a black dotted line. (**D**) Serial sections of one LMP1 + NPC showing a patchy staining of LMP1, whereas the stainings for β_2-m, LMP2 and HLA class I HC (HC10) are diffuse. Numerous inflammatory cells in the background express the components of the HLA class I APM. Magnification × 400.

disease, where LMP1 is expressed at high levels (Murray *et al*, 1998).

As LMP1 is usually expressed focally in NPC, we set out to determine whether the lack of an LMP1 − specific effect was a consequence of this. In serial sections of NPC, we indeed found a focal/patchy pattern of LMP1 expression. Expression of HLA class I APM components, however, was diffuse. Therefore, we concluded that any possible effect of LMP1 on the HLA class I APM was not focal maybe due to paracrine effects (e.g., IL6) of LMP1 or the activation of CD40 expressed on LMP1 − NPC cells (Agathanggelou *et al*, 1995) by surrounding inflammatory cells. Going in line with the *in-vitro* findings, LMP1 + NPC showed increased expression of c-Myc compared with negative cases. This might explain the failure of LMP1 to induce HLA class I APM expression. The histological findings, however, have some limitation because of the small number of investigated NPC cases. However, an earlier observation that STAT3 phosphorylation in NPC biopsies was independent of LMP1 (Buettner *et al*, 2006) indicates that the interacting signalling pathways in NPC are more complex than in the *in-vitro* system. Moreover, the

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phosphorylation of STAT3 might also be transient, so that differences could not be detected by immunohistochemistry.

This model will not explain the entire mechanisms involved in HLA class I APM regulation in NPC as many other factors like the surface expression of CD40 in NPC and the expression of cytokines in the surrounding inflammatory cells will interact with this regulatory circuit. However, it may explain in part the lack of a difference in HLA class I APM expression in LMP1 + and LMP1 − NPC. We speculate that overexpression of c-Myc by LMP1 may counteract or reduce the effects of LMP1 on HLA class I APM expression in epithelial cells.

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