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"NULL" PHENOTYPE OF PERSISTENTLY KSHV-INFECTED B-LYMPHOCYTES

vorgelegt von

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Blood. 2002 Jul 15;100(2):483-90.

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Application of molecular analysis in the diagnosis and therapeutic control of lymphoproliferative disorders, *Balkan Journal of Medical Genetics*, 1999, vol. 2(1), 13-16

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DNA analysis for determining the clonal character of the cell proliferation in B-cell lymphoproliferative disorders, *Balkan Journal of Clinical Laboratory*, 1998, 1, 27-31

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Application of DNA analysis to assess cell proliferative clonality in B-CLL, *Clinical and Transfusion Haematology* 1997**,** vol. 33, N 3-4, p.18-22

1. Summary

Kaposi´s sarcoma-associated herpesvirus (KSHV) is involved in the pathogenesis of Kaposi´s sarcoma (KS) and some atypical B-cell lymphomas. The aim of this study was to investigate the influence of KSHV-infection on cellular gene expression in B-lymphocytes. In order to study the gene expression profile a microarray screen was performed using B-cells that have been persistently infected with KSHV *in vitro*. A considerable number of genes (408) were found to be modulated more than 4-fold by KSHV infection, 275 (67.4 %) were downregulated whereas 133 (32.6%) were upregulated. A multitude of the downregulated genes encoded for B-cell surface markers and several B-cell specific transcription factors (PAX-5, Oct-2 and Spi-B) which was confirmed by RT-PCR and on the protein level. The massive loss of B-cell surface markers or "null" phenotype, was similar to tumor cells isolated from primary effusion lymphoma (PEL). Thus, the loss of B-cell identity is due to KSHV-infection and not to cellular tumor-promoting events. Moreover, this study demonstrated that the "null" phenotype is caused by a soluble factor(s) released from KSHV-infected B-cells. The downregulation of B-cell markers led to severe functional defects, as KSHV-infected B-cells could not be activated by crosslinking of the B-cell receptor. Importantly, KSHV-infected cells could not be lysed by allo-reactive cytotoxic T-cells, suggesting that the "null" phenotype serves as a mechanism for immune escape.

2. Introduction

2.1 Kaposi`s sarcoma-associated herpesvirus

2.1.1 Discovery and phylogeny

KSHV was discovered by Chang and colleagues by representational difference analysis as unique sequences present in more than 90% of Kaposi´s sarcoma (KS) tissues, obtained from patients with acquired immunodeficiency syndrome (AIDS) (Chang et al., 1994). They found that these sequences were homologous to, however, distinct from capsid and tegument genes of gammaherpesviruses and appeared to define a new human herpesvirus, Kaposi´s sarcoma-associated herpesvirus (KSHV) or Human Herpesvirus-8 (HHV-8) (Fig.1).

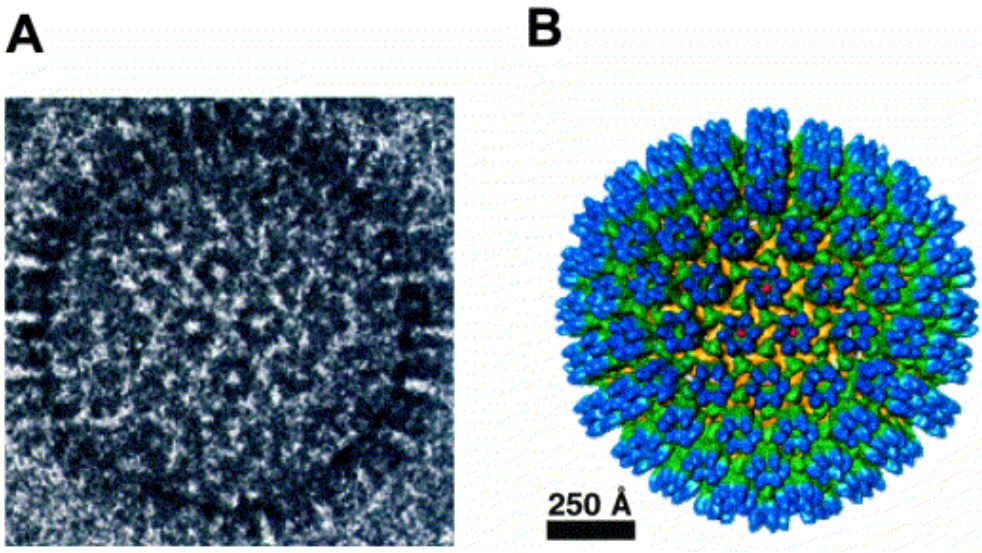
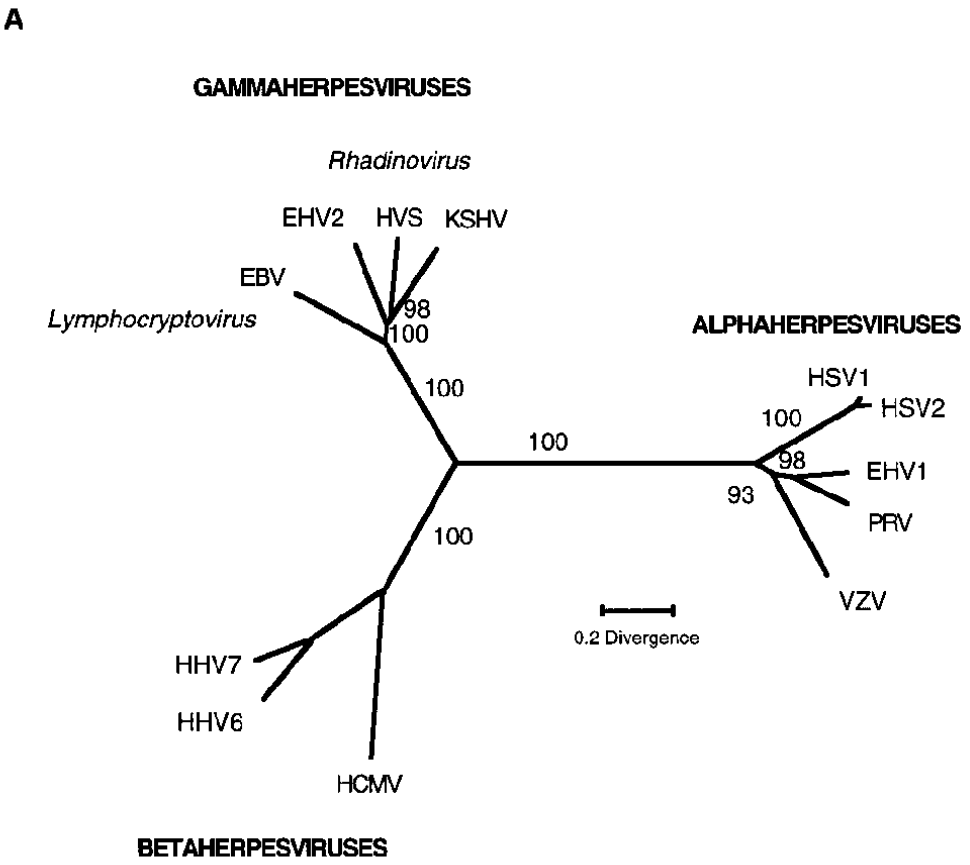


Fig. 1: Three-dimensional (3D) structure of the KSHV capsid (according to Jenner and Boshoff, 2002).
A: Cryoelectron photography of the KSHV capsid. B: 3D reconstruction of the KSHV capsid. The capsid is an icosahedral protein shell made up of pentons and hexons (blue) and triplexes (green). The pentons and hexons are also visible in the photography.

Phylogenetic analyses of molecular sequences showed that KSHV belongs to the gamma-2 sublineage of the *Gammaherpesvirinae* subfamily and thus represents the first human gamma-2 herpesvirus (Moore et al., 1996). Its closest known relative on the basis of available sequence comparisons is Herpesvirus Saimiri (HVS), a squirrel monkey gamma-2 herpesvirus that causes polyclonal T-cell lymphoproliferative disorders in some New World monkey species (Moore et al., 1996). Only KSHV, HVS and Equine Herpesvirus 2 (EHV2)

could be placed on the phylogenetic tree with precision, thus representing a lineage of primate gamma-2 viruses. Previously, McGeoch and colleagues proposed that lines of gamma-2 herpesviruses may have originated by co-speciation of the virus and the host lineages (McGeoch et al., 1995). Based on this view it was concluded that KSHV and HVS have diverged at an ancient time, possibly simultaneously with the divergence of the Old World and New World primate host lineages. Gammaherpesviruses are distinguished as a subfamily by their lymphotropism which is supported by phylogenetic analyses based on sequence data (Roizmann et al., 1992, McGeoch et al., 1995). The biological behaviour of KSHV is consistent with its phylogenetic designation in that KSHV infects B-lymphocytes *in vitro* (Cesarman et al., 1995) and *in vivo* (Ambroziak et al., 1995).



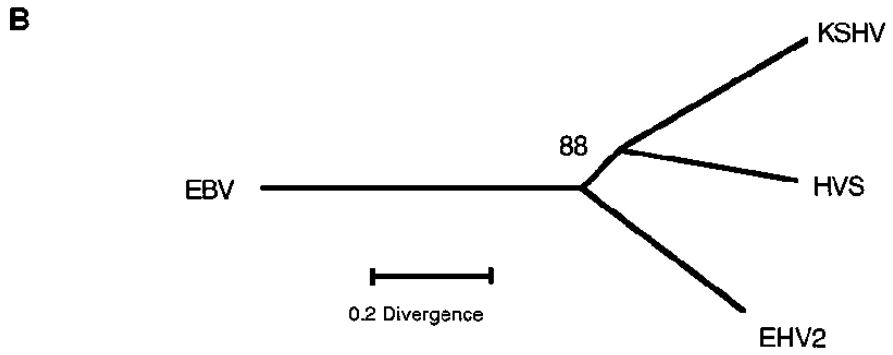


Fig. 2: Phylogenetic trees of herpeseviruses based on a comparison of aligned amino acid sequences (according to Moore et al., 1996).
A: Comparison of major capsid protein (MCP) sequence homologues from KSHV and 12 members of the family *Herpesviridae*. This MCP set assigns the KSHV homologue to gamma-2 sublineage (genus *Rhadinovirus*), containing HVS, EHV2 and bovine herpesvirus 4. B: Phylogenetic tree of gamma herpesvirus sequences based on a nine-gene set CS1 (ORFs 21-26, ORF 29a, -b, 31, 34) demonstrates also that KSHV is most closely related to the gamma-2 herpesvirus sublineage.

2.1.2 KSHV genome

Like other herpesviruses, KSHV is a large double-stranded DNA virus that is located in the cellular nucleus as a closed circular episome during latency. During the lytic phase the virus replicates by a rolling circle mechanism and linear genomes are generated and packaged. During mitosis the KSHV genome is tethered through the terminal repeat (TR) sequences to histone H1 at the host chromatin by the KSHV-encoded latent nuclear antigen LANA-1 (Ballestas et al., 1999, Cotter II and Robertson, 1999, Ballestas and Kaye, 2001). KSHV contains a long unique region of 140 kb size which is flanked by two 20-35 kb terminal repeat regions of 801 bp size and high G+C content (Russo et al., 1996). Currently, 89 genes have been identified in the long unique region, but new open reading frames (ORFs) are still described as previously unknown gene products from alternative reading frames or splicing events (Moore and Chang, 2001).

KSHV shares structural and biological features with Epstein-Barr virus (EBV) but possesses none of its latent genes involved in cell immortalisation and transformation (Russo et al., 1996). KSHV and EBV target many of the same cellular pathways, but use different

strategies to achieve the same effects (Moore and Chang, 2001). Cellular genes like cyclin D2 are induced by EBV (via latent membrane protein-1, LMP-1) but have been pirated by KSHV during its evolution (Arvanitakis et al., 1995). This is not unexpected because both viruses use B-lymphocytes as reservoirs during latency and face similar biological challenges in establishing persistent infections in a B-cell environment. In contrast to EBV, KSHV also infects endothelial cells, as demonstrated in KS tumors (Boshof et al., 1995). Moreover, KSHV is able to infect macrophages and epithelial cells (Blasig et al., 1997, Diamond et al., 1998).

It has become apparent soon after the discovery of the virus that it displays a highly restricted pattern of gene expression in infected cells which is one of the hallmarks of latent infection (Zhong et al., 1996). Since latent KSHV genes are expressed in the majority of spindle and PEL cells, these genes probably play a major role in the pathogenesis of KSHV-associated cancer. The full repertoire of viral gene expression occurs only during lytic replication, when viral progenies are produced and the host cell is destroyed (Renne et al., 1996). The study of KSHV has benefited largely from cell lines established from primary effusion lymphoma (PEL) (see next chapter). These cells harbour the virus in a latent form and can be induced to enter lytic replication by treatment with different agents like sodium butyrate or phorbol-ester-12-*O* tetradecoylphorbol 13 acetate (TPA) (agents normally used for EBV to induce viral replication). A number of studies described the KSHV gene expression in latency and lytic replication (Zhong et al., 1996, Sarid et al, 1998, Sun et al., 1999, Zhu et al., 1999). KSHV transcripts have been categorized into three classes based on their expression in uninduced and lytically induced PEL (BC-1) cells: class I (constitutive), class II (present in uninduced cells but upregulated by TPA), and class III (only present after induction). These categories of viral genes were observed in PEL cells using DNA array technology (Jenner et al., 2001). Latent KSHV genes detected in uninduced cells are v-FLIP (ORF71), v-cyclin (ORF72), LANA-1 (ORF73), K7, T1.1 (*nut*-1), kaposin (K12), vOx-2 (K14) and vIRF-3 (K10.5 and 7). The latent gene products generally act in a cell-autonomous manner (not affecting neighbouring cells) and target the cell cycle and apoptosis. The gene expression pattern of KSHV suggests that the latent genes are involved in the cell transformation process.

The genes expressed during the lytic phase have been categorized in three groups (Jenner et al, 2001). P*rimary lytic genes* include K4 (v-MIP-II), ORF 9 (DNA polymerase), ORF 74 (vGPCR/vIL-6 receptor homologue), K12 (kaposin), ORF 16 (vBCL-2), *secondary lytic genes* are K9 (vIRF-1), vIRF-2, K10.1 (potential IRF homolog), ORF 17 (protease/

aasembly protein), ORF 64 (tegument protein) ORF 65 and ORF 17 (small viral capsid antigens) and *tertiary lytic gene* are K1, K8.1 (glycoprotein gp35-37), ORF 25 (major capsid protein) and ORF 29a (packaging protein). The genes expressed during the lytic replication have two broad functions: production of viral progeny and inhibition of the host antiviral response. The majority of genes, belonging to the latter functional group are homologous to host genes. The products of these genes tend to act in a non-autonomous manner (affecting neighbouring cells) or by inhibiting intracellular signalling. For example, vIL-6, vMIPa and vGPCR are angiogenic, while K1, K3 and K5 may prevent attack by the immune cells. The small fraction of KSHV-infected cells in early stage KS lesions suggests that the phenotypical changes observed in these tumors are caused by paracrine mechanisms.

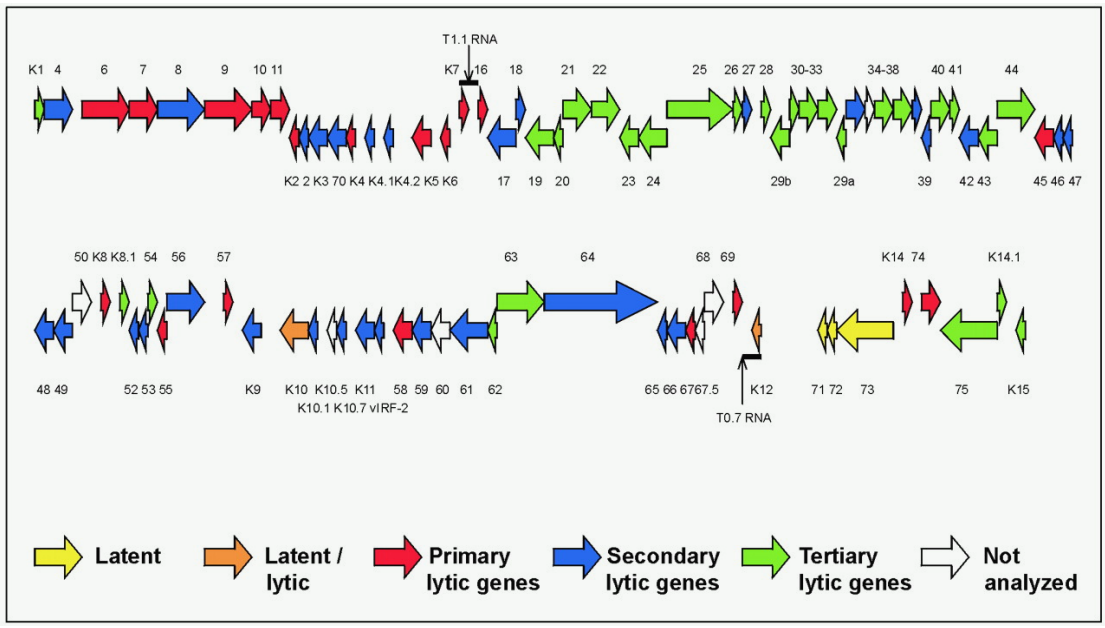


Fig. 3: Map of the different type of KSHV genes (according to Jenner et al, 2001).
Each ORF is color coded according to it´s expression pattern: latent (class I), latent/lytic (induced by TPA), primary lytic genes, and tertiary lytic genes.

2.1.3 Diseases associated with KSHV

The expression profile of KSHV points to viral proteins which are involved in cellular transformation (Jenner et al., 2002). KSHV has been shown to be specifically associated with all forms of KS and has also been detected in most cases of primary effusion lymphoma

(PEL) and in the plasmablastic variant of Multicentric Castleman`s disease (MCD) (Cesarman et al., 1995, Soulier et al., 1995, Dupin et al., 2000).

2.1.3.1 Kaposi´s sarcoma (KS)

An important feature of KS is the occurrence of so-called *spindle cells* and the presence of KSHV in these cells. Recent studies based on gene expression array analysis, proposed that the neoplastic cells from KS are closely related to lymphatic endothelial cells (LECs) (Wang et al., 2004, Hong et al., 2004). KSHV also infects blood vascular endothelial cells (BECs) *in vitro* and *in vivo* and induces reprogramming towards LEC. Spindle cells thus mainly express LECs markers and only low levels of BECs markers (Weninger et al., 1999, Wang et al., 2004, Hong et al., 2004). Some of the spindle cells express proteins characteristic for smooth muscle cells, macrophages and dendritic cells or several of these at once, which on the contrary suggests that the cells most likely derive from a multipotent precursor cell whose progeny gives rise to hematopoietic and endothelial cells (Jenner and Boshoff, 2002). In early KS lesions only around 10% of *spindle* and endothelial cells are KSHV-positive, implicating that paracrine mechanisms are involved in the progression of the disease (Dupin et al., 1999). In late stage nodular lesions around 90% of the spindle cells contain KSHV, suggesting that the virus provides a growth advantage to infected cells (Boshoff et al., 1995, Sturzl et al., 1999). These observations support the hypothesis that at an early stage KS is a polyclonal hyperplasia which develops only into a true clonal malignancy as the disease progresses. A recent report indicated that KSHV TR sequences in nodular lesions display all patterns of clonality (mono-, oligo-, and polyclonal), suggesting that the disease begins as a polyclonal hyperplasia and develops into a monoclonal tumor (Judde et al., 2000).

2.1.3.2 Primary effusion lymphoma (PEL)

Soon after the discovery of KSHV in KS, Cesarman and co-workers found that KSHV is also specifically associated with a rare form of lymphomas, PEL. PEL normally manifests as malignant effusions in the pleural, pericardial or peritoneal cavities, but without significant tumor mass. The majority of PEL patients are also HIV positive with advanced immunosupression. The pathogenesis of PEL is not fully understood. The specific association between PEL and KSHV suggests that the virus is pathogenetically related to the disease (Cesarman et al., 1995a, Pastore et al., 1995, Karcher et al., 1995, Carbone et al., 1996, Nador et al., 1996). This is supported by the fact that these lymphomas show very high amounts of

viral DNA ranging between 40 and 80 copies per cell, in contrast to KS tissues which contain less than one copy per cell (Cesarman et al., 1999) In addition, cases of KSHV-positive PELs in HIV-negative males and females have been identified. However, the factors and mechanisms leading to malignant transformation of B-cells are still unclear. Several lines of evidence suggest that KSHV infection may play a central role in the development of PEL. As mentioned above, KSHV carries several genes, which may act as oncogenes including genes homologues to BCL-2 (ORF16), to cellular D-type cyclins (ORF72/cyclin D) and a G-protein coupled receptor displaying constitutive activation (ORF74/GPCR).

PEL has been recognized as a B-cell malignancy that is morphologically and histologically different from all known B-cell tumors or normal B-cells (Fig.4) . Most PELs are thought to originate from post-germinal centre B-cells due to the presence of hypermutated immunoglobulin genes and markers of a late stage of B-cell differentiation (Gaidano et al., 1997). Most of the cases of PEL have functional immunoglobulin (Ig) gene rearrangements and transcribe Ig mRNA, but most do not express Ig protein (Cesarman et al., 1999). The PEL tumor cells display an intermediate immunophenotype between immunoblasts and plasma cells (Nador et al., 1996, Carbone et al., 1996, Ansari et al., 1996). In most cases they express CD45, consistent with the hematopoietic cell derivation, but lack surface Ig and B- or T-cell associated antigens (Drexler et al., 1998). The tumor cells were also found to express the surface markers CD30, CD38, CD71, epithelial membrane antigen (EMA), CD138-syndecan-1 and MUM1/IFN regulatory factor 4, which is associated with the late stages of B-cell differentiation (Gaidano et al., 1997, Carbone et al., 2000). Based on these findings it can be concluded that PELs posses morphological features similar to transformed plasma cells (Jenner et al., 2003, Klein et al., 2003). PEL is also thought to be monoclonal due to the presence of clonal immunoglobulin gene rearrangements (Nador et al., 1996) and monoclonal viral TRs (Judde et al., 2000). Recently it was shown that PEL cells synthesize the cytokine IL-6, and IL-6 receptors. The application of antisense oligonucleotides inhibited the clonal proliferation of PEL cells, which suggests that IL-6 is an autocrine growth factor for these cells (Asou et al., 1998).

The majority of PEL cells are co-infected by both KSHV and EBV. The EBV infection is monoclonal in PELs, suggesting that EBV infection has preceded clonal expansion of tumor cells (Matolcsy, 1999). The analysis of the pattern of EBV latent gene expression has revealed a restricted latency with expression of EBNA1 (Horenstein et al., 1997). EBNA1 is a gene required for replication and maintenance of viral episomes but not known to be directly tumorigenic in EBV-associated malignancies (Klein et al., 1989). On the

other hand, it has been found that EBNA1 can induce B-cell lymphomas when expressed in mouse B-cells as a transgene, concluding that EBNA1 may contribute to neoplastic transformation in the PEL cells (Wilson et al., 1996). However, the finding that EBV does not express the EBV nuclear antigens EBNA-2 and -3 and only low levels of LMP-1 makes it unlikely that EBV is directly driving PEL cell proliferation (Callahan et al, 1999).

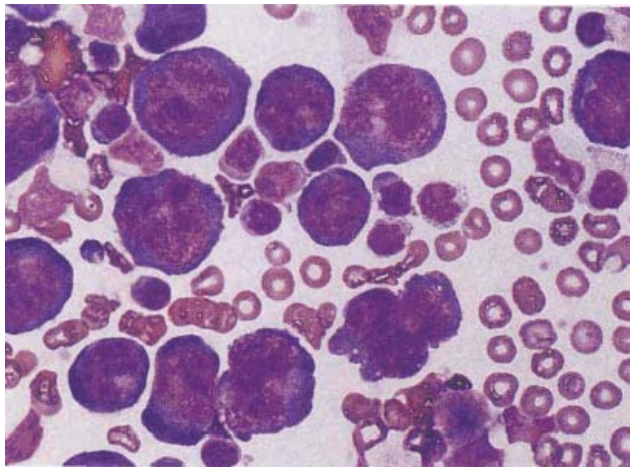


Fig. 4: KSHV in primary effusion lymphoma (according to Nador and colleagues, 1996). Wright-Giemsa-stained air dried cytocentrifuge preparation of a KSHV-positive primary effusion lymphoma (PEL). The cells are considerably larger than normal benign lymphocytes and red blood cells.

2.1.3.3 Multicentric Castleman´s disease (MCD)

MCD is an unusual lymphoproliferative disorder which is more frequent in HIV-infected individuals. MCD is closely associated with KS which has led Soulier and colleagues to search, and find KSHV DNA in MCD tissue (Soulier et al., 1995). KSHV was found to be present in all cases of MCD in AIDS patients and in approximately half of those in HIV-negative individuals. KSHV-positive MCD constitutes a distinct subset of MCD, named plasmablastic MCD, which contains large plasmablastic cells, harbouring KSHV (Dupin et al., 1999, Katano et al., 2000, Boshoff and Weiss, 2001). Unlike PEL cells, coinfection by EBV has not been detected in MCD plasmablasts. Despite the invariable expression of cytoplasmic IgMλ, recent studies examining the number of KSHV TRs or immunoglobulin rearrangements indicate that MCD plasmablasts are polyclonal in origin (Du et al., 2001).

Furthermore, the lack of somatic mutations in the rearranged Ig heavy and light chain genes indicates that they originate from *naive* B-cells (Du et al., 2001, Hamoudi et al., 2004).

2.2 Herpesviruses and immune response

2.2.1 Natural killer (NK) and Cytotoxic T cell (CTL) response

Herpesviruses constitute a family of human pathogens that indefinitely persist in the host organism and often contribute to a variety of disease states. Viral persistence depends on the ability of herpesviruses to establish a latent infection within cells. This process requires the successful evasion of the host immune defence, which usually includes the restricted expression of viral genes during latency. Although such restriction lowers their overall antigenic profile, herpesviruses must employ additional mechanisms to ensure prolonged protection from host immune response. One such mechanism involves the downregulation of immunoregulatory proteins, such as major histocompatibility complex (MHC) class I, on the surface of infected cells. This serves to limit their recognition by cytotoxic T lymphocytes (CTLs) and dampen the inflammatory response to viral infection. Examples of human herpesvirus proteins that downregulate MHC class I include the ICP47 protein of Herpes Simplex Virus 1 (*HSV1*, Ahn et al., 1996, Goldsmith et al., 1998), the vIL-10 protein of EBV (Salek-Ardakani et al., 2002), and the US2,-3,-6 and -11 proteins of human cytomegalovirus (*HCMV*, Ahn et al., 1997, Jun et al., 2000). The virus-specific CD8+ CTL response is probably the main mediator of herpesvirus clearance. A major characteristic of the cytotoxic T-cells is their ability to kill target cells expressing specific MHC class I peptide complexes (Germain et al., 1994, Jondal et al., 1996). They express a range of effector molecules that mediate defence against pathogens. Such an example is the direct cytolysis of target cells mediated by *perforin* release (Kagi et al., 1994, Walsh et al., 1994, Lowin et al., 1994) and *Fas ligand* (Rouvier et al., 1993, Kagi et al., 1995). CTLs also secrete *cytokines* (tumor necrosis factor-*TNF*) and *interferon-*γ, as well as *chemokines* that function to recruit and/or activate the immuno-effector cells such as macrophages and neutrophils (Harty et al., 1999). Many of the effector mechanisms employed by CTLs are also employed by other cells of the immune system, for example Natural Killer (NK) cells. They are also involved in the killing of tumor or virally infected cells (Biron et al., 1997). In addition, NK cells express *perforin* and *Fas Ligand* and are capable of cytolysis (Harty et al., 2000, Taylor et al., 2002). Moreover, they release factors involved in the immune defence including TNF and the chemokine-macrophage inflammatory protein 1α (*MIP-1*α, or *CCL3*, Biron et al., 2001). The

latter are known to have the potential of promoting defence by driving inflammatory processes. NK cells spare normal cells expressing adequate amounts of MHC class I molecules, but kill transformed cells that have a lower level of MHC class I or that have lost its expression. Thus, the altered expression of class I antigens, a common event in tumor transformation or following viral infection, leads to NK mediated target lysis (Garrido et al., 1997, Salcedo et al., 1998, Ploegh et al., 1998).

2.2.2 Important cytokines involved in the immune defence

2.2.2.1 Interleukin 6 (IL-6)

IL-6 is a multifunctional cytokine which is produced by both lymphoid and non-lymphoid cells and regulates immune response, acute-phase reaction and hematopoiesis (Bowcock et al., 1989, Hirano and Kishimoto, 1990). IL-6 has been identified in virus-infected cells and is also known as *interferon-ß2* (*IFN-ß$_2$*; Weissenbach et al., 1980) or hybridoma/plasmacytoma growth factor (*HPGF, IL-HP1*, Aarden et al., 1985, Nordan and Potter, 1986a, Van Damme et al., 1987a,b, Van Snick et al., 1987). Separate studies on the differentiation of human B-cells into immunoglobulin secreting cells has led to its characterization as a B-cell differentiation factor, termed B-cell-stimulatory factor (BSF). One of the important features of IL-6 is the ability to control growth and proliferation of early hematopoietic progenitor cells and its co-stimulatory effect on thymocytes and mature T-cells, which shows that IL-6 may be involved in sustaining the immune response. IL-6 is synthesized by a larger number of cell types including monocytes and macrophages, T-cells and fibroblasts, and epithelial cells.

2.2.2.2 Interleukin 10 (IL-10)

IL-10 has been described first as cytokine synthesis inhibitory factor (CSIF), which is produced by mouse Th2-cells and inhibits activation of and cytokine production by Th1-cells (Fiorentino et al., 1989). The ability of IL-10 to inhibit cytokine production by both T- and NK-cells was found to be indirect via an inhibition of accessory cell (monocyte/macrophage) function (de Waal et al., 1991, Fiorentino et al., 1991, Ding et al ., 1992, Hsu et al., 1992). It was shown that IL-10 profoundly inhibits a broad spectrum of monocyte/macrophage functions and the expression of MHC class II and co-stimulatory molecules such as IL-12 and B7-1/B7-2 (CD80/CD86) (Bogdan et al., 1991, Fiorentino et al., 1991, Ding et al., 1993, Murphy et al., 1994). *In vitro* and *in vivo* studies with recombinant cytokine and neutralizing

antibodies have revealed pleiotropic activities of IL-10 on B-, T- and mast cells (Go et al., 1990, MacNeil et al., 1990, Rousset et al., 1992, Thompson-Snipes et al., 1991). An inflammatory response exhibited by IL-10 deficient (IL10-/-) mice indicated that a critical *in vivo* function of IL-10 is a limitation of inflammatory response. The effect of IL-10 on survival, proliferation and differentiation of human B-cells has been extensively studied as well. IL-10 enhances the survival of normal human B-cells (depending on their activating stage), which correlates with an increased expression of the anti-apoptotic protein Bcl-2 (Levy et al., 1994, Itoh et al., 1995). IL-10 also induces the telomerase reverse transcriptase (hTERT) expression and upregulates telomerase activity in B-cells activated by anti-IgM (Hu and Insel, 1999). Moreover, it is a potential factor for proliferation of human B-cell precursor and mature B-cells activated by surface immunoglobulin (*anti-IgM or SAC*), or CD40 cross-linking (Rousset et al., 1992, Saeland et al., 1993).

2.2.3 KSHV and immune response

KSHV elicits a humoral and cellular host immune response directed against both lytic and latent proteins of the virus (Brander et al., 2001). This response, even in healthy persons, is unable to eradicate KSHV from the organism, suggesting that KSHV, like other herpesviruses, possesses the ability to evade the immune response during infection.

KSHV most often follows the general paradigm of herpesvirus infection, a latent phase of infection marked by a highly restricted pattern of viral gene expression in the majority of cells. The prevalence of KSHV-infected cells undergoing lytic (productive) infection both *in vitro* and *in vivo* is typically low (1-5%), with the remaining infected cells harbouring the virus in its latent form (Katano et al., 1997; Dittmer et al., 1998; Parravicini et al., 2000). Thus, mechanisms of immune evasion upon genes expressed solely during the lytic cycle would protect only a small fraction of KSHV-infected cells. Conclusively, KSHV may require additional mechanisms of evasion that are active during latency. Several KSHV latent proteins have likewise been implicated in viral immune evasion: these include disruption of p53 by the latency-associated nuclear antigen-1 (LANA-1) (Friborg et al., 1999) and LANA-2 (Rivas et al., 2001), inhibition of apoptosis by viral FLIP (Sun et al., 2003, Guasparri et al., 2004), downregulation of the B-cell receptor by ORF K1 (Lee et al., 1998, Lagunoff et al., 1999).

Recent studies have identified several KSHV lytic proteins that exert potential immunoregulatory roles during lytic replication. These include inhibition of apoptosis by viral Bcl-2 (Sarid et al., 1997) and open reading frame (ORF) K7 (Wang et al., 2002), complement

deregulation by ORF4 (Spiller et al., 2003), Th2 polarization by viral MIPII (Weber et al., 2001), and inhibition of the antiviral interferon response by interferon response factor 1 (vIRF-1) (Gao et al., 1997, Li et al., 2000, Lin et al., 2001), vIRF-3 (Lubyova et al., 2000), and vIL-6 (Chatterjee et al., 2002). Furthermore, KSHV expresses two immediate-early proteins, MIR-1 (encoded by ORF K3) and MIR-2 (encoded by ORF K5), that downregulate immunoregulatory cell-surface proteins such as MHC class I and ICAM-1 and thus limiting their recognition by the immune cells (Coscoy et al., 2000, Coscoy et al., 2001). In line with these observations, CTL killing of PEL cells has been shown to be markedly diminished compared with that of EBV-infected Burkitt`s lymphoma cell lines, which the authors suggest to be a consequence of MHC class I downregulation (Brander et al., 2000).

KS research has frequently focused on the role of cytokines in driving spindle-cell proliferation. Early studies performed before the discovery of KSHV have often used KS lesion derived cell lines, which do not harbour KSHV and are of unclear relevance to the tumor. *In situ* studies have demonstrated marked elaboration of inflammatory cytokines directly in tumor tissues, and considerable interest exists in both virus-encoded cytokines and cellular cytokines induced by KSHV infection (Fiorelli et al., 1998). Such an example is the vIL-6 protein, encoded by ORF K2 (Moore et al., 1996a, Neipel et al., 1997a, Nicholas et al., 1997), which has 24.8 % amino acid identity and 62.2% amino acid similarity to human IL-6 (*hIL-6*, Moore et al., 1996), particularly in the regions of interaction with the gp130 receptor, which is responsible for signal transduction for members of the IL-6 cytokine family. There is a difference between vIL-6 and hIL-6 in terms of signal transduction, in that vIL-6 is able to directly activate gp130 signalling without co-receptor usage, suggesting that it has a broader set of target effector cells than the human cytokine (Molden et al., 1997, Hoischen et al, 2000, Mullberg et al., 2000). Despite these differences in receptor usage, no distinct difference has yet been found in downstream signalling pathways between hIL-6 and vIL-6 (Osbourn et al., 1999). Both of them activate STAT-1, STAT-3 and STAT-5 transcription factors (Molden et al., 1997, Burger et al., 1998, Wan et al., 1999) and the Ras mitogen-activated protein kinase pathway (Osbourn et al., 1999, Wan et al., 1999). Functionally, both hIL-6 and vIL-6 induce a B-cell proliferation and prevent apoptosis in susceptible cell lines (Moore et al., 1996a, Nicholas et al., 1997, Burger et al., 1998). Despite some contradictional results, it has appeared that PEL cells in tissue culture are autocrine-dependent on vIL-6, hIL-10 and nerve growth factor, but not hIL-6, for growth and proliferation (Asou et al., 1998, Jones et al., 1999). Human IL-6 is found to play a significant role in the maintenance of EBV-infected lymphoblastoid cell lines (LCL). Although vIL-6 is found to be expressed only in a portion of

PELs and MCD tumor cells, it may have widespread effects since it is a secreted cytokine and is likely to play an important role in the pathogenesis of these diseases. It has also been shown that vIL-6 activates the secretion of vascular endothelial growth factor (VEGF) and enforces an overexpression of vIL-6 in cells, which results in cellular transformation and malignant tumor formation (Aoki et al., 1999).

Assuming all the studies it has appeared that KSHV recapitulates many of the functions of other viruses, particularly EBV. This contributes to the general understanding that there are fundamental properties shared among phylogenetically divergent viruses. Determining the extent of functional similarities, as well as dissimilarities, between KSHV and other viruses has led to major insights into cell-type-specific interactions, viral life cycles and viral disease pathogenesis. Many of the KSHV-regulatory genes can be interpreted to play a functional role in controlling the innate and adoptive cellular immune response. Viral IRF, vFLIP, vIL-6 as well as K3 and K5 proteins directly affect established immune response pathways. The immunoreceptor regulatory proteins, ORF K1, LAMP and ORF4, are also likely to have a direct impact on immune responses that are particular to B-cells. These immune pathways not only affect viral replication, but also regulate cell cycle control, apoptosis and tumor cell immune surveillance. It is understandable, then, that the primary functions of some KSHV "oncoproteins" such as ORF K1, vIL-6 and vIRF, are to alter host-cell immune responses, but these proteins cause cell transformation *in vitro* as well. This has raised the possibility that cellular proteins and pathways that are normally thought of having an immune function, such as pRb and p53, are actually critical in preventing successful persistent viral infection (Moore and Chang 1998).

2.3 B-lymphocytes

As mentioned above, KSHV is detected in PEL tumor cells, which are supposed to be of B-cell origin although lacking basically all B-cell surface markers and having a disrupted B-cell transcriptional program. Therefore, it is important to get some insight into the regular B-cell development and the transcription factors involved in it.

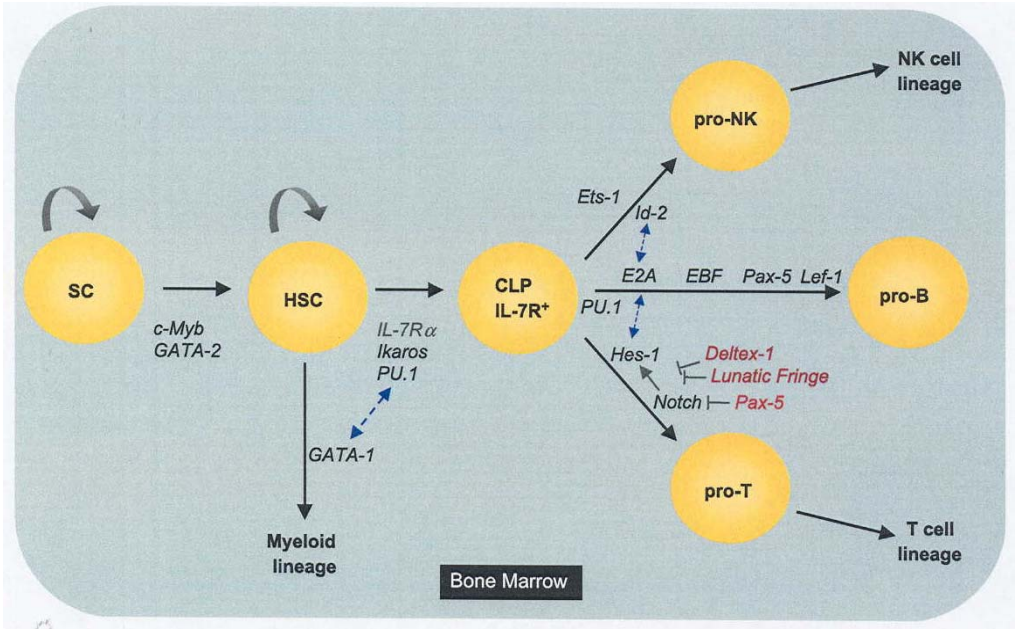
2.3.1 B-cell development

The development of B lymphocytes from pluripotent progenitors is a tightly regulated process that takes place in the fetal liver during embryogenesis and postnatally in the bone marrow (Bartholdy and Matthias, 2004). The B-cell maturation proceeds further in the

secondary lymphoid organs (Fig. 5A, B). The multiple stages of B-cell differentiation are defined by phenotypic and functional changes such as the sequential rearrangement of the immunoglobulin heavy and light chain loci and the expression of various cell-surface molecules. Commitment to the B-cell lineage and subsequent rearrangement of the immunoglobulin (Ig) heavy chain gene is followed by the expression of the pre-B cell receptor (pre-BCR), which is composed of the heavy chain and the light chains λ5 and VpreB. Signalling through the pre-BCR leads to a transient phase of cellular proliferation and triggers the transition from early pre-B cells to late small pre-B cells. Recombination of κ and λ light chain loci is initiated at the pre-B cell stage. Successful pairing of one of the light chains with the heavy chain results in the surface expression of IgM, the hallmark of immature B-cells. IgM-expressing B-cells exit the bone marrow and complete their maturation in the spleen. Antigen encounter leads to an activation of mature B-cells and their differentiation into memory B-cells or antibody secreting plasma cells (PCs), the final mediators of the humoral immune response.

The entire developmental program of B-lymphopoiesis is tightly regulated, and one of the most important regulatory mechanisms is the transcriptional control executed by a large number of cell-specific or ubiquitous factors that drive cells into the B-lineage, lead to their selection and differentiation or to cell death, or maintain them in a defined differentiation stage. Gene targeting studies have revealed that several transcription factors are essential for early B-cell development: Their absence leads to a block in differentiation primarily at either of two stages: (i) at the initiation of B-cell commitment and (ii) at the onset of V_HDJ_H recombination (Fig. 5B). Successful initiation of immunoglobulin gene rearrangement is thus a critical checkpoint in B-lymphopoiesis. Other factors affect the function of mature peripheral cells and several discrete stages of the B-cell development.

A



B

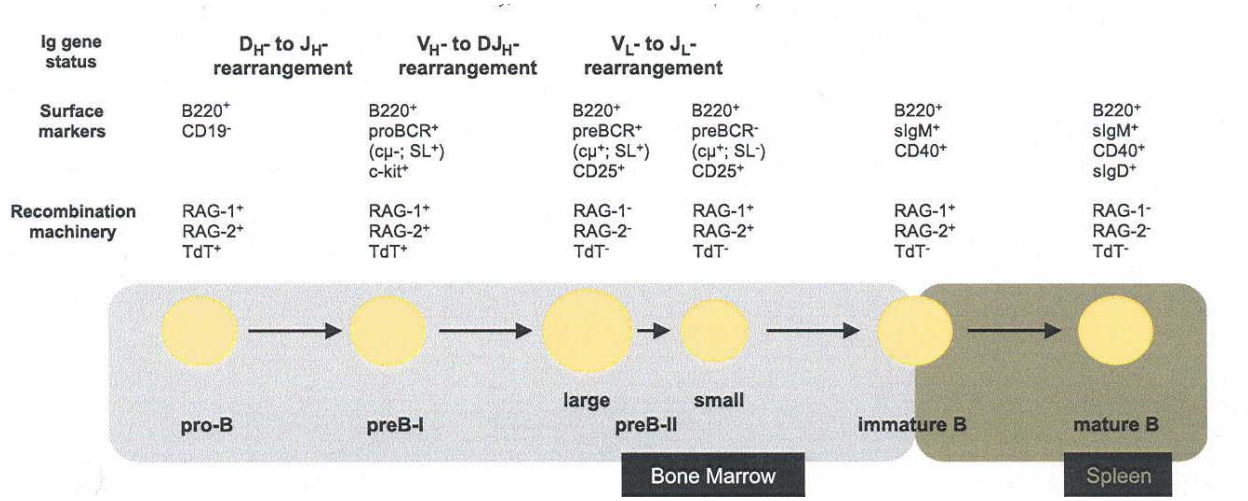


Fig. 5: Lineage commitment in the bone marrow (according to Bartholdy and Matthias, 2004). A: The position of the transcription factors on the scheme represents the stage at which their absence (knockout studies) leads to a developmental block. Proteins normally acting as inhibitors of the B-cell differentiation pathways are depicted in red. Physical interactions between transcription factors are indicated by double pointed blue arrows, SC-stem cell; HSC-hematopietic stem cell; CLP-common lymphoid progenitor ; NK-natural killer cell .B: Murine B-cell development from pro-B stage to stage mature B-cell.

2.3.2. Transcription factors involved in B-cell development and maturation

The main factors driving the maturation of an early B-cell into a plasma cell are the B-cell transcription factors. The process of B-cell development is an orchestra of these factors, acting dependently on each other in a precise hierarchical order.

2.3.2.1 E2A

Early B-cell development is regulated by E47 and E12, members of the basic helix-loop-helix (bHLH) transcription factor family. These proteins are generated by alternative splicing of RNA encoded by the E2A gene (Kadesch et al., 1992). They are known to recognize sequences in the transcriptional control regions of most lymphoid cell-specific genes, either as homodimers or as heterodimers. E12 and E47 proteins are widely expressed and the homodimeric form of E47 is unique to B lymphocytes (Shen et al., 1995). Products of the E2A gene are thought to regulate transcription of the μ, κ and Ig heavy chain genes, since mutation of the E2A binding sites decreases the function of the immunoglobulin gene enhancer (Staudt et al., 1991). It has been found that mice carrying mutations in the E12 and E47 proteins have no mature B-cells and contain CD43+B220+ B-cell precursor arrested prior to D-J_H gene rearrangement (Bain et al., 1994, Zhuang et al., 1994). It has been also shown that mice lacking E12 alone have defects at later stages of the B-cell development, suggesting that E12 and E47 are partially similar but not identical in their influence on B-cell development (Bain et al., 1997). E2A gene products can also regulate some of their targets in conjunction with p300/CBP, HEB and E2-2 (bHLH proteins) (Eckner et al., 1996).

Besides its importance in regulating early B-cell development, E2A may also play a role in later B-cell differentiation. The observation that E2A is upregulated in lymphoid germinal centre cells (Roberts et al., 1993) and that class switching to some isotypes is inhibited in B-cell lines that overexpress Id (inhibitor of development) (Goldfarb et al., 1996), suggests a role for E2A and other bHLH-family factors in the terminal differentiation process.

2.3.2.2 Early B-cell factor-1 (EBF-1)

EBF-1 has been first detected in pro-B, pre-B, and mature mIg+ B cells, but not in terminally differentiated plasma cells or T-cells (Hagman J et al., 1991, Feldhaus A et al., 1992). The fact that both EBF-1- and E2A-deficient mice have defects at a similar stage in B cell development has raised the possibility that these transcription factors might act co-operatively and regulate a common set of genes (Reya and Grosschedl, 1998). Identification

of putative gene targets has suggested that EBF-1 co-ordinately regulates multiple components of the B-cell receptor (BCR) at early stages of B-cell differentiation. In addition to a single site in the *mb-1* promoter (Feldhaus et al., 1992), functionally important binding sites for EBF have been detected in the early promoters of the ψL chain genes λ5 and VpreB1 (Sigvardsson et al., 1997, Martensson et al., 1997, Persson et al., 1998), the B lymphoid tyrosine kinase gene *blk* (Akerblad et al., 1999), and the B29 gene (Akerblad et al., 1999). Binding sites for EBF have also been noted in the human *CD19* promoter (Gisler et al., 1999), and in subsets of mouse and human Vκ promoters, which have been thought to regulate their activation during the early B-cell differentiation. An inhibitory role of EBF has also been identified. One report suggests that it can repress the Igμ intronic enhancer (Akerblad et al., 1996).

EBF-1-deficient mice lack serum Ig and mature surface Ig+ B cells (Lin and Grosschedl, 1995). EBF-deficient progenitor B-cells also do not express transcripts from the *mb-1*, *B29*, *VpreB* or *λ5* genes (Lin and Grosschedl, 1995). Transcripts encoding Pax-5 (see below) have not been detected in *ebf*-/- mice, suggesting that EBF is an upstream regulator of *Pax-5* gene transcription in B-cells. In addition, a functional EBF-1 binding site has been identified 1121 bp upstream of the transcription start sites in the *Pax-5* promoter and in transfection experiments it was shown that EBF-1 directly regulates Pax-5 expression (O´Riordan and Grosschedl, 1999).

Little is understood regarding the regulation of *ebf* gene transcription. One study has demonstrated that ectopically expressed E12 induces EBF-1 expression (Kee et al., 1998). Due to the absence of EBF-1 transcripts from the bone marrow of E2A-deficient mice, it has been argued that E2A-encoded proteins act upstream of EBF-1, potentially by directly activating its transcription in early B-cell progenitors (Lin and Grosschedl, 1995).

EBF-1 also plays a role in the chromatin remodelling. Some observations suggest that EBF-1 activates V(D)J recombination by serving as a docking site for chromatin remodelling complexes since expression of EBF-1 in a kidney epithelial cell line is selectively found to activate some, but not all endogenous IgH D-J and IgL κ/λ chain gene rearrangements (Romanow et al., 2000, Goebel et al., 2001). It is currently not clear how EBF-1 mediates these changes, but Goebel and colleagues speculate that it may recruit histone acetyltransferase proteins that mediate chromatin remodelling.

2.3.2.3 Pax-5 (BSAP, B-cell specific activator protein)

Similar to EBF-1, Pax-5 activity is detectable in mouse and human cells representing early stages of the B-cell differentiation, but is absent from terminally differentiated plasma cells. The Pax-5 gene is alternatively spliced into four different isoforms that are differentially expressed during B-cell development (Zwollo et al., 1997). Except the Pax-5b isoform, most Pax-5 isoforms are found to be downregulated during terminal differentiation, which suggested the possibility that these different Pax-5 isoforms may play distinct roles in B-cell development. Pax-5 directly acts as a transcriptional activator of several lymphoid genes such as *CD19*, *N-myc*, *Igα* and *LEF-1* (Nutt et al., 1998). It has been also shown to bind to *VpreB* and *λ5* promoters (early promoters, characteristic for the immature B-cells), as well as *KI* and *KII* sites upstream of the *J kappa* genes and possibly contributes to their activation (Tian et al., 1997). In addition, Pax-5 is found to act as a transcriptional repressor of lineage-inappropriate genes, including macrophage colony stimulating factor (M-CSF) receptor gene, and thus commits the progenitor cells to the B lymphoid lineage and maintains that commitment by suppressing alternative cell fates (Kee and Murre, 1998, Nutt et al., 1999, O´Riordan and Grosschedl, 1999, Busslinger et al., 2000, Mikkola et al., 2002). Pax-5 also binds to the *mb-1* promoter, which is stabilized by the recruitment of the Ets proteins Fli-1, Ets-1 and GABPα. Other proteins are reported to functionally interact with Pax-5: c-Myb, which binds and activates the RAG2 promoter cooperatively with Pax-5, the runt domain protein CBFα/PEBP2α/AML, the TATA-binding protein (TBP), the Retinoblastoma (Rb) protein and PU.1, which is found to be a direct target of Pax-5 mediated repression and which inversely can inhibit Pax-5 transactivation (Maitra and Atchison, 2000). Other potential interacting partners of Pax-5 in *in vitro* studies are the Id helix-loop-helix proteins Id1, Id2 and Id3, which mostly act as inhibitory factors (Roberts et al., 2001).

Pax-5-deficient mice have been shown to exhibit an early block of B-cell differentiation at the pre-B stage (Urbanek et al., 1994). B-cells from these mice lack the preB-cell receptor (pre-BCR) with a consecutive block in downstream signal transduction, including the BLINK protein which is a signal adaptor protein, essential in BCR signalling. These mice with targeted disruption of Pax-5 genes lack serum Ig and cannot mount a humoral immune response.

2.3.2.4 Spi-B

A family of transcription factors thought to play a pivotal role in hematopoiesis are the Ets DNA-binding proteins, which are monomeric transcription factors binding DNA through

their Ets domain (Macleod et al., 1992, Nye et al., 1992, Gunther et al., 1990, Leprince et al., 1992). Based upon differences within the Ets and other domains, Ets proteins are divided into series of subfamilies, consisting of the Ets, PU.1, Elf-1, Fli-1, and GABPα group. The PU.1 subgroup consists of PU.1 (Spi-1) and Spi-B. Spi-B has been cloned from a Burkitt lymphoma cDNA library using the human PU.1 as a probe (Winandy et al., 1995). Spi-B is mostly expressed in mature B- and T- lymphocytes (Ray et al., 1992, Klemsz et al., 1990, Hromas et al., 1993). It is closely related to PU.1 through structural homology and by its ability to transactivate PU.1 target genes *in vitro* (Ray et al., 1992, Ray-Gallet et al., 1995). However, *in vivo* it has been found that the target genes of Spi-B and PU.1 overlap only partially (Rao et al., 1999). Both PU.1 and Spi-B are known to be required for normal BCR signalling (Garett-Sinha et al., 1999), whereas Spi-B can functionally replace PU.1 in myeloid development, it is not able to replace it in lymphoid development (Dahl et al., 2002). Spi-B deficient mice exhibit defect in BCR signalling and show severe abnormalities in B-cell function and selective T cell-dependent humoral immune responses accompanied by a dramatic effect in germinal centre formation and maintenance.

2.3.2.5 Oct-1, Oct-2 and Bob-1

Protein-DNA interaction assays have identified nuclear factors that bind specific octamer sites, for example in the Ig heavy and light chain variable (V) gene promoters, the enhancer in the intron between the joining (J) and μ constant (C) region, the IgH locus and in promoters of various B-cell specific genes like B29 (Ig beta) and CD20. Such nuclear factors are Oct-1 and Oct-2. Whereas Oct-1 has been identified as an ubiquitous protein, Oct-2 is restricted mostly to lymphoid cells. These factors belong to the family of POU proteins because of the presence of a bipartite DNA binding domain, the so-called “POU” domain. This domain contains helix-turn-helix motifs required for high affinity binding to DNA.

Oct-2 is found to be expressed at low levels in B-cells of early differentiation stages: pro-, pre-B cells, and at higher levels in mature B-cells. Oct-2 is found to transactivate co-transfected reporter plasmids in an octamer site-dependent manner (Müller et al., 1988, Tanaka et al., 1990, Müller-Immergluck et al., 1990). These results have led to the early notion that the B cell-specific activity associated with the octamer site is primarily mediated by Oct-2 and highlighted the functional importance of these sites especially for Ig promoter function and B cell-specific activity.

One protein that was detected to specifically interact with Oct-1 and-2 is OBF-1 (*Oct Binding Factor-1,* Strubin et al., 1995, or *OCA-B*, Luo et al., 1995, or *Bob-1* (Gsteiger et al.,

1995). The expression of OBF-1 is highly B-cell specific and has not been observed in most other cell types examined (Strubin et al., 1995, Luo et al., 1995, Gsteiger et al., 1995). Within the B-cell lineage, OBF-1 expression is found in cells of all stages of differentiation (pro-, pre- and mature B cells) at constant levels (Schubart et al., 1996). By interaction with either Oct-1 or Oct-2, OBF-1 could be recruited to a subset of octamer sites and thereby to co-activate gene transcription. This interaction is highly specific, as OBF-1 interacts efficiently with the POU domains of Oct-1 and Oct-2 *in vitro*, but not with other members of the Oct family. This led to the conclusion that this interaction has an important role in B-lymphocytes.

2.3.2.6 B-lymphocyte-induced maturation protein-1 (Blimp-1)

Blimp-1 is a transcriptional repressor that plays a central role in the terminal differentiation of B-cells into plasma cells (Turner et al., 1994). Ectopic expression of Blimp-1 in lymphoma cell lines or in primary murine splenic B-cells is sufficient to induce differentiation to Ig-secreting plasma cells (Schliephake et al., 1996, Piskurich et al., 2000). Studies in mice lacking Blimp-1 in mature B-cells showed severe defects in pre-plasma memory cells, plasma cells and Ig secretion (Shapiro-Shelef et al, 2003). Therefore, Blimp-1 is considered the “master regulator” of plasmacytic differentiation. It was also suggested that repression of Blimp-1 in germinal center (GC) B-cells is important to delay plasmacytic differentiation and allow affinity maturation and class switch recombination (CSR) (Shaffer et al., 2000, Reljic et al., 2000).

Blimp-1 is detected in all plasma cells and is absent in early bone marrow cells, memory B-cells in spleen and most GC B-cells (Angelin-Duclos et al., 2000). Several B-cell targets of Blimp-1 have been identified. Blimp-1 represses c-*myc* (Lin et al., 1997), *pax-5* promoter (Lin et al., 2002) and class II transactivator (CII) promoter III, required for the transcription of class II major histocompatibility complex (MHC) (Piskurich et al., 2000). In addition, micrarrray studies in B-cells revealed several gene expression programs that are altered in response to Blimp-1 including: repression of proliferation genes, repression of genes required for GC or activated B-cells and induction of genes necessary for Ig secretion (Shaffer et al., 2002).

Aim of the study

PEL is a B-cell malignancy which is morphologically and histologically different from most other B-cell tumors (Gaidano et al., 1997). Almost all investigations regarding KSHV and PEL are based on PEL cell lines originating from patient material. In these tumor cells it is largely unknown which phenotypical changes are caused by viral infection and which by cellular, oncogenic processes. Moreover, PEL cells also contain EBV, so that both viruses might contribute to the transformation processes in doubly infected PEL cells. The aim of this project was thus to investigate the role of KSHV in the phenotypical changes observed in infected B-cells. To this aim *in vitro* KSHV-infected B-cells should be established and analysed. Subsequently, the mechanism and the biological consequences of the phenotypical changes should be investigated.

3. Materials and Methods

3.1 Materials

3.1.1 Equipment

Centrifuge GP Beckman	Palo Alto, USA
Centrifuge J2-21 Beckman	Palo Alto, USA
Centrifuge Varifuge 3.0R Heraeus	Hanau, Germany
Centrifuge Minifuge RF Heraeus	Hanau, Germany
Centrifuge Labofuge T Heraeus	Hanau, Germany
Centrifuge, refrigerated and non-refrigerated Heraeus	Hanau, Germany
Confocal laser scanning microscope Leica	Bensheim, Germany
Elisa Reader Tecan Labinstruments	Crailsheim, Germany
Film developing machine Optimax Typ TR MS Laborgeräte	Heidelberg, Germany
Fluorescence/light microscope Axiovert 35 Zeiss	Oberkochen, Germany
Fluorescence/light microscope Axiovert 200M Zeiss	Oberkochen, Germany
Refridgerator (4°C) Liebherr	Ochsenhausen, Germany
Freezer (-20°C) Liebherr	Ochsenhausen, Germany
Freezer (-80°C) Forma Scientific, Inc., Marietta	Ohio, USA
Cryo 1°C Freezing Container Nalgene Nunc	Wiesbaden, Germany
Gel dryer Bio Rad	Munich, Germany
GelAir drying system Bio-Rad	Munich, Germany
Incubators for cell culture (37°C) Forma Scientific, Inc., Marietta,	Ohio, USA
Inverted microscope TMS Nikon	Düsseldorf, Germany
Laminar Flow Hood Steril Gard II A/B3 The Baker Company,	Sanford, Maine,USA
Magnetic stirrer with heating block Janke & Kunkel	Staufen, Germany
Microwave AEG	Berlin, Germany
Overhead mixer Heidolph	Schwabach, Germany
PCR Thermal Cycler GeneAmp 2400 Perkin Elmer	Weiterstadt, Germany
pH Meter WTW	Weilheim, Germany
Photometer Gene Quant II Pharmacia/LKB	Freiburg, Germany
Pipettes Gilson	Villies Le Bel, France
Eppendorf	Hamburg, Germany

Pipetting aid Technomara	Zürich, Switzerland
Electrophoresis Power supply EPS200 Amersham-Pharmacia	Freiburg, Germany
Sonifier 450 Branson Ultrasonics Corp.	Danbury, USA
Thermomixer Eppendorf	Hamburg, Germany
UV transilluminator (366 nm) Vetter	Wiesloch, Germany
(254 nm) Konrad Benda	Wiesloch, Germany
Vortex mixer IKA Works, Inc.	Wirmington, USA
Water bath Julabo	Seelbach, Germany
GFL	Burgwedel, Germany

3.1.2 Chemicals

Acetic Acid Roth	Karlsruhe, Germany
Acrylamide/Bisacrylamide 37,5/1 Roth (Rotiphorese Gel 30)	Karlsruhe, Germany
Agarose electrophoresis grade Invitrogen	Karlsruhe, Germany
Ammonium persulfate (APS) Sigma	Munich, Germany
Bromophenol blue Serva	Heidelberg, Germany
Bovine serum albumin (BSA) Sigma	Munich, Germany
Calcium chloride Merck	Darmstadt, Germany
Dimethylsulfoxide (DMSO) Merck	Darmstadt, Germany
Dithiothreitol (DTT) Roth	Karlsruhe, Germany
dNTPs Roche Diagnostics	Mannheim, Germany
Dulbecco's modified Eagle's medium (DMEM) Gibco BRL	Karlsruhe, Germany
Ethanol (EtOH) Riedel-de Haën	Seelze, Germany
Ethidium bromide Sigma	Munich, Germany
Ethylenediamintetraacetate disodium salt Roth (EDTA)	Karlsruhe, Germany
Ethylene glycol Sigma	Munich, Germany
Fetal calf serum (FCS) Gibco BRL	Karlsruhe, Germany
Glucose Merck	Darmstadt, Germany
Glycerol Roth	Karlsruhe, Germany
Histogel Linaris	Wertheim-Bettingen, Germany

Hydrochloric acid (HCl) Merck	Darmstadt, Germany
Interferon (IFN) - PBL Biomedical Laboratories	Piscataway, USA
Isopropanol Riedel-de Haën	Seelze, Germany
L-glutamine Gibco BRL	Karlsruhe, Germany
Magnesium chloride Merck	Darmstadt, Germany
Magnesium sulfate Merck	Darmstadt, Germany
2-mercaptoethanol Merck	Darmstadt, Germany
Methanol Merck	Darmstadt, Germany
N-butyrate Sigma	Munich, Germany
Nonidet P40 (NP-40) Fluka	Seelze, Germany
Pefabloc Roche Diagnostics	Mannheim,Germany
Penicillin-Streptomycin Gibco BRL	Karlsruhe, Germany
Phenylmethylsulfonfluoride (PMSF) Roche Diagnostics	Mannheim, Germany
Phosphate buffered saline (PBS) Dulbecco's Gibco BRL	Karlsruhe, Germany
Ponceau S Sigma	Munich, Germany
Rosswell Park Memorial Institute (RPMI)1640 Gibco BRL	Karlsruhe, Germany
Skim milk powder Merck	Darmstadt, Germany
Sodium acetate Riedel-de Haën	Seelze, Germany
Sodium azide Serva	Heidelberg, Germany
Sodium chloride Riedel-de Haën	Seelze, Germany
Sodium dodecylsulfate (SDS) Merck	Darmstadt, Germany
Sodium hydroxid J.T.Baker B.V.	Deventer, Holland
Sorbitol Sigma	Munich, Germany
Tetramethylethylenediamin (TEMED) Amersham-Pharmacia	Freiburg, Germany
12-O-tetradecanoylphorbol-13-acetate (TPA) Sigma	Munich, Germany
Tris(hydroxymethyl)aminomethan (Tris) Roth,	Karlsruhe, Germany
Triton X-100 Serva	Heidelberg, Germany
Trypsin Gibco BRL	Karlsruhe, Germany
Tween 20 Merck	Darmstadt, Germany
Western Blue- Stabilized Substrate for Promega	Mannheim, Germany

3.1.3 Additional materials

autoradiography films	Rochester, USA
cell culture plastic ware Greiner	Greiner, Germany
	Nunc, Germany
Falcon	Becton Dickinson
	Heidelberg, Germany
glass slides	Marienfeld Bad
	Mergentheim, Germany
nitrocellulose	Schleicher & Schuell
	Dassel, Germany
sterile filters 0.2 and 0.4 µm	Millipore

3.1.4 Cell lines

NAME	TISSUE	ORIGIN
721	EBV-transformed LCL	ATCC
BCBL-1	PEL	Don Ganem, UCSF
NKL	NK cell line	Eric Vivier, Mareseille, Framce
NK92	NK cell line	Eric Vivier, Marseille, France
JB4 (HLA-A2)	T cell line	Cristine Falk, GSF, Munich
234 (HLA-A24)	T cell lines	Cristine Falk, GSF, Munich
Bos 4	T-cell origin	Cristine Falk, GSF, Munich
Bos 5	T-cell origin	Cristine Falk, GSF, Munich
Daudi	B-cell origin	Cristine Falk, GSF, Munich
Daudi β2-m	B-cell origin	Cristine Falk, GSF, Munich
K562	monocyte/macrophage origin	Cristine Falk, GSF, Munich

3.1.5 Oligonucleotides

NAME	FORWARD	REVERSE
BCR	CAAAACTCACACATGCCCAC	AGGGCTTTGTTGGAGACCTT
CD19	CAGTCCTATGAGGATATGAGAGGAA	GAAGAGCTCATTGAGTTTATTTAAGG
CD79B	ATCCTCTTCATCATCGTGCC	ACGGATCACCTCATAGCACC
CD80	GTCCTGGACTGCTCTTCCTG	TGCCACATGCAGTGTAACCT
IL-6	AAAGAGGCACTGGCAGAAAA	GAGGTGCCCATGCTACATTT
IL-10	TTACCTGGAGGAGGTGATGC	TGGGGGTTGAGGTATCAGAG
E2A	TGGTAGATGCAAGGGAAACC	TGAATCCACCTGAAAGAGGG
EBF-1	GATTCCAGGTCGTGGTGTCT	CCATAATCGATGGTGGGTTC
PAX-5	CAGAACAGCCAGGTAGAGCC	TCTTGTTTCCCACTTGGTCC
OCT-2	CCTGCTCAGTTCCTGCTACC	CTTGAAGCTCAGGTTGAGGG
SPI-B	GATCCCCCTGGAAGAAAAAG	AGAGGGCCCCAGACATAACT
BOB-1	CCTGAGAAAGTCAGCCAAGG	CCAACTTCCCTTGCACGTAT
BLIMP-1	CCAGCTCTCCAATCTGAAGG	GATTCGGGTCAGATCTTCCA
ACTIN	GACGACATGGAGAAGATCTGG	TGTGGTGGTGAAGCTGTAGC

3.1.6 Molecular weight markers

Gene Ruler 100 bp DNA ladder MBI Fermentas	St. Leon-Rot, Germany
Gene Ruler DNA 1 kb ladder MBI Fermentas	St. Leon-Rot, Germany
See blue plus 2 prestained protein standard Invitrogen, low range	Karlsruhe, Germany

3.1.7 Kits

BCA Protein Assay Pierce	Rockford, USA
Cell proliferation ELISA BrDU (colorimetric) immunoassay	Roche
ECL western blotting detection system Amersham-Pharmacia	Freiburg, Germany
IL6 and IL10 ELISA kits DuoSet ELISA Development System	R&D Systems
RNA midi kit Quiagen	Hilden, Germany

RT Reaction, Superscript II H-	Invitrogen
Qiafilter Plasmid Maxi Kit Qiagen	Hilden, Germany

3.1.8 Antibodies

3.1.8.1 Primary antibodies (*All antibodies were titrated before use*)

NAME	ORIGIN
anti-CD19 mouse anti-human, clone HD37	DAKO
anti-CD71, mouse anti-human, hybrodoma supernatant kindly provided by Walter Muranyi	Heidelberg, Germany
anti-MHC I, mouse anti-human, W6-32	ATCC
anti-CD79b, mouse anti-human ,clone SN8	*Jackson*, Hamburg, Germany
anti-CD138, mouse anti-human	AL-ImmunoTools, Germany
anti-ICAM, mouse anti-human (gp89) kindly provided by Cristine Falk	GSF-Haematologikum, Munich, Germany
anti-CD95, mouse anti-human	*Jackson*, Hamburg, Germany
anti-CD80-FITC conjugated, kindly provided by Elfriede Noessler	GSF-Haematologikum, Munich, Germany
anti-CD86-FITC conjugated, kindly provided by Elfriede Noessler	GSF-Haematologikum, Munich, Germany
anti-B220-FITC, mouse anti-human CD45RA FITC conjugated	Sigma ImmunoChemicals, St. Luis
anti-Pax-5, goat anti-human	Santa Cruz, USA
anti-Spi-B, goat anti-human	Santa Cruz, USA
anti Oct-2, rabbit anti-human	Santa Cruz, USA
anti-phospho STAT-1, rabbit anti-human	Cell signalling
anti-phospho STAT-2, rabbit anti-human	Cell signalling
anti-STAT 1, rabbit anti-human	Cell signalling
anti-STAT 2, rabbit anti-human	Cell signalling

3.1.8.2 Secondary antibodies

NAME	ORIGIN
goat anti-human IgG1-FITC conjugated	*Jackson*, Hamburg, Germany
rat anti-mouse-FITC conjugated	*Jackson*, Hamburg, Germany
goat anti-mouse-FITC conjugated	*Jackson*, Hamburg, Germany

peroxidase-conjugated:

NAME	ORIGIN
donkey anti-goat	*Jackson*, Hamburg, Germany
goat anti-rat	*Jackson*, Hamburg, Germany
goat anti-rabbit	*Jackson*, Hamburg, Germany
goat anti-mouse	*Jackson*, Hamburg, Germany

3.1.9 Enzymes

AmpliTaq Gold® DNA Polymerase — Applied Biosystems, Foster City, CA, USA

3.2. Methods

3.2.1 DNA and RNA techniques

3.2.1.1 Preparation of total RNA

Cellular or tissue total RNA was prepared either with Trizol reagent (*Gibco BRL*) according to the manufacturer's instructions. The cells were washed with PBS, lysed in 1 ml of Trizol reagent solution and incubated for 5 min at room temperature (RT). 0.6 ml chloroform-isoamylalcohol mixture (49:1) were added to the samples, mixed and incubated on ice for 15 min. The samples were centrifuged at 6000 rpm for 30 min. at 4 °C, the aqueous phase was removed and mixed with the same volume of 2-propanol. RNA was precipitated at 4°C for 30 min (or –20 °C for 1 h) and pelleted by centrifugation at 6000 rpm for 20 min. at 4 °C. The RNA pellet was dissolved was washed with 80% ethanol, air dried and dissolved in 50µl DEPC-treated water. RNA concentration was determined on a spectrophotometer by

measuring the A_{260} and samples were stored at –80 °C. The quality of isolated RNA was determined by electrophoresis using a 1% MOPS agarose gel.

3.2.1.2 Determination of RNA and DNA concentration

The concentration and purity of the purified RNA and DNA was determined by measuring the optical density at 260 and 280 nm. The RNA and DNA concentration were calculated with the OD260nm (1 OD260nm = 50 µg/ml dsDNA or 33 µg/ml ssDNA). The purity was estimated with the OD260/OD280 ratio, with a ratio of between 1.6-1.9 indicating a low degree of protein contamination.

3.2.1.3 Reverse transcription - polymerase chain reaction (RT-PCR)

RNA was extracted from 10^7 cells with Trizol (*Sigma, St. Louis*) as specified by the manufacturer. Ten micrograms of total RNA in 10µl of water and 1µg of oligo$(dT)_{18}$ primer were heated to 65°C for 10min and subsequently cooled to 4°C on ice. Twenty microliters of reaction mixture (100mM Tris-HCl [pH 8.3], 6mM $MgCl_2$, 150mM KCl, 1mM each deoxynucleoside triphosphate (*Roche, Penzberg*), 30U of RNase inhibitor (*Fermentas, Vilnius*), 200U of superscript reverse transcriptase (*Invitrogen, Carlsbad*) was added, and the mixture was incubated at 37°C for 1.5h and 67°C for 15min. One (two) microliter of the reverse transcription reaction mixture was amplified with gene-specific primers in a 50µl PCR mixture containing 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 0.2mM each deoxynucleoside triphosphate, 100ng of each primer, and 5U of *Taq* polymerase. Oligonucleotide primers for BCR, CD19, CD79b, CD80, IL-6, IL-10 and actin were used for RT-PCR (see above):

Reaction mixture for RT PCR:

2 µl 10 mM dNTPs (200 µM each)
1 µl forward primer (100 pMol)
1 µl reverse primer (100 pMol)
0.5 (1) µl AmpliTaq Gold® (5U)
0.5µl BSA
1µl cDNA template
44 µl H_2O

3.2.1.4 Agarose gel electrophoresis

Analysis of DNA fragments and plasmids was performed by agarose gel electrophoresis in 1x TAE. In general, agarose concentration was between 1 and 3 % in 1x TAE. The agarose was solubilized by heating in a microwave oven. Ethidium bromide was added to a final concentration of 0.25 µg/ml (2,5 µl stock to 100 ml) just before pouring the gel. Probes were mixed with 0.17x volume loading buffer. Gels (6.5 x 9.5 cm) were run horizontally at 80-120 V. PCR fragments were detected with UV light (254 nm or 366 nm).

loading buffer (6x in water) MBI Fermentas, St. Leon-Rot, Germany
20x TAE: 800 mM Tris
400 mM NaAc
40 mM EDTA
adjusted to pH 7.8 with acetic acid
Ethidium bromide (stock): 10 mg/ml

3.2.2 Tissue culture

3.2.2.1 Generation of persistently KSHV-infected B-cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-treated blood of three healthy individuals by Ficoll-Hypaque discontinuous gradient centrifugation (*Biotest, Dreieich*). Supernatants of BCBL-1 and 721 cells grown at very high densities ($5x10^5$ per ml) were used to infect PBMC as previously described {Kliche, Kremmer, et al. 1998 339 /id}. Supernatants were filtered through 0.4-µm filters and serially diluted with cell culture medium in 48-well plates. Subsequently, PBMC were added to 10^4 cells per well.

3.2.2.2 Infection of KSHV- and EBV- infected B-cells with recombinant vaccinia virus

KSHV- and EBV- infected B-cells were incubated for 1h, 37°C with 20 (25) µl vaccinia virus stock in a final volume 1ml RPMI medium. Afterwards the vaccinia-infected cells were diluted with 10 ml fresh RPMI medium and incubated for additional 18-24 hours.

3.2.2.3 Cultivation and cryoconservation

All suspension cell lines were cultured in RPMI 1640 supplemented with 20% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. For cryoconservation cells were centrifuged at 300 g for 5 min at 4°C. Subsequently, the cells were resuspended in 1 ml FCS/10% DMSO (4°C) with a final concentration of 0.5-1x107 cells/ml, transferred to cryovials and cooled to –80°C in a "Cryo 1°C FreezingContainer". After approximately 24 hours, the vials were transferred to liquid nitrogen for longterm storage. Frozen aliquots were quickly thawed at 37°C in a waterbath and washed with 10ml RPMI to remove the DMSO. Subsequently, cells were resuspended in complete medium and transferred to cell culture dishes.

3.2.2.4 Immunofluorescense and confocal microscopy

The KSHV-positive cells were dried on poly-L-lysine-coated coverslips (*Marienfeld, Bad Mergentheim, Germany*), fixed with ice-cold acetone (10-15 min), and blocked for 20 min with 0.2% gelatine in PBS. After incubation for 1 h with the biotinylated monoclonal antibody kap5C4, directed against KSHV K12 and diluted in PBS/0.2% gelatine, the cells were incubated for 2 h with streptavidin-FITC-conjugated goat anti-rat IgG (*Dianova, Hamburg*) diluted 1:200 in PBS/0.2% gelatine. The cells were washed and examined using a Leica TCS-NT confocal microscope (*Leica, Bensheim, Germany*).

3.2.3 Protein techniques

3.2.3.1 SDS-PAGE

Protein gel electrophoresis with 12 to 20% gels (80 x 50 x 1 mm) was performed using the Protean II system (*Bio-Rad*). After pouring the separation gel, the gel was overlaid with isopropanol to straighten the gel surface. After the polymerization the isopropanol was removed, the stacking gel was poured on top of the separation gel and the comb was fixed. After the polymerisation, the gel was assembled in the gel electrophoresis apparatus. Lysates or precipitated samples were resuspended in 20-50µl 2xSDS protein sample buffer and boiled for 5 min at 95°C. After cooling to RT, the samples were centrifuged for 2 min at 10000g (microcentrifuge) and loaded on the gel together with a protein standard. Separation was performed at a constant current of 400mA for 1-2 h.

Separation gel	**10%**	**12%**
30% Acrylamide/ Bisacrylamide (37.5:1)	3.33	4
1.5 M Tris pH 8.8	2.5	2.5
10 % SDS	100 µl	100 µl
H2O	4.01 ml	3.35 ml
10 % APS	50 µl	50 µl
TEMED	5 µl	5 µl
Stacking gel	**5%**	
30 % Acrylamide/ Bisacrylamide (37.5:1)	650 µl	
0,5 M Tris pH 8.8	1.25 ml	
10 % SDS	50 µl	
H2O	3.05 ml	
10 % APS	25 µl	
TEMED	5 µl	

Electrophoresis buffer (10 x): 50 mM Tris
384 mM glycine
0.1% SDS

3.2.3.2 Western blot analyis

Proteins were blotted onto nitrocellulose membranes using the Protean II system (*BA-85 Schleicher & Schuell*). A piece of nitrocellulose membrane and two pieces of filter paper and two sponges of the same size as the gel were soaked with transfer buffer. A sponge, a piece of filter paper, the nitrocellulose membrane, the gel, another piece of filter paper and again a sponge were packed. Subsequently, air bubbles were removed by rolling a test tube over the sponge and the package was clamped into the transfer tank with the nitrocellulose facing the anode. Blotting was performed at constant current of 400 mA for 1-2 h. Subsequently, the protein transfer was controlled by staining the gel for 2 min with Ponceau staining solution. The membranes were labeled with a pen and washed several times with H_2O to remove the Ponceau staining solution. Unspecific binding was blocked by incubation in TBST (TBS, 0.05% Tween 20), 5% skim milk powder, 0.02% NaN_3 either 1 h at RT or o/n at

4°C. Subsequently, the membranes were incubated with the first antibody in 5-10 ml TBST (used also in the following washing and incubation steps) at 4°C o/n. After five washing steps of 15 min with approximately 200 ml buffer each, incubation with the secondary antibody coupled to peroxidase was performed in 15 ml buffer at RT for 1 h followed by washing 5x 10 min in 200 ml buffer.

The blotted proteins were detected using the ECL Western blotting detection system (*Amersham-Pharmacia*) according to the manufacturer's instructions. The membrane was exposed to BIOMAX-MR autoradiography films (*Kodak*) for different time periods and films were developed using an automatic film developing machine.

Transfer buffer (1l):	Ponceau solution (100 ml):
Tris base 5.8 g	Ponceau S 0.5 g Glycine 2.9 g
SDS 0.37 g	Glacial acetic acid 1 ml
Methanol 200 ml	H_2O 98.5 ml
H_2O up to 1l	

3.2.3.3 Separation of proteins according to the molecular weight

KSHV-positive cells were grown at a high density ($5x10^5$ -10^6 cells/ml) for 4-5 days. Cells were collected by centrifugation 400g for 5min and supernatant containing secreted cellular proteins were filtered through 0,22 μ filters (*MILLEX®-GP, MILLIPOR*). The filtered supernatants were applied to ultracentrifugation cartridges (*Microsep/Filtron*, 100K, 50K, 30K, 10K) and centrifuged at 2000-3000g for 2-2.5h. The fluid passing through the separation filter contains the desired protein fraction, which is used further.

3.2.3.4 ELISA (cytokine capture)

KSHV and EBV-infected B-cell lines from three donors were kept at a high density ($3x10^6$/ml) in a total volume of 4 ml RPMI medium. Supernatants were collected after 48 hours and used for IL-6 and IL-10 detection, performed by IL6 and IL10 ELISA kits (*DuoSet ELISA, R§D Systems*).

A 96-well plate (flat bottomed) was coated with a capture antibody (mouse anti-human IL-6 or IL-10) and incubated overnight at 4°C. After the incubation the coated wells were washed with buffer according to the manufacturer's protocol The plate was blocked by addition of blocking buffer and incubated at RT for 1 hour. Later on the samples together with the standards incubated for 2 hours on the plate in a total volume of 100 μl. The cytokines were

detected by addition of biotinylated goat anti-human IL-6 or IL-10 antibodies and streptavidin HRP antibodies and visualized by ABTS substrate solution. The optical density was determined by using an ELISA reader at 450 nm.

3.2.3.5 Flowcytometry

To analyze the expression of surface markers, 1×10^6 cells were stained with the directly conjugated antibodies anti-CD80-FITC, anti-IgG-FITC, anti-B220-FITC. The washing and antibody incubation steps were performed with FACS buffer (PBS containing 2.5% FCS and 0.02% sodium azide). For indirect immunofluorescence, cells were incubated first with either anti-CD79b, anti-CD19 anti-CD71 or anti-MHC class I antibodies, and subsequently with a secondary, FITC-conjugated anti-mouse IgG antibody. As a negative control, only the secondary antibody was used in each assay. The cells were washed three times with PBS/3%FCS and analyzed on a Coulter Epics flowcytometer with EXPO 32 ADC Software.

3.2.4 Functional assays

3.2.4.1 CTL and NK killing assay

KSHV- and EBV- infected target cells were collected by centrifugation 1500 rpm for 6 min and resuspended in 100µl 10% FCS. This cell suspension was labelled with 5mCi [Cr^{51}] for 1h 30 min at 37°C and 6% CO_2 . After the incubation the labelled cells were washed 2 times with RPMI 1640/10%FCS medium and resuspended in 4ml total volume of fresh medium. Cells were counted using Neubauer chamber and brought to a concentration of 2000 cells/50µl.

The effector NK92, HLA-A2 and HLA-A24 T-cells were distributed in 96-well plates (V bottom). These cells were incubated with the labelled KSHV-positive and EBV-positive target cells at effector : target ratio of 20:1-2,5:1 in a final volume of 100µl. Subsequently, the cell killing assay was performed for 4h at 37°C. After the incubation, 100µl of supernatant were collected from each well and counted in a γ-counter to determine the [^{51}Cr]-release. The percentage specific lysis was calculated by :

$$\text{specific lysis (\%)} = \frac{\text{experimental lysis} - \text{spontaneous lysis}}{\text{maximal lysis} - \text{spontaneous lysis}} \quad \text{X } 100$$

The spontaneous release (Spont) was determined by incubating 50µl labelled targets with 50µl RPMI. The maximal release (max) was determined by adding 10µl Triton X and 50µl RPMI/10%FCS to 50µl labelled target cells. As positive controls for the NK killing, K562, Bos-5 and Daudi cells, as positive controls for the CTL reaction, Daudi-β-2 m cells and as negative controls Bos-5 and Bos-4 cells were used.

4. Results

4.1 Generation of persistently KSHV-infected B lymphocytes

To investigate how the cellular gene expression is altered by KSHV, persistently KSHV-infected B-cell lines were generated by infecting peripheral blood mononuclear cells (PBMC) from three independent healthy individuals. PBMCs were isolated by Ficoll density gradient separation and subsequently infected simultaneously either with KSHV and EBV or EBV alone as previously described (Kliche et al., 1998). As a source of KSHV and EBV, supernatants from the KSHV-positive cell line BCBL-1 and the EBV-positive cell line 721 were used. The presence of KSHV in B-lymphocytes was confirmed by immunofluorescence staining for the latent KSHV protein kaposin A (Fig.6) and Western blot analysis (Fig.7). The KSHV-positive cells were negative for EBV (data not shown).

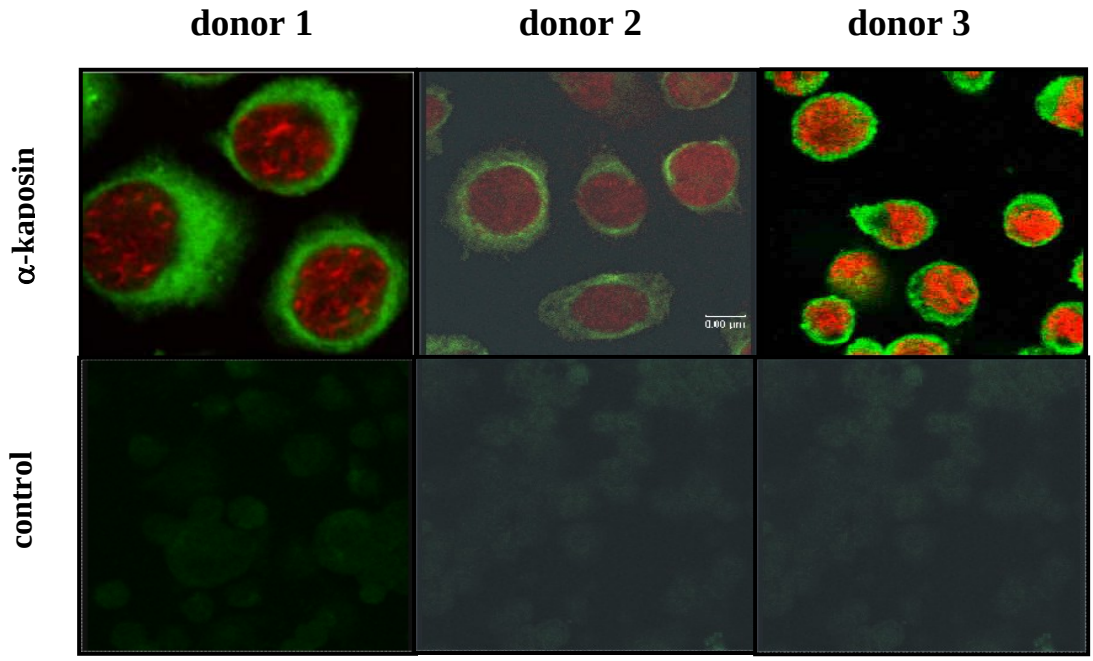


Fig. 6: Generation of persistently KSHV-infected B-cells.
PBMCs were isolated from three different healthy individuals and infected with KSHV. Immunofluorescence staining for detection of KSHV was performed. Antibody against the latent KSHV protein kaposin A (green) and propidium iodide for nuclear staining (red) were used. Control staining with a secondary FITC-conjugated antibody was performed in parallel.

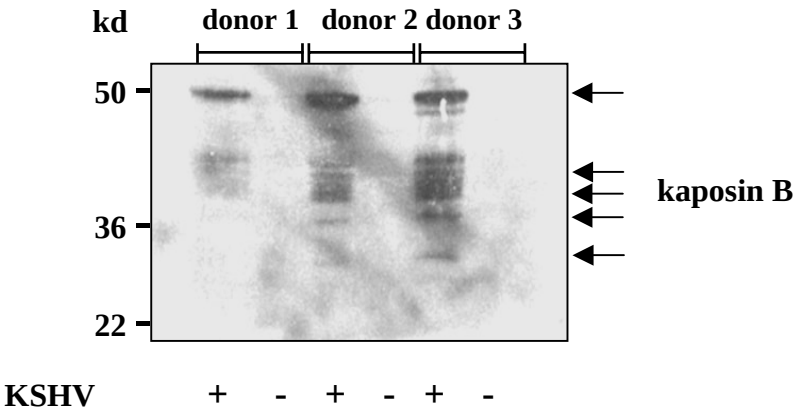


Fig. 7: Western blot analysis for detection of KSHV.
Cell lysates from the three KSHV+ and KSHV- donors were prepared and subjected to SDS-PAGE. The Western blot was performed using specific anti-kaposin B antibody.

4.2 Gene expression profiling of KSHV-infected B-cells

To compare the gene expression profile of B-cells which are transformed with either of the two lymphotropic gamma-herpesviruses, total RNA was isolated from these cells and tested for cellular gene expression by Affymetrix microarrays (U 133). The microarray screen was performed using KSHV-infected B-cells from two independent donors. The expression profile of 24000 cellular genes was determined and subsequently analysed using a variety of different algorithms. A comparison between the gene expression pattern of the two control EBV-transformed cell lines showed little variations (Fig.8 A). In contrast, significant differences in the gene expression level were detected when KSHV+ cells were compared to EBV+ cells (Fig. 8 B). The x axis represents the average gene expression level in the two EBV-infected cells and the y axis represents the average of the gene expression level of the two KSHV-infected cell lines. A major part of the genes in the KSHV-infected cells were downregulated in comparison to the EBV-infected cells (Fig.8 C).

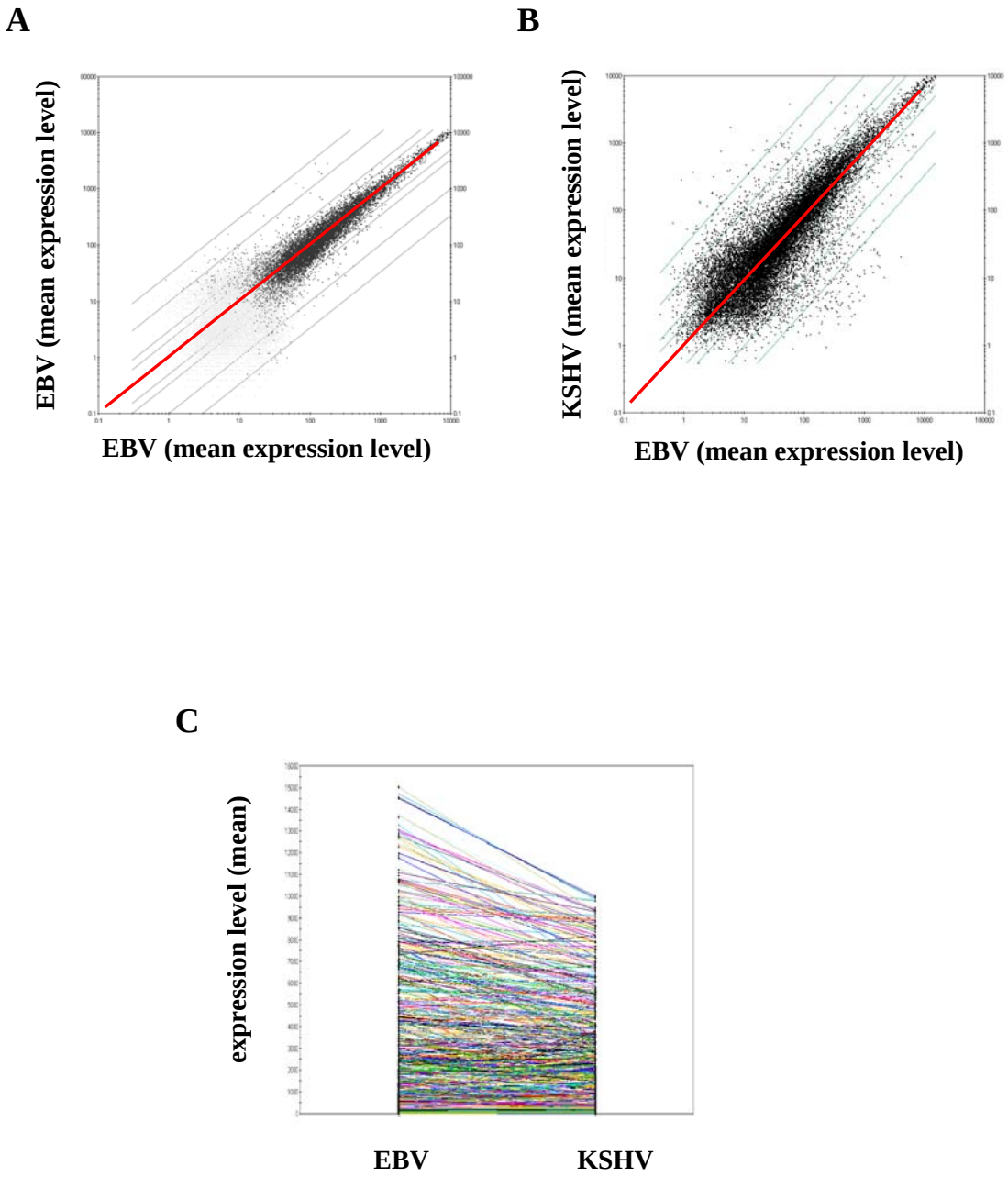


Fig. 8: Microarray analysis for cellular gene expression in KSHV-positive versus EBV-positive B-lymphocytes.
The microarray analysis was performed in duplicates – two different donors infected only with EBV (control) or KSHV. The analysis showed the expression of approximately 24000 cellular genes. A: Comparison of the gene expression profile between the two EBV+ control cell lines. B: Comparison of the gene expression profile between the KSHV+ and EBV+ cell lines. C: Downregulation of a multitude of cellular genes in KSHV +cells lines.

4.2.1 Alterations of cellular gene expression in KSHV-infected B-cells

Significantly down- or upregulated cellular genes are summarized in Table 1. Among the most upregulated genes were granzyme A, S100 calcium binding protein, caveolin, interleukin-10 and interleukin 6 receptor. Granzyme A is a cytotoxic T-lymphocyte-associated serine esterase which facilitates the migration of T-and NK-cells and is involved in the regulation of B-cell proliferation. S100 belongs to the family of calcium binding proteins which are significantly expressed in tumor and high proliferating cells. Caveolin is a main protein which participates in the formation of the lipid rafts in the cell membrane. IL-10 is known to inhibit cytokine production by both T- and NK-cells and the expression of MHC class II and co-stimulatory molecules such as IL-12 and B7-1/B7-2. It also affects the survival, proliferation and differentiation of human B-cells. The IL-6 receptor is plasma membrane receptor , responsible for binding IL-6.

Surprisingly, a multitude of surface molecules including CD19, CD22, CD79a/b (mb-1) and B-cell receptor (BCR) were found to be downregulated. These are molecules which are mainly expressed in B-cells and are essential for the regular function of B-lymphocytes.

KSHV-signal	KSHV+signal	fold change	gene
5,9	1745,85	87,29	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)
4,05	767,1	38,35	G protein-coupled receptor 25
10,7	472	23,6	integrin, alpha 6
5,3	388,4	19,42	immunoglobulin superfamily, member 4
17,7	288,05	14,4	Notch homolog 2 (Drosophila)
0,9	284,7	14,24	integrin, alpha 6
27,8	388,4	13,97	immunoglobulin superfamily, member 4
15,4	277,2	13,86	ELK3, ETS-domain protein (SRF accessory protein 2)
35,2	480,1	13,64	S100 calcium binding protein A10 (annexin II ligand, calpactin I)
22,4	262	11,7	melanoma antigen, family A, 1 (directs expression of antigen MZ2-E)
21,2	215,05	10,14	caveolin 1, caveolae protein, 22kD
4,6	173,05	8,65	B cell RAG associated protein
10,8	169	8,45	interleukin 10
18,25	163,15	8,16	chemokine (C-C motif) receptor 2
33,7	271,45	8,05	BCL2-associated athanogene 3
5,15	149,85	7,49	G protein-coupled receptor 37 (endothelin receptor type B-like)
52,85	330,7	6,26	G protein-coupled receptor, family C, group 5, member D
17	116,75	5,84	caveolin 1, caveolae protein, 22kD
2,4	115,25	5,76	interleukin 6 receptor
8,8	108,7	5,43	G protein-coupled receptor 56
6,6	103,15	5,16	G protein-coupled receptor 48
159,3	16,5	-7,97	tumor necrosis factor receptor superfamily, member 7
160,85	9,3	-8,04	paired immunoglobulin-like receptor alpha
569,05	51,95	-10,95	interleukin 2 receptor, gamma (severe combined immunodeficiency)
221,2	5,65	-11,06	CD22 antigen
230,5	20,7	-11,14	integrin, beta 2 (antigen CD18 (p95)
223,6	16,7	-11,18	Fc fragment of IgG, low affinity IIb, receptor for (CD32)
233,65	6	-11,68	major histocompatibility complex, class II, DO beta
268,75	22,5	-11,94	G protein-coupled receptor 2
317,8	22,45	-14,16	CD79A antigen (immunoglobulin-associated alpha)
383,2	19,75	-19,16	CD19 antigen
392,15	2,7	-19,61	Fc fragment of IgE, low affinity II, receptor for (CD23A)
476,85	22,9	-20,82	T cell receptor alpha locus
622,7	29,8	-20,9	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)
440,15	14,65	-22,01	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)
595,9	23,45	-25,41	T cell receptor alpha locus
530,1	15,65	-26,5	integrin, beta 7
531,7	8,05	-26,59	Fc fragment of IgE, low affinity II, receptor for (CD23A)
536,15	13,05	-26,81	leukocyte membrane antigen
746,4	11,15	-37,32	CDW52 antigen (CAMPATH-1 antigen)
1625,9	23,8	-68,32	major histocompatibility complex, class II, DQ beta 1
3977,35	20,45	-194,49	immunoglobulin heavy constant gamma 3 (G3m marker)

Table 1: Altered expression of cellular genes in KSHV-infected B-cells.
The column "KSHV- signal" shows the mean expression level of cellular genes in EBV-infected B-cells analysed in duplicates. The KSHV+ signal represents the mean expression level of cellular genes in KSHV-infected B-cells analysed in duplicates. The differences in the gene expression level between KSHV+ and EBV+ cells is presented as fold change in the third column. The table summarizes the genes which expression is significantly changed in the range of –5 to –200 and 5 to 90 fold. The genes of interest are depicted in red.

4.3 Confirmation of the microarray data

4.3.1 Transcriptional downregulation of B-cell surface markers after KSHV-infection

To validate the microarray data the expression of some B-cell specific surface markers was tested by RT-PCR (Fig.9). In all KSHV-positive cell lines no BCR, CD19, CD79 and B7-1(CD80) transcripts were detected.

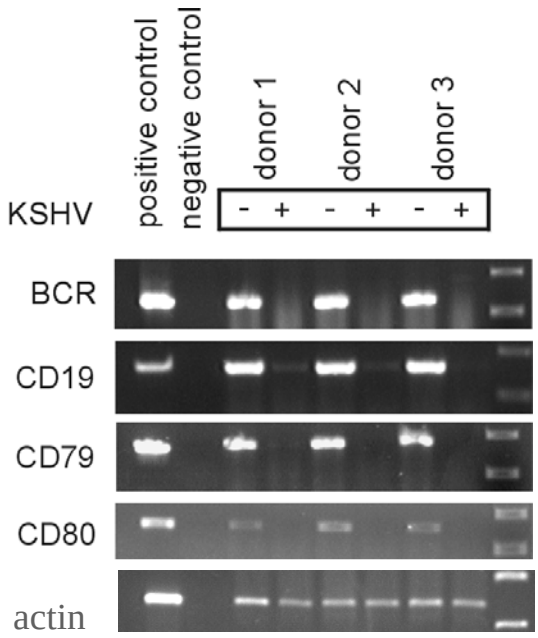


Fig. 9: Transcriptional downregulation of B-cell markers in B-cells infected with KSHV.
Total RNA was isolated from the KSHV+ and the EBV+ cell lines. RT-PCR was performed using specific primers for BCR, CD19, CD79, B7-1(CD80) and actin. The results showed that no PCR product was detected in the KSHV+ samples.

4.3.2 Downregulation of B-cell surface markers on protein level after KSHV-infection

The expression of the B-cell markers BCR, CD19, CD79 and the co-stimulatory molecule B7-1 (CD80) was also tested on protein level by FACS analysis. This analysis confirmed the complete downregulation in KSHV-infected B-cells in comparison to EBV-infected B-cells (Fig.10). In contrast, the transferrin receptor (CD71) as well as MHC class I were still present on the KSHV-positive B-cells. The PEL cell line BCBL (KSHV+) showed a

similar downregulation of B-cell surface markers, indicating that KSHV-infected B-cells develop a similar phenotype as the PEL tumor cells (Fig. 10). In addition, the results indicated that KSHV is responsible for the phenotype of PEL tumor cells.

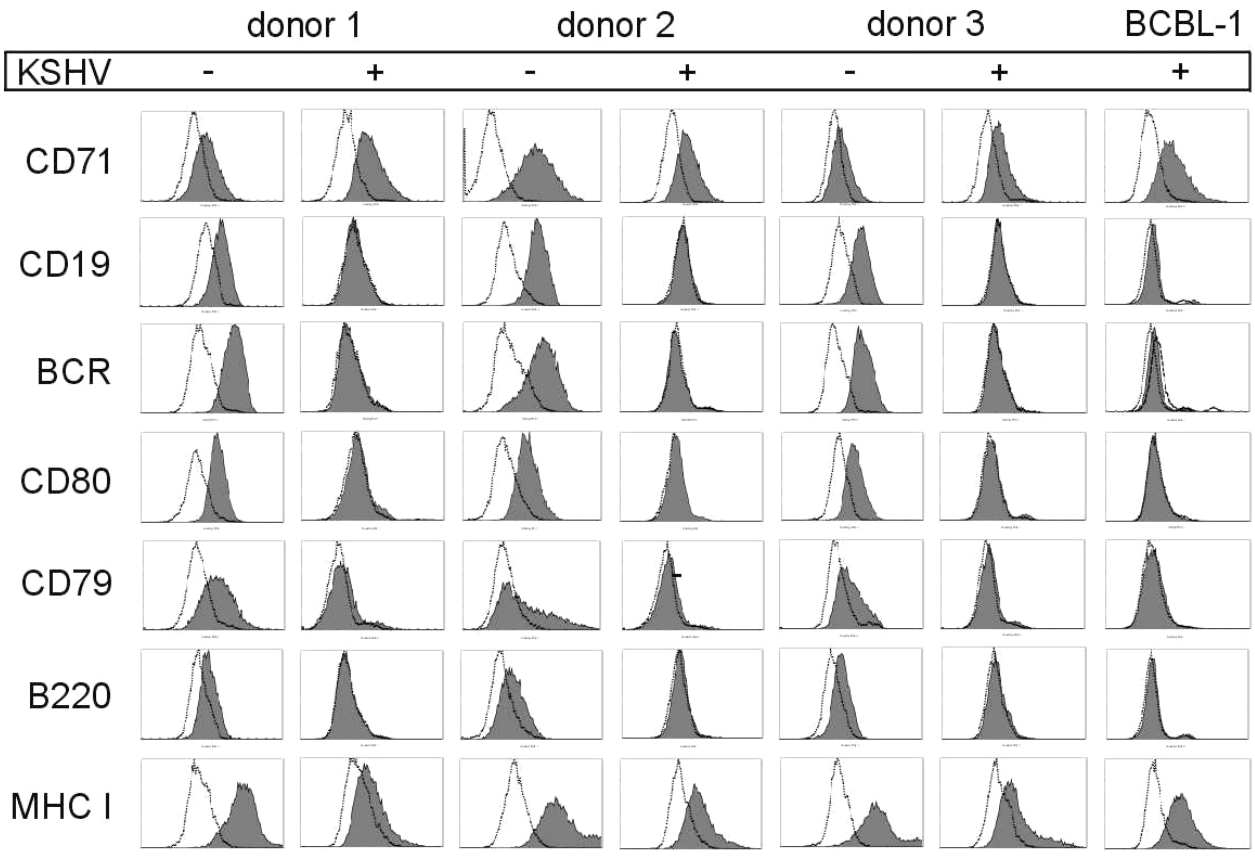


Fig. 10: Downregulation of B-cell markers in KSHV-positive B-cells on protein level.
A FACS analysis was performed using FITC-conjugated antibodies against the BCR, CD79 and B220. The expression of CD19, CD71 and MHC class I was tested using unconjugated antibodies or hybridoma supernatants and secondary FITC-conjugated anti-mouse IgG antibodies. The dotted peaks represent the control stained with a FITC-conjugated secondary antibody and the filled peaks represent the expression level of the surface molecules.

4.3.3 IL-6 and IL-10 are highly secreted from B-cells infected with KSHV

In vivo IL-10 and IL-6 were found to be upregulated in patients with KS and PEL. Their expression was tested on both transcriptional as well as protein level. Both IL-6 and IL-10 transcripts were determined to be significantly upregulated in KSHV-infected cells (Fig 11B).

In addition, cell culture supernatants from KSHV+ and EBV+ B-cells were collected after 2 days and tested for secreted IL-6 and IL-10 by ELISA.

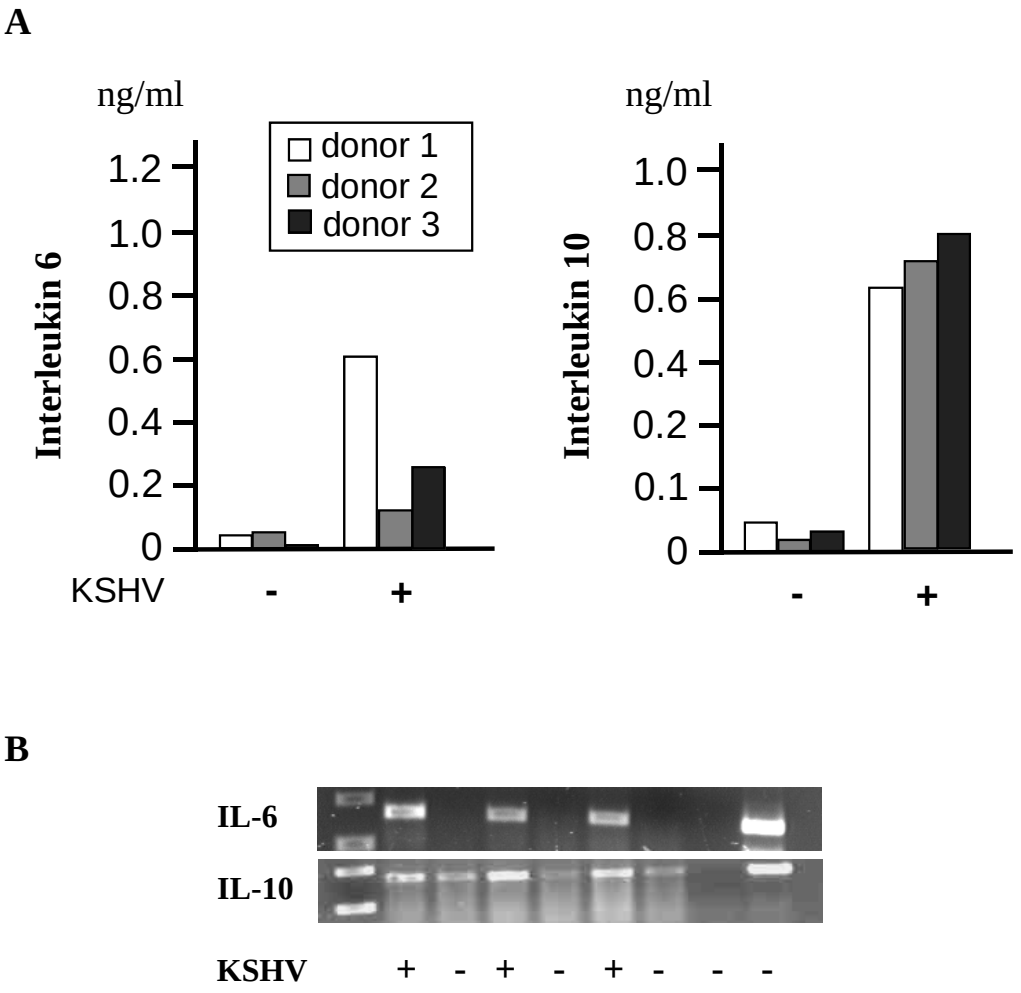


Fig. 11: IL-6 and IL-10 are upregulated on the mRNA and protein level in cells infected with KSHV.
A: ELISA assay for determining the concentration of secreted IL-6 and IL-10. B: RT-PCR analysis showing increased levels of both of the cytokines in KSHV-infected B-cells.

As shown in Figure 11 A, the concentration of both cytokines was significantly increased in KSHV+ B-cells. The concentration of secreted IL-10 was between 600-800 pg/ml, which is approximately 15 to 20-fold higher than in EBV-infected B-cells (20-50 pg/ml). The secreted amount of IL-6 was detected to be in the range of 250-600 pg/ml in KSHV+ (5 to 12-fold higher) and below 50 pg/ml in the control EBV + B-cells. Based on

these results it was concluded that KSHV causes an increased secretion of IL-10 and IL-6 similar to PEL cells.

4.4 Downregulation of B-cell transcription factors in B-cells infected with KSHV

The presented results showed that KSHV-infected cells have a unique phenotype ("null" phenotype), characterized by a complete downregulation of basically all B-cell markers similar to PEL cells. Since B-cell development is driven mainly by B-cell transcription factors, their expression was tested by reverse transcription polymerase chain reaction (RT-PCR) in KSHV-positive cell lines. In the microarray screen the transcription factor Spi-B was found to be downregulated approximately 20-fold (-19.83x). To test the expression of other main B-cell transcription factors including E2A, EBF-1, Pax-5, Oct-2, Bob-1 and Blimp-1 in KSHV-infected cells, a RT-PCR analysis was performed. The results indicated that the B-cell factors E2A, EBF-1 and Pax-5, which appear in the early stages of the B-cell development, were not transcriptionally modulated. Spi-B, Oct-2 and Bob-1, which are highly expressed in mature B-cells, were downregulated in KSHV-positive cells (Fig. 12). Blimp-1, which is a transcriptional repressor of Pax-5 and takes part in the transition to the plasma-cell stage of the B-cell differentiation, was transcriptionally upregulated. Interestingly, Pax-5 was not detected on the protein level in KSHV-infected B-cells, indicating that it might be posttranscriptionally downregulated by a different mechanism (Fig.13).

In summary, it was concluded that in KSHV-infected cells the downregulation of surface markers is related to an altered expression of several B-cell transcription factors.

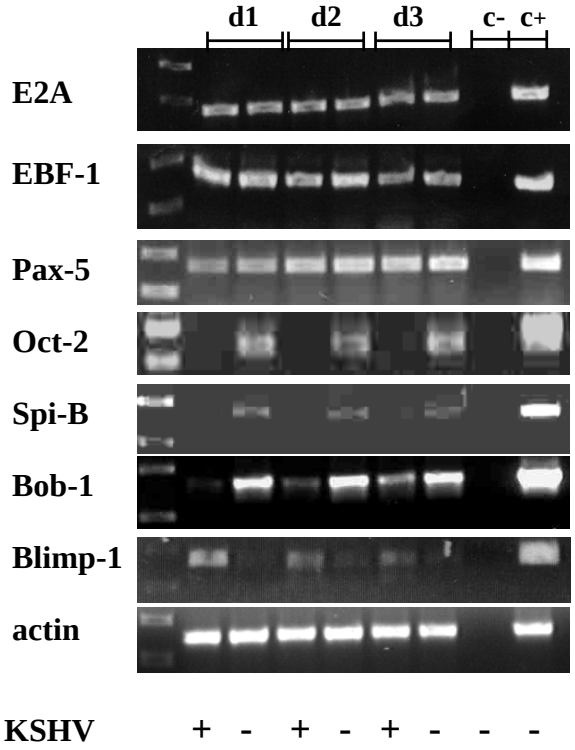


Fig. 12: Downregulation of specific B-cell transcription factors in KSHV-positive cells on the transcriptional level.
Total RNA was isolated from all three donors (d1, d2 and d3) with a following cDNA synthesis. RT PCR was performed using specific primers for the B-cell transcription factors. As a loading control RT-PCR for actin was performed. As a negative control (c-) PCR reaction mixture without template cDNA was used and as a positive control (c+) human cDNA library was used.

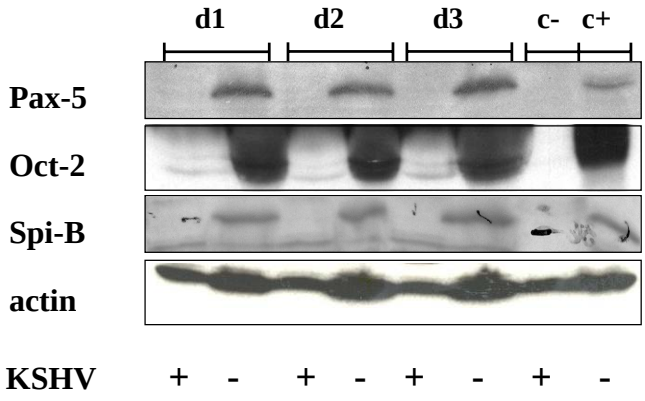


Fig. 13: Downrgulation of Pax-5, Oct-2 and Spi-B on the protein level.
Cell lysates from all three donors (d1, d2 and d3) and the controls (c- or BCBL-1 and c+ or Burkit lymphoma B-cell line DG75) were collected and subjected to SDS-PAGE. The Western blot for detection of Pax-5, Oct-2 and Spi-B was performed using the following antibodies: goat anti-human anti-Pax-5, rabbit anti-human anti-Oct-2 and goat anti-human anti-Spi-B antibodies. In parallel, as a control Western blot for detection of actin was performed using a rabbit anti-human anti-actin antibody.

4.5 Expression of the syndecan (CD138) plasma cell marker on KSHV-infected B-cells

The downregulation of Pax-5 and the upregulation of Blimp-1 suggested that KSHV-infected B-cells have developed a plasma cell-like phenotype. Therefore, they were tested for the expression of CD138 (syndecan) surface protein, which is a marker for plasma cell stage of the B-cell differentiation. The FACS analysis showed the presence of this protein in all KSHV-positive cell lines, confirming their plasma cell-like phenotype (Fig.14).

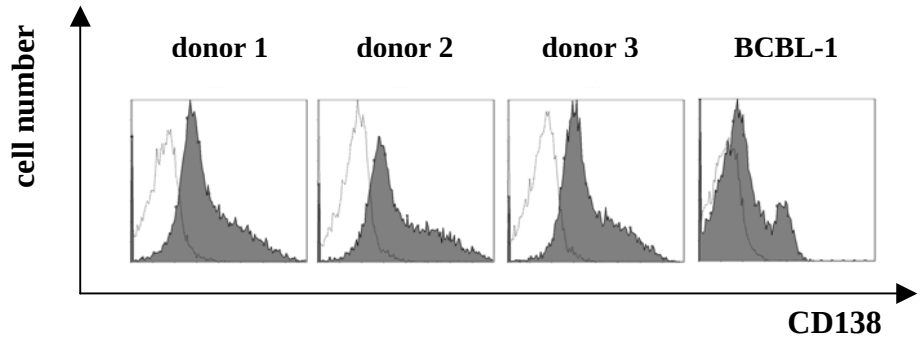


Fig. 14: Expression of CD138 (syndecan) on KSHV positive B-cells and PEL tumor cells (BCBL-1).
KSHV-positive B-cells from the three donors together with PEL tumor cells were stained with a mouse anti-human anti-CD138 monoclonal antibody and subsequently, cells were analysed by FACS analysis. The dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody. The filled peaks represent the expression of CD138.

4.6 Pax-5 is not degraded by the cellular proteasome

Since Pax-5 was present on mRNA but not on the protein level in KSHV+ cells, it was hypothesized that Pax-5 is downregulated due to protein degradation. To test this possibility, KSHV-positive B-cells were treated with the specific proteasome inhibitor MG132 and subsequently tested for Pax-5 expression by Western blot analysis (Fig. 15). The results showed that Pax-5 was not expressed after blocking the proteasome, suggesting that the protein is not degraded via the cellular proteasome system.

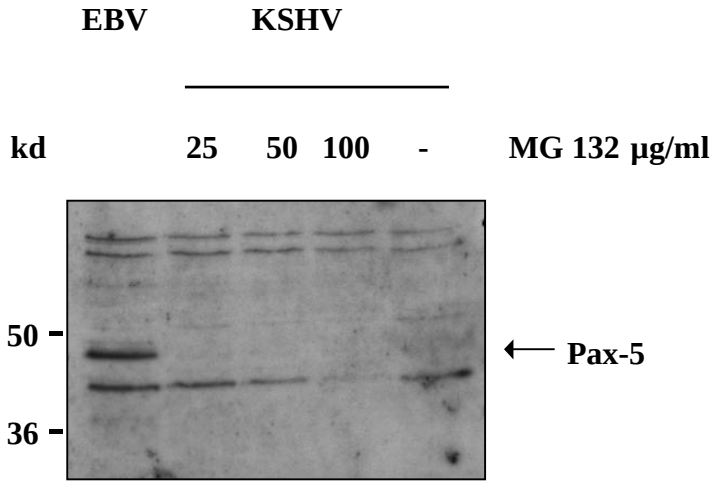


Fig. 15: Pax-5 is not detected after blocking the proteasome pathway.
KSHV-positive B-cells were treated with different concentrations of the proteasome inhibitor MG132, as indicated, and incubated for 3,5 hours. The cells were lysed in 1% NP40 lysis buffer and the cell lysates were subjected to SDS-PAGE on a 12% gel. After blotting onto nitrocellulose membrane Pax-5 was detected by a polyclonal anti-Pax-5 antibody. As a positive control for the western blot EBV-positive cells from the same donor were used.

4.7 A soluble factor(s) released from KSHV-positive B-cells is responsible for the downregulation of B-cell markers

The results presented above indicated that the "null" phenotype is most probably due to an altered B-cell transcriptional program. The persistently KSHV-infected B-cells used in this approach were generated via an infection of PBMCs with supernatant from the PEL cell line BCBL-1 (as described above). To test if a soluble cellular or viral factor is involved in the induction of the "null" phenotype, EBV-positive 721 lymphoblastoid cells were incubated with supernatant derived from the three KSHV-positive B-lymphocytes. Supernatants (SN) of cell lines from three different donors were collected on the 2^{nd}, 3^{rd} and 4^{th} day after the KSHV-positive B-cells have been seeded and subsequently added to the 721 cells. After two days of incubation the cells were checked for expression of the CD19 marker (Fig.16). CD19 was downregulated by all three KSHV-positive supernatants. This was the first hint that a factor(s) present in cellular supernatant is involved in the downregulation of B-cell markers in cells infected with KSHV. This factor could be the virus itself, a viral protein or a cellular factor induced by the virus.

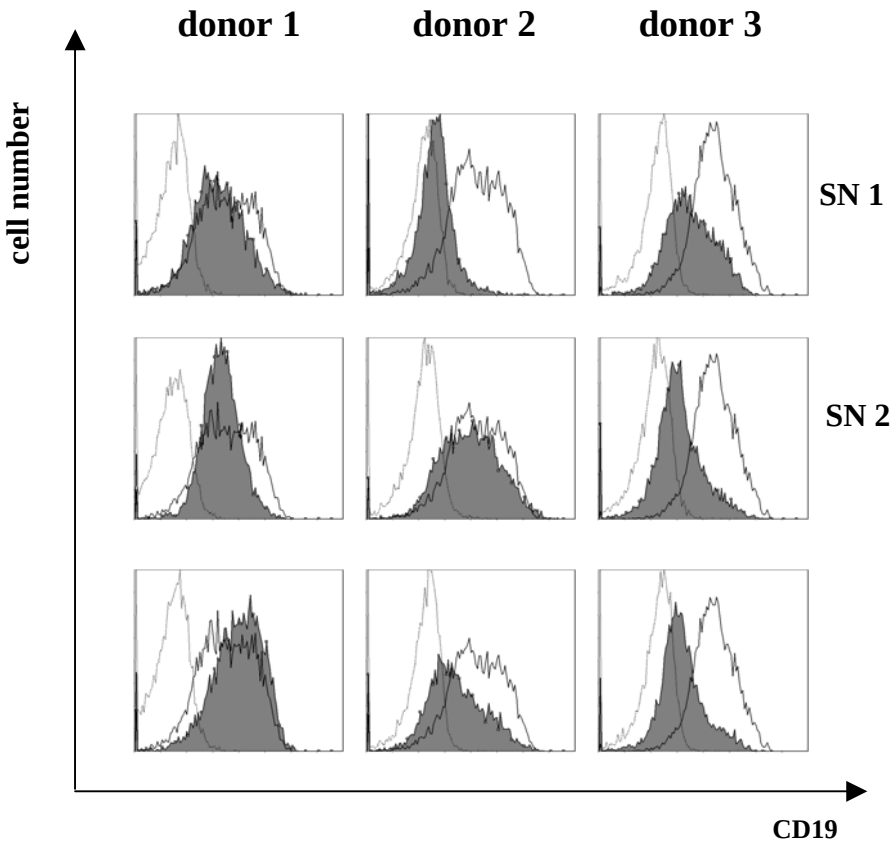


Fig. 16: Downregulation of the B-cell specific marker CD19 in presence of KSHV supernatant.
KSHV-positive cells from three donors were adjusted to a density $0.5x10^6$ cells/ml and supernatants (SN) were collected on the 2^{nd}, 3^{rd} and 4^{th} day. An EBV-transformed lymphoblastoid cell line 721 was incubated for two days with the KSHV-positive supernatant using a dilution of 1:2 in fresh medium. After the incubation cells were stained with an anti-CD19 antibody and analysed by FACS. The filled curves represent the expression of CD19 after the addition of supernatant from KSHV+ cells, the non-filled peaks represent the expression of CD19 in untreated cells and the dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody. The altered CD19 expression was reproducible in two independent experiments.

4.7.1 Characterization of the soluble factor responsible for the downregulation of B-cell surface markers

To test if KSHV viral particles present in the supernatant are responsible for the downregulation of CD19 and the other B-cell markers, or rather a soluble viral or cellular protein, supernatants were fractionated into two fractions >100kd and <100kd by ultrafiltration. The results presented in figure 17 clearly showed that CD19 was

downregulated when supernatant containing proteins <100kd was used. This suggested that the factor(s) is a secreted molecule, rather than the virus itself. This factor(s) is secreted only from KSHV-infected cells, as an altered CD19 expression was not detected in supernatants from EBV-infected cells (Fig.17).

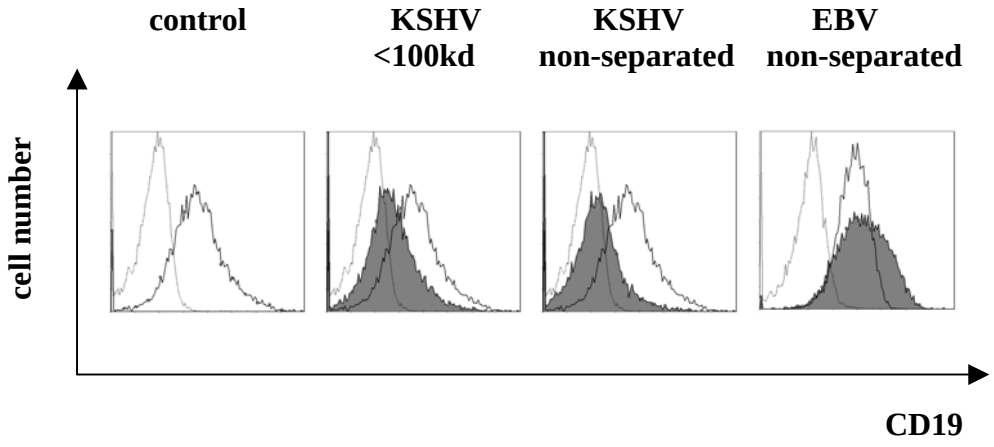


Fig. 17: The downregulation of CD19 is due to a soluble factor secreted from KSHV-infected cells.
The supernatants from the KSHV+ cells was centrifuged at 2500g, 2.5h, using ultrafiltration columns (Microsep/Filtron 100K). The EBV-transformed lymphoblastoid cell line 721 was incubated for two days with either ultrafiltrated or non-separated supernatant from KSHV-positive B-cells or with supernatant from EBV-positive B-cells. After the incubation the cells were stained with an anti-CD19 antibody and analysed by FACS. The filled peaks represent the CD19 expression after addition of KSHV-positive supernatant. The non-filled peaks represent the expression of CD19 in untreated cells and the dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody.

4.7.2 A soluble factor(s) present in PEL effusion fluid downregulates B-cell markers

To ensure that the change of CD19 expression after KSHV-infection is also present *in vivo*, a pleural effusion fluid from an HIV-1 positive patient with PEL was used. The effusion fluid was filtrated through filters with 0.4µm cut-off and cells were incubated with the PEL fluid for 2 and 4 days. The FACS data showed a similar downregulation of CD19 by PEL fluid as in the cells incubated with supernatant from KSHV-positive B-cells (Fig.18 A, B). These results indicated that the downregulation of CD19 is due to a soluble factor secreted from KSHV-infected cells, which is also present in PEL fluid.

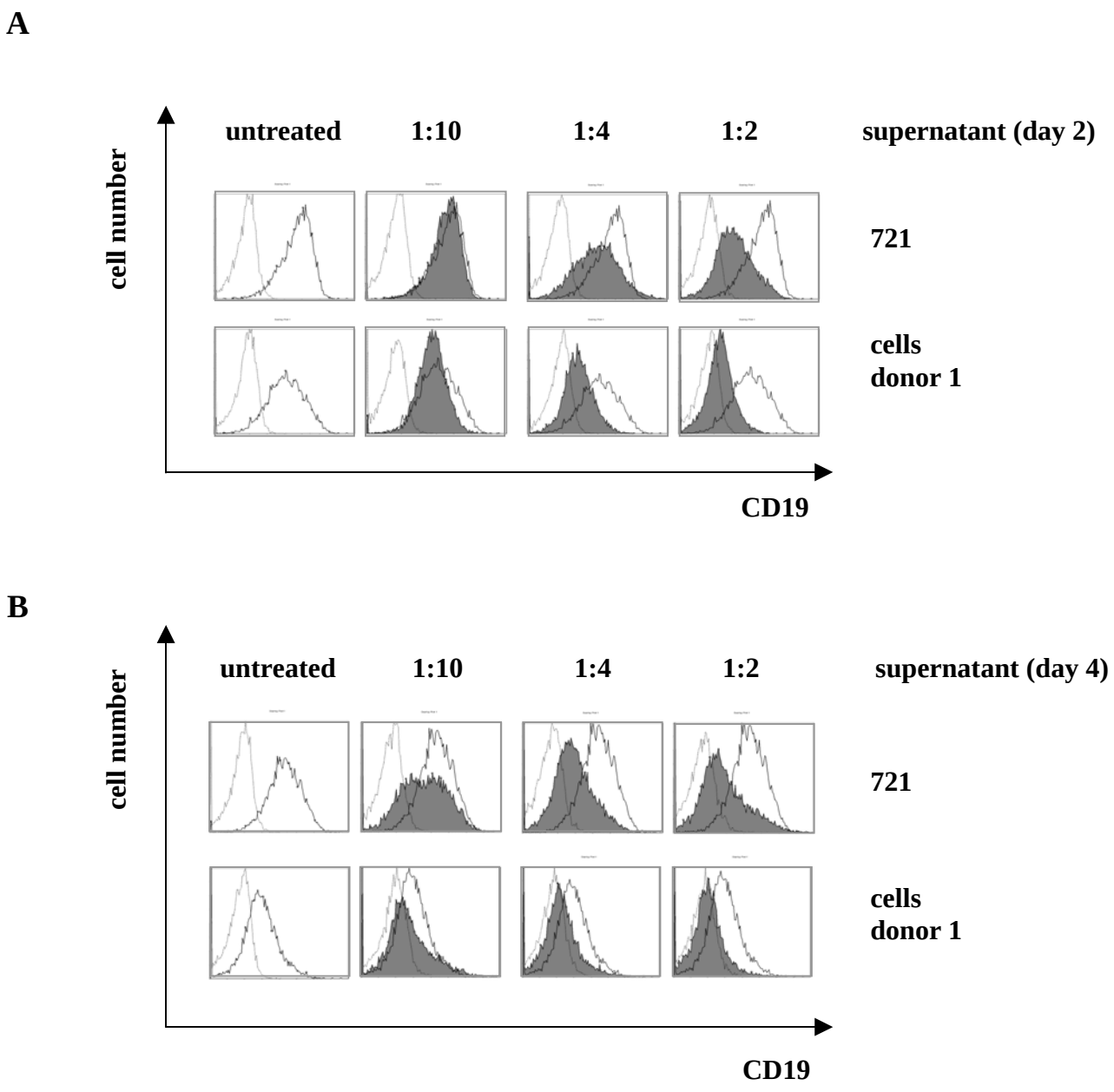


Fig. 18: Downregulation of CD19 in the presence of PEL effusion.
PEL effusion from a patient with both KS and PEL was added to 721 cells and to one of the KSHV-EBV+ donor and incubated for 2 (A) and 4 (B) days. Subsequently, a FACS analysis for CD19 was performed and compared to cells incubated with medium. The filled peaks represent the expression of CD19 after incubation with PEL fluid, the non-filled peaks represent the expression of CD19 in untreated cells and the dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody.

4.7.3 Altered morphology induced in B-cells by PEL effusion fluid

The downregulation of CD19 by supernatants of KSHV-infected cells and PEL effusion fluid was accompanied by a change of morphology (Fig.19). B-cells incubated with PEL effusion fluid grew as single, non-clustered and completely round cells without the protrusions which are characteristic for EBV-transformed B-cells. The control cells were cultured in a control pleural effusion fluid from a patient with heart disease and did not change their phenotype.

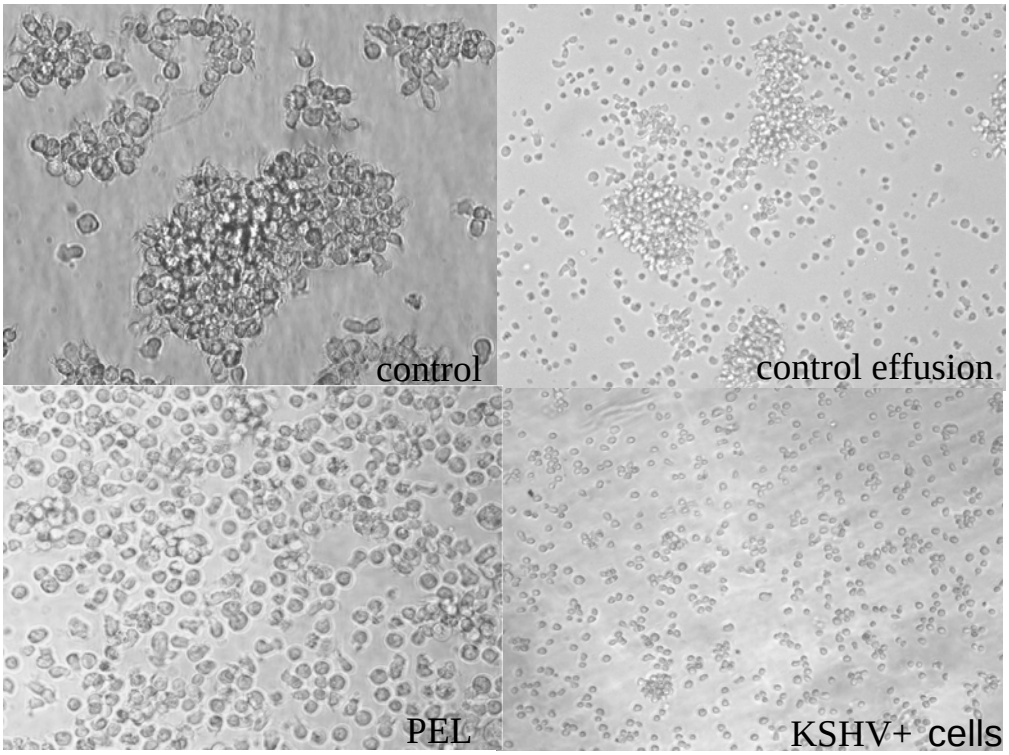


Fig. 19: Altered phenotype of EBV-positive B-cells in the presence of PEL effusion fluid. Microscopical pictures of EBV-positive cells treated with either medium (control), a control effusion fluid or a PEL effusion fluid. Additionaly, KSHV-positive B-cells from one of the donors are indicated.

Taken together these results strongly support the hypothesis that the development of the “null” phenotype is caused by a factor secreted in the PEL effusion and into the cell culture supernatant by KSHV-positive B-cells.

4.7.4 IL-10 is not involved in the downregulation of B-cell markers

IL-10 is one of the soluble factors secreted by KSHV-infected B-cells (see 4.3.3). As shown in figure 11, KSHV-positive cells secrete approximately 15 to 20-fold more IL-10 in comparison to B-cells transformed by EBV. Therefore, we checked if IL-10 would affect the expression of CD19 and respectively the other B-cell markers. For this purpose EBV-positive B-cells were grown in the presence of recombinant human IL-10 for 2 days. Subsequently, the cells were analysed for the expression of CD19 by FACS analysis. The result showed that IL-10 did not have a significant effect on the expression of CD19 (Fig.20). At high IL-10 concentrations, a small shift towards a lower CD19 expression was observed. However, the concentration of the recombinant human IL-10 used in the experiment was considerably higher than the concentration in supernatants of KSHV-infected B-cells (0.6-0.8 pg/ml) (Fig. 20). Based on these results, it was concluded that physiological concentrations of IL-10 do not modulate the expression of B-cell surface markers.

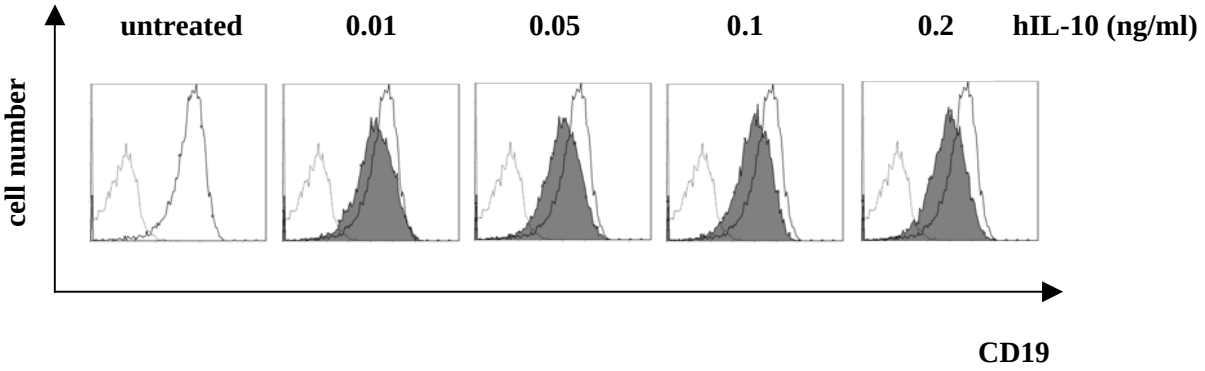


Fig. 20: IL-10 has no effect on CD19 expression.
Recombinant human IL-10 was added in increasing concentrations (0.01-0.2 ng/ml) to EBV-transformed B-cells. The filled peaks represent the expression of CD19 after incubation of EBV-positive cells with IL-10, as analysed by FACS. The filled peaks represent the expression of CD19 after incubation with recombinant hIL-10, the non-filled peaks represent the expression of CD19 in untreated cells and the dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody.

4.7.5 The KSHV homologue of the cellular chemokine MIP-II (vMIP-II) is not involved in downregulation of CD19

KSHV encodes several viral gene products that are homologous to chemokines. The KSHV ORFs K6 (vMIP-1/vMIP-1a/cCCL-1), K4 (vMIP-II/vMIP-1b/vCCL-2) and K4.1 (vMIP-III/vBCK/vCCL3) encode chemokines with homology to cellular CC chemokines such as MIP-1α and RANTES. In contrast to cellular chemokines, the viral MIPs, which are secreted from latently infected PEL cells, are highly angiogenic and possess immunoregulatory functions. Therefore, we checked if the addition of recombinant vMIP-II could affect the expression of B-cell surface molecules by incubating EBV-transformed B-cells for two days with recombinant vMIP-II. The expression of CD19 was subsequently checked by FACS analysis. The results presented in figure 21 clearly showed no significant downregulation of CD19 after the addition of recombinant vMIP-II. These data concluded that vMIP-II is not directly involved in the downregulation of B-cell markers.

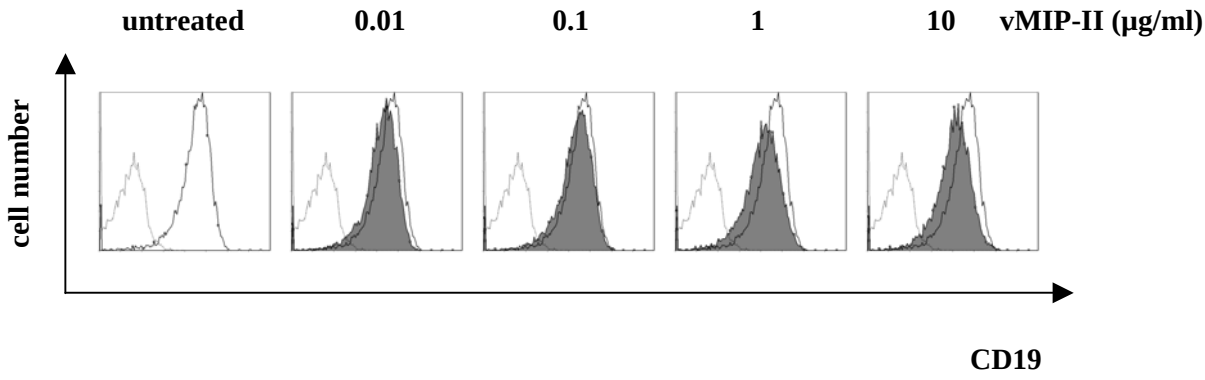


Fig. 21: vMIP-II is not involved in the downregulation of CD19.
Recombinant vMIP-II was added in increasing concentrations (0.01-10µg/ml) to the control EBV-positive (KSHV-negative) B-cells. The expression of CD19 (grey peaks) was checked after 2 days by FACS analysis. The filled peaks represent the expression of CD19 after incubation with recombinant vMIP-II, the non-filled peaks represent the expression of CD19 in untreated cells and the dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody.

4.7.6 Recombinant vIL-6 does not cause a downregulation of CD19

In addition to IL-10 and vMIP-II, PEL cells secrete the viral homologue of cellular IL-6 (vIL-6). The effect of vIL-6 on CD19 expression was tested by incubating EBV-transformed B-cells for two days with recombinant vIL-6. The expression level of CD19 was subsequently tested by FACS analysis. The results presented in figure 22 showed no significant downregulation of CD19 after the addition of recombinant vIL-6. Based on these results it was concluded that the vIL-6 is not directly involved in the downregulation of B-cell markers.

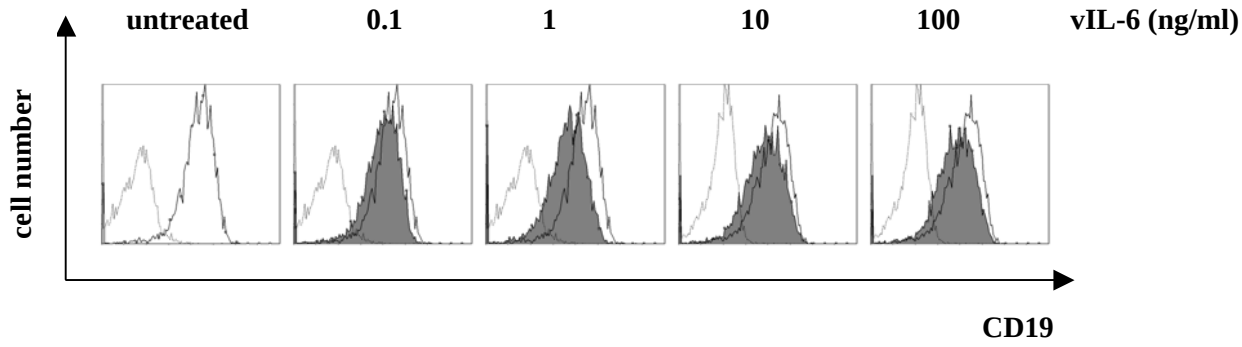


Fig. 22: The viral homologue of IL-6 does not change the expression of CD19.
Recombinant vIL-6 was added in increasing concentrations (0.1-1ng/ml) to EBV-positive (KSHV-negative) B-cells . The expression of CD19 (grey peaks) was tested after 2 days by FACS analysis. The filled peaks represent the expression of CD19 after incubation with recombinant vIL-6, the non-filled peaks represent the expression of CD19 in untreated cells and the dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody.

4.8 Functional characterization of KSHV-infected cells.

4.8.1 KSHV-infected cells cannot be activated by the crosslinking of CD19

This study clearly showed that KSHV-infected B-cells lose their B-cell phenotype as they lack most of the B-cell markers. The next question was thus weather this downregulation is correlated with a functional impairment. For this purpose, the KSHV-positive and the EBV-positive B-cells were treated with an anti-CD19 and a secondary antibody to crosslink the CD19 receptor. Upon crosslinking of CD19, normal B-cells are activated and express co-stimulatory molecules like B7-2 (CD86). As shown in Fig.23, the EBV-infected B-cells could be activated via CD19 crosslinking and showed an increased expression of B7-2. The KSHV-positive cells, however, could not be activated, which correlates with the downregulation of CD19.

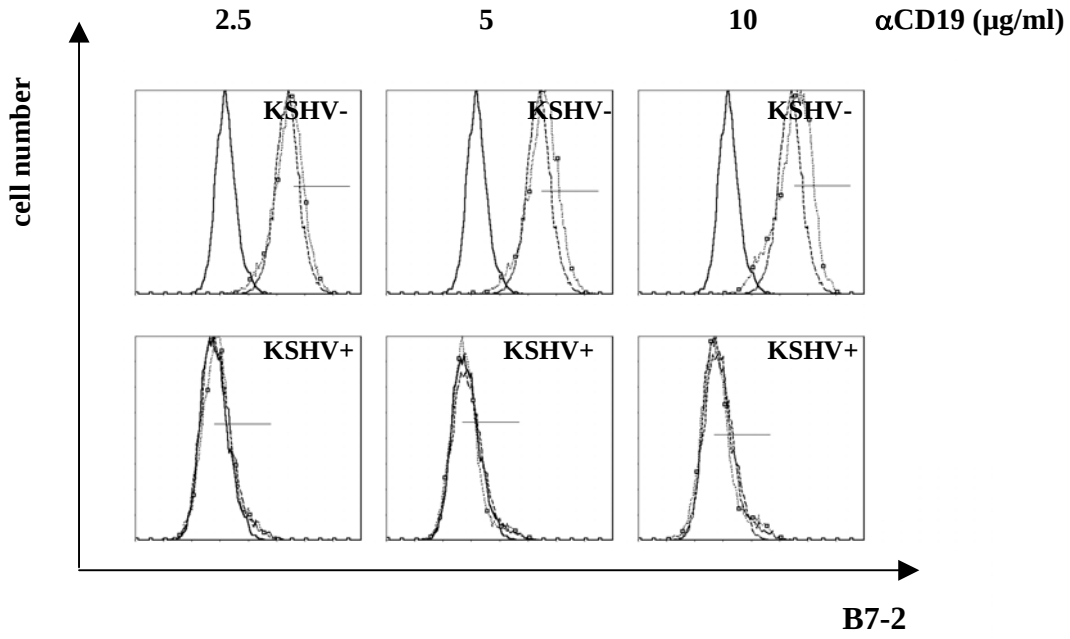


Fig. 23: KSHV-positive cells cannot be stimulated by crosslinking of CD19.
KSHV-positive and EBV-positive cells were incubated with anti-CD19 antibody in increasing concentrations (2.5-10µg/ml) at 37°C. After 24 hours, cells were stained for B7-2 (CD86) using a FITC-conjugated anti-B7-2 antibody and analysed by FACS. The expression of B7-2 could not be stimulated in KSHV-positive cells even if increasing concentrations of the crosslinking CD19 antibody were used.

4.8.2 Blockade of the IFN signalling pathway in KSHV-infected B-cells

The microarray analysis indicated a downregulation of genes involved in IFN signalling in KSHV-infected cells including MxA (-35,44), MxB (-8,35), IRF-7 (-10,61) and

IRF-5 (-2,62) (Fig. 24). To test if KSHV-positive cells can be stimulated by IFN, they were incubated with IFNα for different periods of time (Fig.25 A). After IFN stimulation, the *Jak/ Stat* signal transduction pathway is activated by the phosphorylation of *Stat-1* and *Stat-2* proteins. As shown in figure 25, *Stat-1* and *Stat-2* were phosphorylated after 15 and 25 min in response to IFNα in both KSHV-infected and EBV-infected B-cells. However, MxA was not upregulated in KSHV-infected B-cells after IFNα stimulation (Fig.25 B).These results led to the conclusion that IFN signalling is blocked downstream of *Stat-1/Stat-2* in KSHV-infected B-cells.

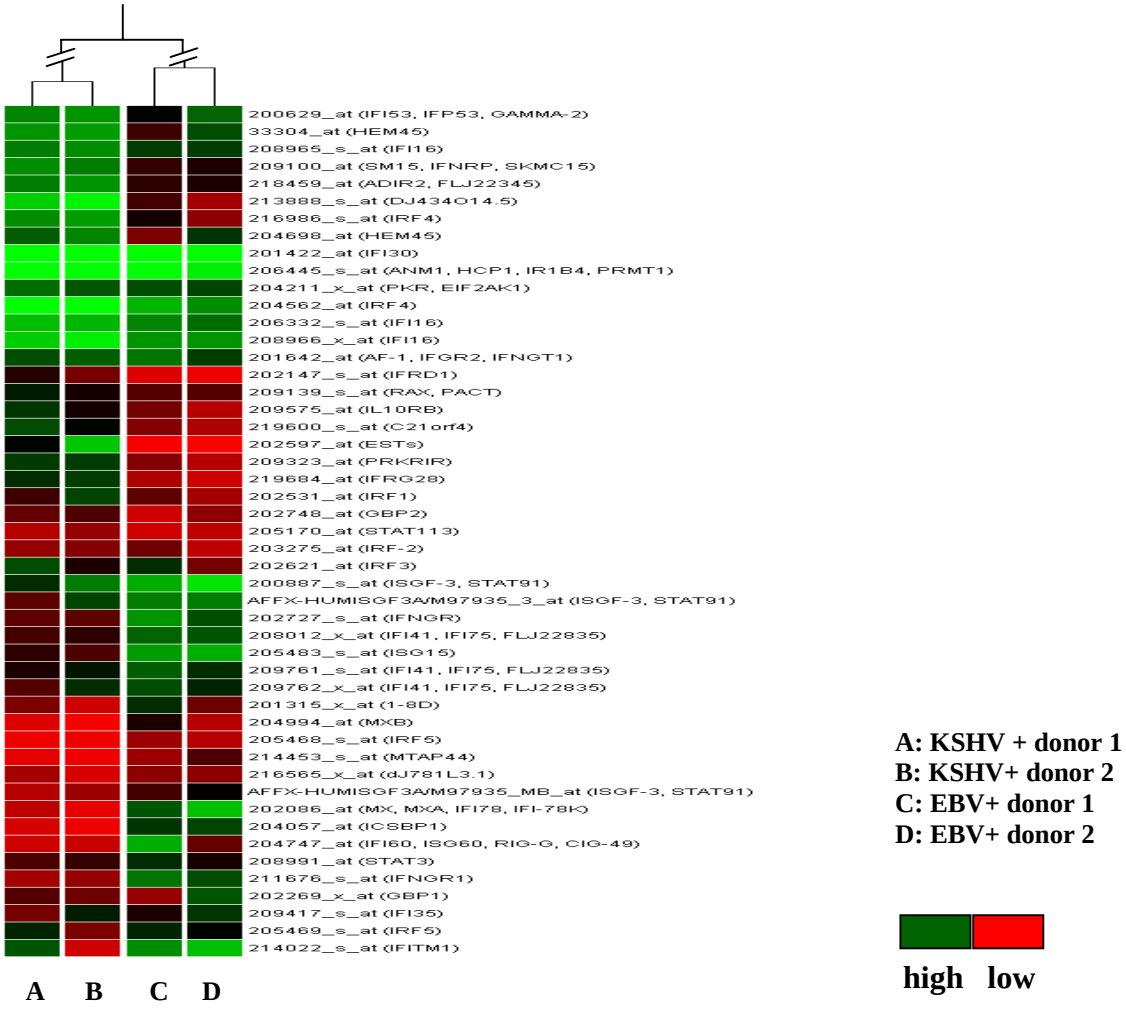


Fig. 24: Impaired expression of IFN regulated genes.
Genes, which expression is downregulated after KSHV- or EBV-infection, are depicted in red, in contrast, the upregulated genes are depicted in green.

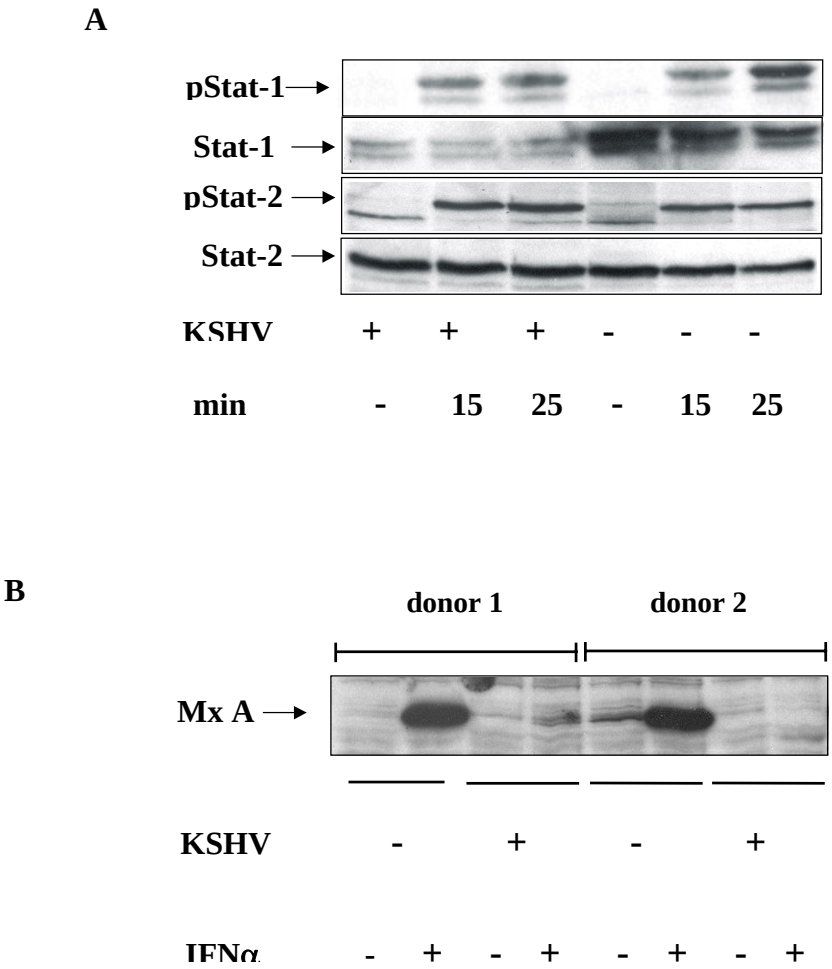


Fig. 25: Stimulation of KSHV-infected B-cell by IFNα.
A: KSHV- and EBV-infected B-cells were stimulated with 1000 U/ml IFNα for 15 or 25 min. Subsequently, the cells were lysed in 1% NP40 lysis buffer and subjected to SDS-PAGE using 10% gel. Western blot analysis was performed with anti-phospho-*Stat-1/2* and anti-*Stat-1/2* antibodies. B: KSHV- and EBV-infected B-cells from two donors were stimulated with 1000 U/ml IFNα for 15 min. Subsequently, the cells were lysed in 1% NP40 lysis buffer and subjected to SDS-PAGE using 10% gel. Western blot analysis was performed with anti-Mx antibody.

4.8.3 Impaired lysis of KSHV-infected cells by Natural Killer (NK) cells

Since a multitude of B-cell markers were downregulated in KSHV-positive B-cells, it was hypothesized that NK-cell recognition is impaired. To test this issue, KSHV-positive B-cells were incubated with NK92 NK-cells in a chromium release cytotoxic assay (Fig.26). The KSHV-positive cells derived from the first and the third donor showed a decreased cell lysis through NK92 cells. The specific lysis was 23% (KSHV+) and 64% (EBV+) for the first donor and 30% (KSHV+) and 45 % (EBV+) for the third donor at an effector: target (E:T) ratio of 10:1. There was no significant difference in the NK lysis between the KSHV+ and

EBV+ cells (43% vs. 37%) of the second donor, suggesting that for this donor stimulatory NK cell ligands are not downregulated in KSHV-positive B-cells.

These data suggested that B-cells infected with KSHV cannot completely evade the innate immune system.

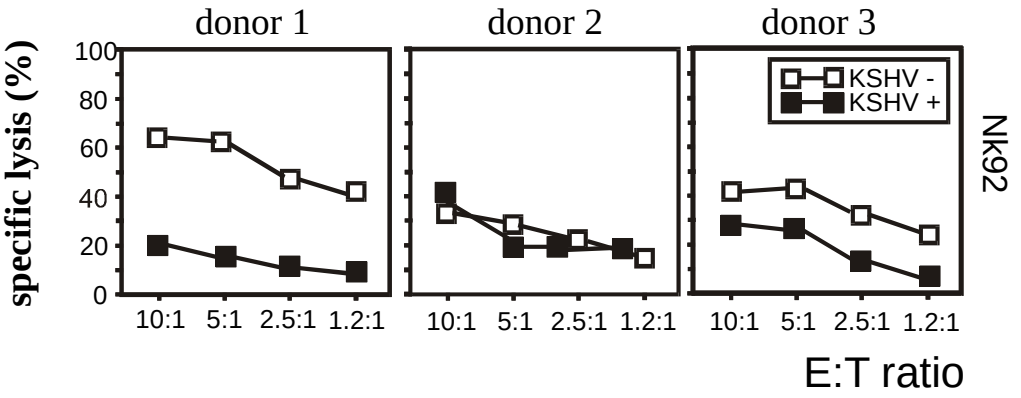


Fig. 26: Differential NK lysis of KSHV-infected B-cells originating from three independent individuals.
KSHV-positive and EBV-positive B-cells from three donors were diluted at concentration of 10^6 cells/100µl and labelled with [^{51}Cr] for 1.5 hours. Subsequently the labelled target cells were washed with cell culture medium to remove the rest of the radioactivity. The target cells were diluted at a concentration $2x10^3$/50µl and added to the NK cell line NK92. The NK92 cells were diluted at a ratio of 10:1-1.2:1. The target and effector cells were incubated for 4 hours. After the incubation supernatants were collected, dried (overnight) and measured for [^{51}Cr] release.

4.8.4 Impaired lysis of KSHV-infected B-cells by allo-reactive cytotoxic T-cells (CTL)

Two different T-cell clones JB4 and 234 recognizing two disctinct haplotypes of the HLA-A, HLA-A2 and HLA-A24, were used to test CTL recognition. KSHV+ and EBV+ cells from each donor were labelled with [^{51}Cr] and incubated with the CTL cell lines. The first and the third donor carried the HLA-A2 haplotype, as the CTL clone JB4 recognized the EBV-positive cells of these donors. The second donor carried the HLA-A24 haplotype, as the EBV-positive cells were recognized by the 234 CTL clone. The results indicated consequently that the cells infected by KSHV could not be recognized and lysed by the CTLs (Fig.27). The specific lysis of KSHV-positive B-cells was at a background level for all three donors. The control EBV-infected cells, in contrast, were efficiently lysed (68-85%). In summary, the data indicated that the CTL lysis of KSHV-infected cells was completely abolished in all donors,

in contrast to NK lysis. This indicates that cells infected by KSHV differ in their efficiency to evade the innate or adoptive immune system.

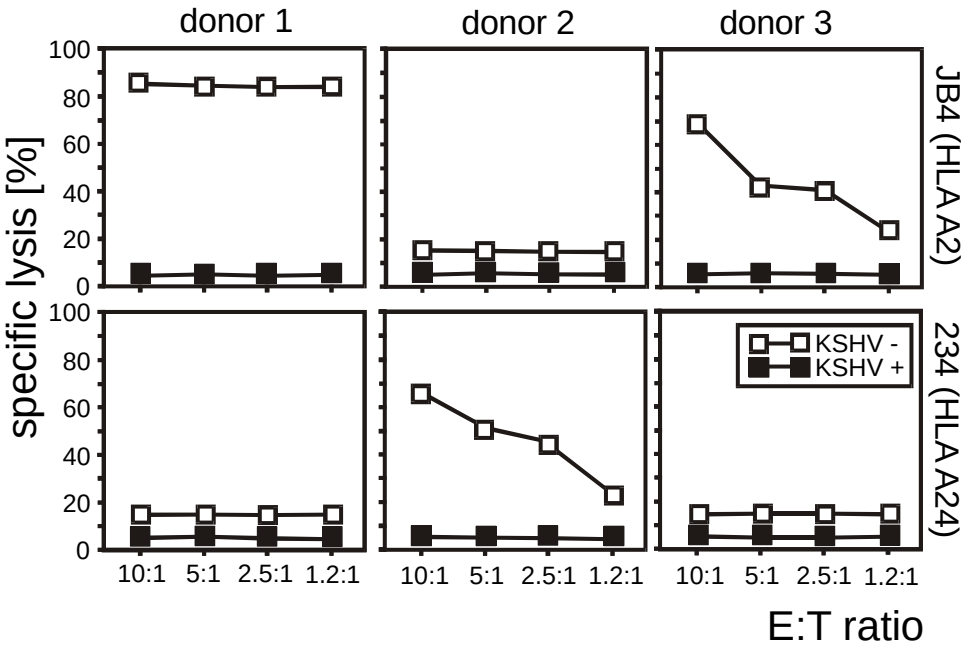


Fig. 27: Complete abolishment of CTL-mediated lysis of KSHV-infected B-cells.
The KSHV-positive and EBV-positive B-cells from the three donors were diluted at a concentration 10^6 cells/100µl and labelled with [^{51}Cr] for 1.5 hours. Subsequently, the labelled target cells were washed with cell culture medium to remove the rest of the radioactivity. The target cells were diluted at a concentration $2x10^3$/50µl and added to the cytotoxic T-cells. The CTLs were diluted at a ratio of 10:1-1.2:1. After 4 hours, supernatants were collected and dried overnight. Subsequently, the [^{51}Cr] release was measured.

4.8.5 Sustained immune escape of KSHV-infected cells after IFN stimulation

Cytotoxic T-cells that recognize and kill virus-infected or tumor cells are restricted by MHC class I molecules expressed on the target cells. The KSHV-positive cells from the three donors express MHC class I, however to a lower extent than the control EBV-infected cells (Fig.10). To test whether the MHC class I downregulation is involved in the immune escape of cells infected by KSHV, the cells were stimulated with IFNα and IFNγ. In both cases MHC class I expression was increased after stimulation (Fig. 28). The stimulated KSHV-positive cells were tested for lysis by the alloreactive CTL cell lines JB4 and 234 in a chromium release cytotoxic assay. The results showed that the IFN stimulation did not have any effect on the lysis of KSHV-infected B-cells (Fig.29). This suggested that the immune

escape of KSHV-infected cells from the CTLs is probably not dependent on MHC class I, but rather on other mechanisms.

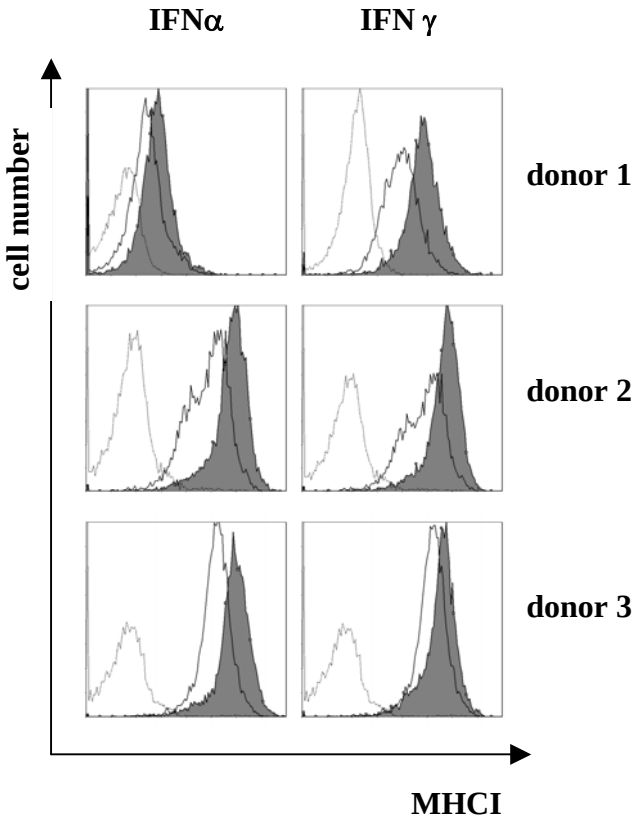


Fig. 28: Increased expression of MHC class I in KSHV+ cells after IFNα stimulation.
The KSHV+ cells were treated with 1000U/ml IFNα. After 20 hours of incubation the cells were stained with anti-MHC class I and FITC-conjugated secondary antibody. The filled peaks represent the expression of MHC class I after IFN stimulation, the non-filled peaks represent the expression of MHC class I in untreated cells and the dotted peaks represent the negative control or cells stained only with FITC-conjugated secondary antibody.

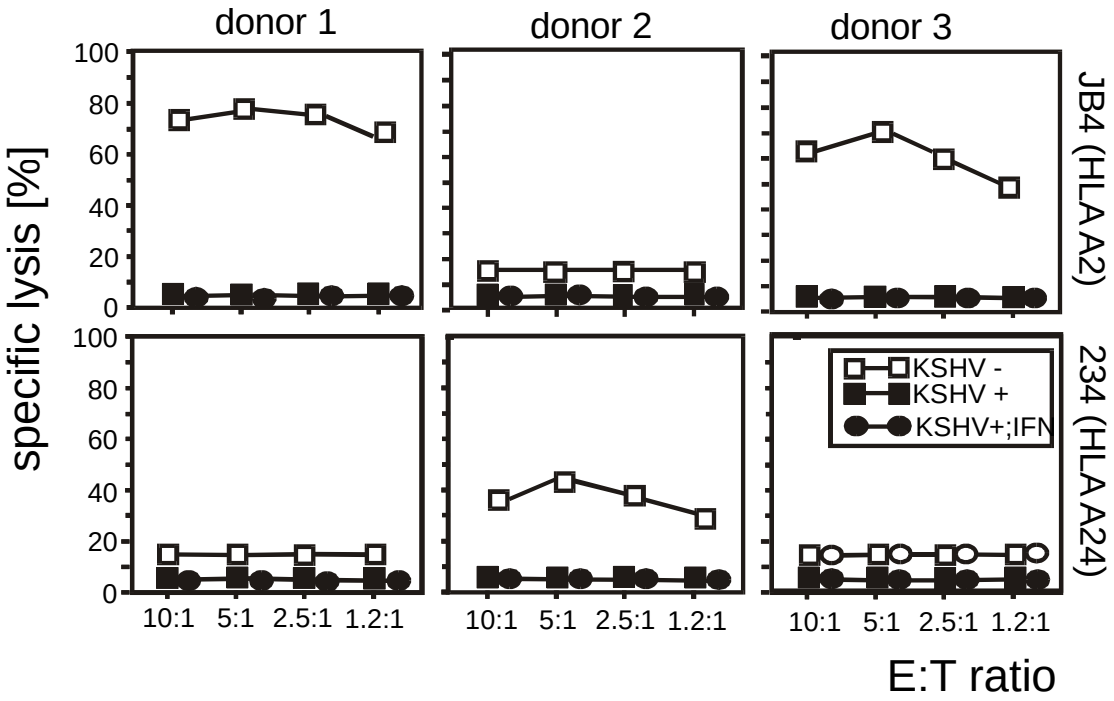


Fig. 29: IFN stimulation did not reconstitute the CTL-mediated killing of KSHV-infected B-cells.
The CTL killing assay was performed with IFNα-induced KSHV-infected B-cells. The specific cytotoxic lysis was tested using a chromium release assay.

4.8.6 Downregulation of co-stimulatory molecules and CD95 in KSHV-infected B-cells

The recognition by CTLs does not only depend on the recognition of a foreign peptide in the context of MHC class I, but also on the expression of co-stimulatory molecules such as B7-1 (CD80), B7-2 (CD86) and ICAM. Therefore, the expression of these molecules was tested in the cells infected by KSHV. The co-stimulatory molecules and as well as the Fas receptor CD95 were downregulated in KSHV-positive cells as detected by FACS (Fig. 30). This suggested that the immune escape of the KSHV-positive cells could be changed by the downregulation of co-stimulatory molecules or the downregulation of CD95.

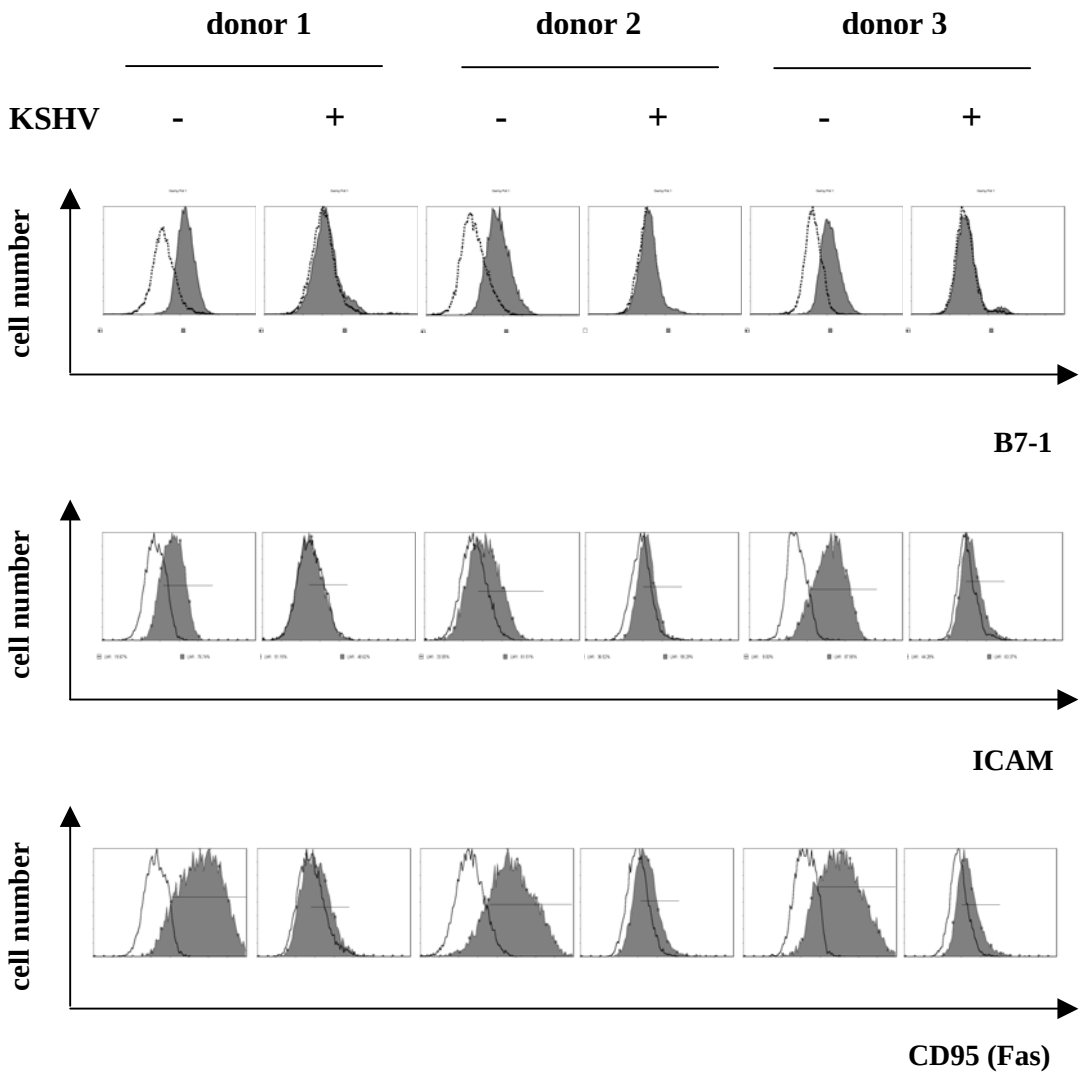


Fig. 30: Downregulation of co-stimulatory molecules (B7-1, ICAM) and CD95 (Fas) receptor in KSHV-positive B-cells.
KSHV-positive and EBV-positive B-cells from the three donors were stained with antibodies against B7-1, ICAM or CD95 and FITC-conjugated secondary antibodies. The filled peaks represent the expression of B7-1, ICAM and CD95, the dotted peaks represent the negative control or cells stained only with FITC-conjugated secondary antibody.

4.8.7 The impaired CTL lysis of KSHV-infected B-cells is not restored by reconstituting B7-1 and B7-2 co-stimulatory molecules

The expression of co-stimulatory molecules is mandatory for the recognition of target cells by CTLs. As shown in the previous chapter, these molecules are downregulated in KSHV-infected cells. To test whether the downregulation of co-stimulatory molecules is responsible for the failure of alloreactive CTLs to lyse KSHV-positive cells, B7-1 and B7-2 were reconstituted by recombinant vaccinia virus. The infection was performed for 18 hours

in parallel with a control vaccinia infection. The cells were tested for the expression of B7-1 and B7-2 by FACS analysis (Fig.31). As an additional control EBV-transformed B-cells were infected with recombinant vaccinia virus expressing B7-1 and B7-2 (Fig. 31). Subsequently, the cells were incubated with HLA-A2 and HLA-A24 restricted alloreactive T-cells and the cytotoxicity was measured in a [^{51}Cr] release assay. The results clearly showed that KSHV-positive B-cells were not lysed after reconstitution of the co-stimulatory molecules, although their expression was significantly increased from 1% to 40-50% and even higher than in the control cells (Fig. 32). There was no significant difference in the CTL lysis of the control EBV cells after infection with recombinant vaccinia virus expressing B7-1 and B7-2. The infection with the control vaccinia virus (vT7) resulted in an increased spontaneous release, which explains the decrease in the specific lysis. The results led to the conclusion that KSHV-infected cells are resistant to CTL-mediated killing neither by downregulation of MHC class I nor by co-stimulatory molecules. (Fig.30).

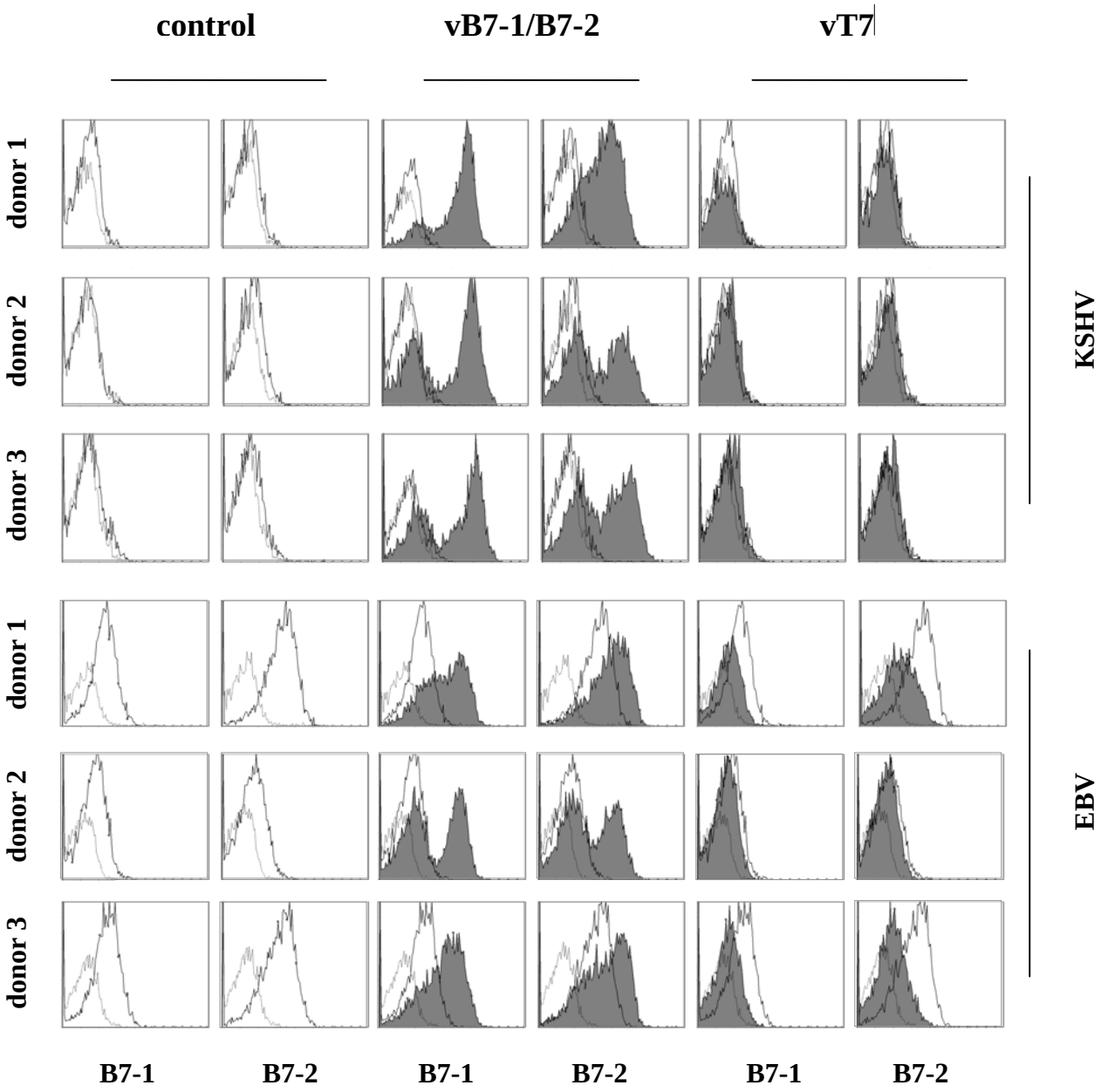


Fig. 31: Expression of B7-1 and B7-2 co-stimulatory molecules after infection with recombinant vaccinia virus.
Infection of KSHV- and EBV-positive B-cells with recombinant vaccinia virus expressing B7-1 and B7-2 constructs and control vaccinia virus. The filled peaks represent the expression of B7-1 and B7-2 after infection, the non-filled peaks represent the expression of B/-1 and B7-2 in untreated cells and the dotted peaks represent the negative control or cells stained only with a FITC-conjugated secondary antibody.

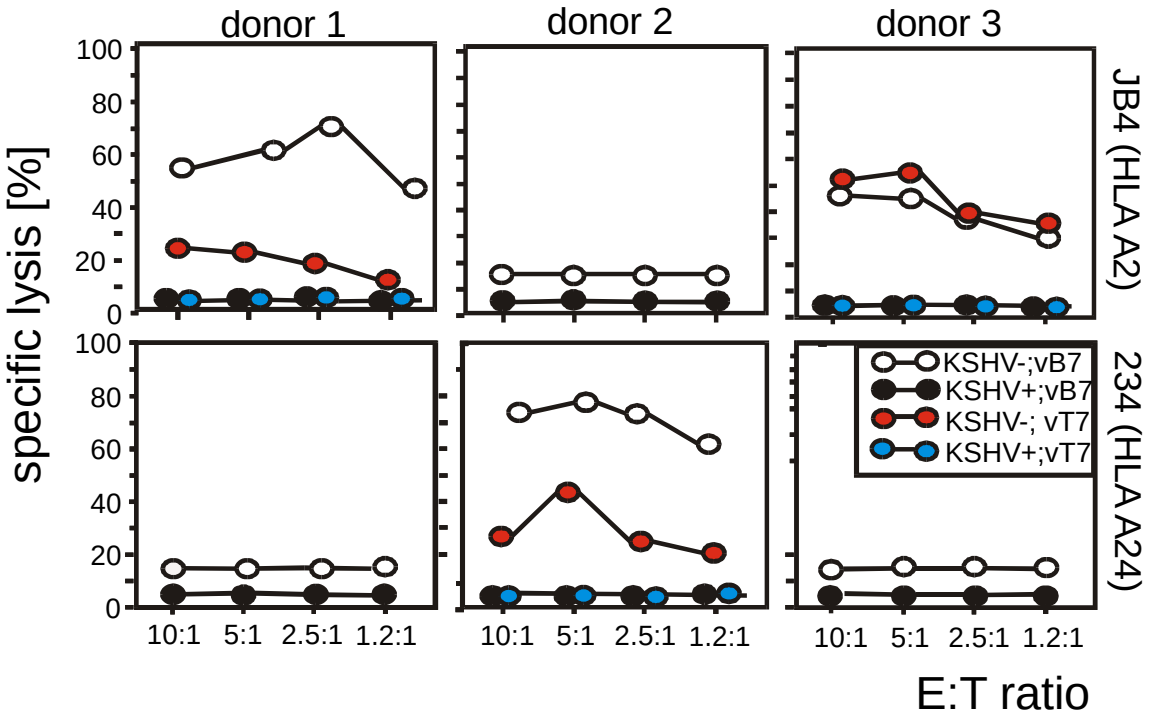


Fig. 32: The impaired CTL lysis of KSHV-infected B-cells is not restored by reconstituting B7-1 and B7-2 co-stimulatory molecules.
Cytotoxic assay of KSHV+ and EBV+ B lymphocytes infected with recombinant vacccinia virus expressing B7-1 and B7-2 and control vaccinia virus. KSHV-infected cells were not lysed by the CTLs as they showed no difference in [^{51}Cr] after infection with recombinant vaccinia virus.

5. Discussion

Kaposi´s sarcoma-associated virus (KSHV) is thought to be related to KS and atypical B-cell lymphomas (PEL and MCD). In KSHV-positive PELs it is largely unknown which phenotypical changes are caused by the viral infection and which by cellular oncogenic processes. Most PEL cells also contain EBV, so that both viruses might contribute to the transformation processes in doubly infected PELs. Therefore, it was interesting to investigate the role of KSHV in B-cell transformation. The present work showed that the KSHV-infected B-cells develop a "null" phenotype, which is observed similarly in the KSHV+ PEL cells. Additionally, this study presented for the first time that a soluble factor(s) released from the KSHV-infected cells is related to the "null" phenotype. Furthermore, the investigations of persistently KSHV-infected B-cells demonstrated that these cells could escape the adoptive immune response by a complete abolishment of CTL-mediated lysis.

5.1 Generation of persistently KSHV-infected B-cells

It was previously shown that KSHV infects normal B-cells only in the presence of EBV (Kliche et al., 1998). Surprisingly, the infected B-cells from the different healthy donors were only KSHV-positive although in previous experiments they could only be infected in the presence of both KSHV and EBV. This shows that EBV is not mandatory to promote infection as previously reported (Kliche et al., 1998). The role of EBV in dually infected cells is currently unclear, but a synergistic effect of both viruses is not excluded as EBV is detected in most cases of PEL (Horenstein et al., 1997, Carbone et al., 2000, Fassone et al., 2000). However, EBV is probably not predominant in the transformation process, as its transforming latent protein LMP-1 is not expressed in PEL cells, although the EBV genome is present and maintained by another latent protein EBNA-1. The model system used in the present study might then be preferable, since the infected cells are only KSHV-positive.

5.2 Gene expression profiling of KSHV-infected B-lymphocytes

Microarray analyses enables the analysis of a multitude of genes at a genome-wide scale. This technology has been used to analyse the effects of a number of viral infections including cytomegalovirus (Zhu et al., 1998). Here, an investigation of the cellular gene expression after KSHV-infection of B-cells was performed using a microarray analysis (*collaboration with the Scottish GTI, UK).* The gene expression was investigated in cell lines from two donors, which led to a higher validity of the results. In a total of 24000 genes a considerable number of genes (408 or 1.7%) were found to be modulated more than 4-fold in

KSHV-positive cells, 275 (67.4 %) were downregulated whereas 133 (32.6%) genes were found to be upregulated. The downregulation of gene expression was detected up to approximately –200-fold. Between the upregulated genes were: granzyme A (87.29), Notch homolog 2 (14.4), S100 calcium binding protein A10 (13.64), interleukin 10 (8.45) and interleukin 6 receptor (5.76) (Table 1). Proteins belonging to calcium binding proteins (calpain, S100 A10-calpactin) are significantly expressed in highly proliferating (tumor) tissues (Hsieh et al., 2003, Kanamori et al., 2004), as well as some lymphomas (Yao et al., 2001) and are recognized as potential tumor markers. IL-10 and IL-6 are synthesized from non-Hodgkin lymphoma cells (Aydin et al., 2002), EBV-transformed lymphoblastoid cells (Bende et al., 1992, Pistillo et al., 1994, Wroblewski et al.,2002) and AIDS-related lymphomas (Foussat et al., 1999, Fassone et al., 2000) and may be involved in autocrine loops. Surprisingly, Granzyme A was highly upregulated in KSHV-positive B-cells. Granzymes A and B together with perforins are cytolitic molecules found in acidic granules of NK and CTLs (Hayes et al., 1989, Peters et al., 1991, Shi et al., 1992). Recently granzyme A was also detected in polymorphonuclear neutrophils (Hochegger et al, 2004). The role of Granzyme A in the pathogenesis of KSHV-associated diseases is currently unknown. The fact that it induces other cytokines such as IL-6, IL-8 and TNFα (Sower et al., 1996) suggests that Granzyme A might be involved in autocrine or paracrine loops in KSHV-infected B-cells.

5.3 B-cell surface markers are downregulated by KSHV-infection

Our results revealed that a significant number of B-cell surface molecules (B-cell receptor, CD19, CD79 and B7-1) were downregulated in KSHV-infected B-cells (Table 1). The B-cell markers tested in our study appear during the regular B-cell development and are still present in mature B-cells. Therefore, it was impossible to classify the B-cell stage of KSHV-infected cells by these markers. The only evidence that these were mature B-cells was the presence of CD71 (transferrin receptor) and MHC class I. Assuming the results we hypothesized that the persistently KSHV-infected B-cells have developed a similar phenotype like the PEL KSHV+/EBV- cell line (BCBL-1), used as a source for KSHV (Fig.10). Thus, persistently KSHV-infected B-cells develop a “null” phenotype, characterized by a loss of surface molecules similar to PEL tumor cells (Fig.33). PEL cells are known to be generally negative for T- and B-cell immunomarkers (except for CD138), but they are genotypically B-cells with rearranged immunoglobulin genes (Drexler et al, 1998, Klein et al., 2003, Hamoudi et al., 2004). Due to gene expression profile and the expression of CD138, PEL cells has been classified as cells with a plasmablastic phenotype similar to Hodgkin´s

lymphoma cells (Arguello et al., 2003). In accordance with previously published results it was concluded that KSHV-infected B-cells develop a phenotype, representing a stage between an antigen-selected Germinal Center (GC) B-cell and a terminally differentiated plasma cell.

Most of the studies on KSHV are performed using immortalized lymphoma cell lines, established from malignant pleural effusion, ascitic fluid or peripheral blood of patients with AIDS- and non-AIDS-associated PEL (Arvanitakis et al., 1996, Drexler et al., 1998, Carbone et al., 2000). Therefore, in PEL cells it is unknown if the phenotypic changes are caused by KSHV itself or by the cellular processes of tumor transformation. More complicated, KSHV-negative PEL cells exist as well (Hisamoto et al., 2003, Nonami et al., 2004). The generation of KSHV-infected cells was performed in conditions where the only transforming factor present was KSHV and (or) EBV. Consequently, the transformation of normal B-cells into "null"-phenotype-cells is due only to the KSHV-infection. In this work it was present for the first time that KSHV itself is able to transform B-cells into PEL-like cells, developing a unique phenotype different from EBV-transformation. The EBV-transformed B-cells are considered to have *mature activated*-B cell phenotype (latency III) (Rochford et al., 1993). In addition, EBV-infected tumors are presumed to arise when virus-induced B-cell development *towards memory B*-cells is not controlled (Thorley-Lawson and Babcock, 1999, Kieff and Rickinson, 2001). In terms of morphology, EBV-transformed B-cells express most of the B-cell markers and co-stimulatory molecules which were downregulated in the KSHV-infected B-cells. Thus, it can be concluded that KSHV and EBV differ in both mechanisms of transformation of B-cells as well as the resulting cellular phenotype.

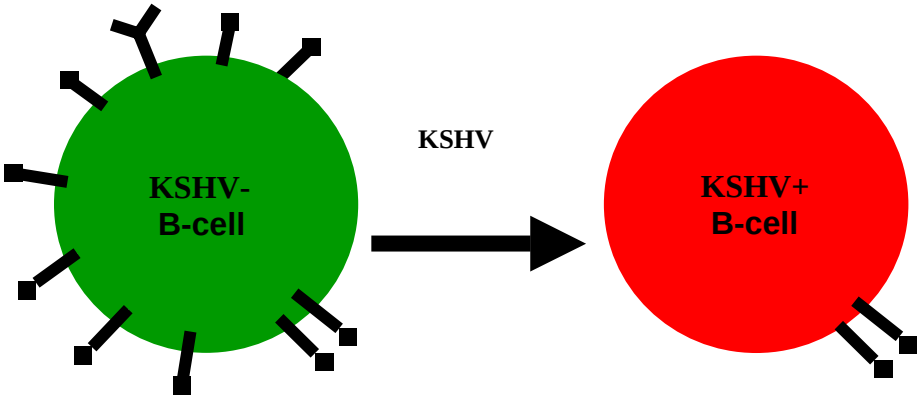


Fig. 33: Development of "null" phenotype of a B-cell after KSHV-infection.
This phenotype is characterized by complete loss of surface molecules.

5.4 Upregulation of IL-6 and IL-10 after KSHV-infection

Among the upregulated genes in KSHV-infected cells were the genes encoding for IL-6 and IL-10 (Fig.11 B). This indicates that also in this regard there is a similar phenotype in persistently KSHV-infected B-cells and PEL tumor cells. In PEL lymphomas IL-6 and IL-10 are important autocrine factors for growth and proliferation of cells (Asou et al., 1998, Jones et al., 1999, Foussat et al., 1999, Fassone et al., 2000). Extensively studied in the KSHV field is IL-6, which is generally acknowledged as the major growth factor for PEL cells. Neutralizing antibodies against IL-6 cause a delay of tumor cell death *in vivo* (in SCID mice) and *in vitro* (Asou et al., 1998, Foussat et al., 1999). This delay was significant but incomplete due to the viral IL-6 as a co-activator of IL-6-dependent cell lines (Moore et al., 1996, Molden et al., 1997). Nevertheless, the role of cellular IL-6 in tumor progression appears to be more important as of vIL-6, as Asou and colleagues showed an inhibition of cellular growth *in vitro* when using human IL-6 antisense oligonucleotides, but not when vIL-6 antisense oligonucleotides were used (Asou et al.,1998). A similar inhibition was observed

by Drexler and colleagues with monoclonal antibodies against the IL-6 receptor (Drexler et al., 1999). Moreover, IL-6 was found to stimulate KSHV replication directly by inducing the immediate early gene ORF50 (transcriptional activator) in the BCBL-1 cell line (Song et al., 2002). Several latent and lytic viral proteins were shown to contribute to the increased expression of IL-6 in *in vitro* studies, including vFLIP (An et al., 2003), LANA-1 (An et al., 2002) and vIL-6 (Mori et al., 2000). IL-10 acts similarly as IL-6 in cellular proliferation. Production of large quantities of human IL-10 occurs frequently in AIDS-related Burkitt´s lymphoma (BL) and correlates with latent EBV-infection in the tumor cell line (Benjamin et al., 1992). A pathogenic role for IL-10 in PEL and AIDS-BL is suggested by the observation that IL-10 neutralizing antibodies and antisense oligonucleotides inhibit proliferation of lymphoma cells (Jones et al., 1999, Fassone et al., 2000). During B-cell development IL-10 has an immunoregulatory function and enhances cell viability and proliferation, immunoglobulin secretion and MHC class II expression. The fact that IL-10 is upregulated in KSHV-positive B-cells and that it has an inhibitory role on co-stimulatory molecules (B7-1, ICAM) in monocytes, suggests that it has a causative effect in KSHV-positive cells. Based on the functions of IL-6 and IL-10 in B-cell development (especially IL-6), it can be hypothesized that their increased production by KSHV-infected B-cells contributes also to their phenotype.

5.5 Disrupted transcriptional program in KSHV-infected B-cells

The “null” phenotype developed by the KSHV-positive cells is characterized by a massive loss of B-cell surface molecules. Therefore, it is rather unlikely that one viral protein is responsible for their downregulation. It was shown that the “null” phenotype is caused by a downregulation at the transcriptional level. In the normal B-cell differentiation the expression of specific B-cell markers and signal transduction molecules (PKC-δ, -ξ, BLNK, Bruton Tyrosin Kinase-[BTK]) is strictly controlled by B-cell specific transcription factors. The results revealed that in B-cells several B-cell specific transcription factors were indeed downregulated after KSHV-infection (Pax-5 [BSAP], Oct-2, Spi-B and Bob-1). As indicated by RT-PCR (Fig. 12), all the transcription factors following Pax-5 in the hierarchical order in which they are expressed in mature B-cells (Oct-2, Spi-B, Bob-1) were found to be downregulated. In the regular B-cell development there is a cascade of B-cell specific transcription factors which sequentially activate each other by binding to the promoter of the next factor. This could be a reason that the factors downstream of Pax-5 are downregulated. Promoter studies show that Pax-5 is not the only factor regulating Oct-2, Spi-B or Bob-1, but

Pax-5 binding sites are present in all of them. As already mentioned, Pax-5 is one of the factors which is essential in forming the B-cell phenotype. Conditional Pax-5 inactivation leads to loss of the B-cell phenotype and previously committed pro-B cells regained the capacity to differentiate into macrophages *in vitro* and to reconstitute T-cell development *in vivo* in RAG-/- mice (Mikkola et al., 2002). Interestingly, Pax-5 was not detected on the protein level in the KSHV-infected cells (Fig. 13). Since it is known that CD19 is a direct target of Pax-5, this explains its downregulation in KSHV-infected cells (Kozmik et al., 1992, Nutt et al., 1998). This finding suggested that the blockade of B-cell specific transcriptional factors in KSHV-infected B-cells originates in the downregulation of Pax-5.

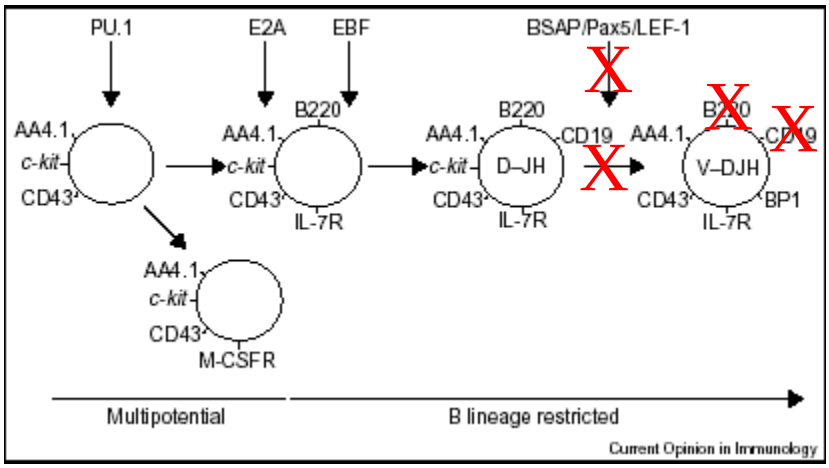


Fig. 34: Diagram of the transcription factors required during commitment to the B-cell lineage (according to Kee and Murre, 2001).
The blockade of Pax-5 expression in the intermediate step between mature B-cells and plasma cells determines a phenotype characterized with a loss of B-cell surface markers.

5.6 Downregulation of Pax-5 and the development of a "null" phenotype

5.6.1 Protein degradation

A possible reason for the downregulation of Pax-5 could be its degradation by the cellular proteasome, which is a normal process in the regulation of protein levels of a multitude of cellular proteins including MHC class I (Rock et al., 2002) and E2A (Kho et al., 1997). Treatment of KSHV-infected cells with the proteasome inhibitor MG132 did not lead to a reconstitution of Pax-5 (Fig.15). This experiment does not completely exclude that the protein is not degraded, as a protein degradation could also involve viral proteases, similar as in the case of p53 (Weidman et al., 2001), octamer-binding protein-1 (Oct-1) and cyclin-AMP

responsive element-binding protein (CREB)(Kliewer et al 1990), which are cleaved and degraded by a viral protease of poliovirus. KSHV possesses two proteins, K3 and K5, which have ubiquitin-ligase activity and which were found to degrade MHC class I (Ishido et al., 2000) and ICAM and B7-2 (Coscoy and Ganem, 2001) in an *in vitro* system via ubiquitination, independent from the host proteasome ubiquitination system. It is not likely that K3 or K5 are directly involved in Pax-5 degradation as they are associated with the plasma membrane and Pax-5 is located in the cellular nucleus. Moreover, K3 and K5 are expressed during the lytic phase whereas the KSHV-positive cells are latently infected.

An alternative way of downregulation of cellular proteins is mRNA instability or degradation of mRNA by a viral protein. Similar event was shown in the case of MHC class I mRNA, which is downregulated by the Bovine-herpesvirus-1 (BHV-1) (Gopinath et al., 2002). Herpes simplex virus, for example, is well known for suppressing the *host-cell* protein synthesis. It causes a marked reduction in the level of the specific mRNA from cellular genes: beta actin, fibronectin, glucose-transporter-1, docking protein (Becker et al., 1993). A *cellular shut-off* was reported recently in the case of KSHV infection concerning the lytical cycle of the virus (Glaunsinger et al., 2004). This *shut-off* was found to be mediated by the viral *shut-off* exonuclease protein (SOX, ORF 37), which promotes degradation of cellular mRNAs (Glaunsinger et al., 2004). Interestingly, the authors found that the only protein able to escape the *shut-off* was IL-6. However, this could not be applied in the present system as the persistently KSHV-infected B-cells are latently infected and lytical viral replication in PEL cells is in 1-5% of the cells. Moreover, the results presented in this study showed that Pax-5 was detected on mRNA level, which excludes the possibility of mRNA degradadtion.

5.6.2 Relation between Pax-5 downregulation, Blimp-1 upregulation and "null" phenotype

During B-cell development the Pax-5 expression is blocked on the transcriptional level. One *transcriptional* inhibitor of Pax-5 is Blimp-1, which is responsible for the stage in which mature B-cells develop into plasma cells by *transcriptional* downregulation of Pax-5 (Lin et al., 2002). In KSHV-infected B-cells an upregulation of Blimp-1 was detected by RT-PCR (Fig.12), which led to the hypothesis that KSHV causes (directly or indirectly) an upregulation of Blimp-1 with a following downregulation of Pax-5 and drives the cells to develop a plasma cell-like phenotype. In the present study cannot be postulated that the altered protein expression of Pax-5 is a direct consequence from Blimp-1 upregulation, as Pax-5 was not *transcriptionally* downregulated. Therefore, in a KSHV-infection another

mechanism might occur. Based on these data could be speculated that KSHV *mimics* the natural processes of B-cell differentiation and drives normal B-cells to develop a plasma cell-like "null" phenotype, by upregulation of Blimp-1 and consecutive downregulation of Pax-5. The role of Pax-5 in the transition into the plasma cell stage is supported by some reports showing that overexpression of Pax-5 inhibits an efficient plasma-cell formation, suggesting that the downregulation of Pax-5 may be a prerequisite for terminal differentiation (Usui et al., 1997).

Other factors driving the B-cell differentiation are IL-6 and IRF-4. KSHV has two homologues of these genes, vIL-6 and vIFN-3. The vIL-6 is secreted from PELs and detected in the range of approximately 300 to 2300 pg/ml in the sera of PEL patients (Aoki et al., 2001). vIL-6 mimics a number of hIL-6 activities including stimulation of IL-6 dependent B-cell growth (Burger et al., 1998) and activation of signalling pathways (Molden J et al., 1997). In contrast to cellular IL-6, vIL-6 does not require co-receptor usage for binding the hIL-6 receptor, which makes it a possible candidate responsible for phenotypic changes. IRF-4 was slightly increased in the microarray analysis, which in comparison to other upregulated genes was not considerable. The viral homologue of IRF-4, vIRF-3 or LANA-2, was shown to be involved in the IFN response and is thus rather unlikely to be directly involved in the phenotypic changes (see below).

The "null" phenotype is morphologically and histologically different to most known B-cell tumors (Jaffe et al., 1996). Hodgkin´s lymphoma cells and Reed-Sternberg cells have similar phenotypic features, as they also lack B-cell markers, such as CD20, the B-cell receptor and CD79, and are thought to originate from germinal centre or post-germinal centre B-cells (Thomas et al., 2004). These cells also lack the immunoglobulin-specific transcription factors BOB-1, Oct-2 and PU.1 (Re et al., 2001, Torlakovic et al., 2001). Controversial data, however, have been reported for Pax-5. Whereas some reports show that Pax-5 is expressed in most cases of Hodgkin´s lymphoma (Schwering et al., 2003), other showed the opposite, i.e. a downregulation of Pax-5 (Hertel et al., 2002). Hodgkin´s lymphoma is a different malignancy with unknown etiology. Although it is clearly related to EBV-infection, the virus is present only in 40-60% of the cases (Morrison et al., 2004). In the present study, it is shown that the agent causing the "null" phenotype of B-cells is KSHV. Possibly, however, KSHV uses analogous way to induce the "null" phenotype, similarly as in Hodgkin´s disease.

Based on the data presented in this work and the reported data can be concluded that the development of the unique "null" phenotype after KSHV-infection is a consequence of the mimicry of the regular B-cell development and the key molecule switching on this process is

Pax-5. It is not clear if the cellular factors or their viral homologues are involved in this process, or this is a combined effect. Here still could be asked the question why the plasma cell-like phenotype is important for the infection. We hypothesize that probably the B-cells are the reservoir of latent KSHV-infection and the development of long-live plasma cell-like B-cells is a self-protection from the innate or adoptive cellular immunity.

5.7 A soluble factor released from KSHV-infected cells is responsible for the "null" phenotype

A typical characteristic of PEL is growth in body cavities. This gave raise to the hypothesis that there is a correlation between the "null" phenotype and the liquid environment (PEL effusion or cell culture supernatant) in which the cells grow. The liquid environment contains viral particles and secreted cellular and viral proteins. The results presented here showed that addition of a supernatant from KSHV-positive B-cells to EBV-transformed B-cells led to downregulation of CD19 (Fig. 16). The separation of viral particles from the soluble proteins by ultrafiltration showed that the factor(s) causing the "null" phenotype is a secreted protein. The downregulation was not detected if supernatants from EBV-transformed B-cells was used, confirming that the soluble factor is exclusively produced by KSHV-infected B-cells. The downregulation of CD19 was also detected with PEL effusion. This effect was accompanied by a change of the morphology of cells, incubated with the effusion fluid. EBV-transformed B-cells have a typical morphology characterized by growth in big clusters and cellular protrusions. When these cells are grown in PEL effusion for 2 or 4 days their morphology was similar as in KSHV-transformed PEL cells including smooth surface, absence of protrusions and less cellular conglomerates. The typical smooth shape and growth as single cells is probably due to the loss of surface receptors, co-stimulatory and adhesion molecules. The EBV-transformed B-cells are a convenient model for studying the morphological changes caused by KSHV. It is rather unlikely that EBV takes part in these changes, as the KSHV-infected B-cells, generated in this study, are in fact EBV-negative.

In this study it is presented for the first time that KSHV-infection is responsible for the "null" phenotype of PEL tumor cells and that the liquid environment in which these cells grow is essential for the development of this phenotype. In addition to this, recent reports indicated that KSHV-infected *solid* immunoblastic lymphomas *express* B-cell associated antigens (CD20 and CD79a) and immunoglobulin more often than PELs (Chadburn et al., 2004, Carbone et al., 2005).

5.8 IL-10, vMIPII and vIL-6 do not have an effect on the development of "null" phenotype

5.8.1 IL-10

A wide spectrum of cytokines and soluble cellular proteins is secreted from PEL cells including IL-6, IL-6 soluble receptor, IL-10, oncostatin M (OSM) (Drexler et al., 1999). In most cases these proteins have been found to be significantly higher than the levels produced by Burkitt or other non-Hodgkin´s lymphoma cell lines. In the present study, the levels of IL-6 and IL-10 secreted by KSHV-infected cells were approximately 15 to 20-fold higher than those secreted by EBV-transformed cells (Fig.11). As already mentioned, the main function of IL-6 and IL-10 is to support cellular growth and proliferation. IL-10 also possesses an inhibitory effect on co-stimulatory molecules (B7-1/B7-2), chemokines (MIP-I, MIP-II) in macrophage/monocyte cells or TNF in T-cells. (Moore et al., 2001). Therefore, we examined whether IL-10 participates in the downregulation of surface markers in KSHV-infected B-cells. As shown in figure 20, a downregulation of CD19 was not detected in the presence of recombinant human IL-10 even if used in high concentrations. This suggests that IL-10 is probably not involved in the development of the "null" phenotype and has a distinct role in other processes, e.g. immune escape by inhibition of Th1-response.

5.8.2 vMIP-II

The viral homologues of MIPs (see above) are chemokine-like proteins expressed during the lytic phase of viral infection which are biologically active in immunosuppressed KS patients (Boshoff et al., 1997, Benelli et al., 2000). The vMIP-II protein binds to the CCR3 chemokine receptor and results in a potent inhibition of HIV entry and the activation and chemotaxis of eosinophils (Boshoff et al., 1997). Both vMIPs induce angiogenesis in chicken embryos. Since nothing was known about an effect of the secreted viral chemokines on cellular phenotype, we tested if recombinant vMIP-II could cause a change of the expression of surface markers. A significant change of the expression of CD19 was not detected, excluding a role of vMIP-II in the development of the "null" phenotype (Fig.21).

5.8.3 vIL-6

Significant amounts of vIL-6 are secreted from PEL cells (Asou et al., 1998). vIL-6 antisense oligonucleotides were found to decrease the clonal growth of these cells (Asou et al., 1998). It was not known whether vIL-6 induces phenotypic changes in B-cells. As a

homologue of hIL-6 it shares some similar functions (see above) and based on that can be hypothesizes that vIL-6 participates in the development of mature B-cells into plasma cells. Therefore, the influence of the vIL-6 on CD19 expression was checked. As shown in figure 22, a significant downregulation was not detected, which suggests that vIL-6 is not involved in phenotypic changes.

Since neither IL-10, vMIP-II nor vIL-6 had an influence on the B-cell phenotype, the question which induces "null phenotype" is still open and will be investigated in future studies.

5.9 Functional characterisation of KSHV-infected B-cells

5.9.1 Impaired signal transduction pathways

The data from the microarray analysis showed that cells infected with KSHV lose most of the characteristic features of mature B-cell and develop a "null" phenotype. These cells also lack important signal transduction molecules delivering the signal to downstream events. For instance, the signalling from the B-cell receptor (BCR) in a normal B-lymphocyte includes kinases as PKC beta 1 which is significantly downregulated in the KSHV-positive B-cells (-20.19x). PKC beta is specifically required for the activation of NFkB by BCR in normal B-cells (Su et al., 2002). NFkB is also downregulated in KSHV-infected B-cells (-3.27x). In addition, the signal from the BCR requires CD19 (Tedder et al., 1994) which is downregulated –19.16x in KSHV-infected B-cells. The Bruton tyrosin kinase (Btk) which is requisite for the regular B-cell development and which is dependent on signals from both BCR and CD19 is similarly downregulated (-12.91x). The crosslinking of the BCR in normal B-cells leads to a cellular activation with an increased expression of co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) (Kozono et al., 1998). The KSHV-infected B-cells could not be activated via crosslinking of CD19, as the expression of B7-2 was not increased (Fig. 23). This confirms that KSHV-infected B-cells are generally impaired in BCR signal transduction .

5.9.2 Blockade of the IFN signalling pathway in KSHV-infected B-cells

Inhibition of IFN signalling is a common event during viral infection. Viruses may either block IFNα/β or IFNγ pathways or both. KSHV is known to encode four homologues of the IRF family (vIRF-1, vIRF-2, vIRF-3, vIRF-4) that repress transcriptional responses to IFNα/β and IFNγ (Gao et al., 1997, Zimring et al., 1998). vIRF-1 does not appear to act at the

level of IFN signalling, but rather inhibits the function of the IFN-inducible cellular factor IRF-1. In the present study, KSHV-infected B-cells stimulated with IRFα responded with a phosphorylation of *Stat-1* and *Stat-2*, despite their slight transcriptional downregulation in the microarray analysis (*Stat-1* -5.16x, *Stat-2* -1.31x) (Fig. 25). Interestingly, downstream proteins of the IFN signalling pathway were significantly downregulated in KSHV-infected cells, e.g. the GTPases MxA (-35.44x) and Mx-B (-8.35x), known as the best-characterized IFN-inducible gene products with antiviral activity (Aebi et al., 1989, Staeheli et al., 1993). This implies that the blockade in the IFN signalling is downstream of *Stat-1-Stat-2*.

Cells that constitutively express the human MxA protein show a high degree of antiviral activity and are resistant to several members of the Orthomyxoviridae including influenza A and C viruses and also other viruses such as Thogoto virus (Arnheiter et al., 1996; Frese et al., 1995, Haller et al., 1998, Marschall et al., 2000). Naranatt and colleagues showed a similar downregulation of Mx-1 in the BJAB B-cell line infected with KSHV (Naranatt et al., 2004). In contrast, the authors found that Mx-1 expression is increased in KSHV-infected primary endothelial cells. Thus, they hypothesized that the Mx-1 modulation is cell-type specific.

Besides vIRF-1, KSHV encodes other IRF homologues that disrupt the host antiviral response: vIRF-2 (Burysek et al., 1999, Burysek and Pitha, 2001) and vIRF-3 (*LANA-2*, Lubyova et al., 2000). The vIRF-2 is constitutively expressed in PEL cells and is known to associate specifically with cellular IRFs and p300 (Burysec et al., 1999). Moreover, it binds double-stranded RNA-activated protein kinase (PKR) and inhibits its activity and also blocks the phosphorylation of the eukaryotic translation initiation factor 2 by PKR (Burysek et al., 2001). LANA-2 which is a latently expressed nuclear protein binds to p53 (Rivas et al., 2001). It was recently found to directly interact with the cellular interferon regulatory factor (IRF) IRF-3, IRF-7 and the transcriptional co-activator CBP/p300 in stably transfected B-cells (Lubyova et al., 2004). In addition, it stimulates the IRF-3- and IRF-7-mediated activation of type I interferon (IFNα and IFNβ) promoters and the synthesis of biologically active type I interferons. In persistently KSHV-infected B-cells IRF7 was significantly downregulated (-10.61x), so that it is rather unlikely that LANA-2 interacts with IRF-7 and causes an activation of type I IFN promoters. However, the involvement of LANA-2 in IFN signalling is not excluded, taking into account the fact that it is B-cell specific. As another latent KSHV protein, LANA-1 was also found to upregulate IFN-inducible cellular genes in stably transfected B-cells, although the IFN genes themselves were not induced by LANA-1 (Renne et al., 2001). Such genes were Staf-50, Evi5, STAT1 (ISGF-3 -subunit), IFI 6-16, MxA, IFI 9-

27, IFNα, IFNβ1, IFNγ. In the microarray screen MxA, IFI-6-16 and Stat-1 were downregulated (MxA -35.44x, IFI-6-16 -3.11x, Stat-1 -5.16x) in persistently KSHV-infected B-cells which is controversial to the data presented by Renne and colleagues. This is most likely due to the fact that in the present study KSHV-infected B-cells were used, whereas Renne and colleagues investigated stably transfected B-cells, constitutively expressing LANA-1. Moreover, the results presented in this study indicated that MxA is not induced after IFN stimulation (*collaboration in the laboratory),* although *Stat-1* and *Stat-2* were phosphorylated (Fig. 25 A, B). Thus, IFN signalling is impaired but at a downstream level.

5.9.3 Innate and adoptive immune response to KSHV-infected B-cells

5.9.3.1 NK-cell lysis

The presented data indicated that at least some KSHV-infected cells could be recognized and lysed by NK-cells (Fig. 26). The specific lysis of KSHV-infected B-cells from two donors was decreased in comparison to the EBV-infected B-cells. However, there was not a significant difference in the NK lysis between KSHV-positive and EBV-positive B-cells of the third donor. This could be due to various quantitative expression of NK-cell ligands on the B-cells. A variable sensitivity of different PELs to lymphokine activated killer (LAK) cell-mediated lysis was shown also by Suscovich and colleagues (Suscovich et al., 2004). The authors suggested that this is a consequence from the different expression of co-stimulatory molecules or other cell line-specific factors on the target cells. Sirianni and colleagues have shown an efficient lysis of PEL cells by PBMCs or purified NK cells from normal blood donors (Sirianni MC et al., 2002). This study examined only the ability of one PEL cell line to be lysed by NK cells and did not discuss possible variations. They also showed a decreased NK lysis if PBMCs from AIDS patients with progressing KS were used. The decreased sensitivity of the PBMCs was restored after disease regression and clearance of KSHV from the peripheral blood. In the present study, instead of PBMCs an NK cell line NK92 was used and it was investigated whether KSHV-infected B-cells can be lysed. Persistently KSHV-infected B-cells could be recognized and lysed by NK92 cells but to a lower extent than B-cells transformed by EBV. NK-cells discriminate between normal cells and cells that do not express adequate amounts of MHC I molecules (Morreta A et al., 2001). The expression of MHC class I on EBV-transformed B-cells (Salek-Ardakani et al., 2002) and PELs is downregulated in comparison to normal B-lymphocytes which explains why they are recognized and lysed by NK-cells. KSHV expresses two proteins, K3 and K5, that down-regulate MHC class I and co-activation molecules, enabling productively infected cells to

escape both CTL and NK cell responses (Coscoy and Ganem, 2000, Rimessi et al., 2001). However, it unclear if K3 and K5 are directly involved in the MHC class I downregulation, as they belong to the lytic KSHV genes and the B-cells used in the current study are latently infected.

5.9.3.2 CTL recognition

Furthermore, the influence of MHC class I downregilation on the lysis by CTL was investigated. The results showed that the CTL-mediated cell lysis of KSHV- infected B-cells was completely abolished in contrast to NK-mediated lysis (Fig. 27). Similar studies were performed recently by Brander and colleagues who examined T-cell activation by PEL cells (Brander et al., 2000, Sirianni et al., 2002). They confirmed that PELs display lower MHC class I surface expression in comparison to EBV-infected cells. The lower MHC I surface expression was reflected by an increased peptide concentration required for T-cell recognition and the resistance of PEL cells to CTLs directed against intracellular antigens. The expression of MHC class I on persistently KSHV-infected B-cells and on PEL cells (BCBL-1) was lower in comparison to the EBV-infected cells, but MHC class I was present on the cell surface. Thus, it was doubtful whether MHC class I expression itself determines the CTL evasion of KSHV-infected B-cells. Our hypothesis that reduced MHC class I expression is not responsible for immune escape was confirmed by IFN stimulation of the persistently KSHV-infected B-cells. As shown in figure 28, the expression of MHC class I on KSHV-positive cells was increased after IFN stimulation, however the cells were still not recognized by the alloreactive CTLs (Fig. 25 B). By CTL analysis with different PEL target cells, Suscovich and colleagues proposed that CTL recognition and activation rather than target cell lysis might be impaired (Suscovich et al., 2004) and that immunoregulatory factors other than MHC class I could be modulated on PEL cells and be a reason for the abolished CTL-mediated recognition.

In addition to MHC class I and the T-cell receptor, in the CTL-mediated recognition are also involved co-stimulatory molecules such as B7-1, B7-2 and ICAM. A complete downregulation of these molecules was detected in KSHV-infected B-cells (Fig.30). The death receptor CD95 which is an important ligand for the CTL-mediated killing of virally infected or transformed cells (Linkermann et al., 2003) was also downregulated (Fig. 30). The target cells require a close cell-to-cell contact to generate a death-signal via this receptor. The introduction of B7-1 and B7-2 molecules by recombinant vaccinia virus into KSHV-infected cells did not reconstitute the CTL-mediated lysis, although the expression was even

higher than the physiological expression in EBV-transformed cells (Fig. 31, 32). This led to the hypothesis that KSHV-infected B-cells have developed a major resistance to CTL-mediated apoptosis. This resistance can be a consequence of a more general mechanism such as the downregulation of the CD95/Fas ligand.

Conclusions and perspectives

The present work showed that B-cells can be transformed *in vitro* by KSHV and develop similar phenotypic and functional features as PEL tumor cells. This phenotype is caused by a soluble factor (s) secreted by the KSHV-infected B-cells. The identification of the secreted factor would give the possibility to develop new therapeutic approaches, for example by specific neutralizing antibodies. Since an introduction of co-stimulatory molecules was not sufficient to reconstitute the CTL-mediated lysis, one of the steps could the investigation whether the downregulation of Fas ligand and the secretion of high levels of Granzyme A is responsible for the CTL esacape.

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7. Abbreviations

AIDS	acquired immune deficiency syndrom
BEC	blood vascular endothelial cells
BCR	B-cell receptor
BCBL-1	Body cavity-based lymphoma cell line 1
bp	base pairs
CTL	cytotoxic T lymphocytes
DNA	deoxyribonucleic acid
BSAP	B-cell specific activator protein
dNTP	deoxynucleoside triphosphate
EBF-1	Early B-cell factor-1
EBV	Epstein-Barr virus
EBNA	EBV nuclear antigen
EH2	Equine Herpesvirus 2
ELISA	enzyme-linked immunosorbent assay
et al.	et alli (Lat. = and others)
FCS	fetal calf serum
Fig.	figure
FACS	Fluorescence-activated cell sorting
FITC	fluoresceine isothyocianate
FLIP	Fas-associated death domain-like IL-1 beta-converting enzyme-inhibitory protein
g	gram
g	gravitation constant
GPCR	G-protein-coupled receptor
h	hours
HCMV	human cytomegalovirus
HLA	Human Leukocyte Antigen
HLH	helix-loop-helix
HHV-8	human herpesvirus-8
HIV	human immunodeficiency virus
HVS	herpesvirus Saimiri
ICAM	intercellular adhesion molecule 1
IL	interleukin
IFN	interferon
IRF	interferon regulatory factor
Ig	immunoglobulin
kd	kilodaltons
µ	micro (10^{-6})
KS	Kaposi´s sarcoma
KSHV	Kaposi´s sarcoma associated herpesvirus
LEC	lymphatic endothelial cells
LCL	lymphoblastoid cell line
LANA-1	Latency-Associated Nuclear Antigen-1
LMP-1	latent membrane protein-1
m	milli (10^{-3})
MHC	major histocompatibility complex
MCD	multicentric Castleman´s disease
min	minute(s)

MIP	macrophage inflammatory protein
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger RNA
n	nano (10^{-9})
NK	natural killers
OBF-1	octamer-binding factor-1
ORF	open reading frame
p	pico (10^{-12})
Pax-5	paired box protein 5
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Rosswell Park Memorial Institute
RT	room temperature
RT	reverse transcription
s	second
TAE	Tris-Acetate-EDTA
TBST	Tris buffered saline with Tween 20
TEMED	N, N, N`, N`-tetramethylenediamine
TPA	12-0-tetradecanoylphorbol-13-acetate
TR	terminal repeat

8. Curriculum Vitae

Personal Particulars

Name	Guergana Iotzova
Date of birth	29. April 1973
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Education:

05/2001 – present	***Ludwig-Maximilians University, Munich, Germany*** **Pre-doctoral fellow/Ph.D Research In Virology** **Doctoral thesis:** "Null" phenotype of persistently KSHV-infected B-lymphocytes **Supervisor:** PD Dr. Jürgen Haas, Max von Pettenkofer Institut
03/2000 – 03/2001	***GSF-Hämatologikum, CCG Leukemia, Med.III, University Clinic Grosshadern, Munich***, Germany Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. **Supervisor**: Dr. G. Behre (funded by the International Federation of Clinical Chemistry -IFCC)
March 1999-June 1999	***Department of Radiation Oncology, Klinikum Rechts der Isar Technical University of Munich, Germany*** Practical work in molecular biology **Supervisor**: Dr. Karin Greulich
01/1998 – 03/2000	In Laboratory of Molecular Pathology, Sofia, as molecular biologist, Involved in the current diagnostic of some hereditary disorders

10/1992 – 10/1997	***University of Sofia, Bulgaria, Faculty of Biology***
	Master of Science
	Speciality: Molecular Biology
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	Msc thesis : "DNA analysis of B-cell Lymphoproliferative Disorders", In Laboratory of Molecular Pathology, University Hospital of Obstetrics and Gynecology, Medical University, Sofia
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09/1980 – 06/1992	**Previous education**
	Primary school and College of chemistry and biotechnology in Sofia

Attended conferences and seminars

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Poster presentaton: “Null” phenotype of persistently KSHV-infected B-lymphocytes

Gene Center Retreat, 2003, Wildbadkreuth, Germany

Presentation: “Null” phenotype of persistently KSHV-infected B-lymphocytes

Annual Meeting of the “Gesellschaft für Virologie”, 2004, Tübingen, Germany

Poster presentation: “Null” phenotype of persistently KSHV-infected B-lymphocytes