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EXPRESSION AND FUNCTION OF THE SMALL IMMUNE ADAPTOR PROTEIN SAP

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To my parents

Szüleimnek

ABSTRACT

The Epstein-Barr virus (EBV) carrier state is widespread in humans. Primary infection can cause infectious mononucleosis (IM) that presents with variable clinical severity, but almost always subsides. For 50% of boys with a rare hereditary immunodeficiency, X-linked lymphoproliferative disease (XLP), primary infection with EBV has a fatal course. In addition, XLP patients have elevated (200 times) risk for development of lymphomas. The underlying cause of the syndrome is lack of the function of a protein SAP, due to mutations of the encoding gene named also DSHP or SH2D1A. SAP binds to a group of cell surface proteins belonging to the SLAM-family.

We found an increase of SAP in T and NK cells after their activation. This correlated well with the inadequacy of cell mediated immune functions in the affected individuals, indicating the importance of SAP.

SAP was absent in B cells infected with EBV and in the established lymphoblastoid cell lines (LCL), in SAC+IL-2 or CD40L+IL-4 activated B cells.

EBV carrying - but not EBV negative - Burkitt lymphoma (BL) lines with the Type I EBV expression pattern were SAP positive. The type III lines had no SAP. The difference between the EBV negative BLs and the group I EBV positive lines is the first marker that distinguishes these 2 groups. Our results suggest that CD40 ligation can be the physiological signal for SAP down-regulation in B cells. We have also detected SAP in 5 EBV negative cell lines derived from Hodgkin's disease.

In the continuation of our studies we focused our attention on the high incidence of lymphoma development in the XLP patients. Since lymphomas appear with high frequency even in EBV negative XLP patients, we considered that in addition to the immunological defect, the absence of SAP may lead to an intrinsic B cell dysfunction. This could possibly affect apoptosis/cell cycle control/DNA repair. We identified SAP as one of the targets of p53. We used a temperature sensitive p53 system and a panel of cell lines with endogenous wt p53. With the help of chromatin immunoprecipitation assay (ChIP) we have proven the functionality of one of the 2 possible p53 binding sites. DNA damage induced by γ-irradiation, induced SAP expression in primary T cells. Induction of SAP by p53 was tissue specific. Our results suggest that SAP contributes to the execution of some p53 functions.

We have introduced SAP by retroviral transduction to various cell lines and found that SAP elevated the sensitivity of cells to DNA damage. This was established by cell survival and colony formation assays. We also showed that the proportion of apoptotic cells was higher in the SAP positive populations. Thus SAP has a pro-apoptotic role.

We found that in activated T cells the SAP level is increased in the late phase of activation. The high levels of SAP showed a negative correlation with cell proliferation. Furthermore, clones of the T-ALL cell line with low and high SAP expression showed that the latter are more prone to activation induced cell death. Altogether, we have shown that absence of SAP function modifies cell proliferation / cell survival and by this we have introduced a new dimension to SAP and to XLP. Loss of this function can contribute to the maintenance of over-activated, proliferating T cells in the fatal IM and also to the development of lymphomas.

LIST OF PUBLICATIONS

I. Noémi Nagy, Cristina Cerboni , Karin Mattsson, Akihiko Maeda, Péter Gogolák, János Sümegi , Arpád Lányi, László Székely, Ennio Carbone, George Klein and Eva Klein. SH2D1A and SLAM protein expression in human lymphocytes and derived cell lines. *International Journal of Cancer, 2000, 88: 439-447.*

II. Noémi Nagy, Akihiko Maeda, Kentaro Bandobashi, Loránd L. Kis, Jun Nishikawa J, Pankaj Trivedi, Alberto Faggioni, George Klein and Eva Klein . SH2D1A expression in Burkitt lymphoma cells is restricted to EBV positive group I lines and is down-regulated in parallel with immunoblastic transformation. *International Journal of Cancer, 2002, 100: 433-440.*

III. Loránd L. Kis, Noémi Nagy, George Klein and Eva Klein. Expression of SH2D1A in 5 classical Hodgkin's disease-derived cell lines. *International Journal of Cancer, 2003, 104: 658-661.*

IV. Noémi Nagy, Miki Takahara, Jun Nishikawa, Jean-Christophe Bourdon, Loránd L. Kis, George Klein and Eva Klein. Wild type p53 activates SAP expression in lymphoid cells. *Oncogene. 2004, 23: 8563-8570.*

V. Noémi Nagy, Ludmila Matskova, Janos Sumegi, Arpad Lanyi, Loránd L Kis, George Klein and Eva Klein. Lack of functional SAP may lead to evasion of apoptosis, a clue to the clinical picture of XLP. *Manuscript.*

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LIST OF ABBREVIATIONS

ACAD	Activated T cell autonomous death
AICD	Activation induced cell death
BL	Burkitt lymphoma
CBP	CREB binding protein
CRACC	CD2-like receptor activating cytotoxic cells
CREB	cAMP response element-binding protein
CTL	Cytotoxic T cell
Eat-2	Ewing's sarcoma-associated transducer
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ERT	Eat-2 related transducer
FIM	Fatal infectious mononucleosis
GC	Germinal center
HL	Hodgkin lymphoma
IFN	Interferon
IM	Infectious mononucleosis
ITSM	Immunreceptor tyrosin-based switch motif
JMY	Junction-mediating and regulatory protein
LCL	Lymphoblastoid cell line
LCMV	Lymphocytic choriomeningitis virus
LMP	Latent membrane protein
MDM2	Murin double minute
NK	Natural killer
SAP	SLAM associated protein
SLAM	Signaling lymphocyte activation molecule
Strap	Serine/threonine-kinase-receptor-associated protein
XLP	X-linked lymphoproliferative disease

X-LINKED LYMPHOPROLIFERATIVE DISEASE (XLP)

The rare X-linked lymphoproliferative disease (XLP) has been reported in 1975 by D. Purtilo [1] based on a family in which 6 male cousins died of a lymphoproliferative disorder. Two of the six boys had laboratory evidence of acute Epstein-Barr virus (EBV) infection. Based on the clinical findings and inheritance pattern, the disease was termed X-linked recessive progressive combined variable immunodeficiency, or Duncan's disease, after the family's name. In the ensuing years the syndrome became known as XLP. It is exceptional among the six human X-linked immunodeficiencies in that the critical events usually occur after EBV infection. Affected males of the X-linked trait have normal responses to other herpesvirus infections, including varicella-zoster virus (VZV) and show only slight immunodeficiency prior to EBV infection, most frequently hypogammaglobulinemia [2].

XLP registry was established in 1978 and has approximately 300 patients registered from over 85 families.

The phenotypic spectrum of manifestation of XLP may vary. The most common are: fatal infectious mononucleosis (50% of cases), malignant lymphomas (30%) and dys-gammaglobulinemia (30%). However, rare cases of aplastic anemia, virus associated hystiocytic syndrome and vasculitis have also been reported. Patients often develop more than one phenotype over time and XLP mortality is 100% by the age of 40.

Infectious mononucleosis

More than 90% of adults worldwide are infected with EBV (henle g, henle w, J nat Cancer Inst., 1969) and carry the virus as a life-long persistent infection, with latent infection of B lymphocytes [3] and virus production into saliva [4]. In most cases primary infection occurs subclinically during childhood [5] often by spread between family members via salivary contact [6]. If primary infection occurs during adolescence or in young adults, infectious mononucleosis (IM) develops in between 50% and 74% of cases [7].

IM normaly presents with the accute onset of fever, sore throat, accompanied by lymphadenopaty, splenomegaly and hepatocellular dysfunction. The symptomatology of IM reflects the involvement of the immune system. It is characterized by a striking lymphocytosis as a result of dramatic increase in the numbers of NK and CD8+ T cells. The EBV specific T cells are directed against lytic, and to a lesser extent against latent, viral epitopes [8] and are thought to control the acute infection by eliminating infected B lymphoblasts. The activated CD8 lymphocytes, which can account for >50% of the total circulating lymphocyte population [9], release cytokines that are predominantly of a Th1 type (IFN-γ and interleukin-2) [10] [11] and are thought to induce the clinical features. Upon resolution of the illness, the absolute number of circulating T cells as well as the relative proportion of CD4+ and CD8+ cells returns to normal [12]. Mononucleosis can manifest with variable severity [13], but it regularly subsides.

Fatal infectious mononucleosis (FIM)

There is a very different course for primary EBV infection in X-linked lymphoproliferative syndrome (XLP) patients. In the affected boys, infection results in overwhelming cytotoxic T cell activation and proliferation, leading to lymphoid infiltrates associated with extensive necrosis in the liver, bone marrow and other organs [14]. Examination of hepatic tissue revealed periportal infiltration of CD8+ T lymphocytes. EBV induced fatal infectious mononucleosis (IM) is the most important characteristic of XLP. The life threatening immunological defect is thus characterised by the erosion of the normally watertight protection against the proliferation of EBV-transformed B cells [15].

The deficient control of primary EBV infection in XLP patients is well documented. EBV specific, MHC class I restricted CD8+ CTLs can be generated in vitro in lymphocyte cultures from EBV seropositive individuals. In such experiments T cells from XLP patients responded adequately. When exposed to autologous lymphoblastoid cell lines (LCL), they were stimulated for DNA synthesis and acquired specific cytotoxic potential. This test shows thus that cells endowed with EBV specific cellular memory can be generated in the XLP patients. On the other hand in a different in vitro test, which reflects the response to EBV infection, T cells of the patients did not function, they did not show inhibition of the EBV driven outgrowth of B cells [16]. The assays show two different aspects of the immune response against EBV. In the former the existence of specific T cells are demonstrated and these are selected out with the appropriate measures, while the latter reflects the events in the lymphocyte population when exposed to the virus. It is possible that in the former test the LCL cells used for stimulation of T cells could contribute with critical factors (e.g. cytokines) that are necessary for inducing T cell function.

Similarly to the impairment shown in the outgrowth inhibition tests, NK-cytotoxicity [17], [18] and further lymphocyte functions [19] of the XLP patients were also defective. This was not due to a reduction in the number of cells with NK markers (Leu-7 and MO-1). They were present at normal levels, but they did not function adequately. In contrast, antibody-dependent cell-mediated cytotoxicity (ADCC) tests with cells of XLP patients were in the normal range [20]. This reflects the difference between the mechanisms by which the two cytotoxic functions are triggered. While the NK-cytotoxicity is induced through specific surface receptors, ADCC is initiated through interaction of the Fc receptors with the target attached antibodies. The immediate signals of the two types of receptors are different, but downstream they merge in the mechanism that inflicts damage on the targets.

Deficient NK cell activity may facilitate the early spread of EBV in the organism not only in XLP but also in the selectively NK-deficient Chediak-Higashi syndrome and in chronic active EBV infection [21] [22]. The importance of the early viral spread is supported by the observation that acyclovir can block the development of incipient polyclonal posttransplant lymphoproliferative disease (PTLD), but has no effect on the established immunoblastoma. Impaired NK function may facilitate the recruitment of newly infected B cells.

Deficient or unbalanced production of certain cytokines such as IFN-γ, IL-1 or IL-2 may contribute to the pathogenesis of XLP [23] [24].

Malignant lymphomas

The malignancies are mostly non-Hodgkin B cell lymphomas, but rare cases of Hodgkin lymphomas and T cell lymphomas have also been described. The majority of the lymphomas are of the Burkitt type (53%) [25] [26]. Eighteen % are immunoblastic lymphomas, 12% non-cleaved lymphomas, 12% small cleaved lymphomas and 5% unclassifiable lymphomas [27]. Most lymphomas are extranodal and involve the terminal ileum, liver, kidney, central nervous system, thymus, and tonsils. Malignant B-cell lymphoma in patients with XLP is generally characterized by early age of onset, and a diffuse pattern of growth [27]. It is estimated that XLP patients have a 200 times higher risk for lymphoma development than the general population [28].

In view of the fact that the EBV specific T cell responses are impaired in XLP patients [29], it is likely that the proliferation of EBV infected B cells is not controlled. It was therefore assumed that EBV contributes to the development of the lymphomas. However, there are only limited data confirming the presence of EBV genome in the lymphoma [27] [30]. In many of the cases documented during the 70s'-80s' the available information identifies the patient as being EBV sero-positive, but not the lymphomas

themselves. Since genetic diagnosis of XLP became available, it has become evident that lymphomas appear before EBV infection [26] [31].

Dysgammaglobulinemia

The degree and pattern of humoral immune dysregulation are quite variable. Generally, besides cellular immune response, EBV infection induces humoral responses as well. Heterophile antibody is present in 85% of adolescents and adults with IM [32]. IgM to the EB viral capsid antigen (VCA) generally persists for about 1–2 months [33], while IgG to VCA along with antibodies directed to EBV nuclear antigens persists for life. However, post EBV infection in XLP patients, lack of IgG antibodies to EBV nuclear antigens (EBNAs) and variable production of anti-VCA is characteristic. In addition, patients can present with panhypogammaglobulinemia, IgG1 and IgG3 subclass deficiency and hyper IgM syndrome prior EBV infection [2]. Generally, B cells of XLP patients do not switch to IgG after secondary challenge with bacteriophage Φ X174 and fail to produce normal IgM levels in vitro [34]. A progressive reduction of immunoglobulin levels over the years can be seen in patients with dysgammaglobulinaemia, which indicates a cumulative effect of sequential environmental factors.

GENETIC DEFECT IN XLP: SAP

The search for the gene responsible for XLP spanned over several decades. At the end of the 80s' the locus was mapped to the long arm of the X chromosome at Xq25 [35] [36]. In 1990 it became clear that only one XLP gene locus exist in all families studied [37], but it took 8 more years to clone the gene in question.

An important milestone for understanding the complexity of XLP occurred in 1998, when at Xq25 *SAP / SH2D1A / DSHD* was identified as the gene that is altered or deleted in patients with XLP. *SAP* was identified by three independent groups using positional cloning [38] [39] and a functional/biochemical approach [40]. This latter group identified it in a search for downstream adaptor molecules that bind SLAM (signalling lymphocyte activation molecule), hence the name SAP (SLAM associated protein).

SAP consists of four exons spanning approximately 25 kb. The human SAP gene gives rise to a transcript of 2.5 kb that translates to a 15 kDa cytoplasmic protein (SLAM associated protein). In the first studies, mutations were not detected in every XLP patients. At that time, it was assumed that these patients may harbor mutations in the intronic sequences, regulatory regions or even in another gene. Given the difficulty of diagnosis in XLP, the possibility of misdiagnosis was also considered as an explanation. However, a more extended study detected mutations in the *SAP* gene in 34 out of 35 families with two or more maternally related males with an XLP phenotype. By contrast, in this study, no *SAP* mutations were detected in males with an XLP phenotype but with no family history of XLP [31], designated as sporadic XLP, underscoring the importance of diagnosis. After identification of the *SAP* gene, genetic diagnosis became possible and it is recommended even in cases without the existence of family history [41], as the XLP phenotype can arise from *de novo* mutations [42].

Most of the 50 mutations of *SAP* that have been reported in patients with XLP consist of large or small deletions resulting in complete loss of the gene or nonsense, splice-site and missense mutations that lead to the premature arrest of protein synthesis, to unstable or to a non-functional SAP [31] [43]. Analysis of missense mutations provided information about the functionally important amino-acid residues in SAP. The number of mutations is greatest in the second exon, with codon 55 commonly targeted. These mutational analyzes indicated that there is no correlation between genotype and

phenotype. In fact, identical mutations result in different phenotypes in the same family [31]. It is likely that environmental factors contribute to the disease manifestation.

Characterization of SAP

SAP (SLAM associated protein) contains a single Src homology 2 (SH2) domain flanked by short tails at the N-terminal (5 aa) and at the C-terminal (25 aa). The single-domain structure of SAP is unusual, as typical SH2 domains are found in multidomain signalling enzymes or adaptor proteins that mediate protein–protein interactions [44]. Its unusual structure suggested that SAP regulates one or several signal transduction pathways by masking a phosphorylated tyrosine.

Genes homologous to SAP have been identified in mice and cotton-top tamarin, suggesting its conserved role in mammals. The human and mouse SAP cDNA are 80% identical, and the human and mouse SAP amino acid sequences show 87% identity and 89% overall similarity. The highest similarity is found in the SH2 domain [45]. The promoter sequences have been mapped and contain functional Ets-1 and Ets-2 transcriptional factor binding sites [45], which both are important for T cell function and development. In addition, other putative transcriptional factor binding sites were identified in the mouse SAP promoter: GATA-1, OCT, glucocorticoid receptor, IRF-1 binding sites [46].

SAP is expressed in NK cells, T cells, including thymocytes and mature CD4+ and CD8+ populations [38] [47] [48]. It is also expressed in tumor lines of T, NK and B cell origin [49]. Expression of SAP in B cells is controversial. It seems to be expressed in rare human and mouse germinal center B cells [50] [51] [52], but it has not been detected by others in human peripheral blood or germinal center B lymphocytes [49] [53]. The discrepancy could be a consequence of technical problems or because of the rarity of the B cells that express it. In addition SAP was found in eosinophils and platelets as well [54] [55].

There seem to be species-specific differences in the regulation of SAP protein expression following lymphocyte activation. For example, the levels of human SAP protein increase in response to *in vitro* activation of T cells using phytohemagglutinin, allogeneic LCLs, infection of PBMCs with EBV [49] [47] or a combination of agonistic anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) [56]. Moreover, protein levels are elevated *in vivo* in the setting of acute EBV infection [12]. In contrast, studies in mice have shown that SAP protein levels decrease upon triggering of the T-cell receptor (TCR) [46]. This difference in the regulation of SAP levels suggest that specific variations could occur in the functional responses of human or mouse T cells following activation via the TCR or other surface receptors.

SAP family

SAP is only the first member of a newly established family that contains two other genes: Ewing's sarcoma-associated transcript-2 (Eat-2, or SH2D1B) [57] and in rodents, EAT2-related transducer (ERT or SH2D1C) [58]. All three members are composed of an SH2 domain and a short C-terminal tail. EAT2 and ERT are more closely related to each other than to SAP. They are positioned in tandem on chromosome 1, and were probably generated by gene duplication. In humans ERT has evolved into a pseudo-gene.

EAT2 is expressed in NK cells [58], dendritic cells and macrophages [59]. Similarly to SAP it is also found in platelets [55]. EAT2 transcripts were detected by PCR in B cells and activated human T cells, but protein expression was not documented [59] [60]. The balancing expression of SAP and EAT2 in the different cells of the immune system suggest their complementary role in regulating signal transduction in different cell types.

For example, since SAP is not expressed in myeloid cells, it is likely that SLAM activates macrophages in a SAP-independent manner, perhaps by binding to EAT-2 [61].

SAP family adaptors interact with SLAM family receptors

Many experiments have now shown that the SH2 domains of SAP and EAT-2 preferentially recognize specific tyrosine-based motifs conforming to the consensus Thr-Ile-pTyr-X-X-Val/Ile motif (called immunreceptor tyrosin-based switch motif, ITSM) that is present in one or more copies within the cytoplasmic tail of molecules of the SLAM (Signaling lymphocytic activation molecule, CD150) family. A related Thr-Val-pTyr-X-X-Val/Ile is also frequently present in these receptors, but it does not seem to bind SAP or EAT-2 [40]. Members of the SLAM family of receptors are haematopoietic cell-surface glycoproteins. Based on the similarities of their cytoplasmic domains and on common chromosomal localization, these receptors are now subclassified into a separate group of the immunoglobulin superfamily [62] [63]. They are: NTBA (SF2000) [64] [65], CD84 [66], SLAM (CD150) [67] [68], CS1 (CD2-like receptor activating cytotoxic cells, CRACC or 19A) [69] [70], CD229 (LY9) [71] [72], CD244 (2B4) [73] [74].

Several genes within the SLAM family produce one or more transcripts, based on the differential use of exons [75] [76] [77]. They have two (or four, for Ly9) immunoglobulin-like domains in their extracellular region. Nearly all SLAM family receptors are involved in homotypic self-associations through the extracellular domain. The exception is 2B4, which recognizes CD48, a receptor that is broadly expressed on immune cells [78]. SLAM is also the lymphoid-specific receptor for measles virus [79]. SLAM family receptors contain a single transmembrane segment and a cytoplasmic domain bearing tyrosine-based motifs. Splice variants of SLAM family receptors exist, most differing in the cytoplasmic region. Although the purpose of these variants is largely unknown, they might allow coupling to different intracellular effectors.

An unusual property of SAP and EAT-2 binding is that their specificity extends on both sides of the tyrosine residue allowing thus their binding in a phosphorylation independent manner. Such SAP binding to an unphosphorylated motif can occur at least in the case of SLAM [80] [81].

As XLP patients lack functional SAP, it has been speculated that the phenotypes of XLP might reflect perturbed signaling downstream of one or more of these receptors, which could negatively influence the generation of a normal immune response.

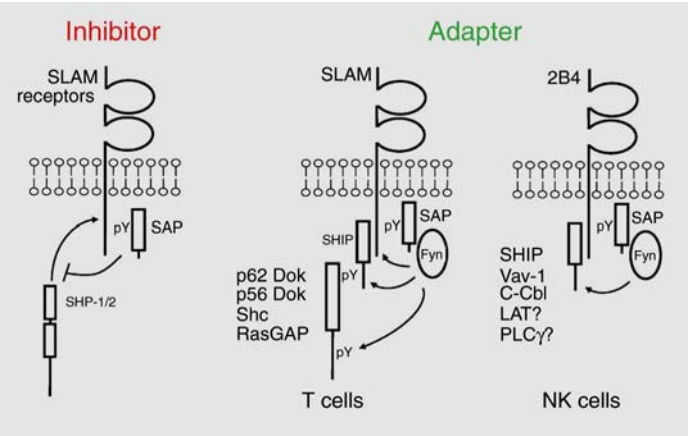

Fig.1 Models of SAP function during SLAM family receptor signaling in T and NK cells. (adapted from [82])

Based on the peculiar structure of SAP, initially it was assumed that it blocks molecular interactions in the signal system. This holds true, since can inhibit the binding

of SHP-2. However, recent evidence indicated that besides this SAP mediates recruitment of the protein tyrosine kinase Fyn T (Fig. 1) [83] [84]. This interaction is mediated by a direct interaction between the SH2 domain of SAP and the SH3 domain of FynT. The existence of this binding surface, in addition to the phosphotyrosine binding fold of the SAP SH2 domain allows a single SAP molecule to bind simultaneously to SLAM related receptors (SLAM, 2B4, Ly9) and to FynT.

Consequences of the absence of functional SAP

Identifying SAP dysfunction as the cause of XLP re-launched the studies concerning the function of immune cells in XLP patients, but this time they were analyzed from the point of view of the receptors to which SAP binds. In addition, studies were done in SAP deficient mice. From these studies it became clear that SAP influences the biology of many types of lymphocytes.

B cells

In XLP patients, the frequency and number of peripheral blood B cells are normal [85]. However, the differentiation of naïve B cells was impaired, as evident by a >10-fold reduction in the frequency and number of memory CD27+ B cells [86]. Moreover, the few CD27+ B cells was that remain in XLP patients express largely IgM, demonstrating that *in vivo* Ig isotype switching is impaired. These studies complement earlier investigations, which revealed that XLP patients fail to switch to antigen-specific IgG following repeated immunization with bacteriophage ΦX174 [34]. Interestingly, in spite of the fact that germinal centers were rare and poorly formed in the spleen of XLP patient, Ig variable region genes expressed by XLP IgM+CD27+ B cells had undergone somatic hypermutation. These affinity matured IgM antibodies could thus protect against pathogens [86].

In a normal situation, CD4+ T cells provide stimulatory ligands, including CD40L, OX40, and inducible co-stimulator (ICOS), and secrete cytokines, such as IL-4, IL-10, and IL-21, that promote B-cell differentiation towards Ig secretion. Further studies with B cells derived from XLP patients revealed that B cells underwent normal proliferation and differentiation when activated by CD40L + different cytokines [53]. However, CD4+ T cells derived from XLP patient expressed low levels of inducible costimulator (ICOS) a potent inducer of IL10 production, which in turn led to insufficient help in vitro. This T cell help could be improved by addition of exogenous IL10 [53].

Investigations of SAP-deficient mice have shown that SAP is critical for the generation and maintenance of long-term humoral immune responses. After lymphocytic choriomeningitis virus (LCMV) infection, *Sap*-/- mice develop an early burst of short-lived antibody-secreting B cells [87]. The number of these cells, however, declines dramatically over time, compared to wt mice. The formation of germinal centers (GC) is appreciably impaired in LCMV-infected SAP-deficient mice. Since GC formation is critical for the generation of long-lived plasma cells, at later time points during infection, there is a dramatic reduction in the number of memory B cells in the spleen and a virtual absence long-lived plasma cells in the bone marrow [87]. A similar phenotype was observed by another group, in which *Sap*-/- animals produced reduced levels of Ig after infection with murine gammaherpesvirus 68, a herpesvirus of wild rodents that is genetically related to EBV [88]. The abnormalities in antibody production in *Sap*-/- animals appear even following *Toxoplasma gondii*-infection, resulting in reduced levels of serum Ig [89]. *Sap*-/- animals have abnormally low-serum IgE following *Listeria major* infection [90]. Defects in antibody production and GC formation are also seen following immunization with specific T-cell-dependent antigens [91].

Similarly to the controversy around expression of SAP in B cells, a controversy regarding the intrinsic B cell defects in the absence of SAP exists as well.

Adoptive transfers experiments into irradiated wild type animals concluded that SAP-deficient B cells are themselves intrinsically normal since SAP-deficient B cells responded normally in the presence of wt T cells [87]. In contrast, wt B cells failed to develop long-term antibody responses in the presence of SAP-deficient CD4+ cells. Another set of adaptive transfer experiments done in Rag2-/- recipient mice revealed the existence of a B cell defect in the absence of SAP, which could not be rescued by wt T cells [51]. The conclusion of this latter experiment was that intrinsic B cell defects, beside T cell defects contribute to defective antibody production in the absence of SAP.

The existence of B cell defects is supported by the work of Al-Alem et al (2005). They have demonstrated that mouse B cells lacking SAP generate decreased circular transcrip levels of Iα, Iγ2a, Iγ2b and Iγ3 after *in vitro* stimulation, which indicate a defective Ig switch recombination. An earlier report suggested already that SAP has an affinity for Ku70 and Ku80 proteins that are known to be involved class switch recombination [92].

In addition to regulating B cell functions, SAP plays a crucial role in CD4+ T cells to mediate T-cell-dependent B-cell help, leading to the formation of GCs and the development of long-lived humoral immunity. Both human and mouse SAP-deficient T cells show defects in the production of Th2-type cytokines, which may contribute at least in part to the perturbed development of long-term humoral immune responses (CD4+ T cells) [90] [89]. On the other hand, recent results suggest that factors other than cytokines are responsible for defects in humoral immunity of mice deficient in SAP [93]. Instead, decreased and delayed inducible costimulator (ICOS) induction and low CD40L expression on SAP deficient T cells is responsible. These defects could be rescued by retroviral transduction of a mutant SAP (R78A). This mutant impairs Fyn binding, thus it could not rescue the Th2 cytokine defect [94], suggesting that SAP can mediate T cell help for B cells by a mechanism that is not dependent on Th2 cytokine production [93].

Except 2B4, B cells express all other members of SLAM family. Still the molecular details of SAP regulated signals initiated from these receptors, are limited. Since SAP is not readily detectable in B cells, studies were done in cell lines. The number of lines that express both SAP and SLAM is low. A few unusual SAP positive lymphoblastoid cell lines (LCLs), Hodgkin lymphoma (HL) derived cell lines or transfectants are available for these studies. It has been shown that in the presence of SAP, SLAM binds SHIP through its second tyrosine motif, but in the absence of SAP only SHP-2, the principal cytosolic mediator of inhibition, can bind to SLAM [50]. Akt phosphorylation, as a consequence of SLAM ligation, was dependent on SAP expression in a transfected cell line, but was not dependent on SAP in HL lines [95], thus questioning the role of SAP in supporting survival signals in the course of malignant transformation.

Another SLAM family member studied in relation to SAP in B cells is CD84. Ligation of CD84 results in its phosphorylation and recruitment of SAP and EAT-2 [96].

T cells

Since massive T cell defects have been described in XLP, it could be envisaged that studies on SAP will reveal its crucial involvement in the activation and function of these cells.

CD4+ T cells

In vitro studies with T cell lines derived from XLP patients were restarted after the cloning of SAP. These experiments revealed that the defect in SAP function causes

aberrant TCR signal transduction in immortalized CD4 T cells. This manifested by transient and rapidly diminished activation of Akt, c-Jun terminal kinases and extracellular signal-regulated kinases upon TCR stimulation [97]. The results were not influenced by immortalization, since experiments performed on non-transformed T cell lines led to similar results. These lines were defective in IL2 production, CD25 upregulation and homotypic aggregation when stimulated via the TCR by anti CD3+ anti CD28 [98]. Mapping the molecular pathways behind this phenotype identified an imbalanced TCR induced signaling with decreased phosphorylation of MAPKs such as ERK-1, -2, JNK, p38 and also a decreased degradation of IκB [98]. Thus besides regulating signals initiated from the SLAM family receptors, SAP interferes/cooperates with the TCR signaling as well.

The best understood function of SAP is Th2 cell priming. This is evident in both, XLP patient derived T cells and in SAP deficient mice as well. However, there is a difference regarding the identity of the cytokine in question. CD4 T cells of XLP patients produce low levels of IL10 [53]. Since IL10 is a potent growth and differentiation factor for human B cells, deficient secretion of this cytokine may contribute to the low levels of memory B cells and decreased serum Ig levels observed in XLP patients. CD4+ T cells of XLP patients produce normal levels of Th1 cytokines (IFN-γ, IL2, TNF-α) in vitro [53].

Similarly, SAP deficient mice were shown to have impaired ability to differentiate into Th2 cells [89]. In SAP deficient mice infection with LCMV or *T. gondii* leads to elevated levels of IFN- γ, paralleled by low levels of IL-4, suggestive of altered Th cell differentiation [90]. As already mentioned, opposite to the human setting, it is IL4 and not IL10 deficiency that marks the defect in Th2 cell differentiation in SAP negative mice. It is possible that a defect in the production of IL-4 and other Th2 cytokines contributes to the abnormalities in GC formation, B-cell differentiation, and long-term Ig production that are observed in SAP deficient mice [87] [91] [51].

Experiments performed with CD4+ T cells lacking SAP or expressing a SAP that is defective in FynT binding, provided evidence that Th2 cell priming relies on the SLAM-SAP-FynT pathway [99].

The impaired production of Th2 cytokines in *Sap*-/- T cells is independent of their increased IFN-γ production [94]. Consistent with their reduced production of IL-4, *Sap*-/- CD4+ T cells show impaired TCR-mediated induction of the Th2-specific transcription factor GATA-3, a transcription factor that regulates Th2 cytokine gene cluster expression. In addition, TCR stimulation led to decreased PKCθ recruitment [94]. Consequently, in SAP deficient CD4 T cells, activation of some NF-κB family members was selective impaired [93].

CD8+ T cells

CD8+ T cells express several of the SAP-associated receptors, including SLAM, 2B4, CD84, Ly9, and NTB-A, which suggests that defects in the functions of one or more of these receptors might influence the CD8+ T-cell response. No functions for CD84, Ly9, or NTB-A have been reported on CD8+ T cells.

The engagement of SLAM on human CD8+ T-cell lines has been shown to enhance TCR-mediated cytotoxicity towards a variety of target cells [100]. It is not known yet, whether SAP-deficient cells are defective in this function.

In mice 2B4-CD48 interaction between the CD8+ T cells and the target cells seems to be unimportant for the cytotoxic effect, but it can enhance the cytotoxic function if the interaction occurs between neighboring CD48 and 2B4 expressing CD8+ T-cells [101].

Recently, two groups re-analyzed the functionality of SAP-deficient CD8+ T-cell lines derived from XLP patients, especially from the point of view of 2B4 [102, 103]. In both studies, SAP-deficient CD8+ T cells exhibited diminished cytotoxicity against

CD48+ target cells, including autologous and allogeneic EBV-transformed B cells [102] [103]. This defect could be restored by retroviral gene transfer of wt SAP into XLP-derived T-cell lines [103] and was not due to impaired expression of the cytotoxic mediators perforin or granzyme. Rather, SAP-deficient T cells were defective in polarization of 2B4 and perforin. This latter defect seemed to be mediated by 2B4 [102]. Thus, following 2B4 engagement on human CD8+ T cells, SAP may influence target cell killing by regulating the assembly of the lytic synapse.

Capacity of these cells to produce cytokines seems to be normal or slightly decreased. XLP-derived T cells exhibited reduced production of IFN-γ in response to 2B4 ligation using mAb or autologous EBV-transformed B cells [103]. However, if cells were activated by anti CD3 + anti CD28, they produced normal levels of IFN-γ, IL-2, IL-4 [102].

In vivo studies performed with mice deficient in SAP, drew attention to another defect in the CD8+ T cells. Following infection of mice with LCMV, Toxoplasma gondii or murine γ herpesvirus-68, there was an increased population of CD8+ cells [90] [88, 89]. More detailed analysis of CD8+ T cells in these mice was done in two recent studies [104] [105]. It seems that SAP is needed for keeping under control their activation and proliferation, since higher frequency of antigen specific CD8+ T cells were generated in the SAP negative mice following infection with both, LCMV or murine γ herpesvirus-68. In the case of LCMV infection, the pathology manifested by damage to lymphoid tissues was more pronounced in the SAP deficient mice and was mediated by CD8+ cells [104]. It is not known yet whether these phenotypes are the consequence of intrinsic CD8+ cell defect or of a regulatory cell defect.

Thus, regarding the function of CD8+ T cells, mouse models recapitulate some of the features seen in XLP patients, e.g. exacerbated proliferation. Other aspects however, paradoxically show the opposite. For example, EBV specific CD8 T cells derived from XLP patients produce normal or low levels of IFN-γ, while mouse CD8 T cells produce high levels of IFN-γ. Similarly, there is a contradiction regarding the efficiency of eliminating specific target cells: XLP patients' CD8+ T cells are deficient in killing while in the mouse system SAP negative CD8+ T cells are more efficient in killing target cells infected with MHV-68 [105], leading to a better clearance of the virus. Differences in the characteristics of the virus-cell interactions and/or possibly the magnitude of the viral load may explain these contradictions.

NK cells

2B4, CRACC and NTB-A are expressed on NK cells and, in humans, activate NK cell responses. NK-cell numbers are normal in XLP patients and *Sap*-/- mice, demonstrating that SAP is not required for the development of this lineage [106]. In *in vitro* assays, human control and SAP-deficient NK cells exhibit equivalent cytotoxicity when triggered through non-SAP-associating receptors, such as CD2 or CD16 [107], thus confirming the old data about their unaltered ADCC function . However, unlike normal cells, the cytotoxic activity of XLP-derived NK cells do not increase when cells are activated through specific receptors known to associate with SAP.

This finding was initially demonstrated for 2B4 [107, 108] [109]. Moreover, in the absence of SAP, 2B4 and NTB-A operate as inhibitory receptors in NK cells derived from XLP patients [110] [65]. Engagement of these receptors on SAP-deficient NK cells suppresses cytotoxicity that is normally induced following ligation of the activating receptors. XLP NK cells were unable to kill an EBV-transformed B-cell line and this property could be overcome by blocking 2B4 and NTB-A using mAbs [65]. The molecular explanation of this effect came from the work of Eissmann et al. (2005). They have shown that SAP can bind to all 4 ITSMs of 2B4 in a phosphorylation-dependent

manner. The phosphorylated third ITSM can additionally recruit the phosphatases SHP-1, SHP-2, SHIP, and the inhibitory kinase Csk. Thus, SAP acts as an inhibitor of interactions between 2B4 and these negative regulatory molecules, explaining how 2B4 inhibits NK-cell activation in the absence of functional SAP.

In mice 2B4 can initiate both positive and negative signals in NK cells, most probably because they express two isoforms: a long form with four tyrosine motives and a short isoform with only one tyrosine motif [111]. However, in vivo studies done in SAP deficient mice attribute an overall activating role to 2B4 and this depends on the presence of SAP [112] resulting in the recruitment of Fyn. In addition, it has been shown that EAT-2 is a negative regulator of 2B4 signaling. Thus EAT-2 and SAP have distinct and sometimes opposing functions in mouse NK cells [58].

The defect in the cytotoxic pathway, normally elicited through the 2B4 receptor, is likely to be relevant for the development of certain features of XLP. For example, CD48, the natural ligand for 2B4, is rapidly upregulated on EBV-infected B cells. Thus, the inability of SAP-deficient NK cells to be activated through 2B4 to produce cytokines or kill CD48-expressing EBV-transformed B cells may contribute to the persistence of these cells during FIM.

Based on the crucial role of the 2B4-SAP pathway in eliminating EBV infected, CD48 positive B cells, Vaidya and Mathew [113] proposed a very interesting hypothesis regarding the evolution of 2B4: it is possible that the solely activating function of human 2B4 (as opposed to the dual roles in mice) evolved due to the selection pressure from EBV infection.

Similarly, to 2B4, one could envisage that SAP-dependent defects in NTB-A signaling may compromise the clearance of EBV-infected target cells. However, recent data may weaken the importance of NTB-A signaling in NK deficiency seen in XLP. Eissmann et al. have shown that homotypic interaction of NTB-A, leads to activation of cytotoxic function even in the absence of SAP and this activity is dependent on EAT-2 [114]. The tyrosine residue necessary to induce cytotoxicity, recruits EAT-2 and no SAP. However the presence of SAP is necessary for INF- γ production.

Interestingly, the third SLAM family receptor expressed in NK cells, CS1 (CRACC), triggers cytotoxicity through an ERK-mediated pathway independently of SAP [70]. Activating signals initiated from this receptor are regulated by EAT-2 [115].

NKT cells

NKT cells represent a peculiar subpopulation of $\alpha\beta$ T cells with immunoregulatory properties. Unlike conventional T cells, most NKT cells express an invariant TCR that is comprised of a specific α-chain rearrangement (Vα14Jα18 in mice and Vα24Jα18 in humans), paired with a limited number of β-chains (predominately Vβ8 in mice and Vβ11 in humans) [116]. This TCR recognizes glycolipid antigens presented in complex with the MHC-like molecule CD1d.

NKT cells are characterized by their ability to secrete large amounts of cytokines such as IFN-γ, IL-4, IL-10 and TGF-β within minutes after TCR stimulation [117]. Upon TCR engagement, NKT cells also have cytotoxic activities through the release of perforins and granzymes and by the expression of membrane-bound members of the TNF family (such as FasL) [118].

The recruitment of FynT to receptors of SLAM family by SAP, and the NKT cell defect observed in FynT deficient mice [119], drew the attention to NKT cells in SAP deficiency. Two independent groups reported that both SAP deficient mice and XLP patients lack NKT cells [120] [121]. This phenotype could be rescued in mice by reconstitution of SAP expression in bone marrow cells. Thus, unlike its role in T and B cells, SAP is a potent regulator of NKT cell development.

Numerous studies have shown that NKT cells can influence the Th1/Th2 balance in immune responses against infectious agents, tumors, alloantigens, and self-antigens. They are an important source of IL-4 production. Thus, it is also possible that the lack of NKT cells in combination with abnormally functioning *Sap*-/- T cells synergistically skews the immune responses towards to a Th1 type. Even though NKT cells can act as effectors, it is likely that regulatory function reflects their true physiological role.

How good is the SAP negative mouse as a model for XLP?

Although SAP -/- mice phenocopy many aspects of XLP, there are limitations to this model of the human disorder. First of all, mice are not susceptible to infection with EBV. Even though it is now clear that XLP can manifest prior to EBV infection, the most devastating phenotypes are still the ones that appear after EBV infection.

There are some clear differences in these two systems that were already mentioned: divergent functions of mouse and human 2B4, different Th2 cytokine defects, more powerful cytotoxic function of mouse SAP negative CD8+ cells when compared with the SAP positive ones (at least in some infections). In spite of these divergences, many molecular details of SAP function in the immune response have been already answered with the help of this mouse model. However, malignant lymphoma, that is a very common manifestation of XLP, does not appear in the SAP negative mice.

EPSTEIN-BARR VIRUS (EBV)

EBV is a B-lymphotropic gamma herpesvirus virus of the Lymphocryptovirus (LCV) genus. It is closely related to other LCVs of Old World non-human primates. The virus was discovered in a cell line established from a Burkitt lymphoma (BL) biopsy in which herpesvirus like particles were detected by electron microscopy [123]. More than 90% of the human population carries EBV. While the virus was shown to induce proliferation and transformation of B lymphocytes in vitro, the virus carrier state is largely harmless. The harmonious host-virus coexistence is the result of a long history with mutual adaptation that used variation in the viral gene expression in different types of infected cells and the finely tuned immune response of the host.

The EBV genome is composed of a double stranded DNA of approximately 172 kb in length, encoding nearly 100 viral proteins [124]. During viral replication, these proteins are important for regulating replication of viral DNA, forming structural components of the virion and modulating the host immune responses. Upon in vitro infection of B lymphocytes EBV establishes a latent infection, when only 9 viral proteins are expressed.

In vitro infection of B lymphocytes with EBV- latency III

EBV shows a high degree of B-cell tropism. B lymphocytes can be readily infected by EBV. Infection of B cell population in vitro results in the development lymphoblastoid cells lines (LCLs). This in vitro transforming capacity for B lymphocytes was EBV's first biological effect demonstrated [125].

The events following infection of B cells by EBV are well known based on in vitro studies. The virus enters B cells by interaction of the major glycoprotein gp350/220, with CD21 (receptor for the C3d fragment of complement), the EBV receptor. MHC class II molecules, serve as a co-receptor for the virus entry. The virus-receptor interaction induces activation of the cell, leading to homotypic cell adhesion, blast transformation, surface CD23 expression, and IL-6 production [126]. Once the viral genome has been

uncoated and transferred to the nucleus, the EBV genome is circularized and is maintained as episome. In these cells expression of six nuclear proteins (EBNAs, EBNA-1 to 6) is regulated by one of two alternative viral promoters (designated Wp and Cp). In addition to the EBNAs, three latent membrane proteins (LMP1, 2A and 2B) are also expressed in LCLs. The LMP transcripts are expressed from separate promoters [127]. In addition to these proteins, 2 small, non-polyadenylated (non-coding) RNAs, EBER1 and 2 are expressed. This type of viral gene expression pattern (9 proteins and 2 RNAs) is known as latency III or growth program. In this proliferation inducing latent viral program, five proteins (EBNA2, 3, 5, 6 and LMP1) are essential for the transforming i.e. activating and proliferation driving effect of the virus.

LCLs show high expression of the B cell activation markers CD23, CD30, CD39 and CD70 and of the cellular adhesion molecules LFA1 (CD11a/18), LFA3 (CD58) and ICAM1 (CD54) [128]. These markers contribute to the immunogenicity of the cells, therefore cells expressing Type III latency can exist only during the acute phase of primary infection, before the EBV specific T-cell response develops, and in patients with impaired immune functions [129].

Other forms of latency

Besides the full latency program seen in LCLs, there are other patterns of viral gene expression. The expression of EBV-encoded proteins differs depending on the type, differentiation and activation status of the target cell [130].

Latency II is seen in EBV-positive nasopharyngeal carcinoma (NPC), Hodgkin lymphoma (HL), nasal NK/T cell lymphomas and some T-cell lymphomas. In this progarm EBNA1, EBERs, BARTs, LMP1 and LMP2 are expressed.

In Latency I only EBNA1, EBERs, LMP2A and BARTs are expressed. It is seen in BL and in peripheral blood B lymphocytes of healthy virus carriers.
In Lat I and Lat II, EBNA1 is expressed from an alternative promoter, known as Qp [131] [132].

Latently infected memory B cells of healthy virus carriers do not seem to express any virally encoded protein [133]. This silent expression is denoted as latency 0. The presence of the virus can be readily detected by the EBERs.

Characteristics and functions of latent viral proteins

EBNA1

EBNA1 is a DNA binding protein that is required for the replication and maintenance of the episomal EBV genome [124]. EBNA1 contains a long glycine-glycine-alanine (gly-ala) repeat sequence, which varies in size in different EBV isolates [124]. This gly-ala repeat domain is a *cis*-acting inhibitor of antigen processing via the ubiquitin/proteosome pathway, thus MHC class I-restricted presentation and subsequent recognition by CD8+ CTLs of EBNA1 is inhibited [134].

In transgenic mice, expression of EBNA1in B cells has been shown to induce B cell lymphomas suggesting that EBNA1 might have a direct role in oncogenesis [135]. Inhibition of EBNA1 in BL lines decreased the survival of the cells, thus suggesting an antiapoptotic role [136].

EBNA2

EBNA2 is a transcriptional activator of both cellular and viral genes, and up-regulates the expression of certain B cell antigens, CD21 and CD23, as well as LMP1 and LMP2 [137] [124]. EBNA2 interacts with a ubiquitous DNA binding protein, RBP-J-

kappa/CBF1, which is responsible for targeting EBNA2 to the RBP-J-kappa binding sequence in promoters [138].

EBNA3, 4 and 6

They are members of the EBNA3 family, also called as EBNA3A, 3B and 3C. EBNA3 and EBNA6, but not EBNA4, are essential for B cell transformation *in vitro* [139].

Similarly to EBNA2, the proteins of EBNA3 family associate with the RBP-J-kappa transcription factor. They work in concert to control RBP-J-kappa regulated expression of cellular and viral promoters that contain J-kappa binding sequences [139].

EBNA 5

EBNA5 (also called EBNA-LP) is not necessary for transformation, but it is required for the efficient outgrowth of LCLs [140].

LMP1 (Latent membrane protein 1)

LMP1 expression induces several phenotypic changes in the EBV infected primary B lymphocytes such as aggregation, increased cell surface expression of CD23, CD39, CD40, CD44, decreased expression of CD10, and increased expression of the cell adhesion molecules CD11a (LFA1), CD54 (ICAM1), and CD58 (LFA3). LMP1 has also been shown to protect B-lymphocytes from apoptosis via the induction of the anti-apoptotic proteins, Bcl-2, Mcl-1, and A20 [141] [142].
LMP1 is oncogenic *in vivo* and induces B-cell lymphoma in transgenic mice [143]. It can transform rodent fibroblast cell lines [144].

It activates nuclear factor-kappa-B (NF-kappa-B), c-Jun NH2-terminal kinase (JNK)/AP-1, and the p38/mitogen-activated protein kinase (MAPK), JAK- STAT pathways [145] [146] [147]. Activation of NF-κB pathway by LMP1 occurs via both known pathways. The canonical pathway leads to the generation of p50/p65 dimers [148], the non-canonical pathway with the generation of p52/RelB dimmers [149] [150].

LMP1 is a functional homolog of CD40 [151]. They are both able to rescue B cells from apoptosis and drive their proliferation. While CD40 requires binding of its ligand, LMP1 is constitutively active [152].

LMP2

The LMP2 gene encodes two distinct proteins, LMP2A and LMP2B. The structures of LMP2A and LMP2B are similar and neither of them is essential for B cell transformation [153]. LMP2A aggregates in patches within the plasma membrane of latently infected B lymphocytes [154].

LMP2A can drive the proliferation and survival of B cells in the absence of signaling through BCR [155]. Therefore it can modify the normal B cell development. It helps to maintain EBV latency in lymphoid tissues and prevents inappropriate activation of EBV lytic cycle [156]. In addition, LMP2A can recruit Nedd4-like ubiquitin protein ligases, resulting in the degradation of Lyn and Syk and in this way LMP2A can modulate BCR signaling [157].

EBERs

The two small non-polyadenylated (non-coding) RNAs, EBER1 and 2 are expressed in all forms of latency.

Reintroduction of EBERs into EBV-negative Akata BL cells restores their capacity for growth in soft agar, tumorigenicity in SCID mice and resistance to apoptotic inducers [158]. Expression of bcl-2 or EBERs in EBV-negative Akata cells enhanced their

tumorigenic potential, but neither bcl-2 nor EBERs could restore tumorigenicity to the same extent as EBV [159].

In vivo EBV infection. Establishment of EBV latency 0 in healthy virus carriers

Following infection with EBV, the virus is carried for lifetime by the individual. Persistently infected cells in vivo do not resemble to the proliferating blasts seen with in vitro infection. The viral genome carrying B-cells in the healthy individuals are found in the memory B –cell compartment in a silent state [160] [3]. The recent view is that these cells usually don not express any viral genes, but they produce EBNA-1 only when they divide and thus ensures the maintenance of the viral episome [133] [161]. These cells do not express the growth-promoting latent genes and have a resting phenotype. The cells do not proliferate and therefore do not constitute a pathogenic threat.

The mechanism how EBV establishes this type of latency has received much attention. Studies were done on different B cell populations that were separated by flow cytometry. These studies led to a scenario proposing that EBV hijacks the normal B cell differentiation program [162].

According to this hypothesis, EBV transmitted through saliva infects in tonsil naïve IgD+ B cells. These cells express all EBV-encoded latent genes (latency III), they transform to blasts and start proliferating (growth program). Since EBV encoded latent proteins are immunogenic, some of these cells will be recognized and eliminated by the immune effector cells. A fraction of the cells will migrate to the follicle, and there its viral transcription program changes [130]. At this stage only 3 latent proteins are expressed: EBNA1, LMP1 and LMP2 (default program). The latent membrane proteins can help the passage of the infected B cell through the germinal center. The low LMP2 levels promote germinal center formation in the absence of BCR, and induce the germinal center regulatory transcription factor bcl-6 [163]. In addition, LMP1 and LMP2 induce activation induced cytidine deaminase and by this initiate immunoglobulin gene mutation and isotype switching [163] [164]. According to this model, expression of LMP1 leads to down-regulation of blc-6, a signal for the memory cell to exit the germinal center. In this way, a naïve EBV infected B cell can pass through the germinal center reaction without encountering cognate antigen. This process enables the infected cell to exit the cell cycle (growth program, latency III) and to gain access to the normal memory B cell pool (resting cell, latency 0) [165]. By shutting down its viral protein expression, the virus escapes the immune surveillance. The size of the virus carrying memory pool is stable over time. Since they do not express proteins that can induce proliferation, their maintenance is probably part of normal memory B cell homeostasis. When the cell divides EBNA1 is expressed, thus securing the maintenance of the viral episome [133].

This model is appealing, but cannot explain the fact that in seropositive donors and also in IM patients, the vast majority of the infected cells are present outside the germinal centers [166]. It is possible that EBV can directly infect memory B cells as well.

Immunity

EBV infection elicits both humoral and cellular immune responses. Antibodies limit the spread of the virus and T cells eliminate the infected B cells that express viral proteins that can be recognized.

Early studies of the CD8+ T cell response to EBV focused on the immunological recognition of cells expressing the latent proteins. CD8+ T cell lines and clones were established in vitro through stimulation with EBV or with EBV-transformed B-LCL. The specificity of these CD8+ T cell lines was determined by assaying their reactivity against fibroblast targets infected with recombinant vaccinia viruses expressing each of the eight

latent membrane proteins [167] [168]. The EBNA3, 4 and 6 proteins were commonly recognized although LMP2 was also an important target [169]. T cell responses to lytic cycle proteins were described several years later [170].

After the development of HLA class I -peptide tetramer technology, the frequency of specific CD8+ T cells was determined within the blood lymphocyte population in IM. These studies revealed that a large fraction of the activated CD45RO+CD8+ T cells were EBV specific and reactive to defined immunodominant epitopes, primarily derived from the immediate early (BZLF1) and some early proteins of the lytic cycle [8] [171] [172]. Individual lytic peptide epitope reactivities could account for 5-50% of the expanded CD8+ T cell population. At the same time, reactivities to certain latent epitopes, mostly from EBNA3,4 and 6, were also detectable, but accounted for only 1–3% of the CD8+ expansion [8]. Both types of reactivity were maintained, albeit at lower levels, in the blood of healthy EBV carriers [173] [174] with less marked differences between the two populations were.

The persistence of the specific CD8+ T cells assures that EBV induced proliferation of cells expressing all immunogenic latent genes (latency III) does not occur as long as the immune system is functional.

EBNA1 is special among the latent proteins in that it does not serve as a target for CD8+CTLs, because its long glycine-alanine repeat prevents the ubiquitin-proteasome dependent processing required to generate peptides that associate with MHC class I molecules [134].

The importance of CD4+ T cells in the control of EBV infection was recognized later. CD4+ CTLs were found in PBMCs of healthy virus carriers that recognized a variety of different EBV proteins expressed in both latent and lytic proteins. CD4+ T cell clones specific for EBNA1 and EBNA2 were found to inhibit outgrowth of EBV-transformed B cells [175] [176] underscoring the important contribution of CD4+ T cells to the control of EBV infection.

Thus, a significant proportion of the high numbers of activated T cells during primary EBV infection actively contribute to the elimination of infected cells. However, another important component of the response during IM is the massive death of activated T cells through apoptosis [177]. This contributes to the resolution of the disease [12] and a pool of resting memory cells emerges.

B cell malignances associated with EBV

Burkitt lymphoma (BL)

The clinical picture and histopathology of BL is unique. BL is a malignancy derived from germinal centers, centroblasts. EBV-positive BLs dominate in the high endemic regions whereas the majority of sporadic BLs is EBV-negative. In both, endemic and sporadic, BLs a constitutive activation of c-myc occurs due to a reciprocal chromosomal translocation between chromosome 8 and either chromosome 14, 2 or 22, that juxtaposes the proto-oncogene to one of the three immunoglobulin loci and provides the drive for proliferation. Cells with Ig-myc translocation have been detected in healthy individuals [178]. Constitutive activation of myc can drive cells into proliferation or apoptosis depending on whether the cells receive or do not receive concomitant growth promoting signals. Survival factors, such as growth stimulatory cytokines, may rescue them from apoptosis. Accordingly, the two conditions associated with Ig/myc translocations carrying BL, chronic hyper-endemic malaria and HIV infections have high levels of B-cell stimulatory cytokines. In addition to cellular factors, EBV expressed genes may counter-balance the pro-apoptotic function of myc, as shown in vitro for EBERs [158] and EBNA-1 [136].

Because EBV negative BLs exist, the precise role of EBV in the pathogenesis of BL remains unanswered. Monoclonal EBV episomes have been detected in virus-positive BL biopsies, suggesting that EBV infection preceded proliferation of the precursor B cells.

Though BL patients are immuno-competent, the EBV-carrying BLs escape from rejection. This escape is partially due to their phenotype, since they lack co-stimulatory surface molecules. Moreover, EBNA-1, the only EBV-encoded protein they express, does not provide MHC class I-associated peptides that could be recognized by CD8-positive CTLs.

EBNA1 and LMP2 were thought to be the only EBV encoded proteins expressed in EBV-positive BL tumors (latency I). Recent results however, suggest that Lat I type should include the expression of BARTs [179] and probably also BARF1 [180]. Expression of variable genes in several cases of BL [181] [182] have been reported.

BL cells (EBV negative and positive) phenotypically resemble centroblasts in that they express high level of CD10 and CD77 [183] [184]. When cells from some EBV-positive BL tumors are passaged in culture, they tend to shift to a more activated phenotype. This is accompanied by change to latency III expression, with EBNA2-6 and LMPs. The EBNA2- and LMP1-induced cell surface antigens, such as CD23, CD30, CD39, LFA1, LFA3, and ICAM1, also are up-regulated [185].

Hodgkin lymphoma (HL)

Hodgkin lymphoma (HL) is a malignancy of unknown pathogenesis. The malignant Hodgkin and Reed/Sternberg (HRS) cells derive from germinal center B cells (or rarely, T cells) but have a heterogeneous phenotype. The HL tissue is a complex granuloma, made up by T, B lymphocytes, macrophages, eosinophils, plasma cells, and less than 2% H-RS cells [186]. In the EBV-positive cases (40-50 % of the classical HL type) the viral gene expression pattern corresponds to latency II, with abundant LMP-1 expression [187] [188]. Based on the clonal rearrangements of the immunoglobulin genes, the cells were assigned to the B-lymphoid lineage [189], however they do not express several of the B-cell specific transcription factors and B-cell markers, CD19, CD20 and surface Ig [190] [191]. Detailed analysis of the gene expression profile of the HL lines and the immunohistochemical analysis of tumor samples showed considerable deviations from the B-lymphocyte pattern. This indicates that the B cell differentiation program of the HRS cell is impaired.

Characteristics of the HL tissue are similar in the EBV-negative and -positive cases. Apart from the expression of EBV-encoded proteins special properties that could be ascribed to the presence of the virus in the H/RS cells have not been discovered. However, the fact that a history of IM is a risk factor for HL development, points to the role of EBV. In addition, approximately 40% of the tumors contain clonal EBV which proves that infection occurrs prior to the tumorigenic process.

Lymphoproliferative disease in immunosuppression

The prototypic EBV-induced lymphoproliferative disorder arises as a result of the iatrogenic immunosuppression of transplant patients (post transplant lymphoproliferative disease, PTLD), although similar disorders occur in some of the primary immunodeficiencies and in patients with AIDS [192]. The appearance of these lymphomas in immunosuppressed individuals underscore the importance of the immune system in controlling EBV-mediated B lymphocyte growth.

It is generally assumed that immunosuppression leads to an increased number of latently infected cells in the blood. These blasts are targets for cytotoxic T cells in healthy carriers [193], but are thought to proliferate unchecked in the blood when the cellular immune response is suppressed. The expansion of these cells may lead to posttransplant lymphoproliferative disease (PTLD). These proliferating blasts survive only because the

immune response is compromised, since PTLD usually resolves upon removing immunosuppression [194] or following adoptive transfer of EBV specific HLA matched CTLs [195] [196].

T CELL HOMEOSTASIS

Upon encounter of Ag, T cells proliferate and exert effector functions such as production of cytokines and lysis of target cells. After elimination of the Ag, T cells are removed and only few survive as memory T cells. This process is highly regulated and requires a switch of the cells from an apoptosis-resistant towards an apoptosis-sensitive state. Removal of T cells occurs by apoptosis. Death in peripheral T cells is induced by 2 mechanisms: activation-induced cell death (AICD) which is induced by death receptor engagement [197] [198] and activated T cell autonomous death (ACAD) [199].

Death receptor mediated AICD

Stimulation of the T cell through the specific TCR leads to activation induced death that involves CD95 or TNF-R1 and probably other members of the death receptor family. This death receptor family (CD95, TNF-R1, DR3, TRAIL-R1, TRAIL-R2 and DR6) is part of tumor necrosis factor (TNF)/ nerve growth factor-receptor superfamily.

Among the death receptors, CD95 (Fas, APO1) is the most extensively characterized death receptor for AICD [198, 200]. The importance of CD95 in AICD is supported by the observation that CD95 and CD95L deficient mice and human patients develop progressive T cell lymphadenopathy and autoimmunity [201] and that these mutant T cells are resistant to AICD in vitro [202] [203]. On the contrary, other reports claimed that on challenge, antigen specific T cells expand and decline in numbers normally in CD95 deficient mice [204].

The ligand of CD95 is regulated at transcriptional level and its expression is induced following TCR engagement [205]. In addition, CD95L is upregulated on non-lymphoid cells in the periphery as well [206] [207]. Binding of CD95L to CD95 will initiate AICD. Downstream activation from CD95 requires activation of caspase-8 by its adaptor FADD (Fas-associating death receptor) [208]. Recruitment of pro-caspase-8 to the DISC leads to autoproteolytic activation of caspase-8 [209]. Activation of caspase-8 at the DISC can be counteracted by cellular (c)-FLIP proteins [210].

The role of TNF-R1 in AICD is not completely understood, but primarily implicated in the late phase of AICD [211].

Most probably CD95 alone is insufficient for depletion of activated T cells, thus involvement of the intrinsic pathway is necessary.

Activated T cell autonomous death, ACAD

In ACAD, T cell death is triggered by growth factor deprivation. It is regulated by the ratio between anti- and pro-apoptotic Bcl-2 family members at the mitochondria [212] [199]. In this pathway, the critical event is permeabilization of the outer mitochondrial membrane. This leads to release of cytochrome C, which together with apoptotic-protease activating factor-1 (Apaf-1) form the apoptosome. At the apoptosome caspase 9 is activated, and leads to activation of executioner caspase 3 and caspase 7 and finally to death.

In ACAD, the pro-apoptotic Bcl-2 family member Bim is determining cell death by the intrinsic pathway [199]. Bim associates with the anti-apoptotic proteins Bcl-2 and Bcl-XL on the mitochondrial membrane, thereby blocking their anti-apoptotic function [213]. Thus, the ratio of Bcl-2 *vs.* Bim regulates T cell death. Following injection of the superantigen SEB, both CD4+ and CD8+ T cells survive longer in Bcl-2 transgenic mice

than normal T cells [199]. Accordingly, retroviral transduction of Bim in SEB activated T cells could accelerate growth factor deprivation-induced apoptosis. Because apoptosis induced by enforced expression of Bim requires Bax and/or Bak, it appears that they are necessary for apoptosis of T cells during the termination of an immune response [214].

TCR stimulation can lead to activation of the intrinsic death pathway as well. While expression of Bcl-2 and Bcl-XL are largely controlled by TCR-mediated NF-κB activation, the up-regulation of Bim by TCR triggering merely depends on p38 and JNK activities [215].

In addition, the extrinsic (death receptor-mediated) apoptosis pathway can be connected to the intrinsic (mitochondria-mediated) pathway by caspase-8-mediated cleavage of the BH3-only Bcl-2 family member Bid towards truncated Bid (tBid).

Usually extrinsic and intrinsic death pathways lead to caspase activation. Recently, non-caspase proteases (*e.g.*, cathepsins released by the lysosomes) have been implicated to complement the extrinsic and the intrinsic death pathway and, thus, might contribute to AICD as well as to ACAD.

P53

The p53 protein was first described in 1979 as a transformation-related protein [216] and as a protein associating with the SV40 DNA tumor virus large T antigen [217] [218]. At first it seemed to be a proto-oncogene, but almost 10 years later, it was discovered that the oncogenic properties of p53 were in fact due to mutations in p53 [219] and subsequent research with wild-type p53 clearly demonstrated that p53 was in fact a tumor suppressor gene.

The p53 gene is one of the most commonly mutated genes in human cancer (approximately 50%) while in remaining tumors p53 function is impaired at other points [220]. Mutations in the p53 gene can result in abrogated function of the protein and it is this dysfunction that is linked to tumor progression and genetic instability.

The genetic knockout of the *Trp53* gene in the mouse [221] underscores p53's central role in tumor suppression. Mice lacking *Trp53* show a dramatic predisposition to cancer development; that is, each mouse born lacking both copies of *Trp53* develops some form of malignant growth within two to 10 months after birth. Most commonly, these animals die from thymic lymphomas, although a significant number of animals develop a range of sarcomas [221]. In the *Trp53* heterozygous mice [222], a tumor predisposition is evident, although the tumor spectrum and latency is different from that seen in the *Trp53* null animals. Whereas *Trp53* null mice are highly predisposed to lymphoma development, heterozygotes develop a greater number of mesenchymal cancers, and these tumors appear at later time points than the malignancies observed in the null mice.

The p53 protein is stabilized and activated in response to a variety of cellular stresses, including DNA damage, hypoxia, growth factor deprivation and oncogene activation [223] [224] [225]. In most cases, DNA damage arises from exogenous sources, but it can happen due to spontaneous base hydrolysis and during natural stress such as inflammation. DNA is the main target of environmental genotoxins (e.g. alkylating compounds, polycyclic aromatic hydrocarbons, biphenyls, heterocyclic amines, UV light and ionizing radiation) and of most cytotoxic anticancer drugs.

Following DNA damage, increase in protein level and activation of p53 can be achieved through several post-translational modifications. The most commonly reported post-translational modifications of p53 include phosphorylation of serines and/or threonines and acetylation, ubiquitylation and sumoylation of lysine residues.

Having a short half-life, p53 is normally maintained at low levels in unstressed mammalian cells by continuous ubiquitylation and subsequent degradation by the 26S proteasome. This is primarily due to the interaction of p53 with the RING-finger ubiquitin E3 ligase MDM2 (also known as HDM2). When the cell is confronted with stress, p53 ubiquitylation is suppressed through phosphorylation and acetylation of p53. p53 and MDM2 are both phosphorylated by ATM (ataxia telangiectasia mutated), which is a kinase that has a key role in the cellular response to DNA damage. ATM is rapidly activated by DNA damage. It phosphorylates MDM2, resulting in MDM2 deactivation, which leads to increased p53 levels [226]. MDM2 initiated degradation of p53 is also regulated by ARF, a protein that binds MDM2 and sequesters it in the nucleolus. However MDM2 is not considered the only regulator of p53 levels. Other factors, such as MDMx, pirh2 or JNK also participate in the stabilization of p53 in an MDM2-dependent or independent fashion.

In addition to stabilizing p53, phosphorylation has been shown to increase its sequence-specific DNA binding [227]. So far, 17 phosphorylation/dephosphorylation sites have been detected in human cells following DNA damage induced by ionizing radiation or ultraviolet (UV)-light irradiation. Significant redundancies are observed in that the same p53 site is phosphorylated by several different protein kinases. In addition, distinct

protein kinases also phosphorylate several sites on p53. It is possible that distinctive combination of phosphorylated residues lead to maximal activation or that a unique phosphorylation pattern induced by specific stimuli could determine the cellular response.

Acetylation of p53 happens in almost every type of cellular stress in a range of cell types. In some cases, previous phosphorylation might be required for subsequent acetylation of p53 [228]. Several proteins act as histone acetyltransferases (most important of them is p300/CBP), which add one or more acetyl groups to p53. Acetylation is important for p53 stability and transcriptional activation [229].

After its stabilization and activation, p53 accumulates in the nucleus, where it forms a homotetrameric complex [230]. Only tetrameric p53 seems to be fully active as a transcriptional activator or repressor of distinct target genes that contain p53 sequence-specific DNA binding sites [231].

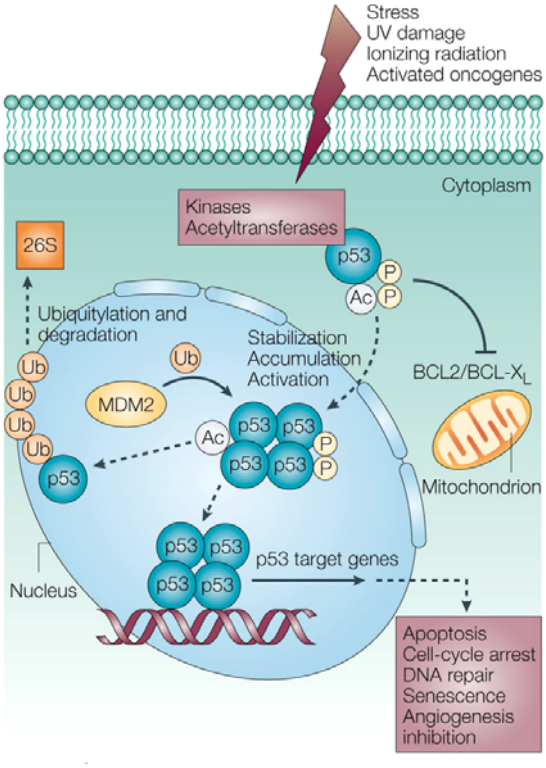

Fig. 2 Activation of p53 and cellular response. (adapted from [232])

While post-translational modification plays an important role in p53 regulation, an increasing array of cofactors is now known to influence p53 activity. The most important cofactors are p300/CBP, that bind to the N- and C- terminus of p53 and enhance the transcriptional activity through their ability to acetylate both p53 and chromatin [233]. ASPP1 and 2 can also specifically increase the transactivation of p53 responsive genes. JMY (junction-mediating and regulatory protein) is a CBP/ p300 co-factor that augments p53 response [234]. Strap (serine/threonine-kinase-receptor-associated protein) was identified as a novel JMY-interacting protein [235] that facilitates the interaction between JMY and p300. In addition, both JMY and Strap interact with MDM2 and prevent downregulation of p53 levels.

A detailed understanding of the interplay between these cofactors and their role in the p53 response will undoubtedly uncover new complexities in p53 control.

P53 effector functions in tumor suppression

DNA damaged cells are more likely to contain mutations and exhibit abnormal cell-cycle control, and present a greater risk of becoming cancerous.
After receiving a stress signal, p53 initiates a number of different cell signaling pathways, including those leading to apoptosis, cell cycle arrest, DNA repair and senescence.

Induction of apoptosis

One of the primary means through which p53 has been shown to suppress transformation in cell culture-based studies is through initiation of the cell death cascade.
The ability of p53 to induce apoptosis through transactivation of its target genes is critical for its function as a tumor suppressor. Proapoptotic genes in which a p53 responsive element has been reported include *Bax*, *IGF-BP3*, *DR5/KILLER*, *Fas/Apo-1*, the *PIG*s, *PAG608*, *PERP*, *PUMA*, *Noxa*, *PIDD*, *DRAL*, *Apaf1*, *Scotin* and *p53AIP1* [224]. The products of these genes may facilitate apoptosis in different ways. For example, many of these proapoptotic gene products such as Bax, Puma, Noxa and p53AIP1 localize to the mitochondria and promote the loss of mitochondrial membrane potential and cytochrome *c* release, resulting in the formation of the apoptosome complex with Apaf-1 and caspase 9 [236] [237].
Another class of proapoptotic genes that can be regulated by p53 such as DR5, Fas and PIDD, are components of the death receptor-mediated cell death pathway [238] [239]. In this case, caspase activation takes place at the plasma membrane following the clustering of death receptors that occurs after they have been bound by their ligands, such as TRAIL or Fas ligand.

Evidence obtained in knockout mice of different proapoptotic target genes (Bax, Noxa, PUMA) indicated that no single p53-induced product can solely explain p53-mediated transcriptionally dependent apoptosis. Thus, it is possible that different combinations of target genes are responsible for p53-induced apoptosis in different tissues. This may largely depend on the induction kinetics and tissue specificity of these target genes in response to the expression of p53.

There is growing evidence to suggest that p53 can induce apoptosis independently of its ability to bind DNA. A number of reports have recently demonstrated the direct localization of p53 to the mitochondria following DNA damage or hypoxia, where p53 can interact directly with antiapoptotic proteins such as Bcl-2 and Bcl-XL [240] [241].

Cell cycle arrest and maintenance of genomic stability

Apart from killing cells, p53 is also able to initiate a cell cycle arrest in response to cellular stress, as well as preserve the stability of a cell's genome.

The best know target of p53 involved in cell cycle arrest is $p21^{waf1/cip1}$. Unlike the p53 knockout mice, mice lacking p21 do not develop tumors [221]. This implies that the tumors seen in p53 null mice do not arise as a result of impaired cell cycle arrest mechanism, but that apoptosis is the dominant mechanism by which p53 inhibits tumor development.

On the other hand, recent experiments [242] suggested that cell cycle arrest may be a significant component of p53-dependent tumor suppression in certain contexts. In this model, the gene encoding a p53 mutant protein, R172P, was introduced into the endogenous Trp53 locus. Previous studies demonstrated that this mutant has lost all apoptotic activity in response to DNA damage, but still retains significant cell cycle arrest function [243]. Quite intriguingly, aged homozygous Trp53R172P mutants develop cancers with a significantly increased latency, and the malignancies that eventually develop do not indicate major genetic heterogeneity characteristic of lymphomas from Trp53 null animals, suggesting that suppression of genomic instability is part of the p53

tumor suppressor function. This function of p53 has also been described previously when tumors lacking p53 expression showed significantly higher levels of aneuploidy than wild-type tumors, as well as numerous regional amplifications and deletions [244]. Together, these findings support the idea that the downstream function of p53 responsible for tumor suppression does not exclusively center on its ability to induce cell death.

DNA repair

Recently, a new role for p53 has come to light, as the tumor suppressor also functions in DNA repair and recombination. In cooperation with its function in transcription, the transcription-independent roles of p53 contribute to the control and efficiency of DNA repair and recombination. In eukaryotes, the five main DNA-repair processes are nucleotide- excision repair (NER), base-excision-repair (BER), mismatch-repair (MMR), non-homologous end-joining (NHEJ) and homologous recombination (HR). p53 is involved in HR regulation only through the transactivation-independent function, but modulates almost all other DNA-repair processes by both transactivation-dependent and -independent pathways [245] [246]. Therefore, p53 might function as the 'molecular node' that lies at the intersection of upstream signaling cascades and downstream DNA-repair and -recombination pathways.

The type and magnitude of the p53 response depends on many factors. A model has been proposed where the outcome of p53 activation correlates with the levels of p53 present in the cell. In circumstances where p53 levels are only slightly elevated, cell cycle arrest occurs through the high-affinity binding of p53 to promoters that closely match the p53 promoter consensus sequence. Death is induced when the cell accumulates high levels of p53, suggesting that promoters regulating apoptosis-inducing genes have a lower affinity for p53.

Additionally, the strength of the interaction of p53 with a particular promoter might be regulated by interactions with other cellular factors. Different chromatin arrangements of target genes according to the cell type or even the position during the cell cycle may also play a role. All these factors can contribute to the heterogeneity of cellular response to p53 activation.

p53 and the immune system

Among the p53 target genes there are a few that are involved in the immune functions. ICAM-1 [247] and IRF-5 [248] were reportedly induced by p53. The immunoregulatory protein CD200 is also a target of p53 and is expressed during dendritic cell apoptosis [249]. In addition, p53 mRNA is stabilized in PHA activated PBLs [250]. These results indicate a role for p53 in immune responses.

Absence of p53 does not affect the development of T cells [251]. However, CTLs are more readily generated from p53-null then from wild type mice [252]. This suggests that p53-null effector cells survive longer after chronic exposure to Ag. On the other hand, uninfected or LCMV infected p53-null mice have only slightly elevated numbers of CD8+ and CD4+CD44^{high} cells and apoptosis of activated T cells is only slightly reduced in p53 mutant mice. It is possible that the role of p53 in lymphocyte homeostasis is more pronounced when the presence of Ag is sustained as it is in primary EBV infection for example.

RESULTS AND HOW DO THEY FIT INTO THE XLP PUZZLE

B cells and XLP

B cells can express SAP

Ever since the discovery of XLP, the major question has been: Why can't patients with XLP control infection with EBV, but can most other viral infections?
Considering that EBV is special because its target is the B cell, the most plausible idea was that the XLP gene (later identified as SAP) has a decisive function in B cells, a function that is lost in XLP.

However, others and we could not detect SAP in peripheral B cells, while it is readily detectable in T cells and NK cells (Paper I). In search for a (maybe rare) B cell, where SAP might be expressed and possibly connected with EBV, we studied a panel of BL lines (paper II).

The vast majority of EBV positive BL lines, that retained the in vivo (resting) phenotype and gene expression pattern, were SAP positive. However, none of the EBV negative BL lines expressed SAP. This result was interesting first of all from the point of view of BL studies, because the endemic, EBV-positive and the sporadic, mainly EBV-negative BLs are regarded as being phenotypically identical [253], they both carry markers of the germinal center. SAP expression is the first difference that was found to distinguish these two groups. Thus, it is possible that the two BL groups originate from B cells with a slightly different phenotype.

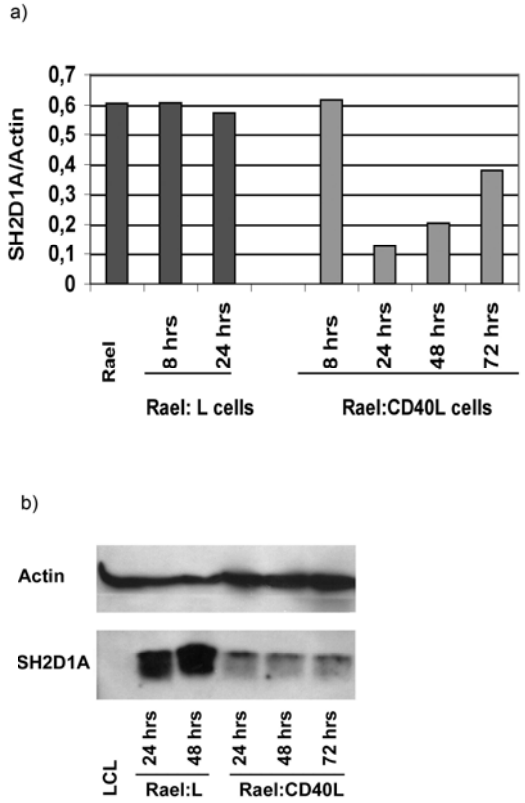

Fig.3 Regulation of SAP (SH2D1A) levels by CD40 crosslinking
The type I BL line Rael was co-cultured with irradiated CD40L expressing L cells or with the control L cells at a Rael:CD40L ratio of 1:1. At the indicated time points total RNA and protein lysate was prepared and tested by Northern blot (a) or immunoblot (b)

Alternatively, SAP expression can be the consequence of a subtle EBV-induced change in differentiation stage. *In vitro* EBV infection of DG75 line seemed to support this idea, since in the convertant lines (that expressed latency I) SAP was induced. However, we were not able to identify the viral gene responsible for this. Transfections with single genes

expressed in latency I (EBNA1, EBER1,2) did not induce SAP expression. It is possible that other genes (BARTs, BARF1) or even EBV encoded microRNAs could be responsible for this.

The results with BL lines indicated that SAP may be expressed in a very narrow window of B cell differentiation, possibly in GC cells. However the direct evidence for expression of SAP in normal B cells is still controversial, recent data shows that in the absence of SAP there is an intrinsic B cell defect during GC formation [51].

Our results with BL lines also identified CD40 signaling as a possible regulator of SAP in GC cells. Crosslinking CD40 on type I BL lines induced down-regulation of SAP and upregulation of SLAM. Kinetic studies done at the level of mRNA (by Northern blott) (Fig. 3a) indicate that this regulation occurs at transcriptional level. Parallel studies done at protein level suggest that in addition to the transcriptional regulation, enhanced protein degradation is also induced by CD40 signaling (Fig. 3b), allowing thus rapid downregulation of otherise long lived SAP at protein level.

XLP-B cells may be deficient in shutting down the latency III program

The results obtained with the BL lines have an additional implication for XLP. We have seen that EBV positive cells with the GC phenotype express SAP and this expression is downregulated while cells change their phenotype and EBV latency program.

According to the current model (introduced by the Thorley-Lawson laboratory) of how EBV establishes the latency 0, EBV infected naïve B cells go through such conceivable SAP positive stage: an EBV positive cell having a GC phenotype. By going through the GC the EBV infected B cells shut down the growth program (exit the cell cycle) and establish latency 0 in a resting memory B cell. In vitro data supports the possibility that such latency switch from the full lymphoblastoid (lat. III) to a more restricted latency program exists [254].

It is possible that SAP is involved/necessary in shutting down the growth program (lat. III) because it is expressed at least in one particular phase of the process. In XLP patients, however, SAP is mutated/deleted, thus non-functional. For that reason, the exit from the cycle of EBV infected blasts can be impaired. As a consequence, these proliferating blasts would persist. However, disappearance of the proliferating blasts is important for the resolution of IM. In addition, cytotoxic function of CTLs and NK cells is also impaired in XLP patients, thus the high numbers of EBV-B blasts are maintained. In turn, these proliferating (latency III) EBV infected B cells maintain the pool of over-activated T cells leading to exacerbated immonupathology. The immune system is thus trapped in a vicious circle eventually leading to extensive tissue damage and death.

Data regarding poor GC formation in XLP patients [86] and SAP negative mice [51] support our hypothesis regarding this additional layer of complexity of the fatal IM seen in XLP.

Beyond XLP: IM versus silent primary EBV infection

Based on this hypothesis and on the clinical picture of fatal IM in XLP, it seems thus logical that shutting down the growth promoting latency III program through GC reaction is extremely important for the resolution of the primary EBV infection. As a consequence/follow up of this line of ideas, we put together a hypothesis on why primary infection has a different outcome depending on the age of the individual. In children, EBV infection generally occurs without any clinical manifestation (marked only by sero-conversion) but if infection happens at a later age (young adults) IM will manifest in about 50% of the cases.

The present model of how EBV enters the memory B cell compartment is based on the assumption that EBV infects primarily naïve B cells. Through germinal center reaction these

cells will then differentiate to memory cells and switch from the EBV growth program (latency III) to latency 0 (no proliferation).

However, there is no known reason why memory B cells would not be infected by EBV. In fact, data exist about the presence of such cells in the IM tonsils [255]. In the normal B cell biology, memory cell activation predominantly leads to plasma cell development [256]. Since EBV directs naïve B cells to follow the normal differentiation program, one could assume that when infecting a memory cell, EBV would also allow them to follow the same path as uninfected memory cells do. Thus infection of a memory cell could lead to plasma cell differentiation. However, plasma cells are the site of virus replication [257]. Thus infection of a memory cell would generate plasma cells that would produce new virus.

Our "IM hypothesis" is based on this different outcome of EBV infection depending on the differentiation state of the infected cell. The number of memory B cells available for infection or the relative ratio between the infected naïve and memory cell would decide the outcome of the primary infection.

According to our hypothesis, when a child encounters EBV, more naïve B cells get infected, simply because they have more naïve B cells. This leads to prompt differentiation from blast (lat III) to memory (lat 0) B cells, where no EBV genes are expressed and they are not recognized by the immune system. Thus, in this case there is limited time for activating the T cells because gene expression is shut down efficiently. The degree of T cell activation is constrained, thus no consequent immunopathology occurs.

When a young adult gets infected, it is possible that more memory cells will be infected, because their relative number is higher. These cells cannot exit the growth program (do not enter GC reaction). They can even become plasma cells and initiate the lytic cycle. The newly produced virus increases the number of infected cells that in turn become blasts and activate T cells. This amplification in numbers of infected cells could lead then to activation of the immune system to a level when pathological manifestations of IM occur. Generation of specific T cell response would finally eliminate the infected cells and IM would come to an end.

The production of new viral particles during IM is supported by the fact that an overwhelming fraction of EBV specific CTLs in IM are directed against lytic antigens.

T cells and XLP

New type of T cell deficiency in the absence of SAP: resistance to AICD

Following the successful resolution of any infection, a contraction phase occurs, during which the majority of virus-specific CD8 T cells die and a pool of resting memory cells emerges. The death of antigen-stimulated lymphocytes during the shut-down of an immune response probably functions to minimize the immunopathology that results from the potently destructive effector molecules produced by these cells. Elimination of activated T cells by apoptosis is probably even more important for the resolution of IM considering the existence of marked lymphocytosis which together with a Th1 dominated cytokine storm is the cause of the disease manifestation. Indeed, in IM a massive apoptosis of T cells is seen.

Our results indicate that the high level of SAP, that is present in the late phases of T cell activation, is necessary for an efficient elimination of activated T cells by activation induced cell death (AICD). In our model system (sublines of the T-ALL tumor line expressing "low" or "high" level of SAP) cells with high SAP were more sensitive to AICD induced by PMA+ionomycin and also by anti CD95 antibody (manuscript).

Thus the course of IM in XLP patients is so often tragic not only because the EBV infected B cells are not efficiently killed by CTLs and NK cells (well known deficiency), but also because the activated T cells do not die.

This new proapoptotic function of SAP in T cells may help to better understand why IM turns fatal in XLP patients.

Beyond XLP: T cell homeostasis under the control of p53?

Based on the fact that activation of T cells leads to upregulation of p53 and that SAP is a target of p53, it is possible that upregulation of SAP during T cell activation is partly under the control of p53. Since SAP contributes to the depletion of activated T cells, this would link p53 with T cell homeostasis. Data exists showing that CTLs were more readily generated from p53-null then from wild type mice indicating that p53-null effector cells survive longer after chronic exposure to Ag and that p53 does play a role in this regulatory process [252]. However, uninfected or LCMV infected p53-null mice have only slightly elevated numbers of CD8+ and CD4+CD44^{high} cells and apoptosis of activated T cells is only slightly reduced in p53 mutant mice. It is possible that the role of p53 in lymphocyte homeostasis is more pronounced when the presence of Ag is sustained as it is in primary EBV infection for example.

Malignant lymphomas in XLP

Pro-apoptotic function of SAP

A very striking manifestation of XLP is the high lymphoma incidence. It is estimated that XLP patients have a 200 times higher risk for lymphoma development than the general population.

It has been assumed that lymphomas appear in XLP patients due to deficient NK and CD8+ cytotoxic T cell control of EBV infected B cells that have proliferating potential. However, after the genetic diagnosis of XLP was possible, it became evident that lymphomas appear in EBV sero-negative XLP patients with similarly high frequency [31]. Thus, in addition to the EBV specific immunodeficiency, intrinsic B cell defect may occur in the absence of functional SAP, a defect that would facilitate the frequent development of B cell lymphomas.

We have therefore searched for EBV independent mechanisms that may be responsible or contribute to lymphoma induction. Since p53 plays a key role in the control of cell proliferation and apoptosis and p53 knockout mice show a high incidence of lymphoid malignancies, we searched for possible connection between SAP and the p53 network.

As a result of this work we identified SAP as a target of p53 (paper IV) and as such, new functions of SAP could be envisaged. As a follow up of this finding, we have shown that in DNA damaged cells SAP has a proapoptotic function (manuscript). Altogether, these results indicate that p53 induced SAP may help to eliminate cells with compromised DNA. The mechanism of this process is not yet clarified.

The introduction of double-strand breaks into DNA triggers a complex set of responses, including cell cycle arrest, relocalization of DNA repair factors and in some cases apoptosis. Failure to induce these responses following DNA damage can lead to a high level of genomic instability that may increase the probabilities for oncogenic transformation.

DNA damaged lymphoid cells that normally would die, might escape death in the absence of functional SAP. These surviving, but damaged cells are likely to accumulate mutations that eventually could lead to lymphoma development.

Why SAP negative mice do not develop lymphomas?

There are a number of possible reasons for this:

-Studies done on p53 KO mice with different genetic background underscored the importance of genetic modifiers present in individual mouse strains [258]. These (still unknown modifiers) are able to exert a strong influence on the cancer spectrum seen in mice

in the case of p53 studies. The same mechanism could also explain the lack of lymphomas in SAP negative mice.

-Another potential explanation is the inherent genetic differences between humans and mice.

-In addition, it is possible that pathways through which SAP contributes to the control of lymphoma development are redundant in mice.

-It is also possible that more oncogenic hits are necessary and that these mice would be susceptible to radiation- or oncogene-induced cancers, but are significantly protected from spontaneous tumorigenesis.

Beyond XLP: SAP is induced by p53 in a tissue specific manner

The failure of wt p53 to induce SAP in non-lymphoid cells (MCF-7, A549, HCT116) suggests that the regulatory processes in which SAP participates are restricted to lymphoid cells. This is in line with the fact that malignant lymphoma is the only tumor type described in XLP patients. To our knowledge, this is the first p53 target gene shown to have a tissue specific expression.

CONCLUSIONS

As XLP patients lack functional SAP, an apparently simple immune adaptor, it has been assumed that the phenotypes of XLP reflect the impairments of signaling initiated by one or several SLAM-family receptors. Thereby the immune responses would be perturbed.

The intense work of the last few years, including the creation of SAP KO mice, provided explanation for several defects seen in XLP patients. These were all involved in effector functions, such as cytotoxicity, cytokine and antibody production of T, NK and B cells. These functions are pivotal for the control of infection, elimination of EBV infected B cells.

However, in spite of all these developments, many questions regarding XLP remained unanswered.

By identifying SAP as a target of p53 and a pro-apoptotic protein, my work introduced a new dimension to SAP and to XLP research. Loss of its pro-apoptotic function could operate both in the fatal IM and also in the development of lymphomas in these patients. It can be summarized as follows:

1. AICD of T cells (homeostasis) is impaired in XLP patients. This allows extended survival of over-activated T cells in IM, cells that in individuals with normal SAP die massively during IM. Persistence of these T cells leads to the massive tissue infiltrates and organ failures seen in fatal IM.

2. DNA damaged lymphoid cells that normally would die, might escape death in the absence of functional SAP. These surviving, but damaged cells are likely to accumulate mutations that eventually might lead to lymphoma development. This function of SAP could help to explain why XLP patients have a 200 times higher risk for lymphoma development than the general population.

With this new function in mind, we can explain better why a defect in SAP leads to clinical manifestation mostly in EBV infection. I propose the following scenario:

Infection of B cells by EBV leads to their proliferation, thus increases the number of antigen presenting cells. In addition, the EBV infected B cells undergo blast transformation and express high levels of co-stimulatory molecules. These B blasts stimulate T cells far beyond the levels of activation seen in any other infection. An efficient homeostasis with rapid elimination of the activated T cells is therefore particularly critical in IM. Loss of the pro-apoptotic capacity of SAP would thus explain why IM turns fatal in XLP patients.

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