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MASTERARBEIT

Titel der Masterarbeit

„Caspase-1 in EBV-infected cells is potentially activated by EBNA1 and causes N-terminal processing of the large tegument protein BPLF1“

verfasst von

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CONTENTS

LIST OF ABBREVIATIONS

ALR	AIM-2 like receptor family
ASC	apoptosis-associated speck-like protein containing a CARD
Asp	Aspartate
BIR	Baculovirus Inhibitor of apoptosis protein Repeat
BL	Burkitt's Lymphoma
BSA	Bovine serum albumin
bZip	Basic Leucine Zipper
CARD	C-terminal Caspase-recruitment domain
CMV	Cytomegalovirus
CRLs	Cullin Ring Ligases
Ctr	control
DAMPs	damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
EBER	EBV-encoded Ribonucleic acids
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EGFR	epidermal growth factor receptor
FCS	fetal calf serum
GFP	green fluorescent protein
gp220/350	glycoprotein 220/350
HA	hemagglutinin
HPV	Human Papillomavirus
HRP	horseradish peroxidase
HSV-1	Herpes simplex virus 1
IFI16	interferon-inducible protein 16
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious Mononucleosis
IP	Immunoprecipitation
kb	kilobase

kD	kilodalton
KSHV	Kaposi’s sarcoma-associated herpesvirus
LMP	latent membrane protein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
N-terminal	amino-terminal
NEM	N-Ethylmaleimide
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	nucleotide-binding oligomerization domain
ORF	open reading frame
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase chain reaction
PRR	pattern recognition receptor
PrV	Pseudorabies virus
ROS	reactive oxygen species
RR	ribonucleotide reductase
SDS-PAGE	sodium dodecyl phosphate Polyacrylamide gel electrophoresis
TGN	Trans-Golgi-Network
TRAF6	TNF Receptor Associated Factor 6
TRITC	Tetramethylrhodamine isothiocyanate
Ub	Ubiquitin

ABSTRACT

Caspases are cellular proteases that play important roles in cellular metabolism, such as programmed cell death or inflammation. Here we found that in cells infected with Epstein-Barr virus, the Caspase-dependent apoptosis pathways are turned off, whereas the inflammatory pathway mediated by Caspase-1 is activated. The large tegument protein of EBV, BPLF1, is processed by Caspase-1, producing a catalytically active N-terminal fragment that translocates to the nucleus where it acts as deneddylase and deubiquitinase. Inhibition of Caspase-1 activation causes accumulation of full length BPLF1 in the cytoplasm and a decrease of viral DNA synthesis. The monitoring of Caspase activity in different cell lines infected with EBV shows a significant upregulation of constitutive Caspase-1 activity, suggesting a potential role for EBV latency proteins in promoting Caspase-1 activation. Here, we show that Epstein-Barr Nuclear Antigen 1 (EBNA1) causes an increase in the processing of Caspase-1 into its active form, pointing to another role of the latency protein in the EBV life cycle.

INTRODUCTION

Inflammation.

Upon invasion by pathogens, the host cell responds with a number of different immune responses. The first and fastest reaction is the innate immune system. It includes physical barriers (skin, mucous membranes, etc), activation of the complement system, but also receptors located on the surface of innate immune system cells that recognize harmful agents and initiate the cellular inflammation reaction (1). The acute inflammatory response is one of the quickest reactions of the body to any kind of cell damage or pathogen invasion that attempts to heal the affected tissue by delivering components of plasma and blood to the site of injury. When infections or tissue damage occur, a signaling cascade is triggered. This results in the production of a number of different inflammatory mediators such as chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades, which are highly concentrated in the proximity of inflamed tissues. These small molecules promote the recruitment of immune cells such as neutrophils, macrophages or T-cells, which attempt to kill and remove the pathogens. Failure to eliminate the invading agent by these cellular factors can lead to chronic inflammation (2).

The immune response to viral infections is an interesting interplay between host and pathogen. It has been known for several years that invasion of a cell by pathogens causes the assembly of a large intracellular protein complex (~700kD) known as inflammasome (3). Assembly of these complexes results in the activation of Caspase-1, and subsequent processing and release of mature pro-inflammatory cytokines IL-1β and IL-18 (4) that eventually contribute to clearance of the invading agent. Inflammasomes are assembled around germline-encoded sensing molecules known as Pattern Recognition Receptors (PRRs). They are expressed by various immune cells and recognize pathogen-associated molecular patterns (PAMPs) such as bacterial or viral proteins, sugars or nucleic acids, as well as endogenous damage-associated molecular patterns (DAMPs), including reactive oxygen species (ROS), cation flux, aluminum

salts, etc. (1,3). These sensors are localized on the plasma membrane (Toll-like receptors), in the cytoplasm ((NOD)-like receptors) or even inside the nucleus, such as the interferon-inducible protein 16 (IFI16) (5). PRRs have the general structure of a C-terminal Leucine-Rich Repeat (LRR) domain for ligand sensing, a nucleotide-binding oligomerization (NACHT or NOD) domain and an N-terminal effector domain (6,7). In humans, the most common effector domains are Caspase-recruitment domain (CARD), pyrin domain (PYD) and baculovirus inhibitor of apoptosis protein repeat (BIR) (8).

Upon sensing of dangerous molecules by their C-terminal ligand-sensing domain, the production of type-I interferons is triggered. For incorporation into the inflammasome complex, the receptor protein oligomerizes and recruits the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) to its N-terminus through homotypic Pyrin-domain interactions (3). The Caspase-recruitment domain (CARD) of ASC then interacts with the CARD of procaspase-1, resulting in auto-proteolytic activation of the inflammasome-mediated caspase pathway. The pro-inflammatory cytokines IL-1β and IL-18 are produced as inactive precursor molecules in the cytoplasm of immune cells. Upon processing by the inflammasome, active Caspase-1, formerly known as Interleukin (IL)-1-converting enzyme, cleaves these precursors in the cytosol or in secretory lysosomes into mature, functioning cytokines, which are released from the cell and migrate to sites of inflammation (9) (Figure 1).

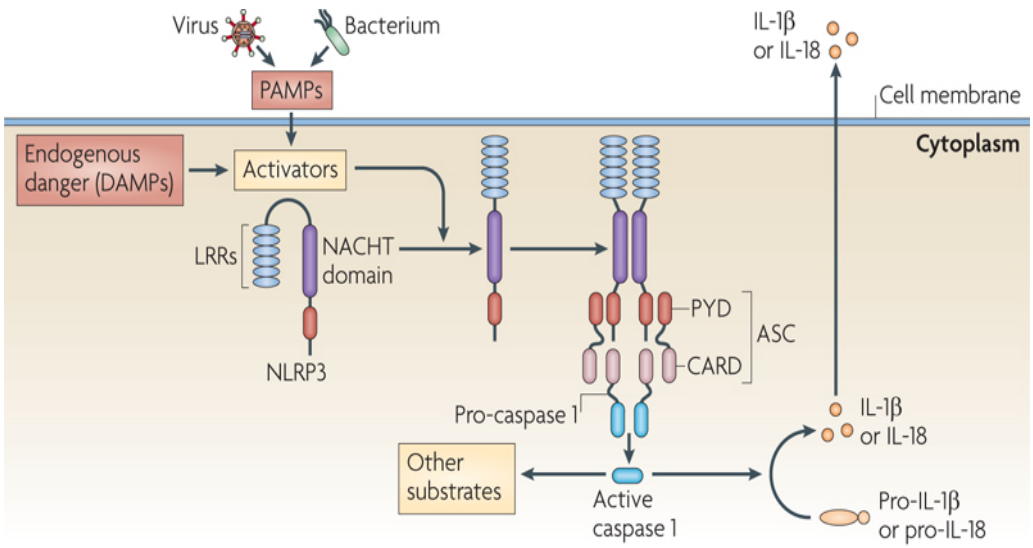

Figure 1. Inflammasome assembly and activation of Caspase-1. Upon recognition of PAMPs or DAMPs by Pattern Recognition Receptors (PRRs), the receptor oligomerizes and binds the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC). This structure constitutes the inflammasome, which recruits pro-caspase-1 and activates it, resulting in maturation and release of pro-inflammatory cytokines IL-1β and IL-18 (8).

Certain viruses have developed mechanisms to evade recognition by these sensing molecules in order to avoid the cellular immune cascade and to establish a persistent infection.

A number of DNA viruses (e.g. Herpesvirus, Poxvirus) and RNA viruses (e.g. Influenza A Virus, Rotavirus) have been shown to cause activation of the inflammasome by stimulating different components of these multiprotein complexes (10,11). Most viruses target the NLRP3 inflammasome, which contains a class of PRRs called nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) (4). More recently, a growing number of viruses such as KSHV (12,13) and HSV-1 (14) have been shown to stimulate a different group of PRRs, the AIM2-like receptor (ALR) family.

Caspases.

The inflammatory mediator Caspase-1 belongs to a conserved family of cysteine-proteases that cleave specifically after aspartate residues in their substrates (11). Caspases are synthesized as inactive precursors, termed procaspases that only after proteolytic activation are able to exert their functions on cellular targets. Pro-caspases consist of a N-terminal domain of varying length and a large or a small subunit. During the activation process, this domain is removed from the N-terminus of the molecule, leaving an active molecule consisting of the two subunits, either as mono- or dimer (15,16).

Caspases are involved in three major cellular pathways: inflammation, apoptosis and necrosis. As mentioned before, inflammatory Caspases are recruited to the inflammasome, where they are autoproteolytically activated, before they exert their function in the inflammatory pathway (16). The second major pathway mediated by Caspases is apoptosis: the programmed cell death. It involves the cleavage and activation of effector Caspases by an upstream initiator Caspase. Apoptosis occurs either through an intrinsic mitochondrial pathway or through an extrinsic pathway in which death receptors on the cell surface are activated by external stimuli. Then mature, processed Caspases activate downstream substrates involved in the apoptotic pathway (16). Apoptosis is an important mechanism occurring under normal cell physiology, during development or after injuries (17), but it is also triggered upon virus infection in order to avoid viral replication and spread.

Viruses have developed strategies to evade those cellular defense mechanisms by expressing proteins that can delay or disrupt host response pathways until enough progeny is produced. But some viruses also use apoptosis to their advantage by encoding products that enhance cell death if it promotes viral spread (18). Up until now, at least 16 viruses have been reported to make use of cellular apoptotic Caspases to cleave viral proteins, resulting in change of viral replication (19). For example, it was shown that viral proteins of human Papillomavirus (HPV) (20) or Kaposi sarcoma-associated herpesvirus (KSHV) (21) are processed by Caspases, which enhances replication of viral DNA.

Herpesviruses and Epstein-Barr virus.

Herpesviruses belong to a large family of DNA-viruses that are classified into three subfamilies based on their host range and replication characteristics: alpha-, beta-, and gamma-herpesviruses (22,23).The characteristic of herpesvirus infection is its potential to establish a lytic infection with a massive production of viruses, or a latent infection followed by a life-long persistence in the host without causing any symptoms. The infection depends on the cell type for alpha-, beta-, and gamma-herpesviruses groups (24).

In some cases however, infection may cause severe diseases. The herpesvirus genome is composed of 120-220 kb double-stranded DNA, enclosed by an icosahedral capsid, a tegument protein layer, and a lipid double membrane envelope with embedded glycoproteins (23) (Figure 2).

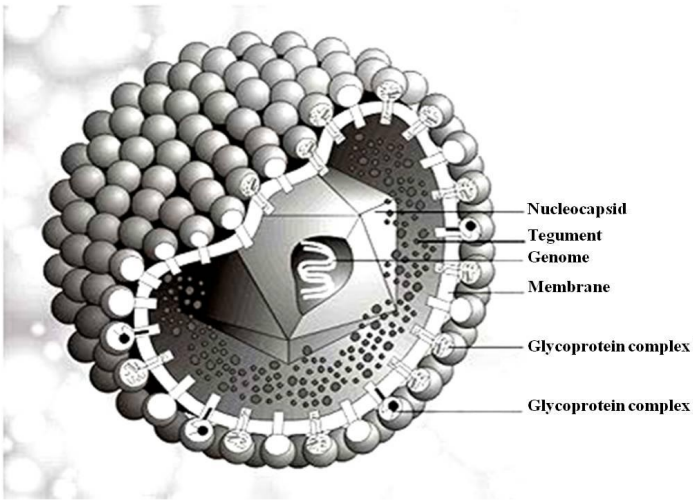

Figure 2. The herpesvirus particle using the example of Varicella Zoster virus, an alpha-herpesvirus.
(Image modified from and courtesy of Dr. Marko Reschke in Marburg, Germany).

The herpesvirus attaches to a host cell by interaction of envelope glycoproteins with the surface receptors on the targeted cell. This binding entails aggregation of receptor molecules within the membrane and thus internalization of the virus. Subsequently, the virus particle is disassembled: tegument proteins are released into the cell cytoplasm and the nucleocapsid is transported to the nuclear membrane via cellular microtubules. The viral nucleocapsid enters the nucleus through the nuclear pore (25), and consecutively, the viral genome is in-

stantly circularized in order to avoid eliciting the cellular DNA-damage-response (26). At this point, using the cellular replication machinery, few copies of the viral episomes are produced and only selected genes are expressed (named latent genes). The newly synthesized circular DNA molecules remain inside the nucleus, but don't integrate into the host cell genome and no infectious virus particles are produced at this stage. In this way the virus establishes latency and can reside in the host cell for an indefinite amount of time.

At any point, unknown mechanisms can trigger the switch into lytic phase. If this happens, the virus uses its own replication machinery in order to express all genes necessary to produce new infective virus particles. The genome is then copied by a rolling circle replication mechanism. The portions of viral genomes are cut off from the long roll and packaged into newly formed capsids. Viral mRNA is synthesized and translocates to the cytoplasm where a pool of viral

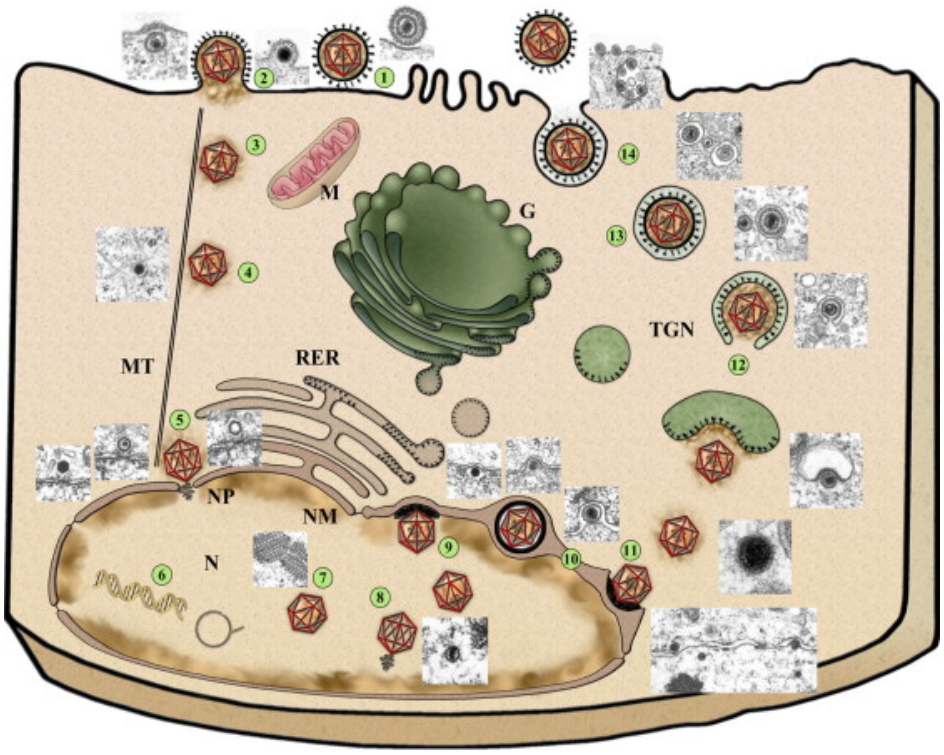

Figure 3. The herpesviridae life cycle by the example of alpha-herpesvirus pseudorabies virus (PrV). Upon attachment to the target cell with glycoproteins the viral envelope fuses with the cellular membrane. The capsid is released into the cytoplasm and transported to the nuclear membrane via cellular microtubules. The DNA is translocated through the nuclear pore, multiplied and packaged into a new capsid, which buds from the nuclear membrane. mRNA is produced and transported to the cytoplasm where it acts as template for the synthesis of structural proteins. After release into the cytoplasm, the capsid buds through the TGN, which contains structural proteins, and acquires tegument and final envelope before leaving the cell through exocytosis (PrV) or cell lysis (EBV) (27).

proteins is produced. The newly assembled capsids containing the viral DNA bud from the nuclear membrane into the cytoplasm and acquire the tegument layer and the final envelope encompassing the surface glycoproteins formed in the Trans-Golgi-Network (TGN). Eventually, the newly synthesized virus particles leave the cell through exocytosis or cell lysis (25,27,28) (Figure 3).

EBV particles were first identified in 1964 by Anthony Epstein and his group in a cell line derived from Burkitt's lymphoma (29,30) (Figure 4). Normally, exposure to the virus frequently occurs during childhood, but is usually asymptomatic. Primary infection may happen during adolescence, leading to the lymphoproliferative disorder Infectious mononucleosis (IM) (31). Despite an EBV-directed immune response, the body is not able to entirely get rid of the virus, because of its ability to hide in B-cells without being detected by immune cells. This allows the virus to establish a silent, life-long infection within 90-95% of the human population (31,32).

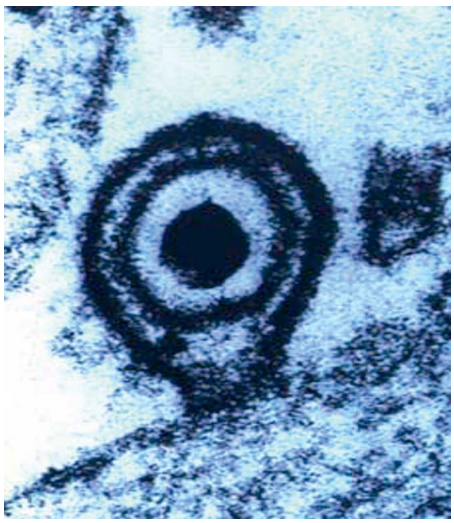

Figure 4. Electron microscopy of the Epstein-Barr virus particle as it buds off an infected cell (24).

Further in-depth studies identified the virus as the causative agent of several malignant human tumors, such as Burkitt's Lymphoma (29), Nasopharyngeal Carcinoma (33) and Hodgkin's Lymphoma (34,35). Its persistence in the human population as well as its associations with severe human diseases makes the

virus a very intriguing model to study. Further, discovering how the virus affects and manipulates cellular pathways is an interesting way to understand these mechanisms in more detail.

The ~184 kb EBV genome encodes about 90 ORFs (36–38). The virus infects both B-cells and epithelial cells but establishes latency in B-cells only (25). During latent infection, there is no production of new infectious particles and the virus expresses only a limited number of latency genes. Three latency types have been characterized, based on the products of these genes that have been detected in different tumor tissues: latency I, latency II, and latency III. Thus, the latency types are classified according to their associated diseases (table 1) (39).

Latency Type	Viral products expressed	Associated Malignancies
Latency I	EBNA1 EBERs BARF0	Burkitt's Lymphoma
Latency II	EBNA1 EBERs LMP1 LMP2A BARF0	Hodgkin's lymphoma Nasopharyngeal Carcinoma Peripheral T/NK lymphoma
Latency III	All EBNAs EBERs LMP1 LMP2A BARF0	AIDS-associated lymphomas Post-transplant lymphoproliferative disorders

Table 1. EBV latency types and associated diseases. Modified from (39).

Common to all three latency types is the expression of EBNA-1, a protein that, among other things, tethers the viral episome to the cellular DNA during cell division in order to ensure equal distribution of viral DNA to the daughter cells (40,41). However, not all latency genes are translated into proteins. A few encode the non-translated EBV-encoded RNAs (EBER-1/-2) and a number of microRNAs (38,42).

In contrast to the selective gene expression observed in the latency stages, lytic phase allows full expression of the entire EBV genome. Periodic spontaneous reactivation causes the switch into lytic phase in resting B-cells. However, the exact mechanism of how and why it occurs is not clearly understood up until now. During this phase, infectious virus particles are produced and about 80 proteins are expressed from the viral genome (32). Lytic genes are grouped into three categories, because they are expressed in consecutive stages during the productive cycle: immediate early (IE), early (E) and late (L) genes (43) (Table 2).

IE	E	L
BZLF1 BRLF1	BMRF1 BALF2 BALF5 BBLF2/3 BSLF1 BORF2 **BPLF1** etc.	gp320/220 BcLF1 BDLF1 BVLF1 etc.

Table 2. EBV lytic phase transcription programs and expressed genes. Modified from (43)

Immediate early genes are the first to be expressed after virus induction and they are important transcription factors necessary for transcription of the early genes (30). The immediate early gene product BZLF1, a member of the bZip family of DNA binding proteins, is a key factor in the switch to productive virus infection and it is the first viral marker detected upon induction of the lytic phase in B-lymphocytes infected with EBV (44). Early gene products are involved in transcription and replication of viral DNA, while the late genes encode structural proteins such as components of capsid and tegument, that will later be assembled to produce mature viral particles (24).

It has been shown that during both latent and lytic cycle, the virus takes advantage of or manipulates the host cell pathways in order to establish a more favorable environment for its survival and maturation. Two examples of major cellular processes being hijacked by EBV are the Ubiquitin Proteasome System

and the cellular Neddylation pathway (45,46).

Ubiquitin Proteasome System and Cellular Neddylation Pathway.

These pathways involve the attachment of a small molecule - Ubiquitin and Nedd8, respectively - to a target protein substrate by a consecutive cascade reaction of three enzymes: an activating enzyme (E1), a conjugating enzyme (E2) and a protein ligase (E3) (47) (figure 5).

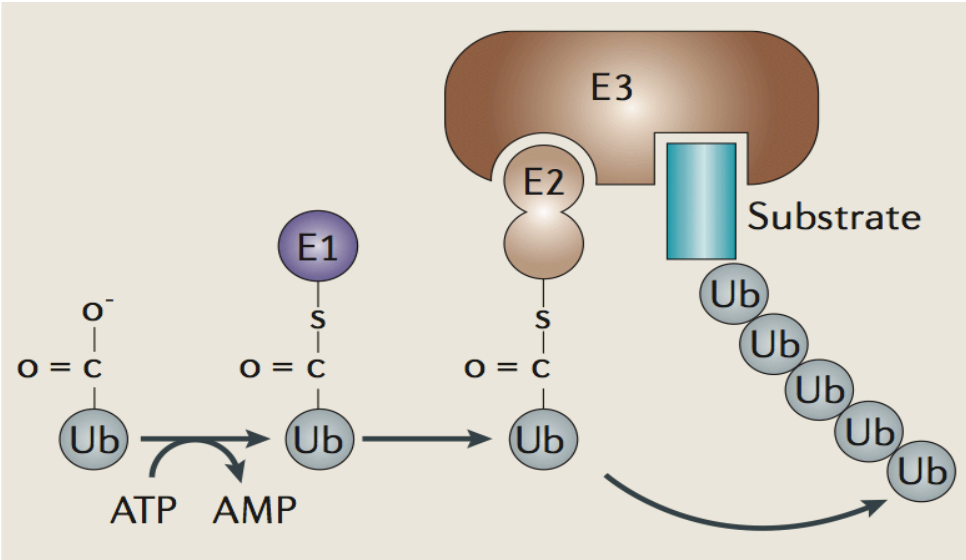

Figure 3. The three steps of Ubiquitination. The Ubiquitin molecule is activated in an ATP-dependent manner by a ubiquitin-activating enzyme (E1), transferred onto a conjugating enzyme E2, and attached to the substrate by a ubiquitin protein ligase E3. After at least 4 cycles of Ubiquitination, the substrate is degraded in the 26S-proteasome. Modified from (49).

The conjugation of Ubiquitin and Nedd8 to the substrates alters their properties and functions, and viruses have developed mechanisms to change the fate of cellular proteins to their advantage. Ubiquitination predominantly leads to protein degradation by the 26S proteasome (48), but it can also have non-degradative functions and it plays a role in DNA repair, transcription, signal transduction, endocytosis and sorting (47,49).

Nedd8 conjugation alters the structural properties of a limited set of substrates

such as Cullin proteins, a class of E3 Ubiquitin ligases, and the best characterized Nedd8 substrates so far. The observation that a large number of NEDD8 substrates are E3 Ubiquitin ligases emphasizes the strong connection between Ubiquitination and Neddylation.
A constant equilibrium between neddylation and deneddylation of substrates is important to maintain also several non-proteolytic cellular events (50): neddylation of a number of ribosomal proteins promotes ribosomal protein stability (51), p53 neddylation inhibits its transcriptional activity (52) and neddylation of the EGFR (epidermal growth factor receptor) causes downregulation of the EGFR machinery (53), etc. A balanced neddylation-deneddylation cycle keeps processes such as these in a stable and normally functioning state.

The Epstein-Barr virus tegument protein: BPLF1.

It was demonstrated that in EBV, the large tegument protein BPLF1 interferes with both the Ubiquitin and Nedd8 pathways because of its deubiquitinating and deneddylating activity within the N-terminal 225 amino acids (45,54,55). The BPLF1 gene is transcribed during the viral lytic cycle and produces a long transcript of about 9.5 kb that codes for a mature protein of 3149 amino acids (46)(56). Since the transcript is detected in infected cells 6 h after infection, it belongs to the class of early proteins. It is crucial for productive virus replication, deregulation of the cell cycle and establishment of an S-phase-like cellular environment that favors productive viral infection (45).

This phenotype is induced because BPLF1 binds to the C-terminal domain (CTD) and removes NEDD8 from cullin proteins. Cullins function as molecular scaffolds for the assembly of the multisubunit Cullin Ring Ligases (CRLs), which constitute the largest group within the RING finger E3 family. Most of the cell cycle regulators including p21, p27 and CDT1 are poly-Ubiquitinated by nuclear Cullin Ring Ligases and become substrates for proteasomal degradation.

Expression of BPLF1 has been demonstrated to stabilize those cell cycle regulators, which synchronizes the infected cells into a continuous S-phase that is

required for viral replication. BPLF1 is further specifically associated with the selective degradation of nuclear Cullins and stabilization of nuclear CRL substrates (45). The BPLF1 N-terminus also plays a role as deubiquitinating enzyme of the cellular DNA polymerase processivity factor PCNA (57) and the viral ribonucleotide reductase (RR) (55), causing downregulation of RR activity, inhibition of PCNA monoubiquitination and attenuation of Polη at DNA damage sites. Furthermore, BPLF1 has been shown to deubiquitinate TRAF6, causing the inhibition of NF-kB signaling during lytic infection (58).

AIM

Virus infection potentially leads to inflammation, cell death and carcinogenesis in infected cells, but how viruses exploit these and other pathways to promote their own survival is still not yet understood. Given the fact that in EBV, BPLF1 is required for efficient viral DNA replication, it is very intriguing that the virus produces this large protein even though the first N-terminal 225 amino acids seem to be necessary and sufficient for its functions. The major aim of this thesis is to understand how the 3149 amino acid tegument protein BPLF1 acts as a deneddylase and deubiquitinase in infected cells to promote virus replication.

MATERIALS AND METHODS

Antibodies.

The following antibodies were used: anti-V5 (1:2500); anti-β-actin and anti-Flag (1:5000); anti-human IgG (1:1000); anti EBV EA-R (BORF2, 1:1000), BZLF1 (1:1000); anti-EBV gp220/350 (1:100) and EBNA1 (OT1-X, 1:1000), anti-LMP1 (1:100), anti-Caspase-1 (1:1000), anti-Caspase-3 (1:1000), anti-Caspase-9 (1:1000), anti-BPLF1 (1:100), anti-HA (1:3000), anti-NEDD8 (1:1000), anti-Ub (1:3000), human serum (1:200). Secondary antibodies: anti-mouse-HRP (1:5000), anti-rabbit-HRP (1:5000), anti-human HRP (1:3000).

Cell lines and media.

The primary cell line used in our experiments is Akata. The Akata cell line was established from a Japanese Burkitt's lymphoma patient. The cell line carries the Burkitt's type chromosome translocation t(8q-; 14q+) (59), causing the typical dysregulation of the *myc* oncogene (60). In this chromosomal abnormality, accounting for 85% of cases, the *myc* gene from the long arm of chromosome 8 is translocated to the Ig heavy chain region on the long arm of chromosome 14, causing overexpression of *myc* (60,61). Akata cells retain BL-type EBV expression (Latency I, expressing EBNA1, EBERs, BARF0). It was discovered that treatment of Akata cells with anti-Immunoglobulin antibodies results in production of a large quantity of virus particles (62), which makes them an excellent cell line to study reactivation of latent EBV in B-cells. We use Akata Bx-1 cells, carrying a recombinant EBV with the thymidine kinase gene replaced by a CMV immediate-early promoter driven GFP (63). BJAB, BL41 and Ramos cell lines are EBV-negative, human Burkitt's lymphoma B-cell lines, growing non-adherently in suspension. Permanent conversion to EBV-positive sub-lines can be achieved by in vitro infection. Akata Bx-1 cells were cultured in Roswell Park Memorial Institute Medium (RPMI-1640, R8758 SIGMA-Aldrich, UK), supplemented with 10% Fetal Calf Serum (10270, GIBCO-Invitrogen, Carlsbad CA),

Penicillin-Streptomycin (P0781, SIGMA-Aldrich, UK), L-Glutamine (G7513, SIGMA-Aldrich, UK) (complete medium) and Geneticin (10131-027, 250 µg/ml, GIBCO-Invitrogen, Carlsbad CA). BJAB, Ramos and BL41 cells were grown in Roswell Park Memorial Institute Medium (RPMI-1640, R8758 SIGMA-Aldrich, UK), supplemented with 10% Fetal Calf Serum (10270, GIBCO-Invitrogen, Carlsbad CA), Penicillin-Streptomycin (P0781, SIGMA-Aldrich, UK), L-Glutamine (G7513, SIGMA-Aldrich, UK) (complete medium) and Geneticin (10131-027, 200 µg/ml, GIBCO-Invitrogen, Carlsbad CA).

Immunoblotting.

Cells were washed in 1x PBS at 3000rpm and lysed in buffer (25 mM Tris-HCl pH 7.6, 50 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail tablets; Roche) and 10 mM NEM (Sigma), and protein concentration was measured with a Protein Assay kit (Bio-Rad Laboratories). Equal amounts of proteins were fractionated in a polyacrylamide Bis-Tris 4–12% gradient or in polyacrylamide Tris-acetate 10% linear precast gels (Invitrogen). After transfer to poly(vinylidene difluoride) membranes (Millipore), the filters were blocked in PBS saline buffer containing 5% non-fat milk and 0.1% Tween 20 and incubated with primary antibodies overnight at 4 °C followed by incubation for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibodies. The complexes were revealed by chemiluminescence (ECL; GE Healthcare).

Immunofluorescence.

Akata Bx-1 cells were treated with anti human-IgG to induce the lytic cycle. Cells were harvested and washed with 1x PBS and resuspended in 1x PBS. Clamps with slides, filter paper and cover slips were assembled and 100 µL of samples were placed on glass slides by cytospin centrifugation at 5000 rpm. Cover slips were washed with PBS in 6-well plates, fixed with 4% Paraformaldehyde for 15 minutes, and washed again with PBS. Cells were permeabilized

with 0.5% v/v Triton X-100 in PBS for 20minutes, and slides were treated with a PBS + 0.5% BSA blocking solution for 2 h at room temperature. Slides were incubated with anti-BPLF1 antibody overnight at +4°C, followed by washing and incubation with the anti-rabbit-TRITC secondary antibody for 1 h. The slides were mounted with Vectashield mounting medium containing DAPI (H-1200, Vector Laboratories) and images were captured with a Zeiss LSM510 META confocal microscope and analyzed with the ImageJ 1.42q software (Wayne Rasband, National Institutes of Health, USA).

Deconjugase activity assays.

Deconjugase activity was assayed by labeling with the HA-Ub-VS and FLAG-NEDD8-VS functional probes (Boston Biochem, Boston, MA) as described (64). $10x10^6$ cells were lysed in 300 µl of buffer containing 50 mM Tris-Cl pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM PMSF, 0.5% NP40, 250 mM Glucose, 5mM $MgCl_2$ (lysis and labeling buffer, LLB) followed by 20 strokes through a G30 needle. Functional labeling was performed by addition of 2.5 mg of HA-Ubiquitin-VS or FLAG-NEDD8-VS followed by incubation for 45 min at 37°C. The cross-linked proteins were immunoprecipitated with Anti-HA-agarose or Anti-FLAG-agarose beads for 4 h at 4°C with rotation and eluted by competition with 25 µg/ml of the HA or FLAG peptides in LLB. Enzymatically active proteins were detected in western blots probed with anti-HA or anti-FLAG antibodies.

Induction of the EBV productive cycle, quantification of viral DNA and titration of infectious virus.

The productive virus cycle was induced in Akata-Bx1 by incubating cell pellets for 1 h at 37°C with polyclonal rabbit anti-human IgG (1:50, DAKO, Denmark) and monitored by the increase of GFP fluorescence or by probing western blots with antibodies specific for the EBV transactivator BZLF1. For induction of the productive cycle in the B95.8 cell line the cells were placed in a six well plate at the density of $5x10^4$ cells/ml in 5 ml medium supplemented with 2% FCS and 20

ng/ml TPA and the supernatant was harvested after 2 weeks (65). Where indicated, the caspase-1 inhibitor VX-765 was added to the culture medium.

Quantitative-PCR was performed on DNA extracted from the cell pellets and the culture supernatant. Total DNA was purified with the DNAzol reagent (Invitrogen) and EBV DNA content was assayed by qPCR with the KAPA SYBR FAST qPCR Kit (KK4604, KAPA Biosystems, Cape Town, South Africa) and an AbiPrism 7000 Sequence detection system using 100 ng of DNA and the probes: EBNA1: 5'GGACGTGGAGAACAGTCATC3'/5'CACTCCTGCCCTTCCTCACC3', product 364 bp; BZLF1: 5'CACTACCAGGTGCCTTTTGT3'/5'GAGACTGGGAACAGCTGAGG3', product 364 bp; GAPDH: 5'AAGGTCGGAGTCAACGGATT3'/5'CTCCTGGAAGATGGTGATGG3', product 224 bp. The cycling conditions were: initial step 50°C 2 min, denaturation 95°C 10 min, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 min, 95°C for 15 seconds, 60°C for 20 seconds and 95°C for 15 seconds. A final extension for 10 min at 72 °C and melting curve between 65°C to 90°C, 1°C/second transition were incorporated. Optical raw data were exported to Microsoft Excel for analysis. All qPCR reactions were performed in triplicate and Ct values were averaged. The fold change in the target gene relative to the GAPDH endogenous housekeeping control gene is determined by: Fold Change = $2^{-\Delta(\Delta Ct)}$ where $\Delta Ct = Ct_{target} - Ct_{GAPDH}$ and $\Delta(\Delta CT) = \Delta Ct_{induced} - \Delta Ct_{control}$, according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. Production of infectious virus was induced by culturing the B95.8 cell line in medium supplemented with 2% FCS and TPA in the presence or absence of the indicated concentrations of the caspase-1 inhibitor VX-765. The presence of infectious virus in the supernatants of the B95.8 cell line was assayed by infection of the EBV negative cell line BJAB. The percentage of EBNA2 positive cells was assessed by immunofluorescence using the PE2 mouse monoclonal antibody (1:150, NCL-EBV-PE2, Leica, Wetzlar, Germany) 48 h after infection.

RESULTS

Processing of BPLF1 by Caspase-1, bioinformatics and validation approaches.

Although the first N-terminal 225 amino acids seem to be necessary and sufficient for BPLF1 functions, EBV produces a large 3149 amino acid long protein. This intriguing fact suggests that BPLF1 is processed to release the catalytic N-terminus, which then acts as promoter of viral replication. In order to understand more about how BPLF1 is processed after expression, bioinformatics algorithms were used to find potential cleavage sites of cellular peptidases in the full BPLF1 amino acid sequence. This analysis yielded several putative sites for protease cleavage.

However, we focused exclusively on the Caspase cleavage sites because they are the main proteases involved in the primary response to virus infection, such as inflammation and apoptosis. Moreover, other examples support that viruses employ cellular Caspases to process viral proteins essential for viral amplification (20,21), which makes these proteases promising candidates for the processing of BPLF1 in EBV.

The result obtained from the bioinformatics assay, strikingly, found a unique Caspase-1 cleavage site at position Asp222 (Figure 6).

The observation that BPLF1 primarily targets nuclear Cullins, suggests that only

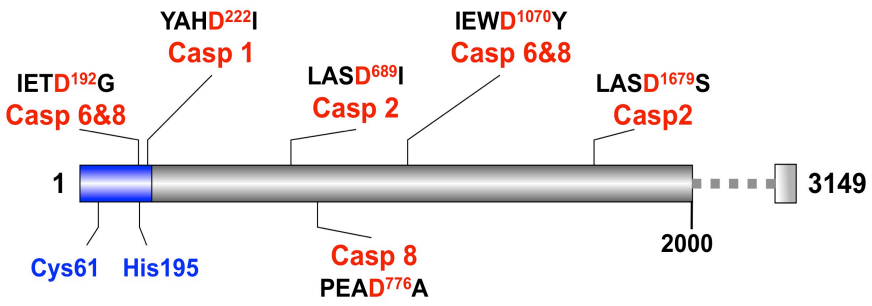

Figure 4. Predicted Caspase cleavage sites in the N-terminal 2000 amino acids in BPLF1.

the N-terminal fragment derived from the full-length protein carries out its enzymatic functions in the nucleus. We investigated this hypothesis using a polyclonal rabbit serum raised against the catalytic N-terminus of BPLF1 (amino acids 1-325). Lysates of non-induced and induced Akata Bx-1 cells were incubated with the functional probes HA-Ub-VS and FLAG-Nedd8-VS. These probes bind to the BPLF1 N-terminus catalytic cysteine. Following anti-HA and anti-FLAG immunoprecipitation, Western Blot analysis was performed probing with anti-HA and anti-FLAG antibodies. The blot shows two additional bands in the induced cells compared to untreated cells in both experiments. One band at ~300kD corresponds to the full-length BPLF1, and another strong band at 38kD, which most likely corresponds to the processed form (Figure 7A).

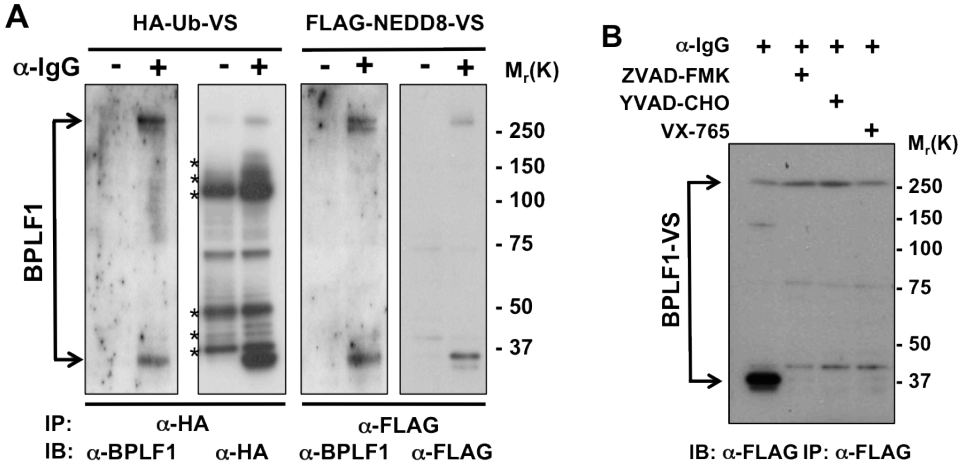

Figure 5. Cleavage by Caspase-1 causes release of the catalytic BPLF1 N-terminus. A. Lysates of induced and non-induced Akata Bx-1 cells were labeled with the functional probes HA-Ub-VS and FLAG-Nedd8-VS and immunoprecipitated with HA- and FLAG-specific antibodies. Western Blots were stained with anti-HA and anti-FLAG antibodies and with an affinity purified rabbit serum raised against the N-terminus of BPLF1. Cellular DUBs labeled with the HA-Ub-VS functional probe are indicated by asterisks. A non-specific band of approximately 65kD is detected by the anti-HA antibody in both untreated and induced Akata-Bx1. **B.** Inhibition of Caspase-1 impairs production of the catalytically active N-terminal fragment of BPLF1. Lysates of induced Akata Bx-1 cells were treated with the indicated Caspase-1 inhibitors and labeled with the FLAG-NEDD8-VS functional probe; FLAG immunoprecipitates were probed with anti FLAG-specific antibody.

The possibility that this fragment is the N-terminus of BPLF1 is further supported by the existence of the Caspase-1 cleavage site at position Asp222 of the protein (Figure 6). In parallel, both HA- and FLAG-IP samples were stained with the purified antibodies to BPLF1, yielding the same additional bands as shown in the HA- and FLAG-staining (Figure 7A), confirming that the 300kD protein is the full-length BPLF1 and the 38kD fragment most likely corresponds to the processed N-terminus. This further shows that the fragments are functional, as they both bind to the Ub- and NEDD8-probes.

Treating induced Akata-Bx1 cells with pancaspase and specific Caspase-1 inhibitors supported the hypothesis of a BPLF1 cleavage mediated by Caspase-1 since the 38kD band practically disappeared compared to untreated control cells (Figure 7B). These results give strong evidence that BPLF1 is N-terminally processed by Caspase-1.

Caspase-1 is activated in EBV-positive Akata cells.

To investigate the role of Caspase-1 in BPLF1 processing, we looked at changes in the activity of Caspase-1 in induced Akata Bx-1 cells during the 36 hours after induction. The Procaspase-1 (45kD) and the processed active Caspase-1 (~20kD) were detected after induction of viral replication (Figure 8). Upon treatment with a Caspase-1-specific inhibitor (YVAD-CHO) before induction, there is a significant decrease of active Caspase-1, compared to the non-treated cells. Interestingly, we could detect activation of Caspase-1 even at the time point of induction, suggesting that the lytic cycle doesn't promote the activation of Caspase-1.

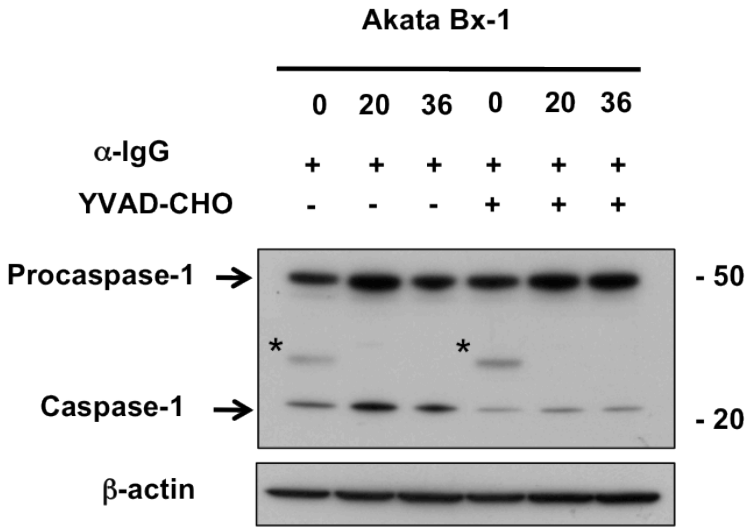

Figure 6. EBV-infection increases activation of Caspase-1 in Akata Bx-1 cells. Akata Bx-1 cells were induced in presence or absence of a Caspase-1 inhibitor and Western Blots were probed with Caspase-1 specific antibodies. Asterisks indicate residual IgG heavy chains detected by the secondary antibody. Caspase-1 activation increases after 20h post induction. In cells treated with the Caspase-1 inhibitor the amount of cleaved Caspase-1 is highly reduced.

Caspase-mediated apoptosis is shut off during EBV replication.

In order to exclude the possibility of the involvement of apoptotic Caspases in the processing of BPLF1, we were interested in ensuring that apoptosis is indeed shut off during productive EBV infection. Akata Bx-1 lysates from not induced and induced cells were fractionated in a SDS-Page gel and membranes were stained with antibodies to the Caspases that mediate apoptosis, Caspases-3 and Caspase-9. The blot shows that the Procaspase-3 at 32kD and the Procaspase-9 at 46kD, respectively, are highly expressed in the all samples (Figure 9).

However, there is no detection of the expected active cleaved fragments for both Caspase-3 at 17kD and the cleaved Caspase-9 at 37kD, in all analyzed samples (Figure 9). Since these two Caspases are normally activated during apoptosis, these results suggest that this process is shut off during the lytic cycle in Akata Bx-1 cells. U9371 human monocytes treated with LPS were used as positive apoptosis control, where both cleaved Caspase-3 and Caspase-9

are detected.

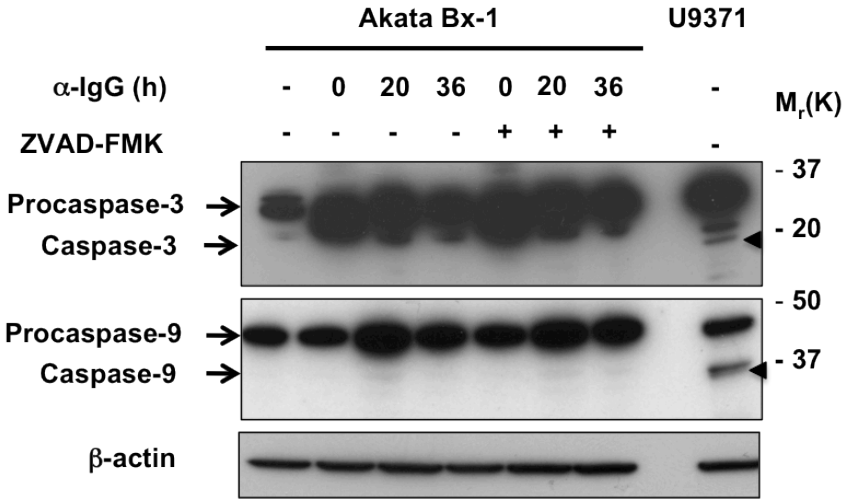

Figure 7. Caspase-mediated apoptosis is turned off during viral replication. Akata Bx-1 cells were induced in presence or absence of a pancaspase inhibitor and Western Blots were performed monitoring the amount of inactive and active apoptotic Caspases, Caspase-3 and Caspase-9. Human monocyte cell line U9371 induced with LPS was used as positive control for Caspase activation. In Akata Bx-1 cells no activation of either of the Caspases could be observed.

Inhibition of Caspase-1 impairs nuclear localization of the BPLF1 N-terminus and viral DNA replication.

The large tegument proteins of herpesviruses are primarily localized in the cytoplasm because the viral tegument is assembled in the cellular cytoplasmic compartment. However, as BPLF1 targets nuclear Cullins and their substrates, it looks like the enzymatic activity of BPLF1 is predominantly carried out in the nucleus by the N-terminal fragment of the protein.

To investigate the role of Caspase-1 in processing of the BPLF1 N-terminus further, we aimed to compare the cellular localization of the protein in presence or

absence of a Caspase-1 inhibitor. For this purpose, we treated Akata Bx-1 cells with the Caspase-1 inhibitor YVAD-CHO and induced the lytic cycle.

48h after induction, immunofluorescence was performed using the specific antibody raised against the BPLF1 N-terminus domain. The primary antibodies were then detected with anti-TRITC-conjugated secondary antibody and visualized by immunofluorescence. BPLF1 cellular localization is shown in Figure 10A. In normal conditions (Ctr), the BPLF1 fluorescence is equally distributed in the nuclei and cytoplasm of induced cells. When cells are treated with the specific Caspase-1 inhibitor YVAD-CHO, the fluorescence is predominantly (>90%) localized in the cytoplasm, suggesting that inhibition of Caspase-1 activity impairs BPLF1 N-terminus cleavage and its nuclear localization.

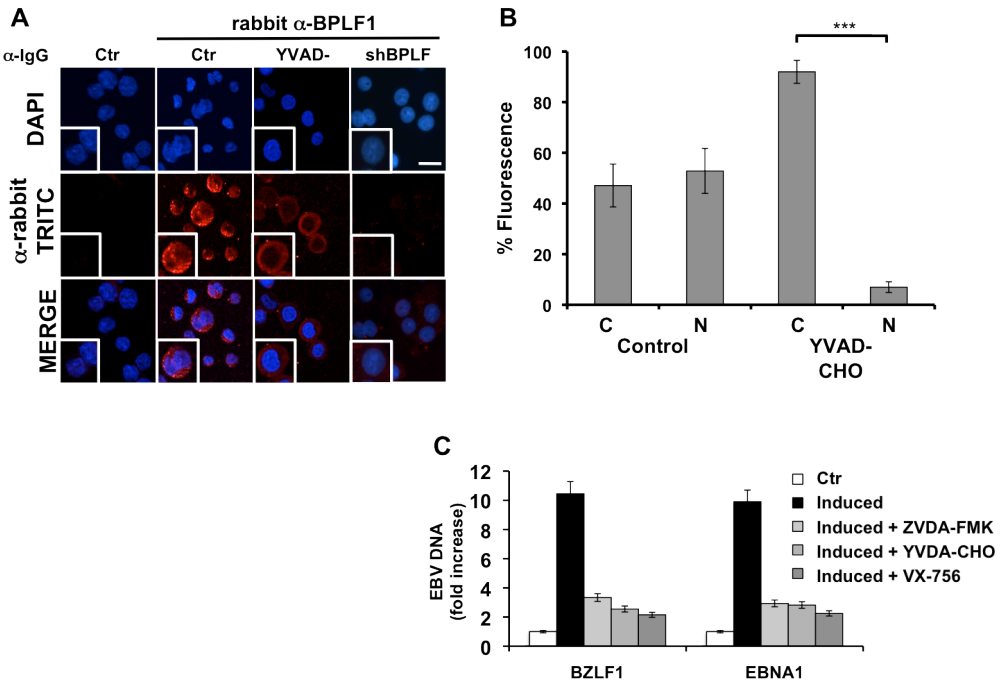

Figure 8. Inhibition of Caspase-1 impairs accumulation of BPLF1 in the nucleus. **A.** Fluorescence microscopy of induced Akata Bx-1 cells in presence or absence of a Caspase-1 inhibitor and a BPLF1-directed shRNA, stained with the antibody to BPLF1. Inhibition of Caspase-1 causes increase in cytoplasmic BPLF1. **B.** Quantification of nuclear and cytoplasmic fluorescence in 50 cells from 2 independent experiments, using the ImageJ software. **C.** Inhibition of Caspase-1 impairs viral DNA replication. The fold increase of EBV genes BZLF1 and EBNA1 was measured by specific qPCR using control and induced Akata Bx-1 cells and induced cells in presence of indicated Caspase inhibitors. Mean ±SE of three experiments.

To confirm that the fluorescence indeed is specific only when BPLF1 is produced after virus induction, induced cells carrying a specific shRNA against the protein were analyzed and no fluorescence signal was detected. Quantification of BPLF1 fluorescence in the cytoplasm and nuclei of cells is shown in Figure 10B.

Since BPLF1 is required for the replication of viral DNA, we wanted to look at the effects of Caspase-1 inhibition on this process. To this end, we performed qPCR analysis on DNA extracted from induced and not induced Akata Bx-1 cells pretreated with Caspase inhibitors.

The experiment showed a clear decrease in the expression of the lytic genes BZLF1 and EBNA1, two indicators of viral replication, in cells treated with pan-caspase inhibitors (ZVAD-FMK) or caspase-1 specific inhibitors (YVAD-FMK, VX-756) (Figure 10C).

Caspase-1 activation is independent of the lytic cycle and it is not specific for Akata Bx-1 cells.

In our results we observed processing of Caspase-1 in EBV-infected cells after induction of the lytic virus cycle. Interestingly, we also see Caspase-1 constitutively activated in non-treated cells, suggesting that in EBV-infected cells, Caspase-1 is always highly activated.

To investigate this in more detail, we examined the Caspase-1 processing in Akata Bx-1, BJAB, BL41 and Ramos cell lines and compared results in cells infected and not infected with EBV. We tested the BJAB, BL41 and Ramos cells because they express viral proteins associated with type III latency.

Cell lysates were used for Western Blot analysis where membranes were stained with the anti-Caspase-1 specific antibody. The corresponding Western Blot result shows a significant increase in processed Caspase-1 in all EBV-positive cells, which was confirmed by the quantification performed in three independent experiments (Figure 11A and 11B).

This interesting result suggests the possibility that EBV latency genes play an

important role in enhancing the processing of Caspase-1.

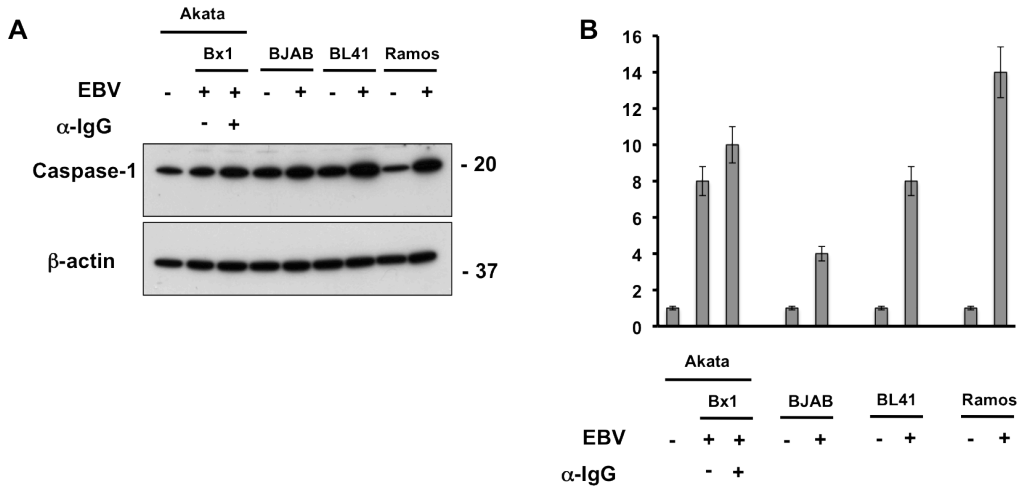

Figure 9. Caspase-1 activity is enhanced in EBV-positive cells. A. Western Blot of EBV positive and EBV negative cell lines Akata, BJAB, BL41 and Ramos. **B.** Quantification was done by measuring the band intensity of Western Blots in three different experiments and normalizing with the corresponding EBV negative sample.

Effect of EBNA1 on Caspase-1 activation.

In order to investigate the putative role of EBV latency gene products in the processing of Procaspase-1, we separately overexpressed the EBV latent proteins EBNA1, EBNA4, EBNA6, LMP1 and LMP2A in BJAB and Ramos cell lines. After transfection, cells were harvested and Caspase-1 proteins were examined by immunoblotting, using the anti-Caspase-1 specific antibody. The expression level of the EBV latent proteins was tested by immunoblotting with specific antibodies directed to the tested EBV latency proteins. Overexpression of EBNA1 (88kD) and LMP1 (45kD) in BJAB is shown in Figure 12A. The overexpression of EBNA1 in the Ramos cells produced a strong band around 45kD, instead of the expected band at 88kD (Figure 12A), which could represent a truncated form of EBNA1.

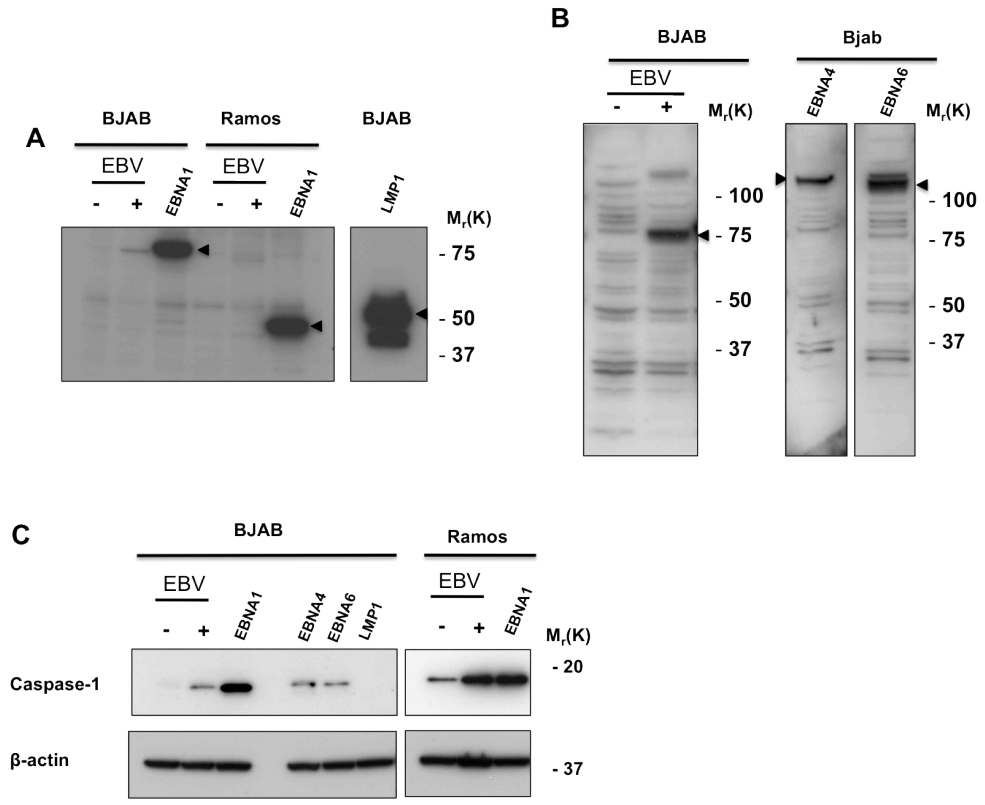

Figure 10. EBNA1 causes cleavage of Caspase-1 in EBV-infected cells. A. Western Blots verify overexpression of EBV latency genes EBNA1 and LMP1. **B.** Overexpression of EBV latency genes EBNA4 and EBNA6. **C.** Western Blot probed with Caspase-1 specific antibodies show activation of the Caspase in EBNA1 over-expressing BJAB and Ramos cell lines.

Due to the lack of specific antibodies directed to EBNA4 and EBNA6, human serum was used as primary antibody to detect those EBV latent proteins. EBNA4 (102kD) and EBNA6 (109kD) were detected at their correct molecular weights (Figure 12B). Same lysates were stained with anti-Caspase-1 specific antibody. EBV-positive and EBV-negative BJAB and Ramos cells were used as controls for Caspase-1 activity. In BJAB cells overexpressing EBNA4, EBNA6, LPM1 and LMP2A, the processing of active Caspase-1 seems to be very ineffective (Figure 12C). However, BJAB cells overexpressing only EBNA1 show a very high amount of active Caspase-1.

The result is further confirmed in Ramos cells overexpressing EBNA1. This suggests that EBNA1, among its many other functions, may also play an important role in the processing of Procaspase-1 into its active form.

DISCUSSION

Virus infection triggers different kinds of defense mechanisms in infected cells. Viruses are further known to exploit cellular mechanisms and pathways and use them to their advantage. With this experimental thesis, we demonstrated that Epstein-Barr virus (EBV) activates the cellular inflammatory pathway in order to promote its replication in infected cells. Further, we found that the EBV latency protein EBNA1 is possibly responsible for activation of the cellular inflammasome that participates in producing the active Caspase-1. This protease subsequently processes the major tegument protein BPLF1 of EBV into an active nuclear deneddylase that promotes viral replication.

There are numerous examples of how viruses manipulate cellular host networks and use cellular enzymes to process and activate viral proteins and how at the same time, viruses employ their own proteins to act on cellular targets to establish a more favorable environment for their survival and spread.

We discovered that the large tegument protein of Epstein Barr virus, BPLF1, is processed by Caspase-1, a key player in cellular inflammation. Being a very large protein, and considering the fact that the catalytic activity lies in the N-terminus of the protein, we hypothesized that the full-length BPLF1 is somehow processed before nuclear translocation.
Since cellular Caspase proteins play important roles during viral infection in both apoptosis and inflammation, we decided to focus on these proteases. Additionally, bioinformatics screening of BPLF1 revealed interesting Caspase cleavage sites along the protein. By producing a polyclonal rabbit serum raised against the catalytic N-terminus of BPLF1, we developed a highly convincing experiment in order to show BPLF1 processing. Using this new antibody as well as FLAG- and HA-Vinyl Sulphone functional probes, we succeeded in visualizing the processed N-terminus of BPLF1 in induced Akata Bx-1 cells (Figure 7). We obtained the first evidence for Caspase-1 dependent cleavage by treating induced Akata-Bx-1 cells with Caspase inhibitors. The results clearly showed that only in non-treated cells could the active N-terminal fragment be visualized

on a Western Blot, as the 36kD fragment binds to Ubiquitin and Nedd8 functional probes (Figure 7B).
The activity of Caspase-1 in EBV infected cells was later confirmed by Western Blot analysis (Figure 8). The active Caspase-1 was detected in EBV-positive Akata Bx-1 cells during lytic cycle, while a significant decrease in processed active Caspase-1 protein was observed after the cells were treated with pan-caspase and specific Caspase-1 inhibitors. In order to confirm that only Caspase-1 is involved in BPLF1 processing, we tested the production and maturation of apoptotic Caspases during lytic viral infection. We confirm that apoptosis is shut off during productive EBV infection, because the two pro-apoptotic Caspases-3 and -9 were not activated (Figure 9), suggesting that both intrinsic and extrinsic apoptosis pathways are inactive in this cellular model.

To further investigate and validate the effect of Caspase-1 cleavage on BPLF1 cellular localization we performed immunofluorescence analysis on EBV induced cells treated and not treated with Caspase-1 inhibitors. Under normal induction conditions, BPLF1 is distributed both in the cytoplasm and in the nuclear compartment of the infected cells. However, upon treatment with Caspase-1 specific inhibitors, the majority of the protein is retained in the cytoplasm (Figure 10A, B). This clearly shows that Caspase-1 is responsible for processing of BPLF1 and that effective translocation of the N-terminus into the cytoplasm is possible only after processing.

Since BPLF1 is known to be an important factor that promotes viral DNA synthesis, it does not come as a surprise that inhibition of BPLF1 processing impaired the synthesis of the EBV genome (Figure 10C).
We observed that the level of the active Caspase-1 is highly elevated in EBV positive cells. This suggests that the latent phase of EBV-infected cells promotes the activation of Caspase-1, and that the induction of the lytic phase is not required for this process (Figure 8). Moreover, latency genes may directly play a role in this activation. We monitored the levels of active Caspase-1 in four different cell lines: Akata, BJAB, BL41 and Ramos, where we compared the EBV-positive cells with EBV-negative cells. The level of active Caspase-1 was highly elevated in the EBV-positive cell lines (Figure 11A, B). This indicates that

Caspase-1 activation is dependent on the presence of the virus and it is induced during latency by some of the latent proteins.
In order to identify the factors that are responsible for this induction, we used cell lines individually overexpressing different EBV latency proteins. We found that EBNA1 (Epstein Barr Nuclear Antigen-1) is the only one of the tested proteins that promotes activation of Caspase-1 (Figure 12).
This observation is very interesting because EBNA1 is also known to be the only EBV protein expressed in all the latency states. Apart from its role in distribution of the viral genome in the host cell, our recent results suggest a new function for EBNA-1 in the cellular inflammatory response. We have found that EBNA-1 plays a role in the processing of Caspase-1, possibly by directly or indirectly manipulating the inflammasome or a different component upstream of Caspase-1 processing, in order to regulate activation of the protease. Based on this discovery, it is now very interesting to understand the mechanism behind this interaction.

As discussed, activation of Caspase-1 is the consequence of a functioning cellular inflammasome complex. Many viruses found ways to modulate the inflammasome and regulate the inflammatory response in order to promote survival in the host (4,10,11,66). Here we have found new data showing how EBV tempers with the inflammatory pathway by employing the versatile latency protein EBNA1 in activation of Caspase-1. It is another very interesting example of how perfectly viruses combine the functions of cellular components and viral proteins during their life cycle.

The inflammasome has been of growing interest to researchers because it is a potential target for viral gene products. Manipulation of the inflammasome would affect activation of Caspase-1 by either enhancing or inhibiting processing of the protease. The resulting changes in the amount of secreted inflammatory cytokines can cause problems for the virus-infected cell. Therefore, dysfunction of this pathway is associated with a number of diseases such as inflammatory bowel disease (67,68), vitiligo (69), gouty arthritis (70) or type 1 and type 2 diabetes (71,72).

Although the NRP3 inflammasome built around NOD-like receptor (NLR) sensor proteins is the most common and therefore best-studied complex activated by viruses in inflammation, a growing number of viruses have recently been found to cause assembly of inflammasomes comprising a sensor protein that belongs to the AIM-2 like receptor family (ALRs). The receptor protein is suggested to sense the presence of the virus in the nucleus, forming an inflammasome complex that leaves the nucleus in order to activate Caspase-1 and proinflammatory cytokines in the cytoplasm (73). This mechanism is very intriguing because inflammasomes were thought to be assembled exclusively in the cytoplasm.

Evidence for viral activation of an inflammasome containing one member of this group of receptors, the nuclear sensor protein interferon-inducible protein 16 (IFI16), was recently observed in cells infected with nuclear-replicating viruses. EBV might be one of the viruses that induce assembly of this newly discovered type of inflammasome. This assumption is supported by the findings described in this thesis and further by recent discoveries by other research groups working with herpesviruses: infection of endothelial and B-cells with KSHV in latent phase (12,13), and even infection of B-cells with EBV (73), caused assembly of the IFI16 inflammasome and activation of Caspase-1.
In the EBV-directed approach, similar to the experiments described in my thesis, different EBV-infected cells were monitored for activation of Caspase-1. It was discovered that during EBV latency I, II, and III, the IFI16 inflammasome and Caspase-1 were constitutively activated, supporting what we discovered. With EBNA1 being one of the factors common to all three latency types, it was suggested that it might be involved in assembly of the inflammasome.
Interestingly, their findings did not coincide with what I observed: While our results clearly show activation of Caspase-1 exclusively in EBNA1-overexpressing BJAB and Ramos cells, in their experiments Caspase-1 activation could not be observed in BJAB cells only expressing EBNA1. Despite their opposing results concerning EBNA1, their experiments support our speculation about formation of an inflammasome complex that is induced by EBV gene products.

These contradicting results confirm that the mechanisms behind the assembly of an inflammasome in cells infected with EBV or other herpesviruses are still

very poorly understood and many questions remain. Nevertheless, this newly discovered possible connection between Caspase-1, BPLF1 and EBNA-1 paves the way for many more interesting experiments that may shed light on the interplay between viral gene products and the inflammasome in the pathogenesis of disease. Further, it reveals new biological aspects of infection and may result in the identification of novel targets for pharmacological intervention.

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APPENDIX

ZUSAMMENFASSUNG

Die Entzündungsreaktion ist eine der schnellsten zellulären Immunantworten auf das Eindringen von Pathogenen wie Viren, Bakterien oder anderen Mikroorganismen, aber auch auf zelleigene Moleküle, die dem Organismus schaden können. Erkennung dieser gefährlichen Strukturen durch Sensorproteine führt zur Bildung des Inflammasoms, ein intrazellulärer Multiproteinkomplex. Diese Struktur katalysiert die autoproteolytische Aktivierung von Caspase-1, welche anschließend zur Reifung von proinflammatorischen Zytokinen beiträgt. Caspasen sind eine Familie von Cystein-Proteasen, welche die wichtigsten Enzyme in Prozessen wie programmierter Zelltod, Nekrose oder Entzündung darstellen.
Viren und andere Pathogene haben Wege entwickelt, diese und andere Prozesse zu manipulieren und zu ihrem Vorteil zu nutzen, um ihr Überleben und ihre Weiterverbreitung zu sichern.

Um diese Interaktionen zwischen Virus und Wirtszelle besser verstehen zu können, arbeitete ich im Zuge dieser Masterarbeit an der weiteren Charakterisierung des Epstein-Barr-Virus, ein gamma-Herpesvirus, welcher unter anderem die Infektionskrankheit Mononukleose hervorruft, aber auch als Auslöser einiger Krebskrankheiten gilt. Wir konnten das virale Tegumentprotein BPLF1 als Substrat von Caspase-1 identifizieren. Die Spaltung des 3149 Aminosäuren langen Proteins BPLF1 durch Caspase-1 ermöglicht die Translokation des katalytisch aktiven N-Terminus des Proteins in den Zellkern, wo er als Deneddylase und Deubiquitinase für spezifische nukleare Substrate fungiert. Weiters erhöht die Prozessierung des Proteins durch Caspase-1 die Produktionsrate viraler DNA während des produktiven viralen Zyklus.

Durch die Beobachtung, dass die Aktivierung von Caspase-1 nicht auf die lytische Phase des Virus begrenzt ist, sondern auch während der latenten Infektion auftritt, konnte festgestellt werden, dass Genprodukte der latenten Phase für die vermehrte Spaltung von Caspase-1 während Virusinfektion verantwortlich sind. Durch Testen verschiedener EBV-positiver Zelllinien, konnten wir tatsäch-

lich EBNA1, Epstein Barr nuclear antigen 1, als potentiellen Faktor in der Aktivierung der Caspase festmachen. EBNA1 gewährleistet unter anderem die korrekte Aufteilung von viraler DNA auf Tochterzellen bei Teilung der Wirtszelle. Unseren Ergebnissen zufolge scheint ENA-1 ebenfalls einen direkten oder indirekten Einfluss auf Caspase-1 Aktivierung durch das Inflammasom zu haben.

SUMMARY

Inflammation is one of the quickest cellular immune responses to the invasion of pathogens such as bacteria, viruses, and other microorganisms. The recognition of invading proteins or nucleic acids by sensor molecules in immune cells results in the assembly of a large intracellular multiprotein complex, the inflammasome. This structure catalyzes the autoproteolytic activation of Caspase-1, which subsequently contributes to the maturation of proinflammatory cytokines. Caspases are a family of Cysteine proteases, which constitute the most important enzymes in processes such as apoptosis, necrosis or inflammation.
Viruses and other pathogens have developed mechanisms to manipulate these and other cellular pathways and to use them to their advantage in order to ensure their survival and spread.

To gain a better understanding of these interactions between virus and host cell, we tried to contribute to a more detailed characterization of Epstein-Barr virus, a gamma-herpesvirus, which is the causative agent of the lymphoproliferative disorder Infectious Mononucleosis, and has also been associated with a number of different cancers. In the experimental approach described in this thesis, we could identify the viral tegument protein BPLF1 as substrate for Caspase-1. Cleavage of the 3149 amino acid long protein by Caspase-1 facilitates translocation of its catalytic N-terminus to the nucleus, where it functions as Deneddylase and Deubiquitinase for specific nuclear substrates. Processing of the protein further increases the rate of production of viral DNA during the productive viral cycle.

Through the observation that activation of Caspase-1 is not restricted to the lytic phase, but also happens in latently infected cells, it became clear that latent gene products might be responsible for the increase in cleavage of Caspase-1. By testing different EBV-positive cell lines, we succeeded in identifying the Epstein-Barr nuclear antigen 1 (EBNA1) as possible determining factor in Caspase-1 activation. EBNA1 ensures the correct segregation of viral DNA into daughter cells during division of the host cell. According to these results,

EBNA1 additionally seems to directly or indirectly influence activation of Caspase-1 by the inflammasome.

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