1 INTRODUCTION

1.1 Apoptosis

Apoptosis is an important control mechanism to balance cell proliferation against cell death in development, differentiation and homeostasis in all multicellular organisms (for an overview see (1)). This cellular suicide program can not only eliminate excessive, but also mutated, infected and damaged cells by an organized degradation. In the mammalian immune system, apoptosis eliminates lymphocytes that fail to properly arrange an antigen receptor, lose affinity to their antigen or develop autoreactive specificity. In addition, apoptosis is involved in the termination of immune responses (for an overview see (2)).

Apoptosis is found in all cell types throughout the metazoans. The key mediators of apoptotic cell death are conserved between *nematodae* (*Caenorhabditis*), *cnidaria* (*Hydra*), insects and vertebrates and some modules of the apoptotic signaling pathways have counterparts in plants and fungi (3,4). Therefore, apoptosis probably evolved in one or several of the very first multicellular organisms (5).

Apoptosis, in contrast to necrosis, is an active ATP-dependent process and the depletion of ATP can cause a switch from apoptotic to necrotic cell death (6). In addition, *de novo* transcription and translation of genes is often needed for the execution of apoptosis. Inhibition of mRNA synthesis, by actinomycin D or blockade of protein synthesis by cycloheximide, can abort or severely impede programmed cell death (7). Furthermore, the balance between pro- and anti-apoptotic proteins (especially proteins of the Bcl-2 family) plays an important role in the decision between cell survival and cell death (see below). Many viruses are able to block apoptosis of their host cell via expression of anti-apoptotic proteins like Bcl-2 family members, viral FLIP homologues (**fl**ice **i**nhibitory **p**rotein, see below) or viral IAP like proteins (**i**nhibitor of **a**poptosis **p**rotein, see below) (for an overview see (8)).

Apoptosis can be caused by diverse signals such as withdrawal of growth factors, loss of cellular adhesion in epithelial cells (anoikis), DNA damage, receptor mediated signals (TNF- and CD95-mediated apoptosis, B and T cell receptor-mediated apoptosis) and external factors like radiation or cytostatic drugs used for example in cancer therapy (for an overview see (9)).

Apoptotic stimuli lead to characteristic morphological changes like cellular and nuclear shrinkage, blebbing of the plasma membrane, condensation of chromatin and DNA fragmentation, which allow a clear distinction of apoptotic from necrotic cells (for an overview see (10)). In addition, the generation of reactive oxygen species by the

mitochondria, a reduction in the mitochondrial transmembrane potential or an intracellular acidification (from pH~7.4 in unstimulated to pH~6.8 in apoptotic cells) can be biochemical characteristics of apoptotic cells (11,12). Modulations in the membrane of apoptotic cells, especially the exposure of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, target the cells for phagocytosis by scavenger cells like macrophages (13). The ingestion by neighboring cells suppresses inflammation by preventing leakage of potentially harmful intracellular components as seen in necrosis and allows recycling of cellular contents by the phagocytic cells (for an overview see (14)).

The deregulation of programmed cell death (too little or too much apoptosis) is the cause for various diseases. The failure of the immune system to eliminate autoreactive lymphocytes by apoptosis can cause autoimmunity (e.g. autoimmune lymphoproliferative syndrome) (15). The constitutive expression of the anti-apoptotic protein Bcl-2 under control of the immunoglobulin enhancer following a translocation into the heavy-chain locus causes tumor formation in follicular lymphomas (16). Several tumors express unusual high amounts of CD95-ligand, which might, together with other factors, enable them to induce apoptosis in anti-tumor lymphocytes and thereby suppress an appropriate immune response (17). Too much apoptosis is also a disease-mediating factor in AIDS (**a**cquired **i**mmuno**d**eficiency **s**yndrome). Pro-apoptotic virus-coded proteins like HIV-1 Tat and gp120, which are expressed by infected T helper cells, affect non-infected CD4 T cells and render them hypersensitive to apoptosis (18). Furthermore, apoptosis is involved in the death of neurons in Alzheimer's disease, Parkinson's disease and stroke (19-21). Apoptosis-modulating therapies are therefore promising tools for the treatment of various human diseases and are currently tested in preclinical and clinical trials (for an overview see (22)).

1.1.1 Molecular pathways of apoptosis

The characteristic apoptotic features described above are the results of complex intracellular signaling pathways. The sensitivity of cells towards apoptosis is mainly regulated by a set of proteins belonging to the Bcl-2 family. This family consists of anti-apoptotic members, including Bcl-2 and Bcl-X_L, and pro-apoptotic members like Bax, Bak and Bid (for an overview see (23)). Members of the Bcl-2 family can form homo- and heterodimers via their Bcl-2 homology (BH) domains (24). The ratio of pro- to anti-apoptotic Bcl-2 family members is important in determining the susceptibility of cells towards apoptotic stimuli. Consequently, overexpression of Bax sensitizes the Burkitt's lymphoma cell line BL-41 for apoptosis after IgM-crosslinking, while Bcl-X_L overexpression

leads to inhibition of IgM-induced apoptosis in the immature mouse B cell line WEHI-231 (25,26). The anti-apoptotic Bcl-2 homolog ced-9 in *Caenorhabditis elegans* protects cells against apoptosis by binding and neutralizing the Apaf-1 homolog ced-4, which otherwise activates the *C. elegans* caspase ced-3 (for an overview see (27)). In mammalian cells, a comparable function for Bcl-2 could not be proved, but Bcl-2 family members seem to be the key regulators for the release of pro-apoptotic factors from the mitochondrial inter-membrane compartment to the cytosol (28,29). Cytotoxic stress (e.g. DNA damage) was reported to activate procaspase-2. The active caspase is involved in redirecting normally cytosolic and inactive pro-apoptotic Bcl-2 family members like Bax, Bad and Bid to the mitochondrial membrane (30). At the membrane of the organelle, they compete with anti-apoptotic Bcl-2 and Bcl-X_L. If the pro-apoptotic fraction gets predominant, it leads to the release of cytochrome c, Smac/DIABLO and the apoptosis inducing factor (AIF) from the inter-membrane compartment of the organelle to the cytosol (Figure 1) (for an overview see (23)). Cytochrome c binds to Apaf-1 and induces its multimerization in the presence of ATP or dATP. The heptameric complex then recruits procaspase-9 molecules via the protein-protein interaction motive CARD (**ca**spase **r**ecruitment **d**omain) in the long prodomain (see below). The procaspase-9 molecules form asymmetric anti-parallel dimers, wherein only one polypeptide is activated (unusual when compared to other caspases, see below). The active complex with a molecular weight of ~1.0 MDa is termed apoptosome and is able to activate caspase-3, the main executioner caspase of apoptosis (31,32). Smac/DIABLO binds to and neutralizes IAPs (**i**nhibitor of **a**poptosis protein), which would otherwise antagonize caspase-3 function (33,34). AIF (**a**poptosis **i**nducing **f**actor) is a phylogenetically ancient mitochondrial inter-membrane flavoprotein. In apoptotic cells, AIF mediates caspase-independent peripheral chromatin condensation and fragmentation of DNA into large pieces of about 50 kb (35). AIF knockout cells are resistant to apoptosis in response to serum withdrawal, while apoptosis in response to several other stimuli like staurosporine and UV irradiation is not affected. Interestingly, AIF is essential for embryonal cavitation, which is performed by apoptosis of the inner cells mass and is a prerequisite for gastrulation and subsequent steps in embryogenesis (36).

Another pathway leading to apoptotic cell death begins with the crosslinking of death-domain containing receptors like CD95 (Fas) in response to binding of their respective ligands. The oligomerization of CD95 receptors leads to the formation of the DISC complex (**d**eath-**i**nducing **s**ignaling **c**omplex), starting with the recruitment of the adaptor protein FADD (**F**as-**a**ssociated **d**eath **d**omain protein) via the death-domains of both proteins (37). FADD also comprises a **d**eath-**e**ffector **d**omain (DED), which binds the death-effector domain of procaspase-8 (FLICE) (in humans also procaspase-10) (38).

Induced proximity (see below) leads to the activation of the procaspase and its release from the DISC, unless a cellular or viral anti-apoptotic FLIP (**f**lice **i**nhibitory **p**rotein) blocks the process. FLIPs can either consist of two DED domains only or resemble an entire caspase-8 molecule without an active site (39). Active caspase-8 directly cleaves caspase-3, thereby starting the executioner phase of apoptosis (Figure 1).

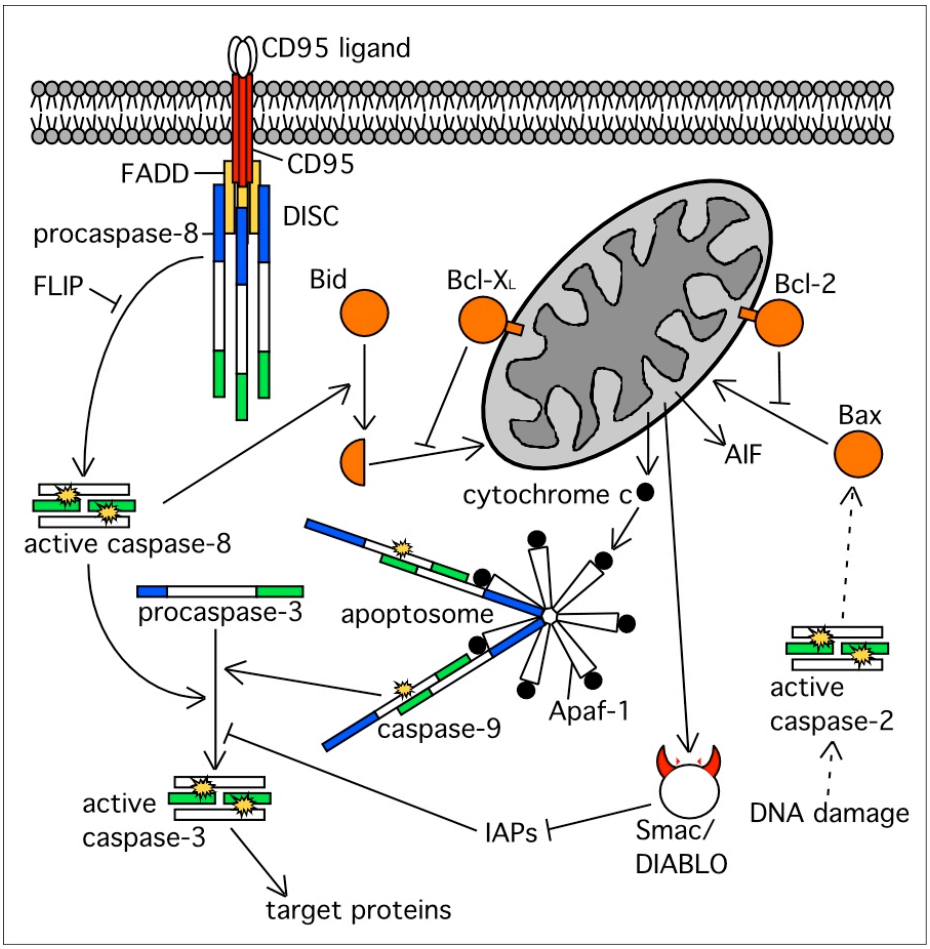

Figure 1. Apoptotic signaling pathways in mammalian cells. Ligand-mediated oligomerization of death-receptor family members like CD95 leads to formation of the DISC complex and activation of procaspase-8 by induced proximity. Active caspase-8 can directly cleave procaspase-3, thereby starting the executioner phase of apoptosis. Alternatively, cleavage of the pro-apoptotic Bcl-2 family member Bid leads to amplification of the apoptotic signal by the mitochondrial pathway. This pathway is also used in apoptosis induced by cytotoxic stress. It leads to the release of pro-apoptotic factors like cytochrome c, Smac/DIABLO and AIF from the mitochondrial inter-membrane compartment to the cytosol. Cytochrome c release leads to formation of the apoptosome, which activates caspase-3.

The direct cleavage of caspase-3 after DISC formation is sufficient to induce a rapid cell death in so called type I cells (40). In type II cells, less DISC is built and too few executioner caspase molecules are activated directly. Therefore, the two pathways described above are intertwined in these cells. Caspase-8, activated by death-receptor crosslinking, cleaves and activates the pro-apoptotic Bcl-2 family member Bid. Truncated Bid translocates to the mitochondria and induces the release of the pro-apoptotic factors

described above, which again leads to activation of caspases. This sideway is thought to amplify the caspase activity in type II cells in response to receptor crosslinking (41,42).

1.1.2 Caspases: the effector proteases of apoptosis

Caspases (**C**ysteinyl **asp**artate-specific prote**ase**s), the executioner proteases of apoptosis, are cysteine proteases with an absolute requirement for aspartic acid in the P_1 position of their four amino acid recognition motif in the target protein. They are synthesized as relatively inactive precursors (zymogens) that are constantly expressed in cells but require proteolytic processing for full activation. Cleavage by another member of the caspase family removes the N-terminal prodomain and divides the remaining protein into a large (~20 kDa) and a small (~10 kDa) subunit. The four subunits generated from two precursor proteins form an active heterotetramer with two independent proteolytic sites (for an overview see (43) and (44)).

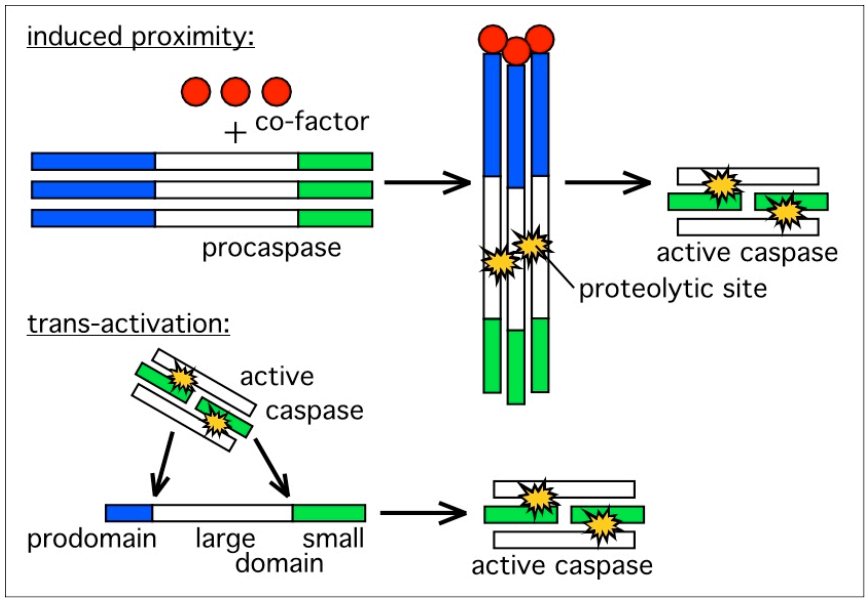

Figure 2. Mechanisms of caspase activation. Caspases can be activated by at least two mechanisms in apoptotic cells. Apical caspases are activated by induced proximity, which enables mutual cleavage of aggregated procaspases by their low intrinsic proteolytic activity. Downstream caspases are trans-activated by cleavage by a more apical family member.

Two different mechanisms can activate caspases in mammalian cells. Oligomerization of specific apical family members in response to an apoptotic stimulus starts the caspase cascade at the onset of apoptosis. Ligation of death-domain containing receptors like CD95 (Fas) or cytochrome c induced oligomerization of Apaf-1 leads to the recruitment of several apical procaspase molecules via protein-protein interaction motifs (DED or CARD, respectively) in their long prodomains. This results in a high local concentration of the

zymogens. The crowded proenzymes can then activate each other by the low level of endogenous catalytic activity harbored in the caspase precursors (induced proximity model) (31,45). Apical caspases thereafter sequentially activate downstream executioner caspases with their characteristic short prodomains by cleavage (trans-activation) (Figure 2). The executioner caspases, caspase-3, -6 and -7, mediate the actual destruction of the apoptotic cell (for an overview see (46)).

It is important to mention that caspases do not lead to an unspecific degradation of proteins. On the contrary, besides the absolute requirement for aspartic acid in the P_1 position of their target sequence, the different members of the family show diverse substrate specificity. The specific consensus cleavage sites are determined by the three amino acids N-terminal to the P_1 position (P_2-P_4), wherein the position P_4 seems to be the most important criterion for specificity. The optimal sequence for each caspase was analyzed by positional scanning of a synthetic combinatorial peptide library, which led to a division of the known caspases into three groups (47). Group I consists of caspases, which prefer hydrophilic amino acids in position P_4. Their optimal target amino acid sequence is WEHD. The members of these group, caspase-1, -4 and -5, are not involved in apoptotic protein degradation, but mediate cytokine processing in inflammatory responses. Group II caspases, including caspase-2, -3, -7 and the *C. elegans* caspase ced-3, prefer the target motive DExD, which appears in many proteins cleaved in apoptotic cells. Group II caspases are the main executioner proteases of apoptosis. The members of group III, caspase-6, -8, -9 and -10, are mainly apical caspases, though caspase-6 does also function as an effector caspase. Caspases from this group need an aliphatic amino acid at the P_4 position of the target sequence (47).

Apart from the ideal cleavage sites mentioned above, caspases also cleave their substrates at sites that were shown to be less optimal targets for the specific enzyme. On the other hand, only a subset of the theoretically optimal consensus sites in target proteins is actually cleaved by caspases in apoptotic cells. Both findings support an additional mechanism determining the sites used by caspases during the process of apoptotic protein degradation. The three-dimensional structure of the target proteins might in part fulfill this function (for an overview see (43)).

Cleavage by caspases can either inactivate vital cellular proteins or activate protein functions by the removal of regulatory domains. Caspase substrates include: other caspases, cell cycle regulators (Rb, MDM2, p21) (48-50), transcription factors (NF-□B, Sp1) (51,52), kinases (Fyn, Lyn, MEKK-1, PAK2) (53-55), inhibitors of apoptosis (XIAP, Bcl-2, Bcl-X_L) (56-58), components of the cytoskeleton (actin, vimentin, cytokeratin 18)

(59-61) and of the nuclear scaffold (lamin A and B) (62,63). In total, about 130 caspase target proteins were hitherto described.

As an important part of the apoptotic destruction, caspases mediate DNA fragmentation by the cleavage of ICAD (**i**nhibitor of **CAD**). This inhibitory protein needs to be cleaved before the endonuclease CAD (**c**aspase-**a**ctivated **D**Nase) can start the degradation of chromosomal DNA (64). In addition, DNA replication proteins, several proteins involved in DNA repair, including PARP (**p**oly **A**DP-**r**ibose **p**olymerase) and DNA-PKcs (**c**atalytic **s**ubunit of **DNA**-dependent **p**rotein **k**inase), are also cleaved by caspases in apoptotic cells (65,66).

1.2 Apoptosis in the immune system

1.2.1 Lymphocyte development

B and T lymphocytes are important components of the mammalian adaptive immune response. They are generated from stem cells in the fetal liver and post-natal bone marrow and differentiate in the central lymphoid organs (B cells in the bone marrow, T cells in the thymus) (for an overview see (67,68)). Mature naïve lymphocytes continually circulate through the blood vessels and the peripheral lymphoid organs including lymph nodes, tonsils and spleen. Specialized **a**ntigen-**p**resenting **c**ells (APCs) such as macrophages and dendritic cells deliver pathogen-derived antigens from the site of infection to the peripheral lymphoid organs and display them to the lymphocytes (69,70). If the antigen fits and crosslinks the surface receptors on a specific lymphocyte and an additional co-stimulatory signal is provided, proliferation (clonal expansion) and differentiation into effector cells is induced. Differentiation of B cells leads to the development of antibody-secreting plasma cells and long living memory cells (for an overview see (67)). Terminally differentiated T cells exist in the form of memory and effector CD4 and CD8 cells. CD4 cells activate macrophages (T_H1 cells) and kill cells infected with intracellular pathogens (T_H2 cells). In addition, T_H2 cells are needed for full activation of B cells recognizing the same antigen. CD8 cells are also known as cytotoxic T cells and kill cells infected by viruses (for an overview see (71)).

It is impossible to produce the immense repertoire of B and T cell receptors (in humans at least 10^{11}) protecting the mammalian organism against infections by pathogens from the same amount of gene loci. Alternatively, B lymphocytes contain a limited number of loci for three gene segments (variable – V, diversity – D and joining – J) for the heavy chain and two gene segments (V and J) for the light chain, which later generate the variable

regions of the antibody. Somatic recombination (V(D)J-rearrangement) under control of the **r**ecombination **a**ctivating **g**enes RAG1 and RAG2 and the ligation with constant region genes leads to antibody formation from a combination of these segments in the developing lymphocytes (72). Additional diversity is created by the insertion or deletion of a few nucleotides at the junctions of the segments. A similar mechanism leads to the formation of T cell receptors. The rearrangement, in general, is very error-prone and the outcome of this process is largely a matter of chance. This, on the one hand, enables the formation of the huge amount of different antibodies and T cell receptors mentioned above, but also leads to the production of lymphocytes with defective receptors as well as autoreactive cells (for an overview see (73)). Both kinds of lymphocytes can go through an additional cycle of receptor editing with extended rounds of light chain gene rearrangements. If this attempt does not generate a functional and non-self receptor, the cell is deleted from the lymphocyte repertoire by apoptosis (74,75).

The affinity of antibodies produced by B cells at the beginning of an immune response is low. To refine the antibody repertoire, B cells go through several rounds of somatic hypermutation. This process induces individual point mutations in the variable regions of the antibody, thereby generating a series of cells expressing related B cell receptors with only subtle changes in affinity towards the antigen (76). B cells in the hypermutation stage produce no or very little functional BCR on their surface. As described above, B cells without a functional BCR normally undergo apoptosis. In addition, the expression of pro-apoptotic proteins like Fas and Bax is upregulated in these cells. Therefore, during this differentiation stage, B cells are very sensitive towards apoptosis (77). After each round of mutation, B cells express a new receptor and a process called affinity maturation, which is probably mediated by competition of B cells for limiting interactions with T cells or dendritic cells, selects cells with high affinity antibodies for survival. These cells undergo the next round of division, mutation, expression and selection, while cells with low affinity antibodies die by apoptosis, a process that can be inhibited by overexpression of Bcl-2 (78).

1.2.2 IgM-mediated B cell apoptosis

To protect the mammalian organism against autoimmune diseases, B and T cells with high affinity to proteins derived from “self” tissues, need to be eliminated. This can be accomplished by apoptosis due to (self)antigen-mediated crosslinking of **B** or **T** **c**ell **r**eceptors (BCR / TCR). The crosslinking of surface IgM molecules can lead to proliferation of cells or apoptosis depending on the cellular context (74).

Major hallmarks in B cell apoptosis are: an early kinase cascade, an increase in cytoplasmic calcium, enhanced expression of a number of immediate early genes, cytochrome c release from the mitochondria, and activation of caspases, the executioner proteases of apoptosis (Figure 3) (for an overview see (79)).

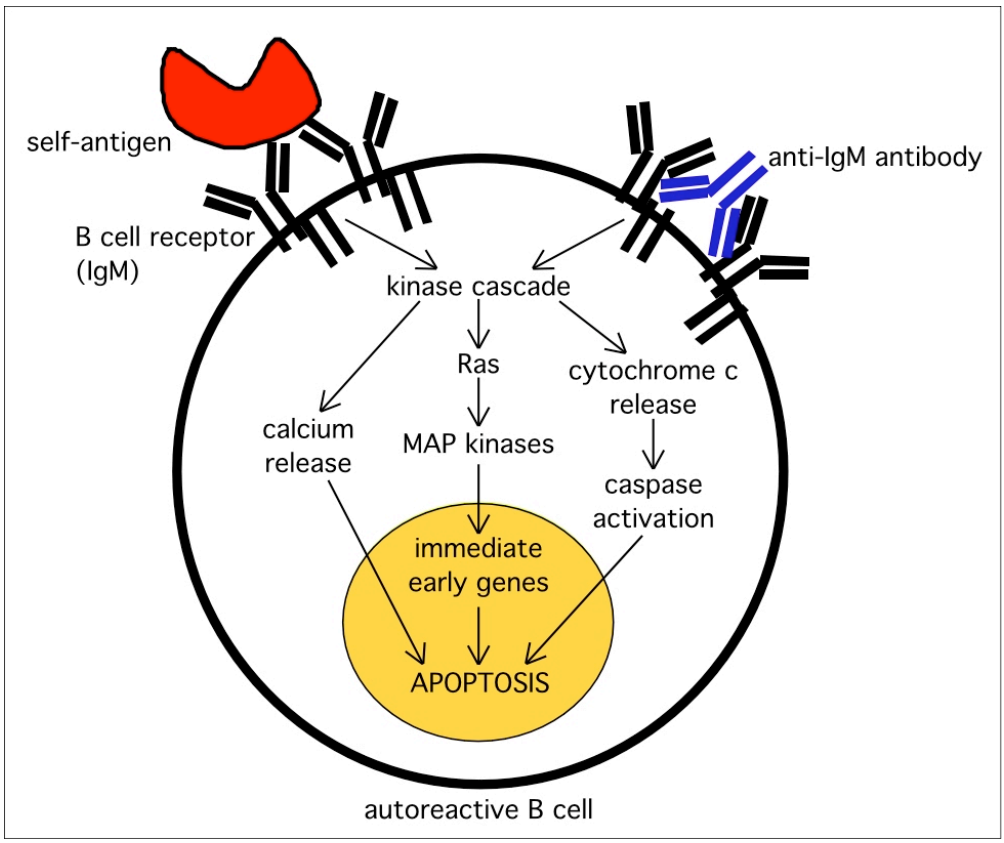

Figure 3. IgM-induced B cell apoptosis. Crosslinking of the B cell receptors (surface IgM) by a self-antigen *in vivo* or anti-IgM antibodies *in vitro* induces a kinase cascade, the release of calcium from the endoplasmatic reticulum, activation of immediate early genes, cytochrome c release from the mitochondria and activation of caspases, which are the main executers of apoptotic cell death.

The kinase cascade begins at the co-receptors (Ig□ and Ig□) of the B cell receptor. In contrast to the surface IgM molecule, the heterodimer has cytoplasmic domains, which contain the **i**mmunoreceptor **t**yrosine-based **a**ctivation **m**otive (ITAM) (80). Phosphorylation of Ig□ and Ig□ at these sites by Src-family tyrosine kinases (Lyn, Blk, Fyn and others) leads to the recruitment and activation of Syk, which then starts several parallel downstream kinase cascades. One of them, the phosphoinositide hydrolysis pathway, leads to the generation of **i**nositol 1,4,5-tri**p**hosphate (IP_3) and **d**i**a**cyl**g**lycerol (DAG), which function as second messengers in mediating calcium release from the endoplasmatic reticulum and protein kinase C activation, respectively (81). An increased cellular calcium level activates the calcium-calmodulin-dependent protein kinase II as well as the phosphatase calcineurin (82,83). Another pathway downstream of Syk leads to the

activation of Ras, which, in turn, phosphorylates MAP kinases. Members of this family were shown to transcriptionally regulate the expression of early response genes like c-fos and EGR-1 (84,85).

Genes induced in a cell in the first few hours following a certain stimulus are referred to as early response or immediate early genes. Activation of these genes is independent of protein synthesis. It therefore requires the function of pre-existing transcription factors. The majority of the immediate early genes upregulated in B cell apoptosis, encode transcription factors including c-myc, c-jun, c-fos and EGR-1. However, some cytokines, growth factor receptors and other genes also belong to this group (86).

1.3 DNA replication

DNA replication is a tightly monitored biological process, as accumulating errors may lead to developmental defects or oncogenesis. Therefore several checkpoints control the successful completion of DNA replication before cells can move on to mitosis. If any damage or stalled replication intermediates are detected, an activated checkpoint delays cell cycle progression until the respective process is completed, induces repair mechanisms or if these attempts fail initiates apoptosis (for an overview see (87)).

The replication of chromosomal DNA is dependent on the coordinated function of a specific set of proteins. As the first prerequisite for DNA replication, the **pre**-**r**eplicative **c**omplex (pre-RC) needs to be assembled at the origins of replication. In *Saccharomyces cerevisiae* three to four conserved sequences of 10 – 15 bp spread over 100 – 150 bp are sufficient to act as origin (88,89). In contrast, origins in higher eukaryotes are not very well defined. They can extend over several kb in the DNA and specific conserved sequence elements equivalent to the ones found in yeast could not be determined (for an overview see (90)). Formation of the pre-RC starts with the binding of ORC (**o**rigin **r**ecognition **c**omplex), a hexamer consisting of six different subunits to the origins of replication. Once ORC is bound to chromatin, the binding of Cdc6 (**c**ell **d**ivision **c**ycle gene 6) and Cdt1 is possible. These two adaptor proteins bind to the ORC complex during G1 and, in turn, are needed to recruit the MCM (**m**ini**c**hromosome **m**aintenance) complex (Figure 4) (for an overview see (91)). The function of this complex in DNA replication is still under investigation, but it is suspected to act as a DNA helicase (92). Once pre-RCs are assembled at the DNA, the chromatin is “licenced” for replication. It is now competent to go through a single round of DNA replication, which is started by cell cycle-regulated kinases at the beginning of S phase (for an overview see (93)).

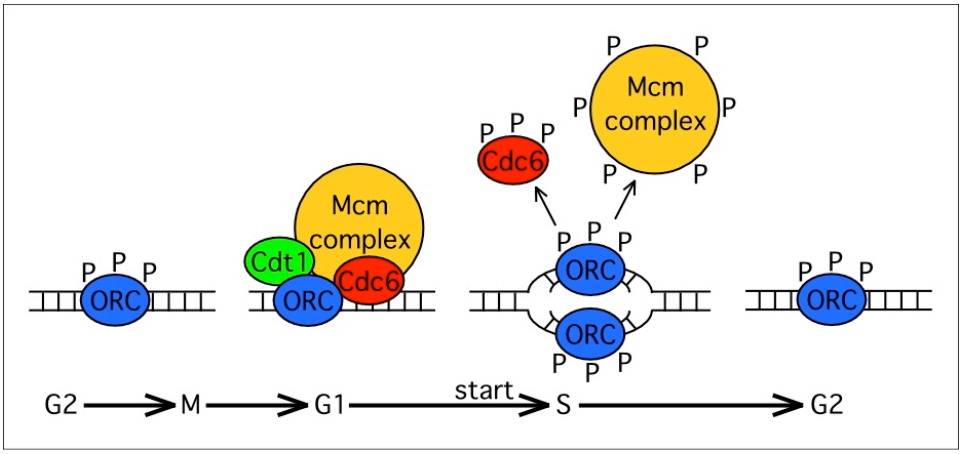

Figure 4. The initiation of DNA replication. The ORC complex, located at origins of replication, is bound by Cdc6 and Cdt1 during the G1 phase of the cell cycle. These proteins are, in turn, needed to recruit the MCM heterohexamer to the pre-replicative complex (pre-RC). Phosphorylation of ORC, Cdc6, and the MCM complex after passage through the START point at the G1/S-boundary, enables DNA replication and leads to dissociation of the pre-RC.

The MCM complex is composed of several different Mcm subunits that do not have an intrinsic DNA binding activity, but were shown to interact with histone H3 (94). One current model, therefore, assumes that the MCM complex, appearing as ringshaped in electron microscopy pictures, binds loosely to chromatin and becomes stabilized with the DNA string in the central cavity - possibly by Cdc6 and Cdt1 (95,96). In this context, it is important to note that Cdc6 belongs to the AAA^+ superfamily of proteins and exhibits significant sequence similarities to subunits of eukaryotic and prokaryotic sliding clamp loaders (see below) (97). All clamp loaders are dependent on ATP binding and hydrolysis to assemble their respective clamps onto DNA (98). Hydrolysis of ATP by Cdc6 is essential for the loading of the MCM complex at least in *Saccharomyces cerevisiae* (99). Once the MCM complex is loaded, its binding to DNA is independent of Cdc6 (100). While Cdc6 is phosphorylated and leaves the complex at the initiation of DNA replication, the ordered assembly of many additional proteins like Cdc45, single strand stabilizing proteins and DNA polymerases begins. The MCM complex is phosphorylated and dissociates from chromatin while DNA replication proceeds (Figure 4) (101).

1.3.1 The MCM complex

The MCM (minichromosome maintenance) complex is a component of the pre-replicative complex (pre-RC). All six MCM subunits (Mcm2 - Mcm7) are essential and highly conserved proteins from yeast to mammals (102). In most of the sequenced archaebacterial genomes, only one Mcm-related gene was found, which has the highest homology to eukaryotic Mcm4. The Mcm-related protein in *Methanobacterium thermo-*

autotrophicum probably exists as a double-hexamer and displays an ATP-dependent 3'=>5' helicase activity (103).

Eukaryotic Mcm proteins probably bind to chromatin as a heterohexamer. Even non chromatin-bound proteins are found mainly in this hexameric status. Degradation of a single subunit in yeast leads to a removal of all other complex members from the nucleus to the cytoplasm, though their protein levels remain unchanged (104). All six Mcm proteins are essential for formation of a functional MCM complex, but complexes consisting of only a subset of the Mcm proteins were also described. For this reason, the exact composition of the MCM complex active in DNA replication is still under debate. Mcm3 and Mcm5 are tightly associated and build one subcomplex. The other subcomplex comprises of Mcm4, Mcm6 and Mcm7 and the weakly associated Mcm2 (94,105). Mcm4 and Mcm6 seem to be the first components of the MCM complex to associate with chromatin (96). All Mcm proteins include Walker A and B ATP-binding motifs (see below) (106). Mutation of these ATP-binding motifs in one MCM subunit dramatically reduces the overall ATPase activity of the complex suggesting a coordinated ATP hydrolysis of the subunits (107).

Yeast Mcm proteins shuttle between nucleus and cytoplasm during the cell cycle. They enter the nucleus at the end of mitosis, but become increasingly cytoplasmic after initiation of DNA replication as the S phase proceeds (108). The nuclear export was shown to be dependent on phosphorylation of the Mcm proteins by cyclin B / Cdc28 (109). In contrast, Mcm proteins in human cells remain in the nucleus throughout the S phase and thereafter, but are excluded from chromatin during this period (108). Like in yeast, chromatin-bound human Mcm proteins are hypophosphorylated, while free Mcms are hyperphosphorylated (110). Phosphorylation inhibits renewed binding of pre-RCs to chromatin after the initiation of DNA replication (109).

The expression levels of human Mcm proteins are unchanged throughout the cell cycle, but quiescent or differentiated cells display much lower Mcm protein levels than proliferating cells. Consequently, the Mcm protein level is substantially higher in malignant cells when compared to their untransformed counterparts (111). The usability of Mcm protein levels as prognostic markers for malignancy in the clinic is currently under investigation (112,113).

1.3.2 The adaptor protein Cdc6

The pre-RC component Cdc6 is a protein of about 66 kDa related to Orc1 (114). It associates with origin-bound ORC in G1 and is, in turn, needed to recruit the MCM complex (see above). Overexpression of Cdc6 in starved human fibroblasts, which

downregulate intrinsic Cdc6 protein, is sufficient to induce stable chromatin association of intrinsic Mcm proteins. A simultaneous addition of Cyclin E / Cdk2 induces the start of DNA replication (115).

In yeast, cyclin B / Cdc28-dependent phosphorylation leads to ubiquitin-mediated degradation of Cdc6 in the S phase. *De novo* protein synthesis is required in each cell cycle (97,109). Yeast cells lacking Cdc6 protein undergo a reducing mitosis without prior DNA replication. Yeast Cdc6 is therefore needed to block premature passage through the M phase (116). On the other hand, constitutively overexpressed Cdc6 causes re-replication and inhibits mitosis in yeast (117). This inhibitory function seems to be dependent on phosphorylation of Cdc6 by Cdc28, as it is disturbed upon the mutation of the three Cdc28 phosphorylation sites or the deletion of the Cy-motive, which is needed for binding to the cyclin subunit of the kinase (116). Although overexpression of Cdc6 in mammalian cells does not cause re-replication (118), a recent report suggests a phosphorylation-dependent function of human Cdc6 in the replication checkpoint pathway, as overexpression of Cdc6 in the G2 phase prevents the entry of HeLa cells into mitosis (119). The replication checkpoint is activated in response to DNA damage or incomplete replication. Its activation inhibits cell cycle progression and by providing time for repair mechanisms prevents a “mitotic catastrophe” (premature mitosis) (120).

The Cdc6 protein level changes during the cell cycle in human cells with the lowest amount in M and G1 phase, when the protein is subject to ubiquitin-mediated degradation (115,118,121). However, an additional mechanism probably has a more important regulatory effect on the protein and prevents re-replication in mammalian cells: phosphorylation of Cdc6 by Cyclin E / Cdk2 in human cells leads to a change in the subcellular localization of the protein. Cdc6, which is mainly nuclear during G1, becomes phosphorylated at the initiation of DNA replication and is cytoplasmic thereafter during S, G2 and M phase (114). Human Cdc6 contains two N-terminal nuclear localization signals (NLS) and a C-terminal nuclear export sequence (NES) (122). The translocation of phosphorylated Cdc6 from the nucleus to the cytoplasm is dependent upon GTP and the function of the nuclear export receptor Crm1 (123).

Cdc6 was shown to slowly hydrolyze bound ATP *in vitro* (124). Cdc6 contains the conserved nucleotide binding motifs Walker A and Walker B. Walker A mediates binding to the α- and β-phosphates of ATP, while Walker B coordinates Mg^{2+} via a H_2O molecule that is needed for the nucleophilic attack in ATP hydrolysis. Cdc6, with a mutation in the Walker A motive is not able to bind ATP. A mutation in Walker B does not inhibit the binding but prevents the hydrolysis of ATP by the protein. An altered sensibility to tryptic digestion of wildtype Cdc6 after ATP hydrolysis implicates a conformational change of the

protein as result of this process. In consequence to their impaired ATP binding and hydrolysis capacities, this conformational change is obstructed in the Walker A and B mutants. Overexpression of the Cdc6 Walker A mutant inhibits the entry into S phase in transfected cells, while the Walker B mutant has a later effect in disturbing the progression through S phase (124).

As mentioned above, Cdc6 is a member of the AAA$^+$ superfamily of proteins with a high homology to sliding clamp loaders. The mechanism of action suggested for these proteins might therefore be also applicable for Cdc6. Biochemical studies of eukaryotic and prokaryotic clamp loaders have suggested the following model: binding of ATP to the clamp loader allows its binding to the respective clamp (here the MCM complex). Binding of the loader (here Cdc6) opens the clamp and enables the insertion of the DNA strand into the open ring. ATP hydrolysis induces a conformational change in the clamp loader (Cdc6), which leads to a closing of the clamp around the DNA and a release of the clamp loader from the complex (Figure 5) (98).

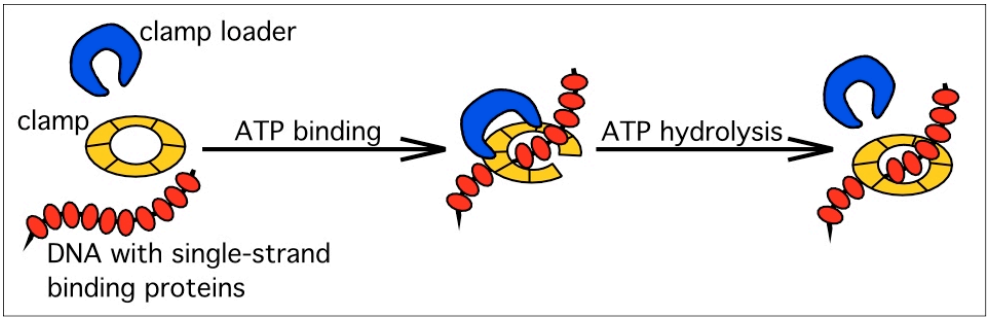

Figure 5. Clamp loading. Binding of ATP induces a conformational change in the clamp loader, enabling binding to and opening of the DNA clamp. Single-stranded DNA can now be introduced into the ring, which is closed after ATP hydrolysis and dissociation of the clamp loader.

Like the sliding clamp loaders, Cdc6 consists of three domains (125). Domain I contains the Walker A and B motifs and the sensor I sequence, which is thought to be involved in mediating the conformational change after ATP hydrolysis. Domain II includes the sensor II motive and together with domain I forms a nucleotide-binding pocket of the AAA$^+$ type. Domain III has a winged-helix structure and is thought to mediate protein-DNA or protein-protein interactions (125). For more details about the Cdc6 domains and special sequence motifs see the discussion part of this work (Figure 26).

1.4 DNA repair

Retaining a complete and undamaged genome is of vital importance for any cell. Therefore, several checkpoints throughout the cell cycle monitor the integrity of the DNA. If DNA damage occurs, these checkpoints turn on DNA repair mechanisms or if the damage is irreparable lead to removal of the cell by apoptosis (for an overview see (87)).

DNA double-strand breaks are primarily caused by cellular recombination processes that occur e.g. in meiosis or V(D)J-recombination in lymphocytes. The most important exogenic cause for DNA double-strand breaks is ionising radiation. Most DNA double-strand breaks are not blunt ended but have short single-stranded overhangs as double-strand breaks mostly result from two nearby single-strand breaks in the different DNA strands. Two kinds of DNA double-strand break repair are known: homologous recombination and **n**on-**h**omologous **e**nd **j**oining (NHEJ). Homologous recombination is the main way to repair DNA double-strand breaks in lower eukaryotes like yeast. This mechanism uses the intact homologous strand as a template thereby enabling a very accurate repair with a low frequency of mistakes (for an overview see (126)).

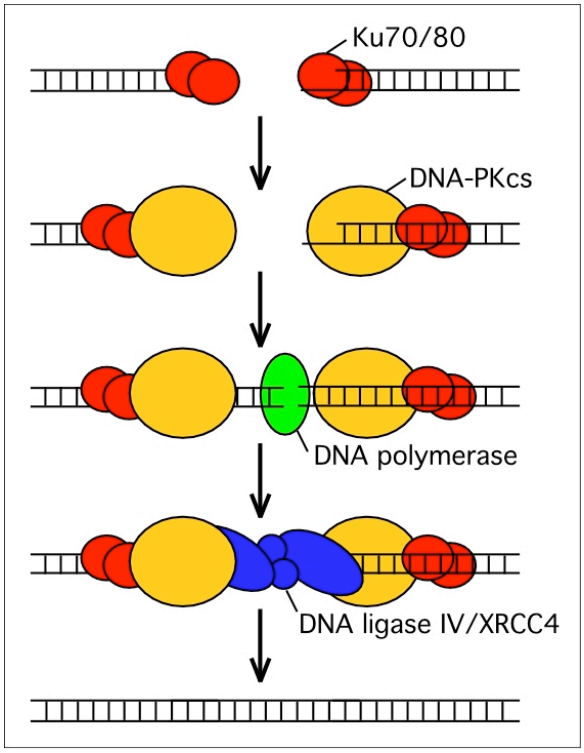

Figure 6. Non-homologous end joining. Non-homologous end joining (NHEJ) is the main mechanism used to repair DNA double-strand breaks in eukaryotic cells. NHEJ starts with the binding of the Ku heteromer to the DNA ends. Ku recruits and activates DNA-PKcs. After DNA polymerases have filled the gap, Ku recruits the DNA ligase IV / XRCC4 complex, which finally seals the break.

Non-homologous end joining (NHEJ) is the fast but more error-prone DNA repair mechanism used most frequently in mammals. NHEJ is supported by microhomologies of 8 - 20 bp if they reside within the first 20 bp at the DNA ends (127). The current model for NHEJ assumes the following repair mechanism: the Ku heteromer, consisting of the two regulatory subunits Ku70 and Ku80 of DNA-PK (**DNA**-dependent **p**rotein **k**inase), binds to the DNA ends and occupies about 20 bp (two helical turns) (128). Ku recruits the catalytic subunit DNA-PKcs to the break. Ku migrates inwards along the DNA strand during this process, while DNA-PKcs remains directly at the break. The active DNA-dependent protein kinase (DNA-PK) now occupies about 28 bp (129). DNA-PK phosphorylates Ku and might thereby activate its ATP-dependent helicase activity. Ku could then unwind the DNA strands, possibly helping to expose regions of microhomology, which were shown to improve NHEJ (130). Gaps are filled by DNA polymerases and DNA ligases seal the breaks (131). The DNA ligase IV / XRCC4 complex needs Ku for effective DNA binding (Figure 6) (132).

In apoptotic cells DNA fragmentation produces double-stranded DNA fragments of polynucleosomal size (133). These fragments are probable targets for DNA repair by NHEJ. DNA repair on the fragments would be counterproductive and would also consume ATP, which is needed to carry out the active apoptotic death. Depletion of ATP was shown to switch apoptotic to necrotic cell death, a scenario which needs to be prevented as it might lead to inflammation (6). It should therefore be advantageous to repress DNA repair in apoptotic cells.

1.5 Aim of the study

Apoptosis of lymphocytes that recognize self-antigens is an important mechanism to prevent the development of autoimmune diseases. To get a better insight into the molecular processes involved in IgM-mediated B cell apoptosis, the objective of this study was to identify differential transcription of genes and posttranslational modification of proteins in response to the induction of apoptosis by B cell receptor (BCR) crosslinking. The oligomerization of BCR molecules (IgM) by self-antigens leads to apoptosis of autoreactive B cells in the mammalian immune system. This effect can be mimicked *in vitro* in the human Burkitt's lymphoma cell line BL60-2 by crosslinking the BCRs with anti-IgM antibodies.

Due to the fact that proliferation is strongly reduced in apoptotic BL60-2 cells, the first aim of the study was to analyze the influence of apoptosis on the pre-replicative complex, which is needed for the initiation of DNA replication. There are two main possibilities to

reduce the amount of functional protein in a cell: downregulation of the transcription level and degradation / inactivation of the protein. Both possibilities should be checked for the pre-RC components Mcm2 – Mcm7 and Cdc6. Initial results revealed a caspase-mediated cleavage of two of the proteins in apoptotic BL60-2 cells. Further analysis in additional apoptotic systems was planned to determine the generality of the effect. In addition, the identification of the cleavage sites and functional properties of the generated apoptotic protein fragments were assessed.

The second aim of the study was to carry out a screen for genes differentially regulated at transcriptional level in BL60-2 cells after induction of apoptosis with anti-IgM antibodies. The screening experiments were performed using three independent experimental systems. The results achieved with Affymetrix GeneChips, cDNA membranes and **R**Nase **p**rotection **a**ssays (RPAs) together with subsequent validation were expected to give insight into the fundamental process of B cell selection in the immune system.

It was anticipated that the screening for changes in gene expression, representing an early apoptotic event, together with the analysis of protein modifications, occurring relatively late in apoptotic cells, would produce a broad spectrum of results that might lead to a better understanding of the mechanisms and pathways activated in apoptotic cells.