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Chlamydia Interferes with Host Cell Death: Molecular Analysis of Apoptosis Signaling Pathways

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Abbreviation List

A

A1	Bcl-2 homologue protein
AIF	Apoptosis-inducing factor
AMC	7-amino-4-methylcourmarin
Apaf-1	Apoptotic protease activating factor 1
APC	Antigen-presenting cell
APC	Allophycocyanin
APS	Ammonium peroxide disulphate
Atc	Anhydrotetracycline
ATCC	the American Type Culture Collection
ATP	Adenosine triphosphate

B

Bad	Bcl-2 antagonist, causing cell death
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-w	B-cell lymphoma protein W
Bcl-x_L	B-cell lymphoma-X long
BH	Bcl-2 homology
Bid	Bcl-2 interacting protein
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting mediator of cell death
Bmf	Bcl-2 modifying factor
bps	Base pairs
BSA	Bovine serum albumin

C

C. elegans	*Caenorhabditis elegans*
C.t.	*Chlamydia trachomatis*
C. trach	*Chlamydia trachomatis*

CAD/DFF40	caspase-activated DNAse
CADD	Chlamydia protein associating with death domains
Caspase	Cysteine-aspartic-acid-proteases
CD	Cluster of differentiation
cDNA	Complementary DNA
Ced-3	*C. elegans* cell-death gene
CHAPS	3-[(3/Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Chl	Chlamydia
cIAP	Cellular inhibitor of apoptosis protein
CPAF	Chlamydia protease-like activity factor
Cy3	Cyanine 3
Cy5	Cyanine 5

D

3D	Three-dimensional
DC	Dendritic cells
DEVD	Asp-Glu-Val-Asp
Diablo	Direct IAP-binding protein with low pI
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
DS	Danger signals
DSR	Danger signal receptors
DTT	Dithiothreitol

E

E. coli	*Escherichia coli*
EB	Elementary body
ECL	Enhanced chemoluminescence
EDTA	Ethylendiamintetraacetate

EGTA	Ethylenglycoltetraacetate
EndoG	Endonuclease G
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase

F

FACS	Fluorescence-activated cell sorter
Fas	Apoptosis stimulating fragment
FCS	Fetal calf serum
Fig	Figure
FITC	Fluorescein isothiocyanate

H

HeLa-fas	HeLa cells stably expressing fas receptor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HMGB1	High mobility group box 1 (protein)
Hrk	Harakiri
HSP60	Heat shock protein 60
HtrA2/Omi	High temperature requirement protein 2

I

IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of CAD
inf	Infection
IP	Immunoprecipitation

J

JNK	c-Jun NH_2-terminal kinase

K

kDa	Kilodalton

L

LB	Luria Bertani
LGB	Lower gel buffer
LPS	Lipopolysaccharide
LSM	Laser scanning microscope

M

M	mol/l
Mcl-1	Myeloid cell leukemia sequence 1
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
MoPn	Mouse pneumonitis
mRNA	Messange RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide

N

NF-κB	Nuclear transcription factor κB
NP-40	Nonidet P-40

O

OD	Optical density

P

p53	Tumour protein 53
PAGE	Polyacrylamide gel electrophoresis
Parp	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Poly(ethylenimine)
pH	Power of hydrogen

PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIPES	Piperazine-N,N-bis(2-ethanesulfonic acid)
PKCδ	Protein kinase C δ
PS	Phosphatidylserine
Puma	p53 upregulated modulator of apoptosis

R

RB	Reticulate body
RelA	v-rel reticuloendotheliosis viral oncogene homolog A
RFX5	Regulatory factor X, 5
RHO	Rhodamine
RLU	Relative luminescence units
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNAsin	RNase inhibitor
rpm	rounds per minute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR

S

SDS	Sodium dodecyl sulfate
Smac	Second mitochondrial-derived activator of caspase
Stauro	Staurosporine
SSC	Saline sodium citrate
STD	Sexually transmitted disease

T

T-REx	Tet-repressor-based expression system
TAE	Tris-acetate-EDTA
tBid	Truncated Bid
TBS	Tris-buffered saline

TBST	Tris-buffered saline with 0.05 % Tween
TCA	Trichloroacetic acid
TdT	Terminal deoxynucleotidyl transferase
Temed	N'N'N'N'-Tetramethylethylendiamine
Tet	Tetracycline
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Trail	TNF-related apoptosis-inducing ligand
Tris	Tris-(Hydroxylmethyl-) Aminomethan
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

U

UGB	Upper gel buffer
UTP	Uridine 5'-triphosphate
UV	Ultraviolet

V

v/v	Volume per volume

W

WT	Wild type

X

XIAP	X-linked inhibitor of apoptosis

Z

zVAD	z-Valine-Alanine-Aspartate

1. Introduction

Cell death or survival has been shown to be important in a number of infectious diseases. The host cell fate may determine the outcome of the infection. It has been suggested that Chlamydia infection can interfere with the host cell apoptosis machinery, although the precise molecular mechanisms are not well understood. We investigated apoptotic signaling regulation during Chlamydia infection and the impact of apoptosis on host immune response and bacterial propagation.

1.1 Apoptosis

Apoptosis is an evolutionary conserved mechanism that plays an important role in various physiological and pathological situations. Apoptosis is essential to keep tissue homeostasis, to deplete unwanted, damaged or infected cells. Apoptosis is also crucial for development of the immune system and its function. Apoptotic cells display typical morphological and biochemical features, such as cell shrinkage, nuclear DNA fragmentation as well as membrane blebbing. Since the time that this unique form of cell death was first named by John Kerr in 1972 (Kerr et al., 1972), the mechanism of apoptosis has been extensively explored. Besides the important impact in physiological conditions, apoptosis has been also implicated in many diseases, including infectious diseases, cancer and autoimmune disease. Specific targeting of apoptotic signaling pathways provides a new therapeutic approach and may have clinical applications in the near future.

1.1.1 Apoptotic signaling pathways

Two apoptosis inducing pathways (the mitochondrial pathway and the death receptor pathway) have been described in the literature to lead to apoptotic cell death (Fig. 1.1). Bcl-2 preventable cytochrome *c* release from the inter-membrane space of the mitochondria into the cytosol is a central step in the former pathway (Kluck et al., 1997; Liu et al., 1996; Yang et al., 1997), which

directly activates effector caspases by formation of the apoptosome (together with caspase-9 and Apaf-1) (Acehan et al., 2002). Several other mitochondrial proteins (Smac, AIF etc.) were also found to be released during apoptosis (Du et al., 2000; Susin et al., 1996). This release is governed by Bcl-2 family proteins (pro-apoptotic BH3-only proteins and Bax/Bak multidomain proteins, as well as anti-apoptotic Bcl-2 like proteins). The death receptor pathway is initiated by ligation of death receptors (CD95, TNFR and TrailR etc.) by their ligands at the plasma membrane. Death receptor signals activate effector caspases either directly by activation of caspase-8 (in type I cells), or via signals through the mitochondrial pathway by cleavage of the BH3-only protein Bid (in type II cells) (Fig. 1.1) (Li et al., 1998; Scaffidi et al., 1998). Therefore mitochondria play a key role in the apoptotic signaling pathway in most cases, which is tightly controlled by upstream Bcl-2 family proteins.

1.1.2 Bcl-2 regulated mitochondrial signaling

Bcl-2 was the first apoptosis regulating protein identified, and can prevent apoptosis (Vaux et al., 1988). Since then many experiments have been done and are in progress to explore the nature of the molecular regulation of cell death. Bcl-2 family proteins are composed of 4 conserved domains, respectively the Bcl-2 homology domains 1, 2, 3 and 4 (BH1, BH2, BH3 and BH4), (Schwartz and Hockenbery, 2006). The anti-apoptotic Bcl-2 like proteins group usually contains all of the 4 domains, except for Mcl-1 that does not contain a BH4 domain. The pro-apoptotic class of BH3-only proteins shares only a short region of the BH3 domain, whereas the multidomain effector proteins Bax and Bak have BH1, BH2 and BH3 domains. Interactions between these proteins have been implicated to control pro-apoptotic or pro-survival decisions. Two distinct functional models (Fig. 1.2) have been proposed (Willis and Adams, 2005). One is called displacement model (BH3-only proteins activate Bax/Bak by removing Bcl-2 like proteins); and the other is the direct binding model, which assumes direct activation of Bax/Bak by BH3-only proteins.

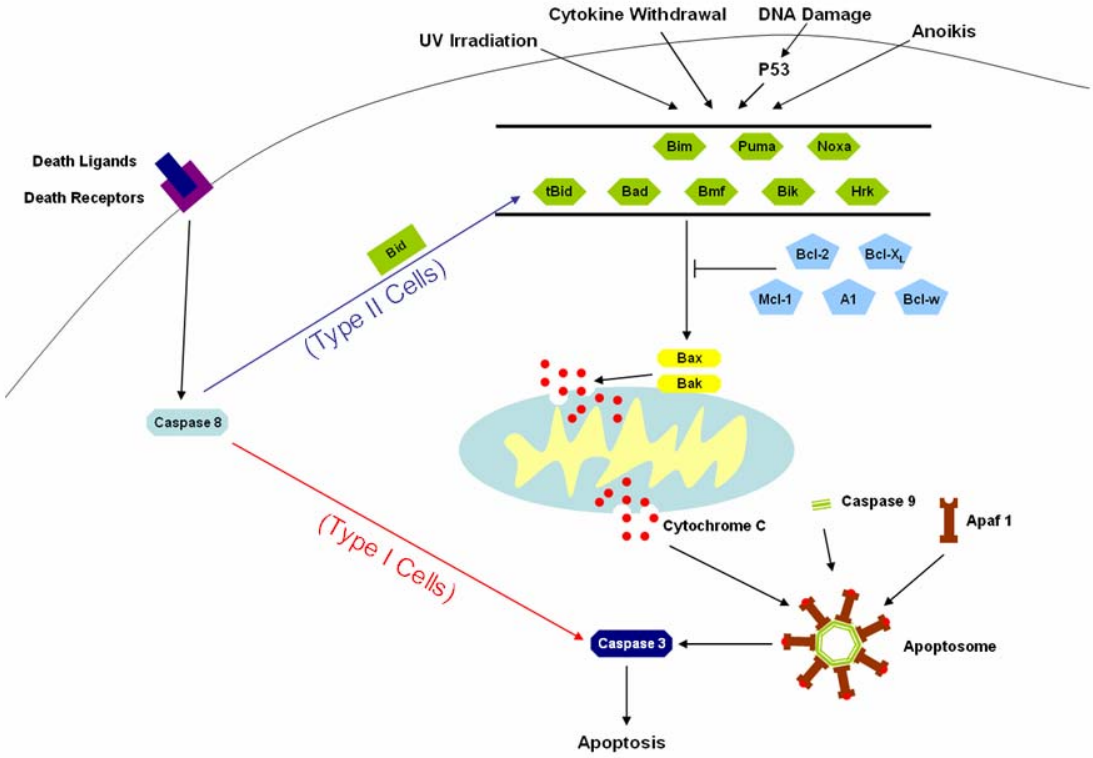

Fig. 1.1 Overview of apoptosis pathways

In mitochondria-regulated apoptosis, BH3-only proteins (green) are activated by extrinsic stimuli and trigger mitochondrial apoptosis by activating Bax/Bak (yellow), a process that may depend on Bcl-2-like proteins (blue) and is inhibited by high levels of these molecules. Activation of Bax/Bak leads to release of cytochrome *c* from mitochondria to the cytosol, where it binds to Apaf-1 and caspase-9, constituting a complex called apoptosome, which leads to the activation of caspase-9 and of effector caspases. Effector caspase-3 is activated by the apoptosome and induces apoptosis. Combination of death ligands and death receptor at the surface of cell membrane leads to activation of caspase-8. Activated caspase-8 can either directly (red arrow) activate caspase-3 in type I cells, or via a mitochondria-controlled loop through cleavage of the BH3-only protein Bid (blue arrow).

In the displacement model, BH3-only proteins bind to several or all anti-apoptotic Bcl-2 like proteins. Bax and Bak are activated by BH3-only proteins through liberation from Bcl-2 like anti-apoptotic proteins from their initial interacting partner Bax/Bak in healthy cells. Studies on protein interactions among Bcl-2 family proteins revealed that several BH3-only proteins, such as Bim, Puma and tBid, can bind to all Bcl-2 like proteins to induce apoptosis. However, others such as Noxa and Bad can only bind to some of the Bcl-2 like proteins, and therefore need to co-operate with each other to induce apoptosis (Fig. 1.3) (Chen et al., 2005). In resting cells, Bak can be sequestered by Mcl-1 and Bcl-x_L. Noxa or Bad are alone not able to release both anti-apoptotic players to activate Bak, but co-overexpression of Noxa and Bad is sufficient to liberate Bak and leads to its oligomerization (Willis et al., 2005).

The direct activation model obviously proposes that BH3-only proteins are able to directly activate Bax/Bak to induce apoptosis, independent of Bcl-2 like proteins. In this model, BH3-only proteins are divided into two subgroups, activators or strong killers (Bim, tBid etc.), and sensitizers or weak killers (Noxa, Bad, Hrk etc.) (Certo et al., 2006), respectively. Cheng and her collegues recently reported that activator BH3-only proteins are released from Bcl-2 like proteins by sensitizer BH3-only proteins (Kim et al., 2006). Furthermore, downregulation of Bim in Puma deficient cells leads to partial resistance to apoptosis induced by combined overexpression of Noxa and Bad, indicating that the activators may be involved in apoptosis induced by the sensitizers (Kim et al., 2006). Another recent study that mutated the BH3-only protein Bim_S at the Bcl-2 binding site revealed that the mutated Bim_S is still inducing apoptosis in the absence of interaction with Bcl-2 like proteins. Co-overexpresion of BH3-only proteins and Bax in yeast (yeast has no Bcl-2 like proteins) further indicated that BH3-only proteins can co-operate directly with Bax to trigger cell death (Weber et al., unpublished data). Liu *et al* demonstrated recently that a Bcl-x_L mutant that does not bind to Bax or Bak still inhibits activated T cell death, which may be considered as indirect evidence against the displacement model (Liu et al., 2006). However, Bax and Bak were shown to be able to induce apoptosis without association with the putative BH3-only proteins, for instance in cells lacking both Bim and Bid plus

reduced Puma (Willis et al., 2007). Further investigations have to be performed to understand how Bcl-2 family proteins achieve apoptosis induction.

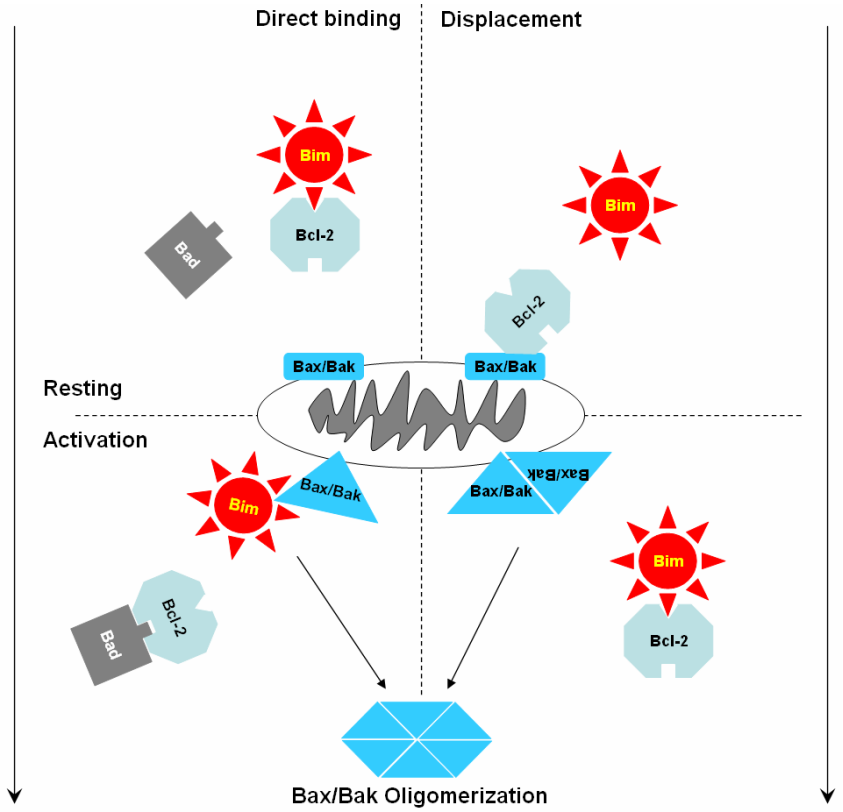

Fig. 1.2 Various models of BH3-only proteins-mediated Bax/Bak activation: the direct activation model and the displacement model

In the direct binding model, sensitizer BH3-only proteins (e.g. Bad) liberate potent BH3-only killers (e.g. Bim) from Bcl-2 like proteins that bind to and inhibit activator BH3-only proteins in the absence of apoptosis. Liberated activator BH3-only proteins directly bind and activate Bax/Bak and lead to their oligomerization. In the displacement model, the BH3-only proteins do not directly impact on the effector proteins Bax/Bak. Here, Bax/Bak can be activated through BH3-only proteins mediated neutralization of anti-apoptotic Bcl-2 like proteins.

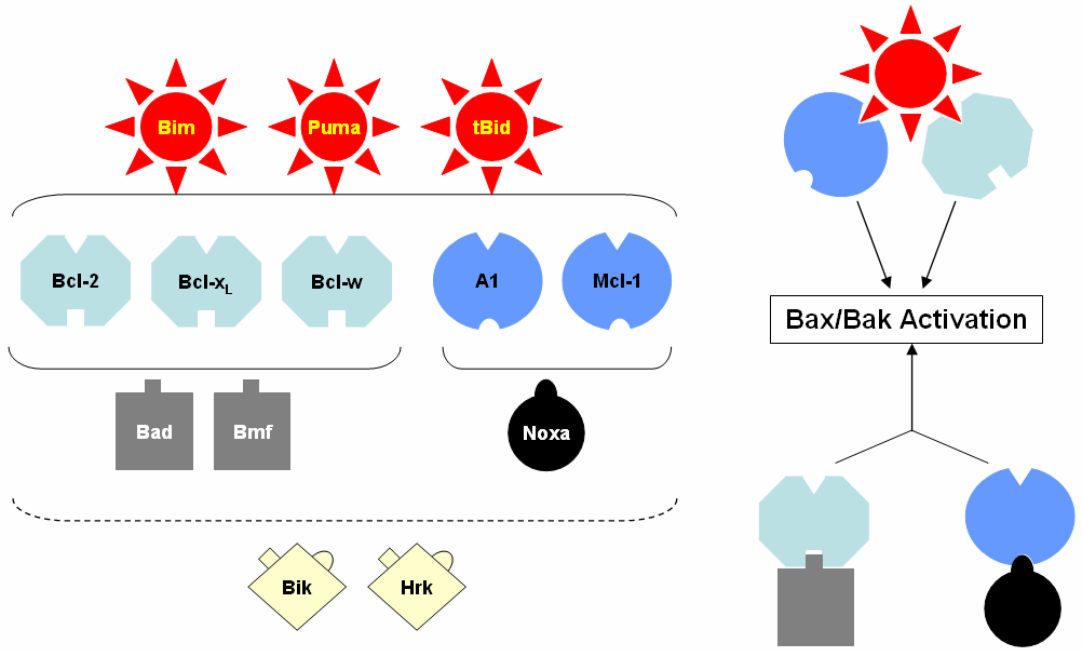

Fig. 1.3 Interaction between Bcl-2 family proteins in the displacement model

Although the potent BH3-only proteins (Bim, Puma and tBid) can bind to all of the Bcl-2 like proteins, some of the BH3-only proteins only selectively bind to a subgroup of the anti-apoptotic Bcl-2 like proteins. For example, Noxa prefers binding to Mcl-1 and A1, while Bad and Bmf only bind to Bcl-2, Bcl-w and Bcl-x_L. The selective BH3-only proteins are weak killers, possibly because of the need for co-operation to overcome the entire anti-apoptotic effects of Bcl-2 like proteins. Not one but several week killers play together to achieve Bax/Bak activation. The nonselective BH3-only proteins, such as Bim, Puma and tBid, are more potent killers, because of their strong association with most of the anti-apoptotic Bcl-2 like proteins. Activation of a single potent killer can cause Bax/Bak activation without involvement of another BH3-only protein.

1.1.3 Regulation and function of BH3-only proteins

The BH3-only proteins share a short region, the so called BH3 domain, and this family has 8 members at present (Bim, Puma, Bad, Bid, Noxa, Bmf, Bik and Hrk) (Fig. 1.4). These proteins initiate pro-apoptotic signals upstream of mitochondria. They are induced or activated in response to various stress

stimuli or intracellular signals. In most cases, one or several BH3-only proteins are probably involved in a given situation of apoptosis.

Bim has 3 isoforms, Bim_{EL}, Bim_L and Bim_S. Bim_{EL} and Bim_L are perhaps sequestered with microtubules through their association with dynein light chain 1 in non-apoptotic cells, and translocate to mitochondria following release upon activation, although this is still uncertain (Puthalakath et al., 1999). Bim_S is the most potent killer among the 3 isoforms (O'Connor et al., 1998). It has been also reported that Bim undergoes transcriptional regulation as well as JNK and ERK mediated phosphorylation (Hacker et al., 2006; Kirschnek et al., 2005; Ley et al., 2005). Bim plays an important role in response to UV irradiation as well as to several chemotherapeutic drugs and is involved in maintaining stable T and B cell populations (Bauer et al., 2006; Enders et al., 2003; Erlacher et al., 2005; Hildeman et al., 2002; Putcha et al., 2001; Puthalakath et al., 1999).

Puma and Noxa are under transcriptional control by p53 and other factors, indicating their involvement in DNA damage and p53 mediated apoptosis (Villunger et al., 2003). Little is known about post-translational modifications of Puma and Noxa. It was reported recently that both are involved in cell death of activated T cells (Bauer et al., 2006). Noxa is also up-regulated in response to TLR stimulation or viral infections (Sun and Leaman, 2005), and is induced upon proteasome inhibition (Qin et al., 2005).

As discussed earlier, in type I cells, death signals that are initiated at the cell surface directly (i.e. without mitochondrial contribution) activate effector caspase-3 via activated caspase-8 to induce cell death. In type II cells, activated caspase-8 cleaves the BH3-only protein Bid that activates the mitochondrial pathway, rather than direct activating caspase-3. Loss of Bid leads to resistance of hepatocytes (type II cells) to apoptosis induced by Fas signaling (Luo et al., 1998; Yin et al., 1999). Bid is therefore important in death receptor pathway mediated apoptosis in type II cells, and is regulated by proteolytic cleavage.

In resting cells, Bad is phosphorylated on several serines (Bergmann, 2002), and probably activated by dephosphorylation. Studies using Bad knock-out mice revealed that Bad $^{-/-}$ cells are partially resistant to apoptosis induced by serum withdrawal and growth factor starvation (Ranger et al., 2003).

It is not well understood how the other BH3-only proteins regulate cell survival and death. Bmf may be regulated by release from its sequestion site at the myosin V actin motor complex (Puthalakath et al., 2001). Bik and Hrk are possibly regulated at the transcription level with no remarkable phenotype in single knock-out mice. Interestingly, the combined loss of Bim and Bik can arrest spermatogenesis (Coultas et al., 2005). More double knock-out mice will have to be generated to understand more fully the roles of BH3-only proteins *in vivo*.

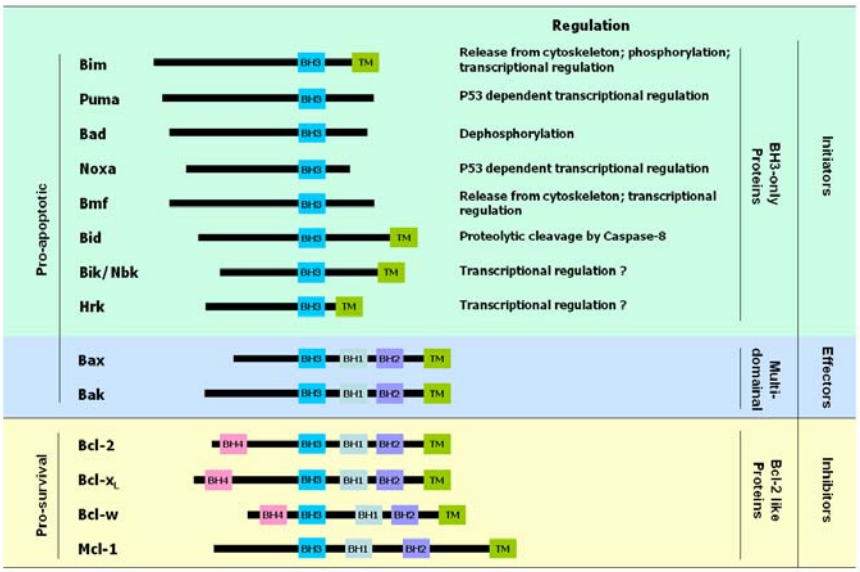

Fig. 1.4 Domain structure of BH3-only proteins

Bcl-2 family proteins are clustered into three groups, based on both structure and function. The anti-apoptotic proteins usually contain all four Bcl-2 homology (BH) domains, except for Mcl-1 that does not have a BH4 domain. Pro-apoptotic Bax and Bak (also known as multi-domain proteins) contain BH-domains 1, 2 and 3, which mediate cytochrome *c* release from mitochondria. Mitochondrial apoptosis is initiated by activation of one or several BH3-only proteins, which share only the BH3-domain. The known or suggested mechanisms for activating BH3-only proteins are indicated.

1.1.4 Mitochondrial events during apoptosis

Two questions are relevant to the involvement of mitochondria during apoptosis. First, what is released from mitochondria during apoptosis and is involved in triggering apoptosis? Some proteins may just be found released due to massive damage of mitochondria membrane after cell death, but play no role in the pro-apoptotic signal transduction. Second, how are those proteins released, by a specific channel, or through loss of mitochondrial outer membrane potential (MOMP)? Are they all released by a same mechanism?

A number of mitochondria-based intermembrane proteins have been described to be translocated to the cytosol or to the nucleus during cell death. These proteins are engaged in different roles to facilitate transduction of apoptotic signaling (Fig. 1.5). As mentioned above, cytochrome *c* release from mitochondria plays a central role in mitochondrial apoptosis. Following release, cytochrome *c* forms a complex called apoptosome, together with caspase-9 and Apaf-1 (Li et al., 1997). The apoptosome in turn activates effector caspase-3, which transmits the signal for instance into the nucleus where the typical apoptotic nuclear morphological changes are observed. However, several other mitochondrial proteins have also been found to be released. Smac/Diablo is released to liberate Inhibitor of Apoptosis Proteins (IAP) from caspases (Chai et al., 2000; Wu et al., 2000). IAPs are reported to bind caspases and inhibit their activation (Deveraux et al., 1997). Thus, Smac may liberate caspases from their inhibitors and facilitate their activation. AIF and EndoG are another two proteins that are released from mitochondria, and are translocated to the nucleus, where some cleavage of DNA may be achieved by those factors in the absence of caspase activation, even though the exact role of these proteins in apoptosis remains under discussion (Arnoult et al., 2003a; Arnoult et al., 2003b; Daugas et al., 2000; Ekert and Vaux, 2005; Li et al., 2001; Susin et al., 1996). Deletion of either AIF or EndoG did not protect the cells from undergoing apoptosis, but caused a mitochondria-related dysfunctional phenotype (Irvine et al., 2005; Vahsen et al., 2004).

It has been under debate for years how proteins are released from mitochondria. One hypothesis is that they are secreted due to the collapse of the mitochondrial membrane potential. Others suggest that those molecules are secreted into the cytosol through a specific channel that is formed by Bax/Bak at the mitochondrial outer membrane. Before clear evidence is provided for the latter hypothesis, some observations argue clearly against the view that mitochondria contained pro-apoptotic proteins are released due to loss of mitochondrial membrane potential. First, caspases are found to cause mitochondria membrane potential loss *in vitro* (Ricci et al., 2003). In addition, loss of caspase-9 or inhibition of caspases by chemical inhibitors can prevent the collapse of the mitochondrial membrane to some extent (Ricci et al., 2003; Samraj et al., 2006). It has been widely accepted that caspase activation is achieved by cytochrome *c*-facilitated formation of the apoptosome (Liu et al., 1996). Loss of Bax/Bak can block cytochrome *c* release as well as caspase activation (Wei et al., 2001). Hence, apoptotic signals initiated from mitochondria, for instance cytochrome *c* release, proceed before massive damage of the mitochondrial membrane occurs. There may be an amplification feedback loop following caspase activation to further damage mitochondria. It has to be further investigated if some of the pro-apoptotic mitochondrial intermembrane space proteins are released after caspases mediated mitochondrial damage at the later stage of apoptosis, which eventually lead to complete cell death. Recent studies demonstrated that cytochrome *c* and Smac were released to the cytosol nearly the same minute, whereas AIF was following some hours later before loss of mitochondrial outer membrane potential became detectable (Munoz-Pinedo et al., 2006). It is thus conceivable that apoptosis is initiated by release of some of the mitochondrial pro-apoptotic proteins (i.e. cytochrome *c*), which leads to caspase activation. Activated caspases may cause mitochondrial membrane degradation and further release numerous mitochondrial proteins, possibly including AIF.

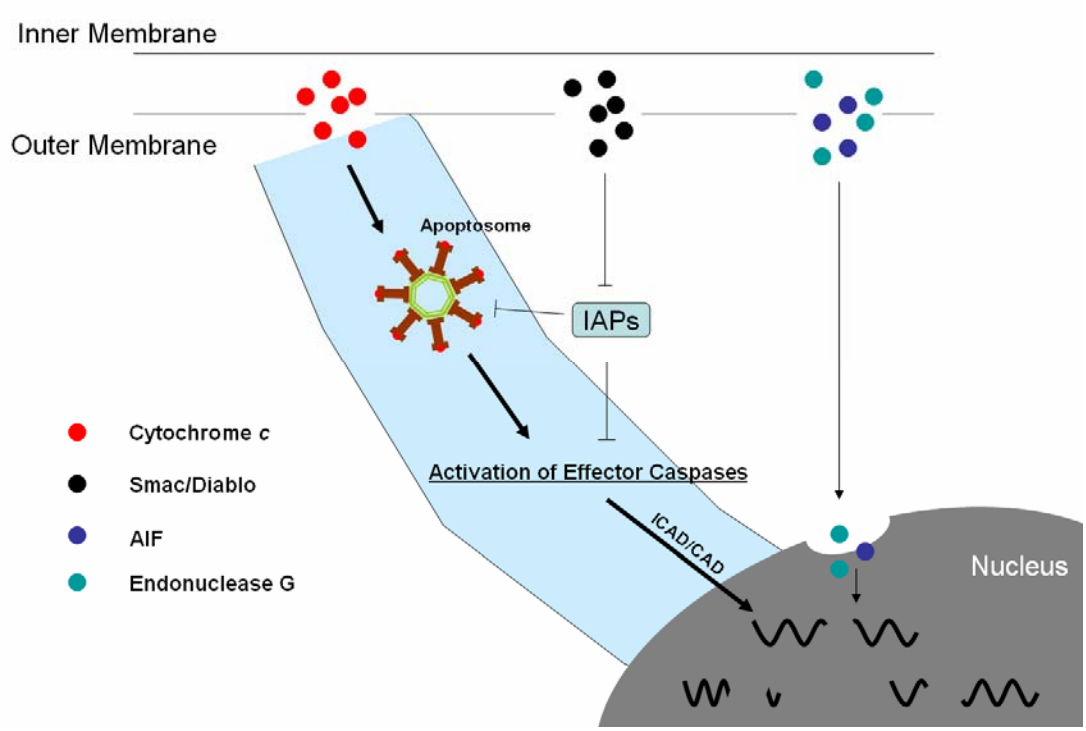

Fig. 1.5 Release of apoptotic proteins from mitochondria

A number of mitochondrial proteins, such as cytochrome *c*, Smac/Diablo, AIF, endonuclease G and HtrA2/Omi, have been found to be released from the mitochondrial intermembrane space during the process of apoptosis. Some possible pro-apoptotic actions of these factors are shown.

1.1.5 Caspase family proteins and their role in apoptosis

Homologous to the *C. elegans* programmed cell death regulator Ced-3, caspases are a family of cysteine proteases. Caspases and their homologous proteins have been found in distant species, including plants, fungi and eukaryotes (Boyce et al., 2004). It is clear that caspases are playing a key role in most cases of apoptosis regulation. Caspase activation is usually described as a signaling cascade, with 'initiator' caspases activating 'effector' caspases. In the mitochondrial apoptosis signaling pathway, caspase-9 is activated and recruited into a so called apoptosome complex that contains two other pro-apoptotic proteins, cytochrome *c* and Apaf-1. Effector caspases (caspase-3, 6 and 7) may be activated either by the apoptosome in mitochondria-mediated apoptosis, or by the initiator caspase-8 following death receptor signaling in

type I cells (Scaffidi et al., 1999). As discussed above, caspase-8 can also indirectly activate effector caspases via a mitochondrial amplification loop in type II cells (Scaffidi et al., 1999), where signals are transduced through BH3-only protein Bid (Li et al., 1998). Effector caspases are required for the activation of the CAD/DFF40 mediated internucleosomal DNA fragmentation (Tang and Kidd, 1998).

Besides their classical roles in apoptosis, other important roles of individual caspases outside apoptosis have been found. Caspase-1 was identified as an IL-1β processing enzyme (Cerretti et al., 1992; Thornberry et al., 1992). Deletion of caspase-1 prevents processing and secretion of the proinflammatory cytokines IL-1β and IL-18, as well as LPS-induced septic shock (Ghayur et al., 1997; Kuida et al., 1995; Li et al., 1995). Caspase-11 $^{-/-}$ mice show resistance to endotoxic shock, and lack of caspase-1 activation and IL-1β secretion, indicating that caspase-11 may work upstream of caspase-1 (Wang et al., 1996). Mice lacking caspase-2 are viable and caspase-2 deficient cells are resistant to only a limited range of apoptotic stimuli (Bergeron et al., 1998). The caspase-9 pathway may have a compensatory function in caspase-2 $^{-/-}$ cells (Troy et al., 2001). A recent study demonstrated that caspase-2 may be regulated by activation of PIDD (p53-induced protein with a death domain) (Tinel et al., 2007). Caspase-12 is found at the ER and may be responsible for ER stress-induced apoptosis (Nakagawa et al., 2000). The function of caspase-14 has been suggested to be unrelated to cell death. Besides its clear role in death receptor mediated cell death, caspase-8 has been shown to substitute for caspase-3 function in caspase-3 $^{-/-}$ mice in a mouse model of stroke by middle cerebral artery occlusion (Le et al., 2002). Highly homologous to caspase-8, caspase-10 is also playing a role in the death receptor pathway at least in some cell types *in vivo*, even though the clear conclusion *in vitro* has yet to be established (Kischkel et al., 2001; Sprick et al., 2002; Wang et al., 2001). Due to the important role of caspase-9 in mitochondrial signaling amplification and in the formation of the apoptosome, caspase-9 $^{-/-}$ cells are resistant to a variety of apoptotic stimuli (Yoshida et al., 1998). Although caspase-3 has been relatively well understood to cause CAD activation, the roles of other effector caspases, i.e. caspase 6 and 7, remain unclear.

Compensation mechanisms between different caspases may also exist (Le et al., 2002; Troy et al., 2001; Zheng et al., 2000).

Although caspases are clearly involved in apoptosis regulation, a number of studies pointed out that cell death may also take place in the absence of caspases or of caspase activation (Lee et al., 2006; Oppermann et al., 2005). Inhibition of caspases can block the typical apoptotic biochemical changes, such as membrane blebbing, DNA degradation and nuclear fragmentation, but frequently direct the cells to non-apoptotic cell death (Vandenabeele et al., 2006).

1.2 Chlamydia

1.2.1 Chlamydial infection and Chlamydia related diseases

Chlamydiae are intracellular bacteria that cause a wide range of diseases in different organisms, including humans and mice. Chlamydia leads to a variety of serious human diseases. *Chlamydia trachomatis* is the most common bacterial pathogen that causes sexually transmitted disease (STD): 4.19 % in young adults in the United States have been tested positive for *Chlamydia trachomatis* in one study (Miller et al., 2004). *Chlamydia trachomatis* also causes trachoma, which represents the leading reason for preventable blindness, accounting for more than 15 % of blindness cases worldwide (Thylefors et al., 1995). *Chlamydia pneumoniae* leads to airway infection, causing approximately 10 % of community-acquired pneumonia (Kuo et al., 1995), and has been found in atherosclerotic plagues (Shi and Tokunaga, 2004). However, whether *Chlamydia pneumoniae* infection is a risk factor for atherosclerosis is still under discussion (de Kruif et al., 2005).

Different species of Chlamydia share a special and unique intracellular lifestyle (Belland et al., 2004). An entire infection cycle is completed by two separate forms of Chlamydia, the elementary bodies (EBs) and the reticulate bodies (RBs). EBs are infectious but metabolically inert, that means one single EB can never divide or proliferate into two before it enters a host cell and is converted to an RB. In contrast, RBs are metabolically active but non-infectious. Therefore, Chlamydia infection is considered to start at the time that EBs are internalized into host cells, such as epithelial cells, macrophages, dendritic cells (DCs) and fibroblasts, via small endosome-like vacuoles, which are dramatically enlarged during the infection process and fail to fuse to lysosomes. Converted from EBs in the vacuole, RBs proliferate in the same inclusion, and after a period of time condense back to EBs. The re-differentiated EBs are at later time point released from the infected cells and promote a next round of infection, spreading Chlamydia into the neighbouring

tissues. Interruption of the above procedure may break the chlamydial infection cycle and block bacterial propagation.

1.2.2 Inhibition of apoptosis during Chlamydia infection

It has been shown several times that Chlamydia infection leads to resistance of the host cell against various experimental apoptotic stimuli, since the first observation was published in 1998 (Fan et al., 1998). Several possible mechanisms (Fig. 1.6) have been suggested to play a role in preventing Chlamydia-infected cells from premature apoptosis.

1.2.2.1 Targeting mitochondrial signals for inhibition of apoptosis

The first observation of protection against apoptosis was published in 1998 (Fan et al., 1998). Zhong and his colleagues demonstrated that apoptosis induced by several stimuli is blocked in *Chlamydia trachomatis* infected cells and that this protection involves inhibition of caspase activation and blockage of cytochrome *c* release from mitochondria. Not surprisingly, *Chlamydia pneumoniae* was soon afterwards found to have a similar effect (Fischer et al., 2001; Rajalingam et al., 2001). The apoptosis inhibition mechanism during infection was further elucidated by evidence that death receptor-initiated apoptosis was not inhibited in type I cells, where mitochondria do not play a role in apoptotic signaling and effector caspase-3 is directly activated by caspase-8. However, apoptosis inhibition was seen in Chlamydia infected-type II cells, where CD95 receptor-initiated apoptotic signaling requires a mitochondrial amplification loop (Fischer et al., 2004a). It is therefore plausible that Chlamydia impacts on or upstream of the mitochondrial step to achieve apoptosis inhibition. As discussed earlier, the release of cytochrome *c* from mitochondria is governed by Bcl-2 family proteins, where the activation of Bax and/or Bak is followed by cytochrome *c* release. Thus, it was also demonstrated that Bax and Bak activation by extrinsic stimuli is blocked during chlamydial infection (Fischer et al., 2004b; Xiao et al., 2004). Activation of Bax and/or Bak is initiated by the pro-apoptotic class of BH3-only proteins and the anti-apoptotic class of Bcl-2 like proteins (see above for a more detailed

discussion). We then investigated these pro- and anti- apoptotic players during Chlamydia infection. One major part in the project of this thesis was the attempt to identify the basic chlamydial anti-apoptotic mechanism.

1.2.2.2 Recruitment of pro-apoptotic molecules by the chlamydial inclusion vacuole

Ojcius and his colleagues found that infection with *Chlamydia trachomatis* leads to activation of PI3 kinase (PI3K) (Verbeke et al., 2006). PI3K activation causes Akt activation that leads to phosphorylation of the pro-apoptotic BH3-only protein Bad (Datta et al., 1997; del Peso et al., 1997). As discussed above, Bad is regulated and activated by dephosphorylation of the protein. Bad functions at mitochondria, where it can lead to Bax/Bak activation and cytochrome *c* release (Chen et al., 2005). In Chlamydia-infected cells, phosphorylated Bad is unexpectedly found localized to the chlamydial inclusion membrane, via interaction with 14-3-3β that is recruited by the Chlamydia-encoded protein IncG (Scidmore and Hackstadt, 2001). Removed away from its traditional functioning site at mitochondria, phosphorylated Bad is inactive and even associated with the Chlamydia inclusion, where Bad is not able any more to induce apoptosis. It is interesting that only the *Chlamydia trachomatis* inclusion recruits Bad but not *Chlamydia pneumoniae*, which does not encode IncG. In addition, inhibition of PI3K sensitizes *Chlamydia trachomatis* infected cells to staurosporine-induced apoptosis but has no effects on cells infected with *Chlamydia pneumoniae*. Besides pro-apoptotic Bad, another pro-apoptotic molecule, protein kinase Cδ (PKCδ), is also observed at the chlamydial inclusion membrane (Tse et al., 2005). As a member of the protein kinase C family, PKCδ accumulates at mitochondria during apoptosis induction, where cytochrome *c* is released (Majumder et al., 2000). Like Bad, PKCδ loses its accession to the conventional functional site at mitochondria.

1.2.2.3 Roles of NF-κB during Chlamydia infection

Several anti-apoptotic proteins, including the IAP family protein cIAP2, have been described to be up-regulated during Chlamydia infection (Paland et al., 2006; Rajalingam et al., 2006). Rudel's group postulated that IAP family members form a complex, the so called IAPsome, in which individual IAPs (cIAP1, cIAP2 and XIAP) may stabilize each other. Knock-down of cIAP1, cIAP2 or XIAP, using siRNA technology, can to some extent reverse the resistance of Chlamydia-infected cells to staurosporine-induced apoptosis (Rajalingam et al., 2006). IAP family proteins, especially cIAP2, are regulated through an NF-κB dependent pathway (Chu et al., 1997; Stehlik et al., 1998; Wang et al., 1998). IAPs have been suggested to bind to caspases and inhibit the activity of this group of proteases (Deveraux et al., 1997; Roy et al., 1997). However, recent findings indicate that human cIAP-1 and cIAP-2 bind but are not able to inhibit caspases (Eckelman and Salvesen, 2006; Huh et al., 2006). Further pieces of evidence have shown that probably only XIAP is a direct caspase inhibitor (Eckelman et al., 2006). The anti-apoptotic effects of IAP family proteins therefore remain to be further investigated.

A number of transcription factors make up the NF-κB family. Members of this family regulate expression of many genes during immune responses. Despite an unclear molecular basis, NF-κB has been proposed to be an anti-apoptotic player, up-regulating a set of anti-apoptotic genes. Although the exact molecular mechanism of NF-κB induced apoptosis inhibition has yet to be determined, at least massive liver apoptosis has been seen in mice deficient in the NF-κB subunit RelA (Beg et al., 1995). Up-regulation of NF-κB has been reported a number of times during infection of monocytes, endothelial cells, macrophages and DC with different strains of Chlamydia (Kol et al., 1999; Prebeck et al., 2001; Wahl et al., 2001). Thus, investigating NF-κB is relevant for the study of Chlamydia anti-apoptotic mechanisms. However, no evidence has been found that NF-κB is activated in infected epithelial cells (Fischer et al., 2001; Xiao et al., 2005), a common chlamydial infection target that is widely used for apoptosis inhibition experiments. This indicates that NF-κB regulation is cell type dependent and may be present in cells that express Toll

like receptors (TLRs, a group of proteins that recognizes various components from infectious agents and initiates innate immune response). Thus, activation of NF-κB during Chlamydia infection may be a result of the recognition of chlamydial components, for example chlamydial HSP60 or LPS, by TLR2 and TLR4. Xiao et al. demonstrated that Chlamydia-infected MEF cells deficient for NF-κB p65 are still resistant to apoptosis (Xiao et al., 2005). To conclude, NF-κB may play a role to an extent in some cell types, but is likely not involved in the potent anti-apoptotic events that are observed in Chlamydia infections.

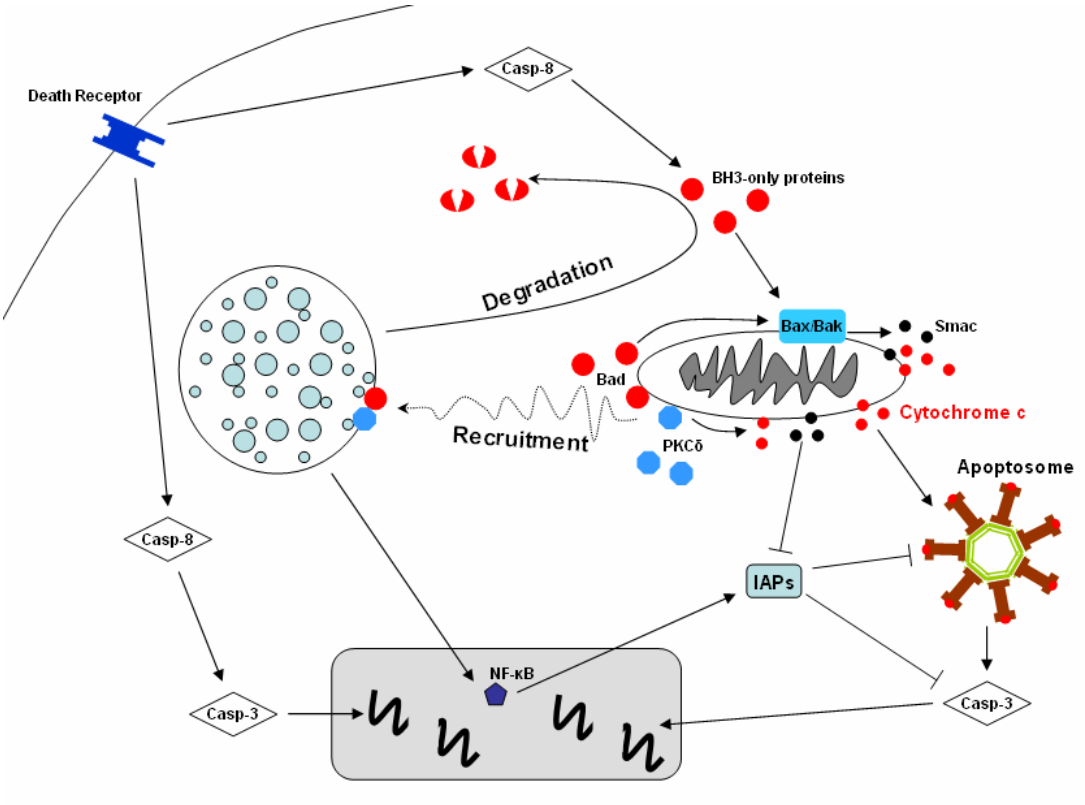

Fig. 1.6 Interference with apoptotic signaling during Chlamydia infection

several mechanisms have been suggested to lead to inhibition of apoptosis in host cells against a wide range of apoptotic stimuli. Pro-apoptotic BH3-only proteins, which during apoptosis cause Bax activation and cytochrome *c* release, are degraded. The Chlamydia vacuole recruits pro-apoptotic molecules, such as PKCδ and Bad, away from their conventional functioning positions. cIAP-2 is up-regulated following activation of transcriptional factor NFκB.

1.2.3 Induction of cell death during Chlamydia infection

Although many studies have demonstrated apoptosis inhibition during Chlamydia infection as described above, apoptosis induction has been also reported from time to time in Chlamydia infected cells. In fact, many pathogens maintain both anti- and pro-apoptotic activities. For example, wild type modified vaccinia virus (MVA) blocks apoptosis in infectious situations in vitro. However, it induces apoptosis when an anti-apoptotic gene F1L is knocked out from the virus (Fischer et al., 2006). In natural infection cases, apoptosis induction quality may thus be hidden due to a strong anti-apoptotic function carried by the same microbes. This is particularly important for intracellular bacteria or viruses to prevent infected cell death at an early stage of infection, and to obtain time and space for growth. Both anti- and pro-apoptotic effects may be generated at different stages of infection to favour and complete the special chlamydial intracellular infectious life cycle. Inhibition of apoptosis may be required to prevent death of cells containing premature Chlamydia, while induction of cell death at the end of infection could be a means to trigger or enhance release of EBs into the intercellular space and therefore propagate infection.

Cell death during Chlamydia infection was reported almost 30 years ago, showing massive damage of cellular organelles (Chang and Moulder, 1978). Since then, several forms of cell death have been described by different groups, including apoptosis, necrosis, and a form named aponecrosis with features of both apoptosis and necrosis (Dumrese et al., 2005; Gibellini et al., 1998; Jendro et al., 2000; Jungas et al., 2004; Ojcius et al., 1998; Perfettini et al., 2003). The major evidence of cell death induction by Chlamydia is based on the appearance of nuclear morphological changes or nuclear damage during infection. However, no caspase activation has ever been observed in any Chlamydia-infected cells (Ojcius et al., 1998; Perfettini et al., 2002). It is important to note that loss of Bax or over-expression of Bcl-2 can partly revert the cell death ratio in infected cells (Perfettini et al., 2003; Perfettini et al., 2002). Phosphatidylserine (PS) exposure is also found in some cases during Chlamydia induced cell death. Besides those apoptotic features, the pro-

inflammatory mediator HMGB1 is found to be released during infection, which is normally taking place in necrosis (Jungas et al., 2004). A chlamydial hypothetical protein called CADD, possibly interacting with death receptors, has been implicated to induce cell death with typical apoptotic features involving caspas activation (Schwarzenbacher et al., 2004; Stenner-Liewen et al., 2002). However, caspase activation has never been observed during Chlamydia infection. The exact role of CADD *in vivo* therefore remains unclear. Based on the mystery of controversial results and conclusions, a set of well established standard assays are applied in this project to further study the molecular process of cell death during Chlamydia infection.

1.2.4 Cell survival and its impact on the immune system

Modulation of host cell survival or death has been proposed for many microbes to create a better environment for their intracellular life, or for the host to eliminate infection. Numerous viruses encode viral Bcl-2 like proteins (Henderson et al., 1993; Sarid et al., 1997) or Bcl-2 like structurally similar proteins (Aoyagi et al., 2006; Kvansakul et al., 2007) to prevent infected cell death. Cytomegalovirus restricts cross-species infection by apoptosis induction (Jurak and Brune, 2006). Induction of apoptosis by Mycobacteria involves a mechanism that mediates crosspriming of CD8 T cells *in vivo* (Winau et al., 2006). Without exception, host cell survival or death will directly decide the outcome of chlamydial infection.

Prevention of premature host cell death will help providing space for the pathogen to replicate, and to avoid early recognition by phagocytes. This is important because cytotoxic T lymphocytes can destroy Chlamydia-infected cells (Fling et al., 2001; Gervassi et al., 2004). Inhibition of apoptosis may also be utilized in another way. Prolongation of life of infected granulocytes may lead to a longer and more persistent immune response against the pathogen or may lead to damage of host tissues (van Zandbergen et al., 2004).

In contrast, cells containing a large vacuole face stress at least from limited physical space, even though it is still unclear how Chlamydia-infected cells

undergo cell death at the end of an infection cycle. Death at the end may be an efficient approach to open the plasma membrane to release reconstituted EBs for propagating the infection into neighbouring tissues and could also be initiating or promoting an immune response. Different forms of cell death of infected cells frequently lead to different results of the infection (Fig. 1.7). Apoptosis generates apoptotic vesicles that wrap up pathogens, and exert ‘eat me’ signals that will be recognized by antigen presenting cells, followed by digestion of apoptotic bodies. Although contrast models have been reported, the general perception is still that apoptosis will not but necrosis will promote or initiate an immune response against antigens contained in the dead cells.

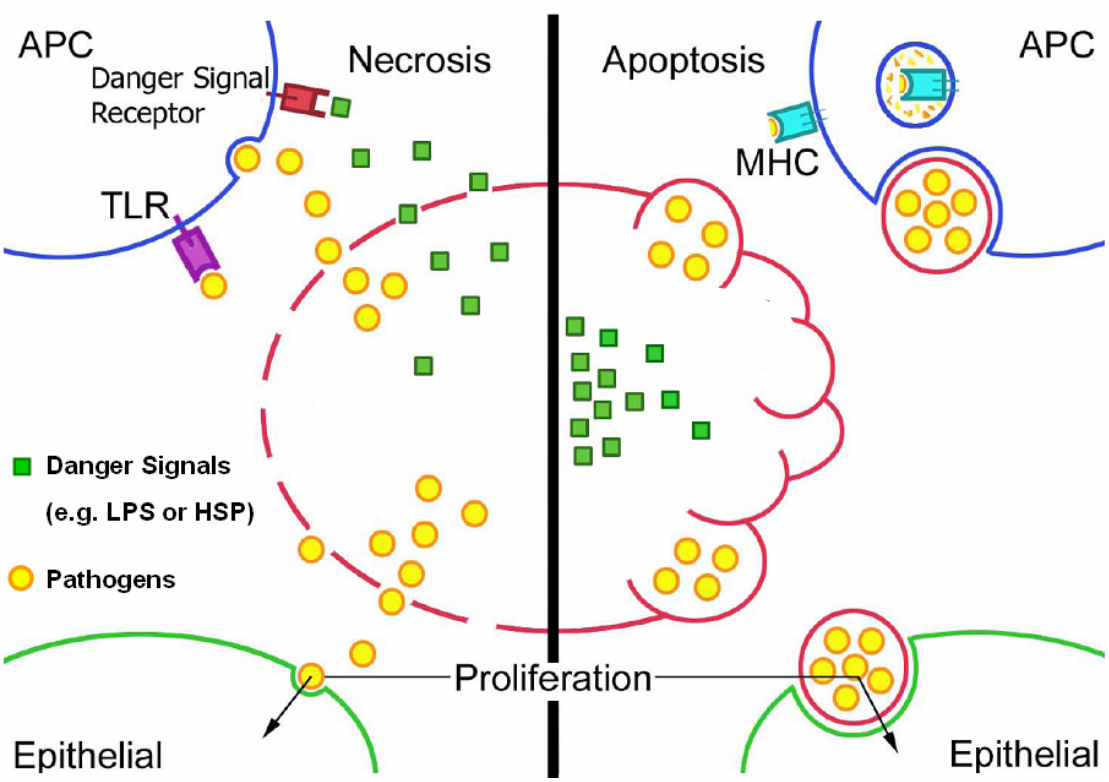

Fig. 1.7 Possible outcome of various forms of cell death in infection

Infection by intracellular pathogens can lead to different forms of host cell death, most likely a combination of apoptosis and necrosis. Necrosis leads to massive release of pathogenic components that can be recognized by Toll-like receptors (TLR) on antigen-presenting cells (APC) and trigger an innate immune response. Danger signals (DS) such as ATP and uric acid are also released during necrosis, followed by binding to danger signal receptors (DSR) on APC, which leads to maturation of dendritic cells (DC) and secretion of pro-inflammatory cytokines. In contrast, cells dying through apoptosis release apoptotic bodies that contain viable pathogens or pathogen-derived antigens. APC that ingest the apoptotic bodies can process microbial antigens and present them on their surface via major histocompatibility complex (MHC) molecules, thus stimulating the adaptive immune system. Viable pathogens in apoptotic bodies can also be internalized by new host cells, and thereby perhaps contribute to propagation of the infection.

1.3 Aim of the study

Different strains of Chlamydia cause severe human diseases. Although it is known that Chlamydia interfere with the host cell death program, controversial results have been reported in different situations of infection, especially infection of different cell hosts with different strains of bacteria. The molecular mechanism of apoptosis inhibition or cell death induction during infection and their physiological role also remain unclear.

This study was designed for better understanding of the molecular mechanisms of apoptosis inhibition and cell death induction throughout Chlamydia infection, as well as for assessing their possible physiological roles *in vivo*.

Previous studies showed that Chlamydia targets mitochondria to induce potent anti-apoptotic effect. In this study, we analyzed expression of Bcl-2 family proteins in Chlamydia-infected cells. A cell line with inducible expression of Bim_S was also used in infection experiments to assess the integrity of downstream apoptosis pathway, and the physiological role of apoptosis inhibition in infection.

To better understand cell death induction by Chlamydia, various cell lines were infected for measurement of a number of standard apoptosis markers (i.e. nuclear morphological changes, caspase activation, cytochrome *c* release etc.). Cells lacking Bax, Bak or both were analyzed. *In vitro* phagocytosis was tested by co-incubation of Chlamydia-infected cells and phagocytes.

2. Material and methods

2.1 Material

2.1.1 Cell lines

Cell lines	Features	Origin	Source/Donor
HEp2 cells	Laryngeal carcinoma cell line	Human	ATCC (Manassas, USA)
HeLa 229 cells	Cervical adenocarcinoma cell line	Human	ATCC
HeLa-fas cells	HeLa cells stably expressing Fas receptor	Human	Dr. H. Wajant (Stuttgart, Germany)
HeLa T-REx cells	HeLa cells stably expressing tetracycline-repressor	Human	Invitrogen (Karlsruhe, Germany)
HeLa T-REx Noxa cells	HeLa T-REx cells with Noxa stably transfected	Human	Dr. B. Seiffert (Munich, Germany)
HeLa T-REx Bim_{EL} cells	HeLa T-REx cells with Bim_{EL} stably transfected	Human	Dr. B. Seiffert
HeLa T-REx Bim_S cells	HeLa T-REx cells with Bim_S stably transfected	Human	Dr. B. Seiffert
HeLa T-REx Puma cells	HeLa T-REx cells with Puma stably transfected	Human	Dr. B. Seiffert
Jurkat cells	T lymphocyte cell line	Human	ATCC
Raw264.7 cells	Macrophage cell line	Mouse	ATCC
DC D2SC/1 cells	DC line	Mouse	ATCC
Wild type MEF cells	Embryonic Fibroblasts	Mouse	Dr. D. Huang (Melbourne, Australia)
Bax $^{-/-}$ MEF cells	MEF cells deficient in Bax	Mouse	Dr. D. Huang
Bak $^{-/-}$ MEF cells	MEF cells deficient in Bak	Mouse	Dr. D. Huang
Bax/Bak $^{-/-}$ MEF cells	MEF cells double deficient in Bax and Bak	Mouse	Dr. D. Huang

2.1.2 Chlamydia strains

Strains	Host	Related Diseases	Source/Donor
Chlamydia trachomatis (L2)	Human	Sexually transmitted Disease Trachoma, blindness	ATCC
Chlamydia muridarum	Mouse	Pneumonitis	Dr. Roger Rank (Little Rock, USA)

2.1.3 Buffer and Solutions

Buffers and solutions were prepared using Millipore Q-distilled water. Chemicals were purchased from Sigma (Deisenhofen, Germany) or Roth (Karlsruhe, Germany), unless indicated otherwise.

PBS		Dulbecco's PBS (1x) (PAA, Pasching, Germany)
TAE	40 mM 1 mM pH 8.3	Tris-acetate EDTA
6x Loading buffer (agarose gel)	1 g/l 20 mM 15 % (v/v) pH 8.5	Orange G Tris(hydroxymethyl)aminomethane Glycerol
Laemmli buffer	2.9 g/l 14.4 g/l 1 g/l pH 8.3	Tris(hydroxymethyl)aminomethane Glycine SDS

Lower gel buffer	182 g/l	Tris(hydroxymethyl)aminomethane
(LGB, Separating gel)	4 g/l	SDS
	pH 8.8	
Upper gel buffer	60 g/l	Tris(hydroxymethyl)aminomethane
(UGB, Stacking gel)	4 g/l	SDS
	pH 6.8	
Running buffer	6 g/l	Tris(hydroxymethyl)aminomethane
(SDS page)	28.8 g/l	Glycine
	1 g/l	SDS
10x Tris/Glycine buffer	30.3 g/l	Tris(hydroxymethyl)aminomethane
	144 g/l	Glycine
Blotting buffer	20 % (v/v)	Methanol
	10 % (v/v)	10x Tris/Glycine buffer
TBS	8 g/l	NaCl
	2.42 g/l	Tris(hydroxymethyl)aminomethane
	pH 7.6	
TBST	1x	TBS
	0.05 % (v/v)	Tween 20
Blocking buffer	1x	TBST
	50 g/l	Nonfat-milkpowder
Fixation buffer	4 %	Paraformaldehyde

Lysis buffer	50 mM	PIPES
	50 mM	HEPES
	1 mM	EDTA
	2 mM	$MgCl_2$
	10 mM	DTT
	1x	Protease inhibitors (Roche)
	1 %	Triton X-100
Permeabilization buffer	1x	PBS
(for confocal)	5 g/l	BSA
	0.1 %	Triton X-100
(for FACS)	1x	PBS
	10 g/l	BSA
	10 g/l	Saponin
Blocking buffer	1x	PBS
(for FACS	3 %	BSA
	0.1 %	Triton X-100
Washing buffer	1x	PBS
(for confocal)	5 g/l	BSA
Washing buffer	1x	PBS
(for FACS)	10 g/l	BSA
NP-40 lysis buffer	150 mM	NaCl
	1 %	Ipegal CA-630
	50 mM	Tris
	pH 8.0	

DEVD assay buffer	50 mM	NaCl
	2 mM	$MgCl_2$
	40 mM	B-glycerophosphate
	5 mM	EGTA
	0.1 %	CHAPS
	0.1 g/l	BSA
	10 mM	HEPES
	10 µM	DEVD AMC fluorimetric substrate
	pH 7.0	

2.1.4 Media

HEp2 cells medium	1x	DMEM (PAA)
	10 % (v/v)	FCS (Biochrom)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
HeLa cells medium	1x	DMEM (PAA)
	10 % (v/v)	FCS (Biochrom)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
HeLa-fas cells medium	1x	DMEM (PAA)
	10 % (v/v)	FCS (Biochrom)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
HeLa T-REx cells	1x	DMEM (PAA)
medium	10 % (v/v)	Tetracycline negative FCS (PAA)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
	5 µg/ml	Blasticidin

Stable HeLa T-REx transfected clones medium (Bim_S, Bim_{EL}, Noxa, Puma)	1x	DMEM (PAA)
	10 % (v/v)	Tetracycline negative FCS (PAA)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
	5 µg/ml	Blasticidin (Invitrogen)
	125 µg/ml	Zeocin (Invitrogen)
Jurkat cells medium	1x	RPMI 1640 (Biochrom)
	10 % (v/v)	FCS (Biochrom)
	1 % (v/v)	Penicilin-Streptomycine (Biochrom)
MEF cells medium	1x	DMEM (PAA)
	10 % (v/v)	FCS (Biochrom)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
Raw264.7 cells medium	1x	RPMI 1640 (Biochrom)
	10 % (v/v)	FCS (PAA)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
DC D2SC/1 dendritic cells medium	1x	RPMI 1640 (Biochrom)
	10 % (v/v)	FCS (PAA)
	10 µg/ml	Gentamicin (PAA)
	50 µg/ml	Vancomycin (Sigma)
Freezing medium	60 % (v/v)	Culture medium
	30 % (v/v)	FCS (PAA/Biochrom)
	10 % (v/v)	DMSO
LB-medium	10 g/l	Bacto-Trypton
	5 g/l	Yeast-extract
	10 g/l	NaCl

Chlamydia transport medium	1x	PBS
	2 %	FCS
	40 µg/ml	Gentamicin (PAA)
	25 %	Sucrose
	(sterile)	
DNA laddering buffer	150 mM	NaCl
	50 mM	Tris-HCl
	0.5 %	SDS
	pH 8.0	

2.1.5 Kit systems

Name	Purpose	Company/Donor
TriFast™	RNA isolation	Peqlab (Erlangen, Germany)
GoTaq	DNA polymerase for PCR	Promega (Madison, USA)
Bio-Rad Protein Assay	Protein concentration measurement	Bio-Rad (Munich, Germany)
TUNEL kit	Detection of fragmented DNA	Promega
PKH26 red fluorescent cell linker mini kit	Cell membrane fluorescence staining	Sigma (Deisenhofen, Germany)
PKH67 green fluorescent cell linker mini kit	Cell membrane fluorescence staining	Sigma
Luminol buffer system	Western blotting development	PerkinElmer
Enliten® ATP assay system	Measuring ATP level	Promega (Waltham, USA)

2.1.6 Antibodies

Name	Clone	Isotype	Source	Company/Donor
B-Actin	Mono-AC-15	IgG1	Mouse	Sigma
Bcl-2	Mono-Bcl-2/100	IgG1	Mouse	BD Pharmingen (Heidelberg, Germany)
Bak	Polyclonal	IgG	Rabbit	Cell Signaling (Danvers, USA)
Bax	Polyclonal	IgG	Rabbit	Cell Signaling
Bad	Polyclonal		Rabbit	Cell Signaling
Bcl-xL	Polyclonal		Rabbit	Cell Signaling
Bid	Polyclonal		Rabbit	Cell Signaling
Bim	Polyclonal		Rabbit	Sigma
Bim	Polyclonal		Rabbit	Stressgen (Ann Arbor, USA)
Bmf	Mono-17A9	IgG2aκ	Rat	Dr. A Strasser (Melbourne, Australia)
Active caspase-3	Mono-C92-605		Rabbit	BD Pharmingen
caspase-9	Polyclonal		Rabbit	Cell Signaling
FITC-conjugated Chlamydial LPS	Mono-ACI	IgG3	Mouse	Progen (Heidelberg, Germany)
cytochrome *c*	Mono-7H8.2C12	IgG2b	Mouse	BD Pharmingen
caspase-7	Mono-B94-1	IgG1κ	Mouse	BD Pharmingen
Fas	CH-11	IgM	Mouse	Cell Signaling
HSP60 (Chlamydia)	Mono-A57-B9	IgG1	Mouse	Affinity BioReagents (Golden, USA)
Noxa	Mono-114C307.1	IgG1	Mouse	Alexis (Läufelfingen, Switzerland)
Puma	Polyclonal		Rabbit	Prosci (Poway, USA)
Puma	Polyclonal		Rabbit	Sigma
PARP	Polyclonal	IgG	Rabbit	Cell Signaling

Smac/Diablo	Polyclonal	IgG	Rabbit	R&D Systems (Minneapolis, USA)
α-Tubulin	Mono-DM 1A	IgG1	Mouse	Sigma

Secondary Antibody:			
Name	Isotype	Source	Company/Donor
Rat	IgG	Goat	Dianova (Hamburg, Germany)
Mouse	IgG	Goat	Dianova
Rabbit	IgG	Goat	Sigma
FITC-Rabbit	IgG	Goat	Dianova
Cy3-Rabbit	IgG	Goat	Dianova
Cy5-Rabbit	IgG	Donkey	Dianova
FITC-Mouse	IgG	Goat	Dianova
Cy3-Mouse	IgG	Goat	Dianova
Cy3-Mouse	IgG	Donkey	Dianova
Cy5-Mouse	IgG	Donkey	Dianova

2.2 Methods

2.2.1 Cell culture

All cells were cultured in the corresponding medium as described above, and maintained in a humidified incubator (Thermo) at 37 °C with 5 % CO_2. 1 % Trypsin-EDTA was used for splitting the cells upon confluence. Trypsinization was stopped after 5-10 minutes by FCS containing medium and cells were thoroughly resuspended. 10-20 % of cells were transferred into a fresh cell culture plate further cultured in fresh medium.

For long term storage, the same trypsinization procedure was performed to detach cells from cell culture plates. Cells were then pelleted and resuspended in freezing medium, and stored either in liquid nitrogen or in a freezer at -80 °C.

Frozen cells were rapidly thawed at 37 °C and added immediately into pre-warmed medium for re-culture. Cells are usually seeded into plates one night before performing experiments.

2.2.2 Chlamydia infection

Chlamydia trachomatis strain L2 and *Chlamydia muridarum* were grown in HEp2 cells. After 2 days of growing, cells were harvested and broken by 5 minutes vortexing with glass beads. Chlamydia EBs were then washed in PBS and purified by ultracentrifugation at 13,000 rpm. Pelleted Chlamydia EBs were then aliquoted in Chlamydia transport medium and stored at -80 °C until use. For experiments, indicated amount of Chlamydia was added to cells cultured in FCS free medium. FCS was re-added to the medium after 2-4 hours of infection.

2.2.3 Apoptosis induction (UV, Staurosporine, Fas)

2.2.3.1 UV irradiation

Culture medium was removed before cells were exposed to UV light (1600 J/m^2) in a transilluminator box (Stratagene). Medium was immediately re-added after UV irradiation. Apoptosis was measured at indicated times.

2.2.3.2 Staurosporine

Staurosporine was directly added to cultured cells at a final concentration of 1 µM. Apoptotic cell death was measured within 6 hours.

2.2.3.3 Fas ligand

Hela-Fas or Jurkat cells were stimulated by anti-CD95 antibody (Cell signaling) at a final concentration of 100 ng/ml. Apoptosis could be detected after 4 hours.

2.2.3.4 Noxa, Bim_S induction in HeLa T-REx cell lines

In HeLa T-REx stable cell lines (HeLaTRex Bim_S, HeLaTRex Noxa), Bim_S or Noxa expression was induced by inactivation of the Tet suppressor upon addition of 1 µg/ml tetracycline or 100 ng/ml anhydrotetracycline (non-antibiotic).

2.2.4 Transfection (electroporation, PEI, lipofectamine, fugene)

2.2.6.1 Transfection by electroporation

Cells (70-80 % confluence) were plated one day before transfection. 5 million cells were harvested, pelleted by centrifugation, and resuspended in 420 µl DMEM with 25 % FCS. 20 µg of designated vectors were added into the cell solution in an electroporation cuvet (Bio-Rad), followed by electroporation at

960 µFD and 230 volts using an electroporation machine from Bio-Rad. The electroporated cells were incubated at room temperature for 10 minutes before washing with 2 ml of DMEM containing 0.5 % FCS. Cells were finally resuspended in normal culture medium in 6 well plates until experiments.

2.2.4.2 Poly(ethylenimine) (PEI) transfection

Cells (70-80 % confluence) were seeded one day before in 24 well cell culture plates. On the next day, cells were washed with PBS and incubated in FCS-free medium immediately before transfection. About 3.5 µg DNA was prepared in 150 µl PBS for each sample and incubated at room temperature for 10 minutes after vortexing. In the meantime, 0.9 mg/ml PEI was prepared in water, from which 7.5 µl PEI solution was added to the vectors in PBS. Samples were vortexed and incubated at room temperature for another 10 minutes. The mixture was eventually added to the cells for transfection. The cell culture medium was replaced after 2-4 hours with normal medium containing 10 % FCS.

2.2.4.3 Lipofectamine™ 2000 DNA delivery (Invitrogen)

Cells (70-80 % confluence) were seeded without antibiotics one day before transfection. For each transfection, DNA and Lipofectamine™ 2000 (volume for different scales see table below) were incubated in FCS-free medium for 5 minutes at room temperature. DNA and Lipofectamine™ 2000 were mixed by incubation at room temperature for 25 minutes (the complex is stable up to 6 hours at room temperature). The mixture was added to the cells designated for transfection, and the plates were gently shaken to mix the medium thoroughly. Medium was replaced after 4-6 hours. In each transfection, the DNA (µg) and Lipofectamine™ 2000 (µl) ratio was usually 1:2 to 1:3 for most cells (As shown in the table below).

Culture plates	Volume of Plating medium	Volume of dilution medium	DNA	Lipofectamine ™ 2000
96-well	100 µl	2 x 25 µl	0.2 µg	0.5 µl
24-well	500 µl	2 x 50 µl	0.8 µg	2.0 µl
12-well	1 ml	2 x 100 µl	1.6 µg	4.0 µl
6-well	2 ml	2 x 250 µl	4.0 µg	10 µl
60-mm	5 ml	2 x 0.5 ml	8.0 µg	20 µl
10-cm	15 ml	2 x 1.5 ml	24 µg	60 µl

2.2.5 SDS-polyacrylamide-gel electrophoresis (SDS-PAGE)

Gels with different density were prepared according to the table below (for 5 gels).

Seperating Gel	15 %	12.5 %	10 %	8 %	Stacking gel	
LGB (ml)	7.5	7.5	7.5	7.5	UBG (ml)	3.75
Acrylamide (ml)	15	12.6	9.9	7.8	Acrylamide (ml)	2.4
H_2O (ml)	7.5	9.9	12.6	14.7	H_2O (ml)	13.8
APS 10 % (µl)	180	180	180	180	APS 10 % (µl)	70
Temed (µl)	30	30	30	30	Temed (µl)	30

Denatured protein lysates were loaded onto the gels, besides the size marker Precise Blue (Bio-Rad). Gels were firstly run at 130 volts for about 10-15 minutes until the bands move into the lower separating gel, followed by a complete run at 180 volts for about 45-50 minutes. Proteins were transferred at 100 volts for 90 minutes in blotting buffer, followed by protein detection that will be described below in more detail.

2.2.6 Western blot

Following the indicated treatment, cells were harvested at the indicated time points, and lysed in a detergent containing lysis buffer (1 % Triton X-100, 50 mM PIPES, 50 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, and complete Roche protease inhibitor, pH 7.0) for 30 minutes on ice. After 10

minutes centrifugation at 4800 rpm, supernatant was collected. Protein concentrations were determined by Bio-Rad protein assay before samples were heated at 95 °C within laemmli buffer for 5 minutes. Lysates were run on a polyacrylamide gel as described above, and proteins were transferred into nitrocellulose membranes. Blocked for 2 hours in 5 % nonfat-milk in TBST, membranes were incubated with primary antibody at an optimal concentration for 1 hour at room temperature (otherwise overnight at 4 °C), followed by 3 times 10 minutes wash in TBST. After washing, membranes were again incubated at the same conditions with proper secondary antibodies. Finally, membranes were washed another 3 times with TBST (TBS for the last wash step), and proteins were visualized by a chemiluminescence detection system (PerkinElmer Life Sciences).

2.2.6.1 Digitonin pre-treatment in Smac/DIABLO release assay

For the Smac/DIABLO release experiment, an additional step was introduced before lysing the cells in the 1 % Triton X-100 containing lysis buffer. Cells were pre-incubated with 200 µg/ml digitonin for 10 minutes on ice to release cytosolic Smac. Cells were then washed with 3 equivalent volumes of PBS and centrifuged at 1500 rpm for 10 minutes. The pellets were then further processed for protein lysis as described above.

2.2.7 Assays for measuring apoptosis

2.2.7.1 Hoechst staining

Following the indicated treatment, cells were stained with 20 µM Hoechst 33258 for 30 minutes at 37 °C before harvesting. After staining, cells were trypsinized and washed with PBS. Nuclear morphological changes (fragmentation or condensation) were determined under a fluorescence microscopy. At least 300 nuclei per sample were counted.

2.2.7.2 DNA laddering

Following the indicated treatment, cells were harvested by trypsinization, washed in PBS and lysed in a detergent containing DNA laddering buffer (150 mM NaCl, 50 mM Tris-HCl, and 0.5 % SDS, pH 8.0) supplemented with 500 µg/ml Proteinase K. The solution was incubated at 37 °C overnight, followed by phenol-chloroform extraction. DNA was precipitated in isopropanol and the pellets were dissolved in EB buffer (Qiagen) containing 10 µg/ml RNAse. After incubation at 37 °C for 1 hour, samples were run on a 1 % agarose gel containing ethidium bromide.

2.2.7.3 TUNEL assay

Following the indicated infection or treatment, cells were fixed either on a cover slip (for confocal microscopy analysis) or in solution (for FACS analysis) with 4 % paraformaldehyde for 25 minutes and washed with PBS. A permeabilization solution containing 0.2 % Triton X-100 was applied to permeabilize the cell membrane for 5 minutes. A TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labelling) kit from Promega was used according to the manufacturer's instruction to label fragmented DNA. Briefly, cells were equilibrated at room temperature with an equilibration buffer provided by the manufacturer and incubated with TdT reaction mix for 60 minutes at 37 °C in a humidified chamber. The reaction was stopped by 2 x SSC for 15 minutes. After washing with PBS, cells were either stained with streptavidin-APC (FL4, for FACS) or Streptavidin-FITC (for confocal microscopy). At the end, cells were washed with and resuspended in PBS until analysis by FACS or detection with confocal microscopy.

2.2.7.4 Caspase-3 activity

Following the indicated treatment, cells were harvested by trypsinization, and washed with PBS. A short spin at a maximum speed (13,000 rpm) was performed to remove traces of PBS before lysis. Cells were lysed in NP-40 lysis buffer (150 mM NaCl, 1 % Ipegal CA-630, 50 mM Tris-HCl, pH 8.0) for 10

minutes on ice. Cell lysate were collected after a maximum speed (13,000 rpm) spinning by a desktop centrifuge (Thermo) at 4 °C. Triplicates of aliquots (10 µl) were added to 90 µl of DEVD assay buffer (50 mM NaCl, 2 mM $MgCl_2$, 40 mM β-glycerophosphate, 5 mM EGTA, 0.1 % CHAPS, 100 µg/ml BSA, 10 mM HEPES, pH 7.0) containing 10 µM DEVD-7-amino-4-methyl-coumarin (AMC) fluorimetric substrate. Reactions were incubated in 96-well flat-bottom plates at 37 °C for 30 minutes, before free AMC was measured.

2.2.8 Phagocytosis assay

MEF cells were stained with PKH26 (red, Sigma, the procedure of membrane staining will be described below) and seeded one day before infection or treatment as indicated. At end of the treatment, PKH67 (green, Sigma) stained DCs or Raw macrophages were added to MEFs culture at 37 °C. The co-culture was stopped after 2 hours by trypsinization, and all cells were collected and fixed by 4 % paraformaldehyde for 10 minutes at room temperature. Following FACS analysis of all cells, the uptake rate was determined by the ratio of green/red double positive cells divided by whole population of red cells.

2.2.8.1 PKH membrane staining

Cells (5×10^6) were harvested, washed with PBS, and dissolved in Diluent C provided by the manufacturer. At the meantime, fluorescence dye was diluted in ethanol and dissolved in Diluent C (final concentration 2 µM). Cells were immediately mixed with the fluorescence dye for 3 minutes at room temperature. The reaction was stopped by PBS containing 1 % BSA for 1 minute. Stained cells were then washed and culture was continued according to the respective experimental design.

2.2.9 Infectivity assay

HeLa T-REx or HeLa T-REx Bim_S cells were seeded one day before infection. Cells were treated with anhydrotetracycline with or without zVAD after 24 hours of infection. Six hours later, cells were washed with PBS and kept in the

incubator for further growing. Following a total of 54 hours of infection, cells were harvested and broken by vortexing (5 minutes) with glass beads. 10 % of cell lysate was transferred into a fresh monolayer of HEp2 cells. One day later, HEp2 cells were fixed with 70 % ethanol and stained by a FITC-conjugated anti-Chlamydia LPS antibody. Infectivity was analyzed by visualization under a confocal microscope.

2.2.10 MTT assay

20,000 cells were seeded in a well of a 96 well plate one day before experiments. Following the indicated treatment, 20 µl MTT (5 mg/ml) was added to each well. Plates were gently shaken to thoroughly mix MTT into the media. Cells were incubated for 1-5 hours at normal cell culture conditions (37 °C, 5 % CO_2) to allow MTT to be metabolized. The MTT metabolic product formazan was resuspended in 200 µl isopropanol containing 0.04 N HCl. The results were read by a photometer (optical density 560 nm, subtract background 670 nm). The optical density can be directly correlated with cell quantity.

2.2.11 ATP assay

Enliten® ATP assay system kit (Promega) was used to measure ATP levels of cells, according to the instruction provided by the manufacturer. Briefly, following the indicated treatment, ATP was extracted from cells by 2 % TCA. RLU value was then determined by a luminometer, after a short co-incubation of reconstituted rL/L reagent with the diluted ATP extracts.

2.2.12 FACS analysis

2.2.12.1 Active caspase-3 staining

Following the indicated treatment, cells were harvested by trypsinization and fixed at room temperature with 4 % paraformaldehyde for 10 minutes. Cells were washed with washing buffer (0.5 % BSA in PBS) before incubation with a

primary anti-active caspase-3 antibody (BD Pharmingen) in washing buffer containing 0.5 % Saponin (permeabilization buffer) for 20 minutes at room temperature. Cells were washed with permeabilization buffer followed by a 20 minutes incubation with a secondary antibody (FITC or Cy3 conjugated anti-rabbit antibody) at room temperature. Finally, cells were washed twice respectively in permeabilization buffer and washing buffer, then analyzed by FACS.

2.2.12.2 Cytochrome c release

Following the indicated treatment, cells were harvested by trypsinization and then treated with 200 µg/ml digitonin for 10 minutes on ice, as described in the Smac release protocol. Cells were then washed with PBS containing 0.3 % BSA. After centrifugation, cells were fixed in 4 % paraformaldehyde for 20 minutes at room temperature. After additional washing with PBS and permeablization in blocking buffer (3 % BSA, 0.1 % Triton X-100 in PBS), cells were incubated with anti-cytochrome *c* antibody (BD Pharmingen) overnight at 4 °C. The next day, cells were washed three times with blocking buffer, before incubation with a Cy3-conjugated donkey anti-mouse antibody (Dianova) for 1 hour. Cells were eventually washed with PBS and analyzed by FACS.

2.2.12.3 Mitochondrial membrane potential

Following the indicated treatment, cells were harvested by trypsinization. Washed with PBS, cells were incubated with 5 µM Rhodamine-123 at 37 °C for 30 minutes. Cells were washed with PBS before FACS analysis.

2.2.12.4 Plasma membrane integrity

Following the indicated treatment, cells were harvested by trypsinization. Washed with PBS, cells were analyzed by FACS after PI (50 µg/ml) staining.

2.2.14.5 Propidium Iodide DNA labelling in fixed cells

Following the indicated treatment, cells were harvested by trypsinization and fixed in 100 % cold ethanol for 20 minutes. After centrifugation, pellets were collected by carefully removing ethanol. FACS analysis was performed after PI (50 µg/ml) staining.

2.2.13 Microscopy

2.2.13.1 Leica Fluoresence microscopy

Nuclear fragmentation or condensation was detected by Leica DM RBE (Bensheim, Germany) fluorescence microscopy, following Hoechst staining.

2.2.13.2 Confocal microscopy

Two confocal microscopes (Zeiss LSM 510 (Jena, Germany) and Leica TCS SP5 (Bensheim, Germany)) were used in this study. Cells growing on a cover slip were washed and fixed, following the indicated treatment. After fixation, cells were permeabilized with PBS containing 0.1 % Triton X-100 for 4 minutes. Before incubation with the primary antibody (40 minutes, at room temperature), cells were blocked with 5 % donkey serum or goat serum (depending on the secondary antibody to be used). Cells were washed with PBS after incubation with the primary antibody, and further incubated with the corresponding secondary antibody (fluorescence conjugated) at room temperature for 40 minutes. Finally, cells were washed 3 times PBS and mounted on a glass slide. Samples were kept at 4 °C until analysis.

2.2.13.3 Electron microscopy

For electron microscopy, cells were harvested after the indicated infection and treatment, fixed, and pictures were taken with a Zeiss EM 10CR electron microscope (Jena, Germany).

3. Results

3.1 Chlamydia infection induces broad degradation of pro-apoptotic BH3-only proteins

Since the first description of chlamydial anti-apoptotic effects by Dr. Guangming Zhong's group in 1998 (Fan et al., 1998), the molecular mechanism has been widely investigated. Following the application of various apoptotic stimuli such as UV light, staurosporine or Taxol, as well as initiation of death signaling on the cell surface, Bax and/or Bak are activated by BH3-only proteins to facilitate cytochrome *c* release from mitochondria into the cytosol. Cytochrome *c* release from mitochondria is an essential step that leads to activation of caspase family members. However, all of the above processes are prohibited during chlamydial infection (Fan et al., 1998; Fischer et al., 2004a; Fischer et al., 2001; Fischer et al., 2004b), most likely the results of a Chlamydia-induced deficiency of signal transduction upstream of Bax/Bak. In most cases of apoptosis, one or several BH3-only proteins are up-regulated or activated by different stimuli, which leads to the activation of Bax and/or Bak. The BH3-only proteins serve as a class of initiative factors that induce apoptosis. This group of pro-apoptotic proteins are therefore candidates for further investigation to explore chlamydial anti-apoptotic molecular mechanisms.

3.1.1 Disappearance of pro-apoptotic BH3-only proteins in Chlamydia-infected cells

In a previous study on global genes expression during Chlamydia infection, no significant change of mRNA level of BH3-only protein Bim had been observed (Hess et al., 2001). However, our Western blotting analysis revealed nearly complete disappearance of endogenous pro-apoptotic BH3-only proteins Bim, Puma, Bad and Bmf (Fig. 3.1) after 24 hours of infection with *Chlamydia trachomatis*.

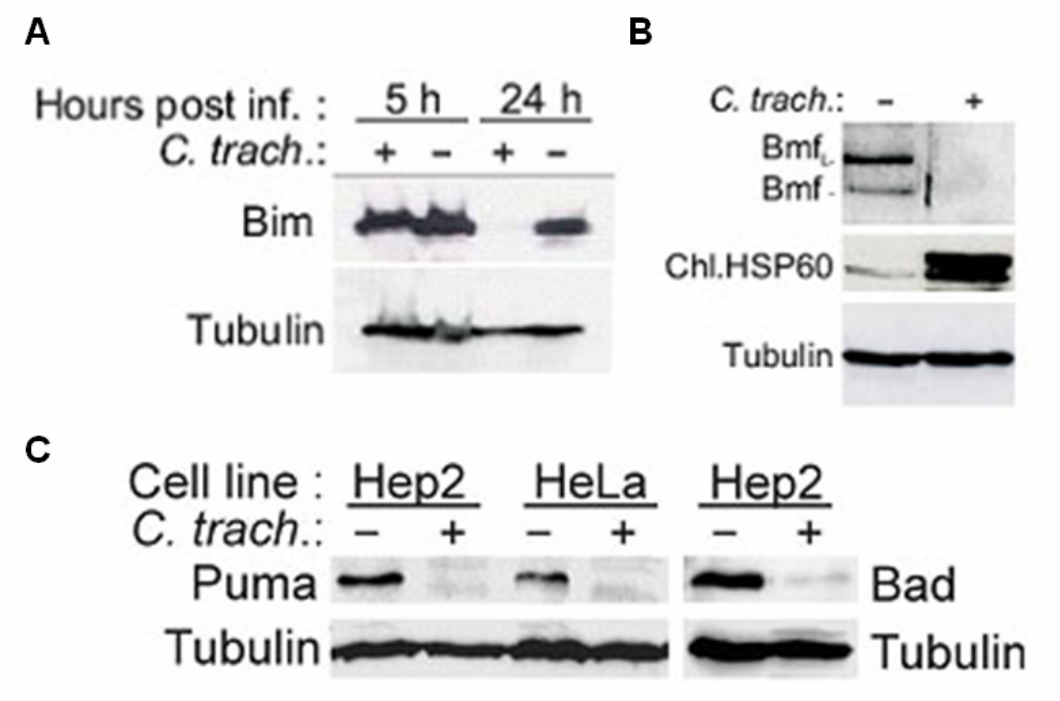

Fig. 3.1 Disappearance of BH3-only proteins Bim, Bmf, Puma and Bad during Chlamydia infection

HEp2 (A, C), MEF (B) or HeLa cells (C) were left uninfected or were infected with *Chlamydia trachomatis* for 24 hours or as indicated, and whole cell extracts were subjected to Western blotting using specific antibodies against Bim, Puma Bad and Bmf. Tubulin and Chlamydia HSP60 served as loading control and infection control, respectively.

3.1.2 Kinetics of the degradation of the BH3-only proteins Bim, Puma and Bad in Chlamydia-infected cells

Although the BH3-only proteins share a short region called BH3 domain, they are regulated by different mechanisms to induce apoptosis. In a time course experiment, disappearance of Bim, Puma and Bad was monitored. The disappearance of all three BH3-only proteins became detectable at a similar time point (around 16 hours post-infection), and was nearly complete after 24 hours of infection (Fig. 3.2). The similar disappearance kinetics of BH3-only proteins suggests that the same mechanism is employed to target different BH3-only proteins for degradation. Interestingly, expression of the other Bcl-2 family proteins (Bcl-2, Bak, Bax and Bcl-x_L), as monitored by Western blotting,

remained constant (Fig. 3.2), which emphasizes the specificity of the degradation of BH3-only proteins.

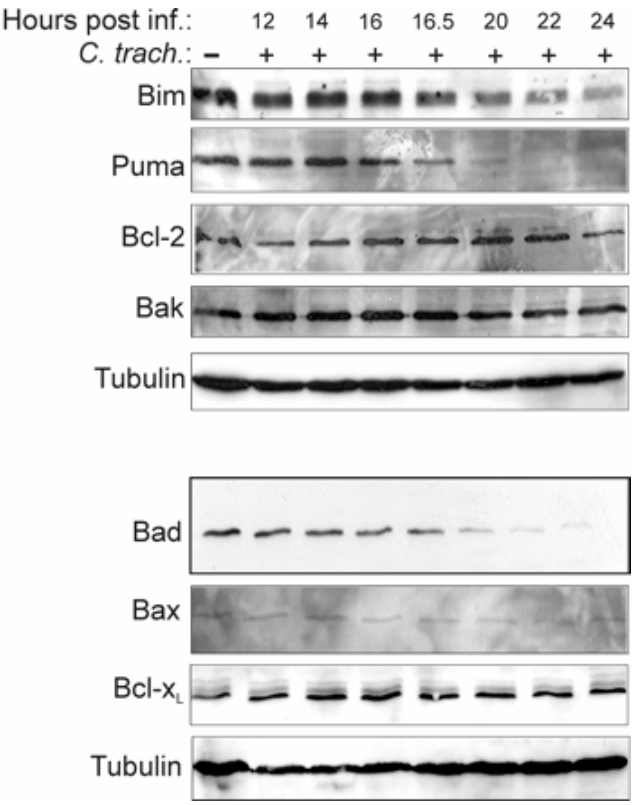

Fig. 3.2 Time course of disappearance of Bim, Puma and Bad during Chlamydia infection

Cell lysates were prepared from HEp2 cells infected with *Chlamydia trachomatis* at the indicated time points. Western blotting using specific antibodies revealed the expression levels of the indicated Bcl-2 family proteins (Bim, Puma, Bcl-2, Bak, Bad, Bax and Bcl-x_L). The separate top and bottom panels of results were obtained by reprobing two membranes from a same set of samples. Tubulin served as loading control.

3.1.3 Proteasome activity is required for degradation of BH3-only proteins

Proteasome-mediated proteolytic degradation of unwanted or misfolded proteins is a major mechanism in the regulation of cellular protein expression levels. Most of the proteins are targeted for proteasomal destruction by ubiquitination (Hochstrasser, 1995). We analysed BH3-only proteins

degradation in the presence or absence of the proteasome inhibitor MG-132. As shown in Fig. 3.3, Bim, Puma and Bad were degraded after 24 hours of infection like described above. The proteasome inhibitor MG-132 blocked the Chlamydia-induced disappearance of all three BH3-only proteins. The inhibition sensitivity was relatively variable, 20 μM MG-132 was sufficient to almost completely block degradation of Bim and Bad, but had no clear effect on Puma degradation. Even double concentration of MG-132 (40 μM) could only save part of the Puma protein from destruction. It is however still unclear how BH3-only proteins are recognized and degraded by the proteasome, at least no clear ubiquitination chains have ever been observed by Western blot against any BH3-only proteins. Inhibition of disappearance was also seen when the proteasome inhibitor Lactacystin was used (not shown).

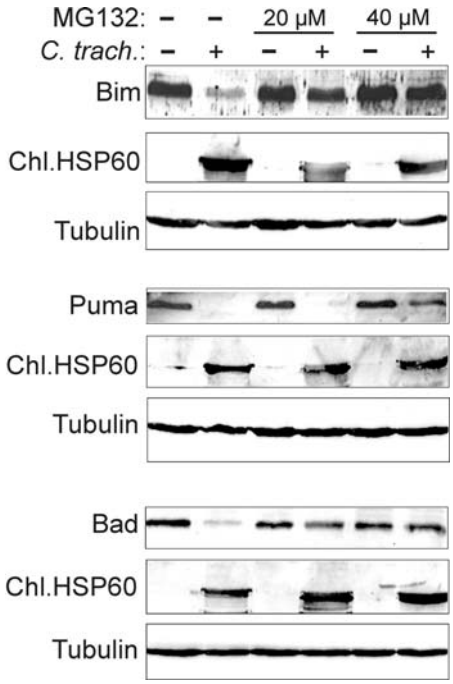

Fig. 3.3 Degradation of BH3-only proteins requires proteasomal activity
HEp2 cells were left uninfected or were infected with *Chlamydia trachomatis* for 16 hours, when the indicated amount of the proteasome inhibitor MG-132 was added for additional 8 hours. Cells were then extracted for Western blotting analysis, using specific antibodies against Bim, Puma, and Bad. Chlamydial HSP60 and Tubulin served respectively as infection and loading control. Results were obtained by running 3 separate gels with samples from one experiment.

3.1.4 Noxa is also targeted for degradation in Chlamydia-infected cells

Another BH3-only protein, Noxa, has been reported to be regulated by transcriptional induction dependently or independently of p53 (Sun and Leaman, 2005; Villunger et al., 2003). Noxa is usually only weakly expressed in HeLa cells. Therefore we used a stable HeLaTRex (HeLa cells expressing Tet repressor) Noxa cell line where Noxa is inducible upon addition of tetracycline (dissociation of Tet repressor and induction of gene expression) (Berens and Hillen, 2004). HeLaTRex Noxa cells were infected with *Chlamydia trachomatis* for 24 hours before tetracycline was added. After additional 8 hours of cell culture, cells were harvested and subjected to western blotting. Fig. 3.4 shows that Noxa was significantly induced upon tetracycline treatment, but the amount of induced Noxa was clearly reduced in the infected sample. We can therefore conclude that BH3-only protein Noxa is probably another target for degradation during Chlamydia infection.

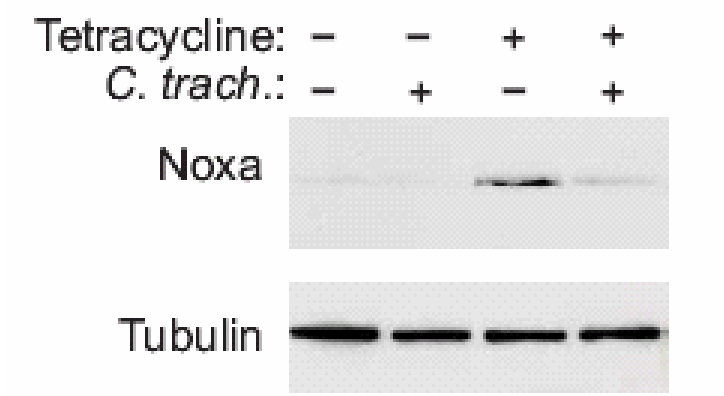

Fig. 3.4 Reduction of tetracycline-induced Noxa levels in Chlamydia-infected cells

HeLaTRex Noxa cells were left uninfected or infected for 32 hours, with or without tetracycline in the last 8 hours for induction of Noxa. Cells were harvested and Western blotting analysis was performed using an antibody against Noxa. Tubulin served as a loading control.

3.1.5 Specific destruction of truncated Bid (tBid) during infection

Our previous study showed that Chlamydia inhibits host cell apoptosis at the level of mitochondria (Fischer et al., 2004a). In type II cells, although death receptor initiated apoptosis as well as cytochrome *c* release is blocked, the upstream signal such as caspase-8 activation and expression of intact Bid are both unchanged during infection. As a member of the BH3-only proteins group, Bid connects the death receptor pathway and the mitochondrial pathway, by transducing the death signal from activated caspase-8 to mitochondria (Li et al., 1998; Luo et al., 1998; Yin et al., 1999). Intact Bid is a weak killer, while tBid (cleaved by active caspase-8) is more active in inducing cytochrome *c* release and apoptosis. Structure studies demonstrated that the BH3 domain (considered to be important for interaction between BH3-only proteins and the other Bcl-2 like proteins, and being the Chlamydia-targeted region of Bim degradation) is hidden in the 3-D structure of intact Bid, and is exposed on the surface of truncated Bid after caspase-8 mediated cleavage (Chou et al., 1999). It has been shown that the BH3 domain is required and sufficient for Chlamydia induced degradation of Bim (Fischer et al., 2004b). This may explain that intact Bid is not targeted for degradation because of the BH3 domain hidden in the 3D structure. We then performed experiments to study tBid, where the BH3 domain is exposed on the surface of the protein, during Chlamydia infection. Both Jurkat cells (lymphoid) and HeLa-Fas cells (HeLa cells stably transfected to express the Fas death receptor on the cell surface) were infected with *Chlamydia trachomatis* for 24 hours, followed by additional 6 hours of stimulation with a monoclonal antibody to the Fas death receptor. As expected, tBid was only generated by stimulation of the death receptor pathway in uninfected cells, and its levels were strongly diminished in infected cells (Fig. 3.5). The proteasome inhibitor MG-132 was also included in the HeLa-Fas experiments; these experiments provided further evidence that the same mechanism was employed as in the course of the other BH3-only proteins degradation, since tBid disappearance was prevented in the presence of MG-132 (Fig. 3.5 B).

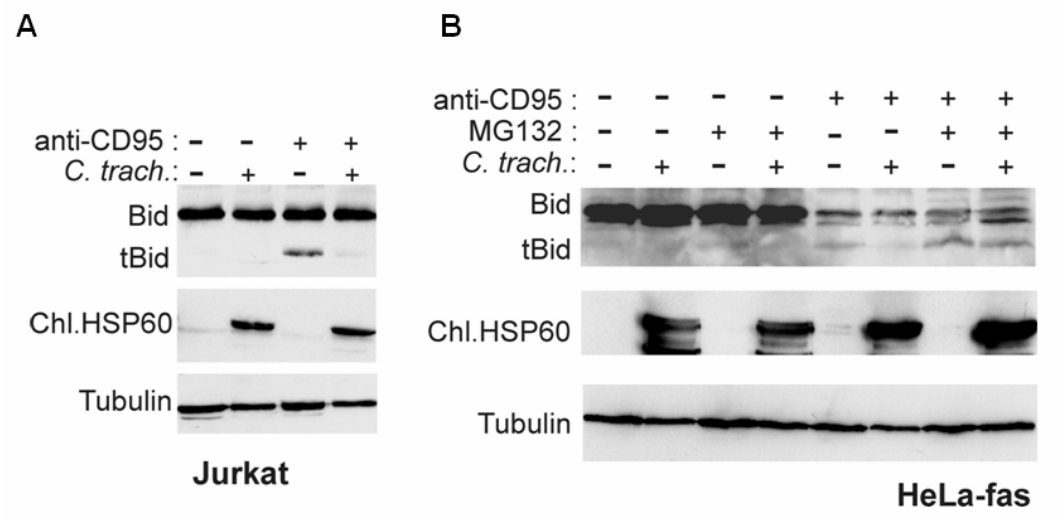

Fig. 3.5 Chlamydia infection leads to proteasome-mediated destruction of tBid

Jurkat (A) and HeLa-fas (B) cells were uninfected or infected for 24 hours, when monoclonal anti-CD95 antibody (to the Fas death receptor) was added for an additional 6 hours. The proteasome inhibitor MG-132 was included in some wells of the HeLa-Fas cells together with anti-Fas monoclonal antibody in the last 6 hours. After a total 30 hours of infection, cells were extracted and subjected to Western blotting. An antibody recognizing both intact Bid and truncated Bid was used to detect the expression of both forms. Chlamydia HSP60 and tubulin are infection and loading controls, respectively.

3.2 Host cell apoptosis induced by Bim_S blocks Chlamydia development and propagation

As discussed above, all investigated BH3-only proteins are targeted for degradation during Chlamydia infection. To further investigate the biological relevance of those events in infected cells, we performed experiments in another HeLa TRex cell line (HeLa TRex Bim_S cells) that stably expresses tetracycline-inducible Bim_S. As one of the BH3-only proteins, Bim has 3 isoforms, Bim_{EL}, Bim_L and Bim_S (O'Connor et al., 1998). Bim_S is the most potent killer among those 3 isoforms. Transcriptionally upregulated Bim_S translocates to mitochondria, causing activation of Bax and/or Bak and release of cytochrome *c*. To test the integrity of downstream pathways during Chlamydia infection, we here characterized this cell line under either uninfected or infected conditions.

3.2.1 Characterization of Bim_S-induced apoptosis, in comparison with staurosporine-induced apoptosis

Before performing Chlamydia infection experiments and to characterize the cells, we performed an analysis of HeLa TRex Bim_S cells under various conditions of tetracycline or staurosporine stimulation. Fig. 3.6 shows a similar ratio of nuclear morphological changes induced by either tetracycline-induced Bim_S or by staurosporine, reaching around 90 % apoptosis after 6 hours. Caspase activation was involved in apoptotic cell death. To characterize the apoptosis pathway in HeLaTRex Bim_S cell line, we performed a time course experiment. Following 1, 3 or 6 hours treatment with tetracycline or staurosporine, cells were harvested and subjected to either Western blotting or to FACS analysis. These assays showed that caspase-9, caspase-7 and caspase-3 are all activated upon Bim_S induction and staurosporine treatment, followed by Poly (ADP-ribose) polymerase (PARP) cleavage (Fig. 3.7 A, C). The same time points were further adopted for analysis of the release of apoptotic proteins from mitochondria. Again, a similar time course of

cytochrome *c* and Smac/DIABLO release was observed by FACS analysis and Western blotting experiments (Fig. 3.7 B, D).

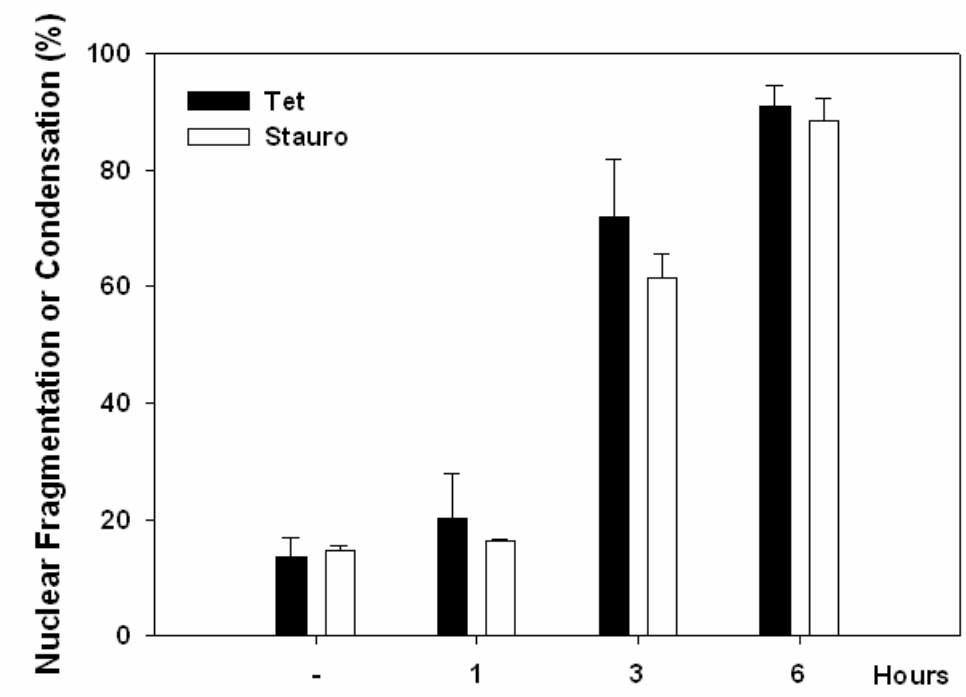

Fig. 3.6 Comparison of nuclear apoptosis induced by Bim_S and staurosporine

HeLaTRex Bim_S cells were left untreated, treated with tetracycline or staurosporine. After the indicated times, all cells floating or attached to the plate were harvested followed by Hoechst staining. At least 300 cells were counted under fluorescence microscopy for each sample. Standard deviation was calculated from two separate experiments.

3.2.2 Bim_S over-expression induces apoptosis and loss of ATP in infected cells

As discussed above in Chlamydia-infected cells, caspase activation, cytochrome *c* release, and Bax/Bak activation are blocked, accompanied with broad degradation of BH3-only proteins. To clarify the biological relevance of pro-apoptotic BH3-only proteins destruction for chlamydial anti-apoptotic effects, we used HeLa T-REx Bim_S cells for infection experiments. Interestingly, while Chlamydia infected cells were resistant to UV induced apoptosis, tetracycline induced Bim_S over-expression killed infected cells to a

comparable extent as uninfected cells (Fig. 3.8 A). This sensitivity to Bim_S in the infected cells directly indicates that the downstream pathways remain unaffected during Chlamydia infection. Although the low concentration of tetracycline we used in those experiments should not affect Chlamydia activity, since tetracycline-treated HeLa T-REx cells are still resistant to UV induced apoptosis, an additional control was performed to exclude any possible role of the antibiotic effects of tetracycline. Anhydrotetracycline, which does not have any antibiotic effects against Chlamydia was used in further experiments. As expected, anhydrotetracycline-induced Bim_S killed cells infected for 24 hours with *Chlamydia trachomatis* (Fig. 3.8 B), at which time point Chlamydia had already established an anti-apoptotic system that blocked UV- or staurosporine- induced apoptosis. In addition, inhibition of caspases completely blocked Bim_S-induced apoptosis (nuclear fragmentation) in infected cells (Fig. 3.8 C). Apoptosis of infected cells was accompanied by a dramatic decrease in cellular ATP level (Fig. 3.9).

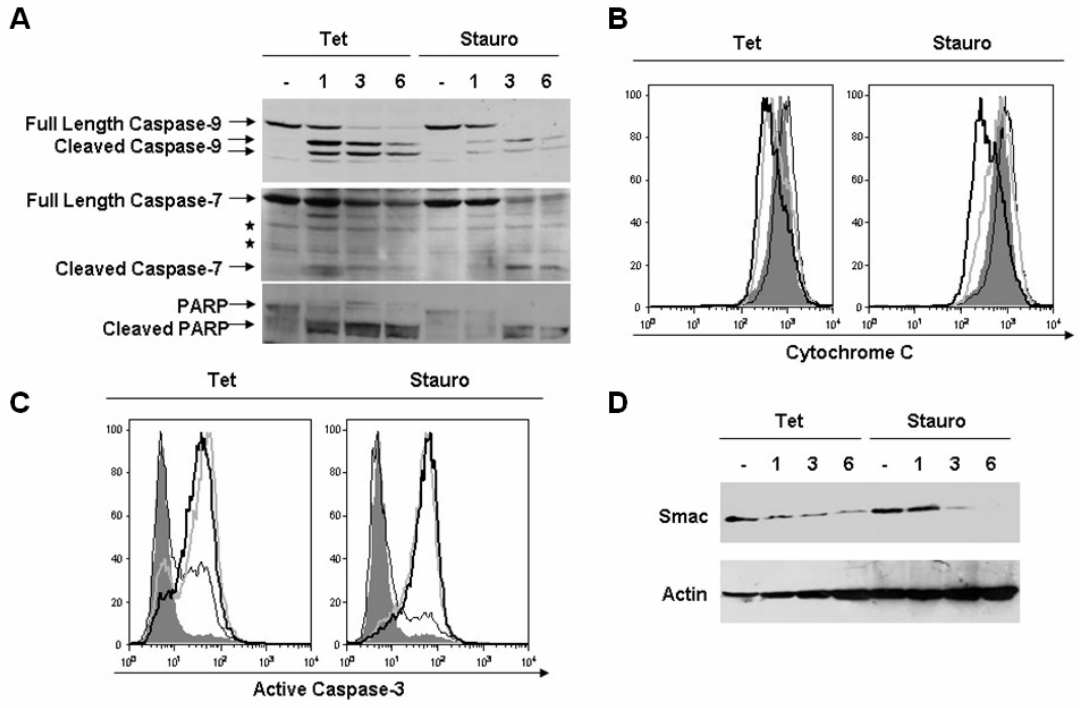

Fig. 3.7 Characterization of Bim_S- and staurosporine-induced apoptotic signal transduction

HeLaTRex Bim_S cells were either left untreated or treated with tetracycline or staurosporine for the indicated period of time. After indicated time points, cells

were harvested and incubated with 200 ng/ml digitonin on ice for 10 minutes to release cytosolic proteins. B) Cells were then stained with anti-cytochrome *c* antibody and analyzed by FACS. Weaker staining appeared in cells whose cytochrome *c* had been released. D) Another set of cells were lysed and subjected to Western blotting detecting Smac/DIABLO. Before digitonin treatment, some cells were directly either extracted for Western blotting (A) or fixed for FACS analysis (C). A) Antibodies against caspase-9, caspase-7 (both recognize intact and cleaved forms) and PARP were used in the Western blotting experiment, C) while an antibody specifically recognizing only active caspase-3 was applied for FACS analysis. Grey solid, untreated; light black line, 1 hour; strong grey line, 3 hours; strong black line, 6 hours.

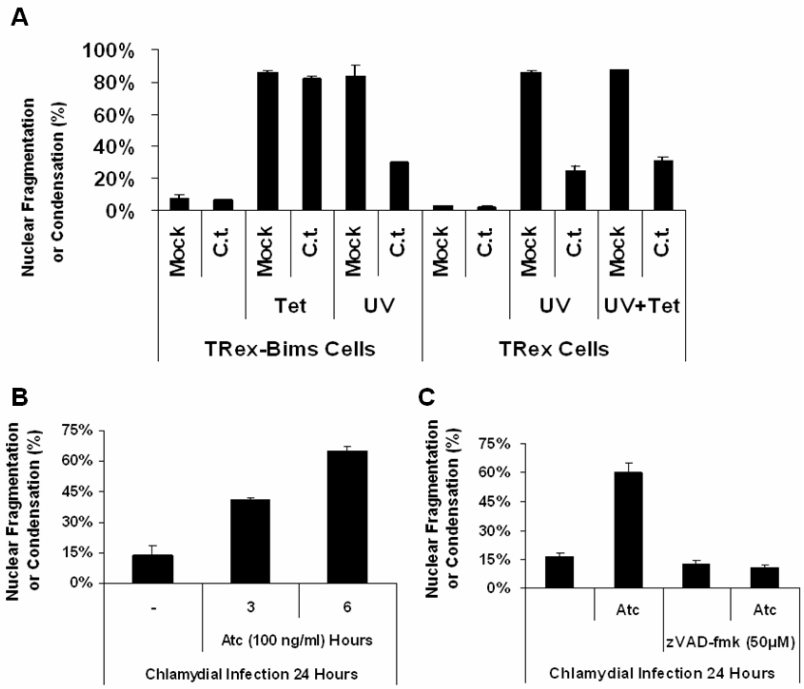

Fig. 3.8 Chlamydia-infected cells maintain sensitivity to the BH3-only protein Bim_S-induced apoptosis

HeLaT-REx or HeLaT-REx Bim_S cells were infected with *Chlamydia trachomatis* at an MOI of 3. After 24 hours, some cells were treated with tetracycline (A) or anhydrotetracycline (B, C), in the presence or absence of the broad-spectrum caspase inhibitor zVAD (C). Some cells were irradiated with UV light as a control for establishment of chlamydial anti-apoptotic effects (A). Cells were stained with Hoechst following 6 hours treatment, or as indicated. Apoptotic cells were detected by counting cells containing

fragmented or condensed DNA by fluorescence microscopy. Percentage of Hoechst-positive cells is shown with standard deviation from 2 seperate countings. The experiments have been performed 3 times with similar results.

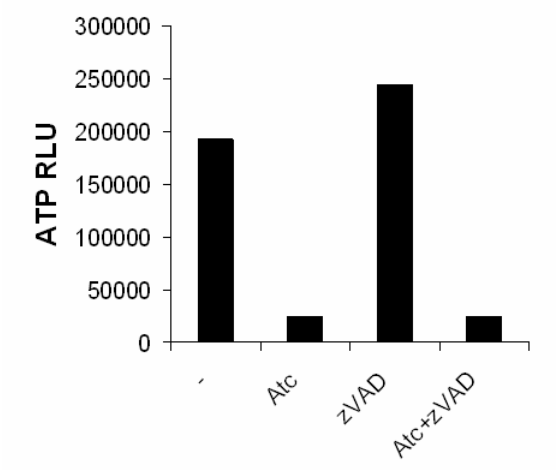

Fig. 3.9 ATP content is dramatically decreased in apoptotic cells
HeLaT-REx Bim_S cells were infected with *Chlamydia trachomatis* at an MOI of 3. After 24 hours, some cells were treated with anhydrotetracycline for additional 6 hours, in the presence or absence of the broad caspase inhibitor zVAD. ATP was extracted at 30 hours post infection and the levels were measured by the Promega ENLITEN® ATP assay system. Experiments with the same settings have been performed twice, showing similar results.

3.2.3 Apoptosis induced at early but not late infection stages blocks chlamydial propagation

Although apoptosis inhibition in Chlamydia-infected cells was reported several years ago, it is still unclear why Chlamydia depends such activity during infection. Several possibilities have been discussed and reviewed (Byrne and Ojcius, 2004; Miyairi and Byrne, 2006; Ying et al., 2007). For example, inhibition of cell death may help Chlamydia to be ignored by the host immune system, and therefore help establishing chronic diseases. One plausible possibility is also that Chlamydia depends on host cells for replication, and therefore blocks host cell death. Although reasonable and perhaps obvious, the latter hypothesis has not been confirmed, because it is close to impossible to induce apoptosis in Chlamydia-infected cells due to the strong anti-apoptotic

effects provoked by the infection. Since we were able to induce apoptosis in infected cells by over-expression of Bim_S as described above, the HeLa T-REx Bim_S cell line was used to test this hypothesis. Cells were infected, and cell death was induced by expression of Bim_S. Chlamydia in host cells that were killed after 24 hours of infection (middle stage of infection cycle (entire infection cycle takes about around 50 hours), for RBs replication) where not able to propagate into fresh monolayers of cells, whereas no influence on propagation was observed when host cells were killed after 48 hours (late stage of infection cycle, EBs had already been generated) (Fig. 3.10). This result shows that apoptosis can be a defence mechanism against Chlamydia infection at least during the early and middle stage of infection, when EB reconstitution has not yet been achieved.

3.2.4 Inhibition of caspase activity restores EBs production in apoptotic host cells, but fails to rescue propagation

To better understand the role of host cell apoptosis in Chlamydia development, we analysed infected cells by electron microscopy, in the presence or absence of apoptosis induction. The caspase inhibitor zVAD was included in some cases. While most RBs were converted into EBs in infected cells after 2 days of infection, EBs were hardly detectable in cells undergoing apoptosis upon anhydrotetracycline treatment (Fig. 3.11). In contrast, a large number of RBs were found to be released into the intercellular space. RBs are non-infectious. Deficiency in EB production is therefore a conceivable explanation for the blockage of Chlamydia propagation by apoptosis induction. Inhibition of caspases can block Bim_S-induced apoptosis in infected cells, and can surprisingly restore EB production up to a level similar to that in untreated infected cells (Fig. 3.11). However, although EB production is rescued by inhibition of caspases, this cannot rescue chlamydial propagation (Fig. 3.12). A second factor that does not depend on caspase activity may thus contribute to chlamydial infectivity.

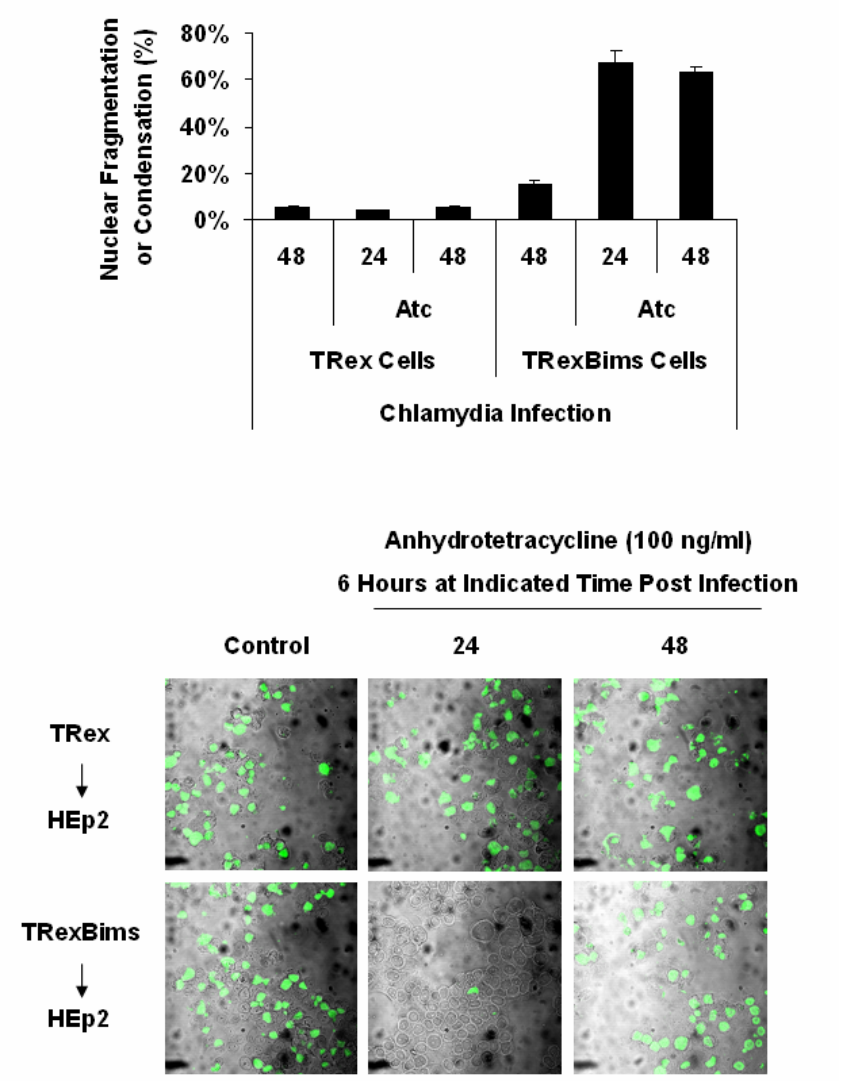

Fig. 3.10 Apoptosis induction in host cells prevents Chlamydia propagation

HeLa T-REx or HeLa T-REx Bim_S cells were infected with *Chlamydia trachomatis* at an MOI of 3. Anhydrotetracycline was added at the indicated time points to induce Bim_S and therefore apoptosis in infected cells. After 6 hours, medium was changed and cells were kept in culture until a total infection of about 54 hours (roughly a complete infection cycle for *Chlamydia trachomatis*). Ten percent of material from broken cell lysate was added to pre-seeded HEp2 cells for propagation. HEp2 cells were fixed and stained with an anti-Chlamydia LPS antibody the next day, and chlamydial inclusions were observed under a confocal microscopy.

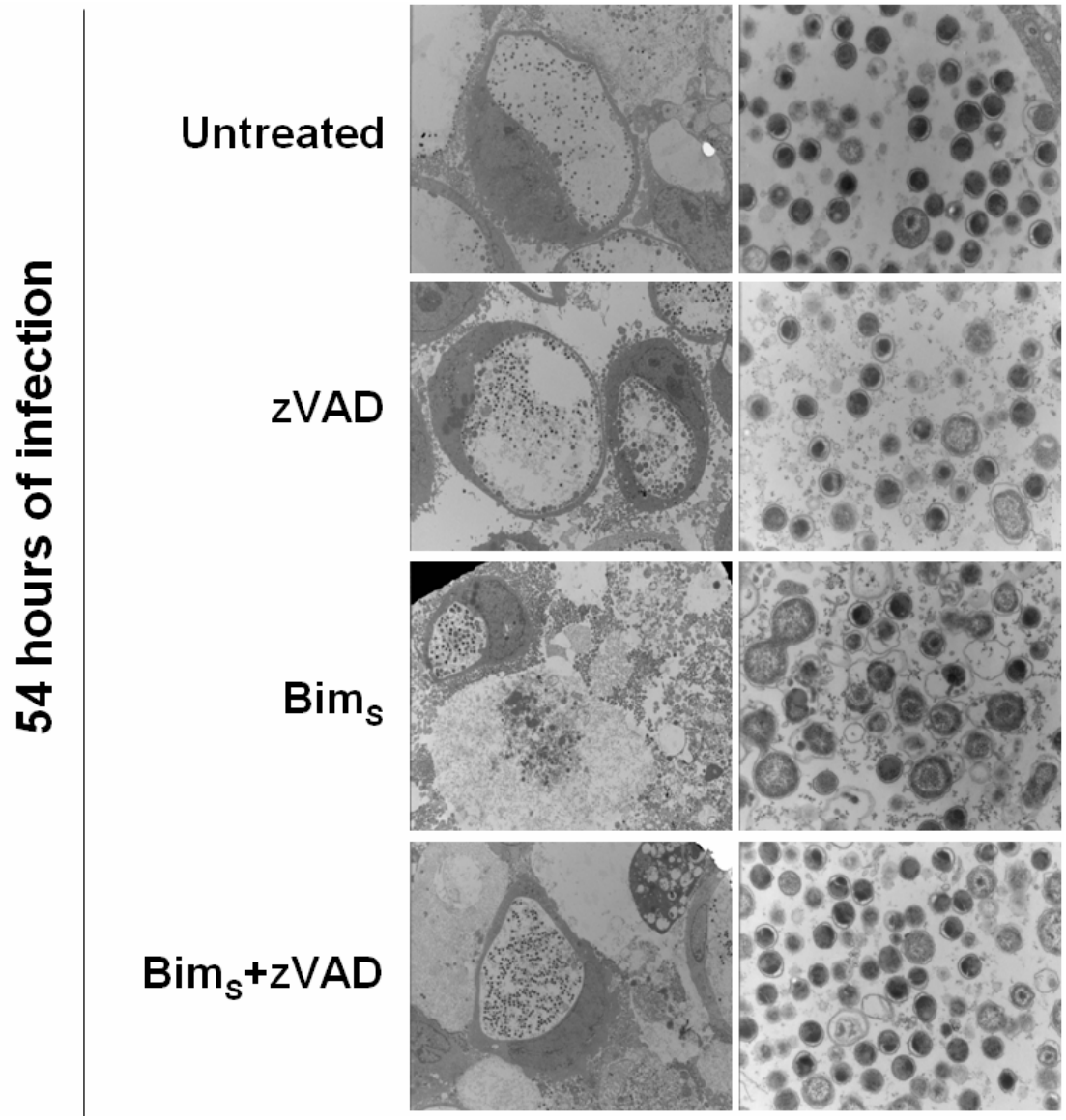

Fig. 3.11 Electron microscopic analysis of Chlamydia in apoptotic host cells

HeLa T-REx Bim$_S$ cells were infected with *Chlamydia trachomatis* at an MOI of 3. Anhydrotetracycline was added at 24 hours post-infection to induce Bim$_S$ and therefore apoptosis in infected cells, with or without the broad caspase inhibitor zVAD. After 6 hours, medium was changed and cells were kept in culture until a total infection time of about 54 hours (roughly a complete infection cycle for *Chlamydia trachomatis*). Cells were harvested and fixed for electron microscopy analysis. The infected cells containing chlamydial inclusions as well as EBs and RBs are shown.

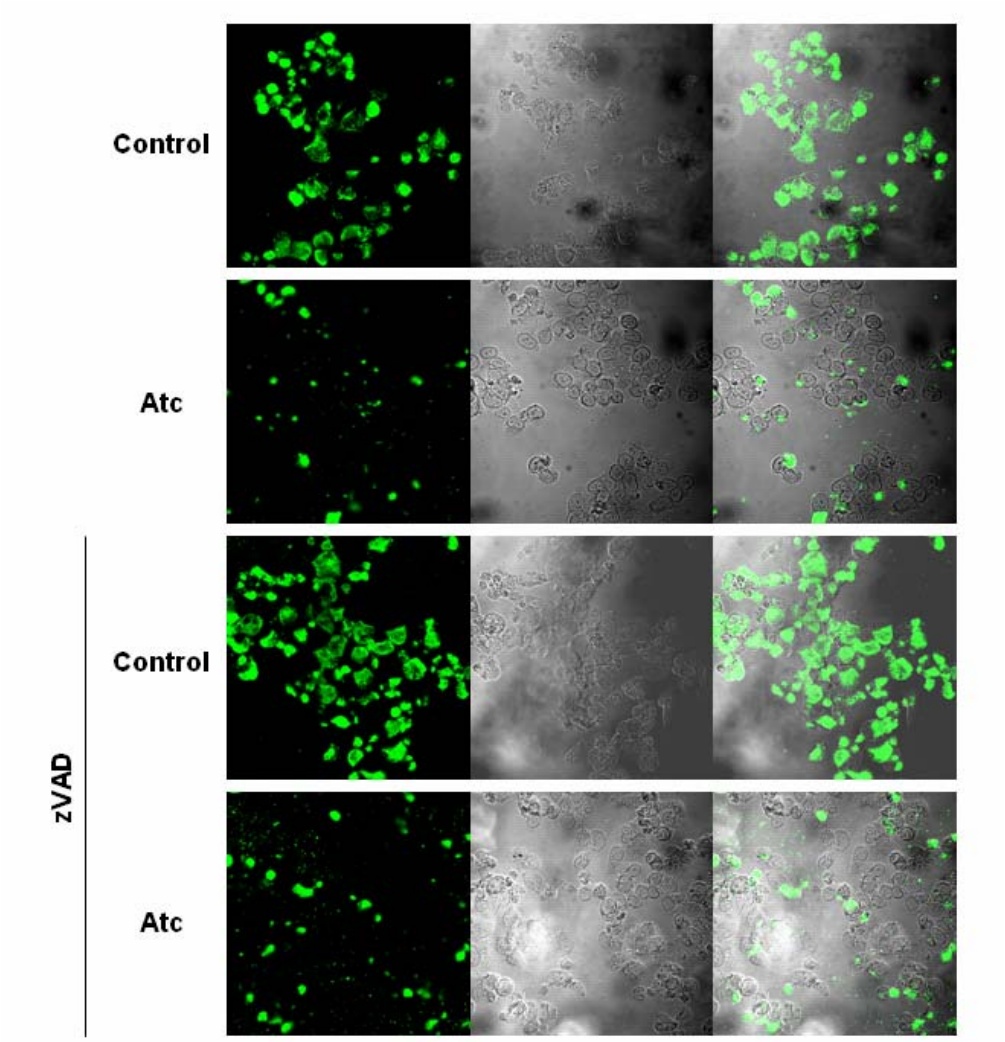

Fig. 3.12 Caspase inhibition fails to rescue Chlamydia propagation in apoptotic cells

HeLa T-REx Bim_S cells were infected with *Chlamydia trachomatis* at an MOI of 3. Anhydrotetracycline was added at 24 hours post-infection to induce Bim_S and therefore apoptosis in infected cells, with or without the broad caspase inhibitor zVAD. After 6 hours, medium was changed and cells were kept in culture until a total infection time of about 54 hours (roughly a complete infection cycle for *Chlamydia trachomatis*). Ten percent of material from broken cell lysate was added to pre-seeded HEp2 cells for propagation. HEp2 cells were fixed and stained with an anti-Chlamydia LPS antibody on the next day, and chlamydial inclusions were observed under a confocal microscopy.

3.3 Chlamydia causes cell death with features similar to apoptosis

Although Chlamydia-induced anti-apoptotic effects are clear, it has been proposed that Chlamydia-infected cells may undergo apoptosis at the later stage of infection. In fact, cell death in Chlamydia infections has been observed for decades (Todd et al., 1976; Todd and Storz, 1975), although the mechanism and role *in vivo* remain unclear. Several previous studies have shown that Chlamydia infection leads to host cell death, demonstrating features of both apoptosis and necrosis (Dumrese et al., 2005; Ojcius et al., 1998). The issue remains inconclusive due to different Chlamydia strains and various cell types used in studies by groups worldwide. Also, a wide range of different assays used by investigators could be another reason for the mixture of results. To better understand the reported death-inducing activity of Chlamydia during infection, we performed experiments in typical Chlamydia target cells, using a series of well established standard apoptosis assays to test for cell death induction at the late stage of infection.

3.3.1 Chlamydia infection induces nuclear morphological changes

Nuclear morphological changes, such as DNA fragmentation and condensation, are one of the most distinctive phenotypes that investigators use to define apoptosis. In infected MEF cells, we found that high doses of Chlamydia induced nuclear changes indistinguishable from changes induced by UV irradiation (Fig. 3.13 A). In agreement with previous findings, apoptosis induction by Chlamydia was found to be cell type dependent and more pronounced in E1A transformed MEF cells (Fig. 3.13 B). We were, however, also able to induce nuclear morphological changes in HEp2 and HeLa cells, albeit at relatively high infection doses (data not shown). Kinetic studies revealed that nuclear morphological changes occurred at a relatively late stage, only after 2 days (at the time of the completion of an infection cycle) with an MOI of 1 in MEF cells (Fig. 3.13 D). Infection with another species, *Chlamydia muridarum*, previously known as MoPn strain of *Chlamydia*

trachomatis, was analysed to compare induced nuclear morphological changes with those induced by the *Chlamydia trachomatis* L2 strain. There were no differences in nuclear morphological changes observed following infection with those two strains. *Chlamydia muridarum* also induced nuclear morphological changes at an MOI of 5, but not at low doses at 24 hours post infection (Fig. 3.13 C).

3.3.2 Chlamydia infection induces genomic DNA breaks

TUNEL staining has been widely used in the past to detect apoptosis, because of its specific recognization of the single or double-stranded DNA breaks, induced by apoptotic DNA fragmentation (Trimarchi et al., 2004). The TUNEL reaction adds labelled nucleotides to the free end of damaged DNA, which can then be detected by various means. We performed TUNEL assays in Chlamydia-infected MEF cells, where nuclear morphological changes were observed (Fig. 3.14 A). Interestingly, MEF cells infected with *Chlamydia trachomatis* at an MOI of 5 showed clear TUNEL positive signals that were indistinguishable from that induced by UV irradiation. To exclude the possibility of reagents crossreacting with the Chlamydia inclusion, we co-stained TUNEL assayed cells with an antibody recognizing Chlamydia LPS. No clear overlap between Chlamydia inclusions and the TUNEL signal (in the nucleus) was seen, suggesting that DNA breaks were induced in *Chlamydia trachomatis* infected cells (Fig. 3.14 B). An atypical Chlamydia inclusion shape may be due to centrifugation of infected cell onto glass slides after harvesting of all cells (both attached and floating cells were collected to avoid loss of dead cells).

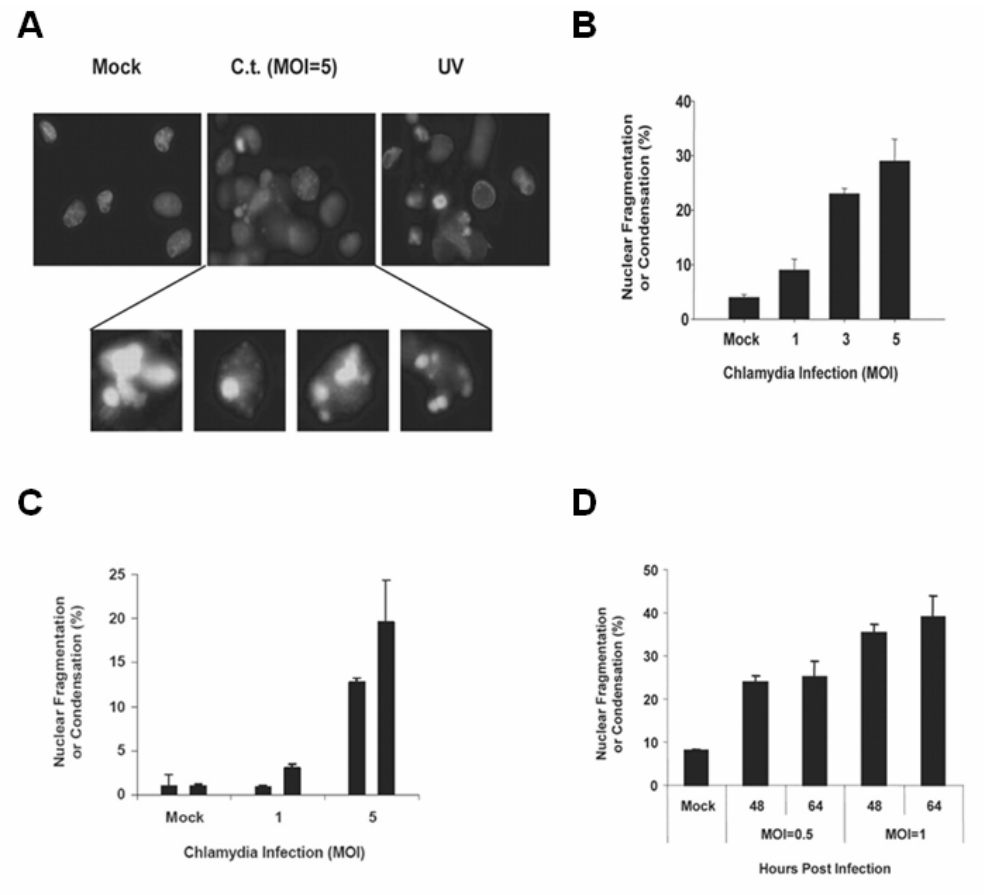

Fig. 3.13 Nuclear morphological fragmentation and condensation during infection with *Chlamydia trachomatis* and *Chlamydia muridarum*

MEF cells were infected with *Chlamydia trachomatis* (A, B, D) or *Chlamydia muridarum* (C) at the indicated MOI for 24 hours unless otherwise indicated. Following infection, all cells (attached or floating) were stained with Hoechst and harvested by trypsinization. Nuclear morphological changes (fragmentation or condensation, shown in A) were determined and scored in at least 300 nuclei for each experimental condition by fluorescence microscopy. Examples of typical nuclear morphological changes following Chlamydia infection or UV irradiation (as a positive control) are shown in A. Standard deviation was calculated from two scorings of one single sample, and similar results were reproduced in two to five independent experiments. Two separate experiments are shown in C.

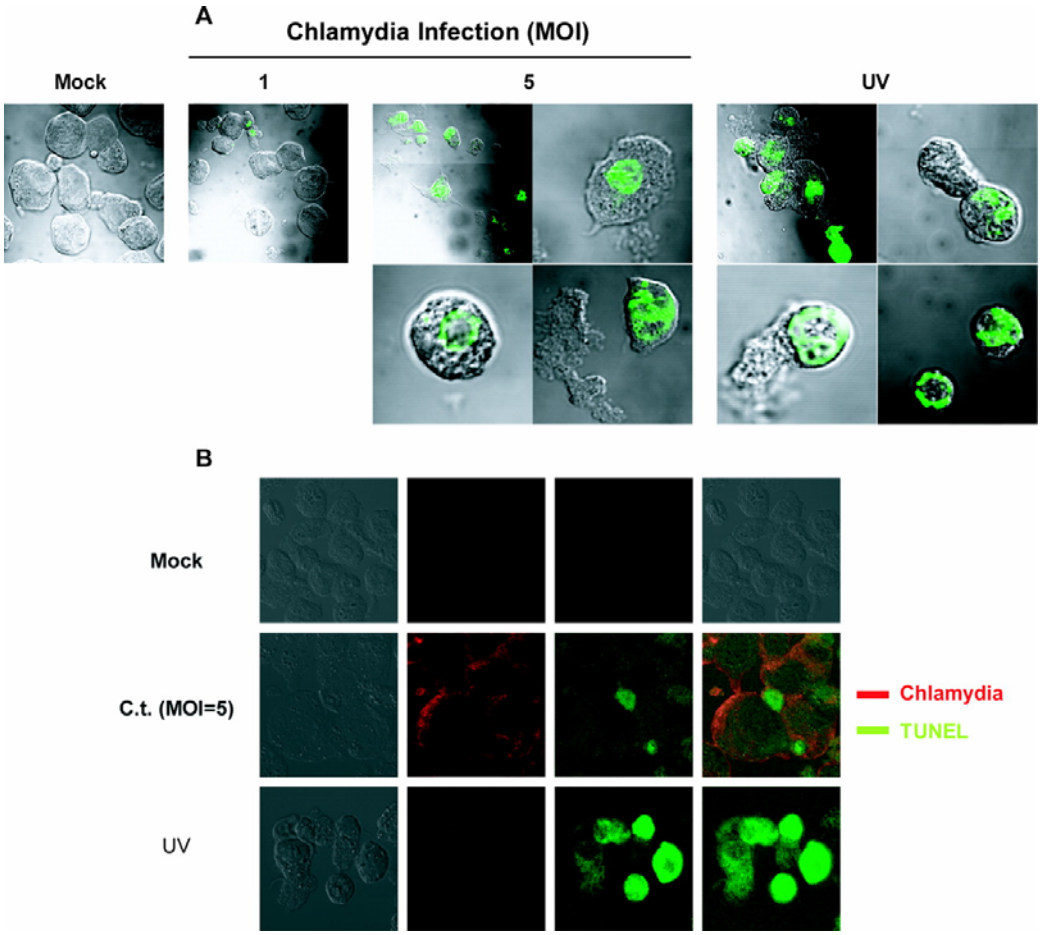

Fig. 3.14 Chlamydia-infected cells are TUNEL positive

MEF cells were infected with *Chlamydia trachomatis* at the indicated MOI for 24 hours, while some cells were irradiated with UV as a positive control. All cells floating in the medium and attached to the plate were harvested and stained with TUNEL kits according to the instruction provided by the manufacturer. Additional chlamydial LPS staining was performed for the experiments shown in B. Cells were analysed by confocal microscopy.

3.3.3 Atypical DNA laddering pattern during Chlamydia infection

Fragmentation of genomic DNA during apoptosis leads not only to DNA breaks detectable by TUNEL assay, but should be also detectable through gel electrophoresis separation of DNA fragments. However, a TUNEL-positive signal is not equal to the results of gel electrophoresis-revealed genomic fragmentation. The latter is the results of cleavage during apoptosis at easily

accessible nucleosomal boundaries, which generates DNA fragments of multiples of 180 bps. However, any sort of DNA damage rises a positive TUNEL result. In DNA from UV irradiated cells, gel electrophoresis revealed cleaved DNA bands that appeared as rungs of ladders as well as a smear at higher molecular weight, a result of typical apoptotic DNA fragmentation induced by UV irradiation. In samples from Chlamydia infected cells (Fig. 3.15) (where nuclear morphological changes and TUNEL positive signals were observed), although several small DNA bands were seen, there was no indication of larger fragments; this pattern was different from the pattern shown in the UV irradiation sample. This result suggests that TUNEL-positive signals from Chlamydia-infected cells and UV-irradiated cells were induced by different mechanisms, and that Chlamydia infection does not lead to apoptotic DNA fragmentation.

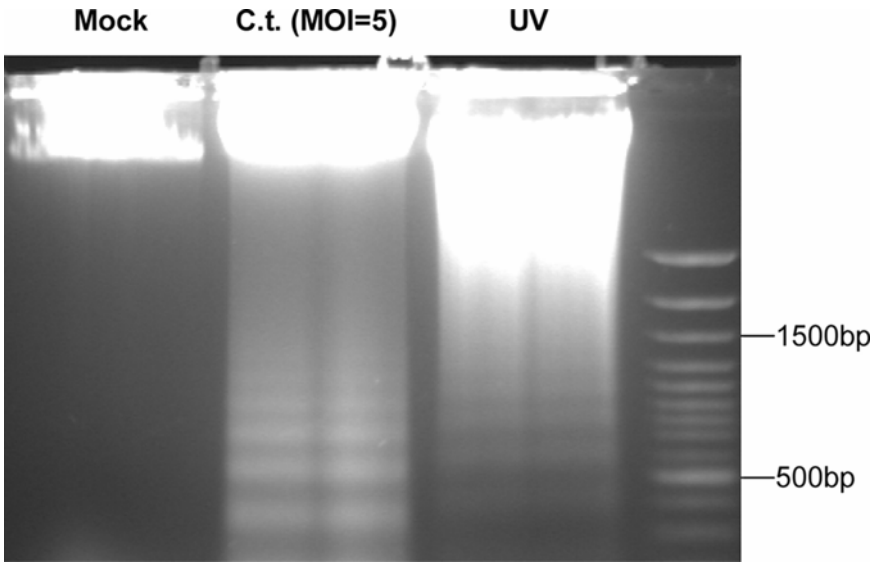

Fig. 3.15 Comparison of DNA fragmentation patterns between Chlamydia infection and UV irradiation

MEF cells were infected with *Chlamydia trachomatis* at an MOI of 5 for 24 hours, or treated with UV irradiation and cultured overnight. Chromosomal DNA was then extracted as described in material and methods. DNA fragmentation patterns were revealed by gel electrophoresis on a 1 % agarose gel containing ethidium bromide. Similar results were seen in 3 independent experiments.

3.3.4 No involvement of caspase-3 in Chlamydia-induced cell death

Caspase activation is a classical and indispensable event of apoptosis induction. Therefore, measurement of caspase activation or activity (for example, caspase-3, an effector caspase involved in all or nearly all conditions of apoptosis) is one way to define a form of cell death as apoptosis. Here, we applied different assays to test both caspase-3 activity and caspase-3 activation during Chlamydia infection (Fig. 3.16). As a positive control, UV-irradiated cells again showed a dramatically increased caspase-3 activity by enzyme assay, which measures cleavage of a substrate with the recognition sequence DEVD, a preferred caspase-3 cleavage site. Flow cytometry analysis using an antibody specifically recognizing active caspase-3 fragments demonstrated a population of cells that were positively stained with the active caspase-3 antibody following UV irradiation. However, in Chlamydia-infected samples, neither caspase-3 activity nor active caspase-3 staining was observed (Fig. 3.16 A, B). This indicates that Chlamydia-induced cell death does not involve caspase-3 activation. To further test this conclusion, the broad caspase inhibitor zVAD was included in an experiment where cell death was induced by *Chlamydia trachomatis* infection. As expected, caspase inhibition almost completely blocked apoptosis induced by UV irradiation, but had no effect on Chlamydia infection-induced cell death (Fig. 3.16 C). This result again supports the conclusion that caspases are not involved in cell death induction by Chlamydia.

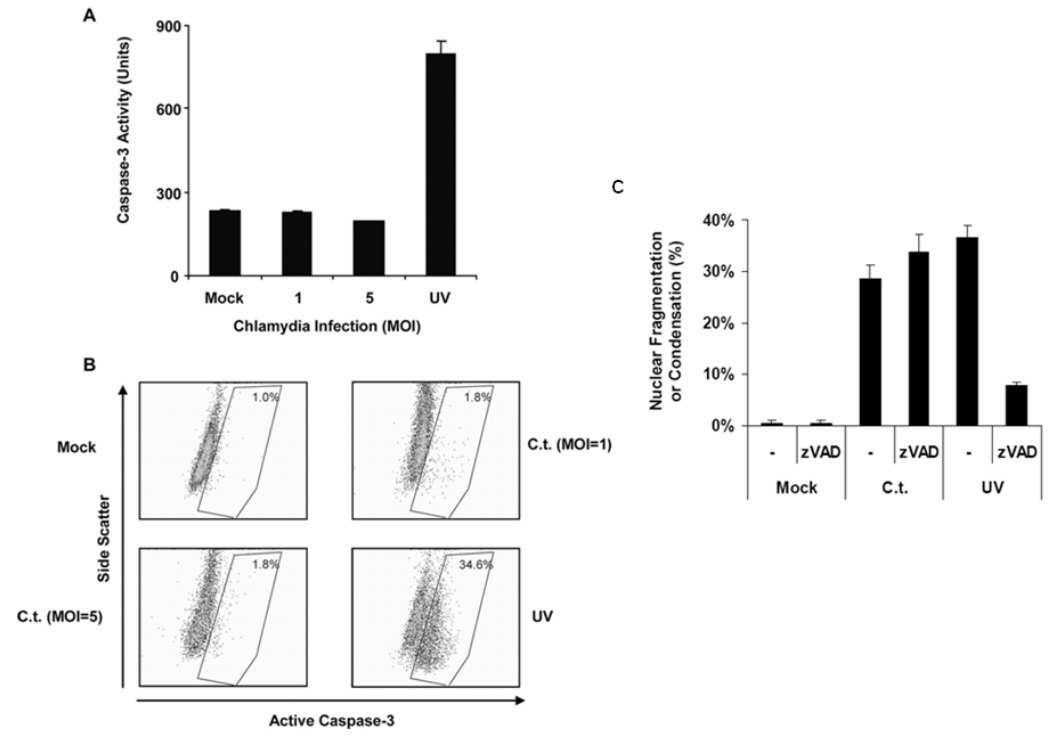

Fig. 3.16 No involvement of caspase activation or activity during Chlamydia-induced cell death

(A) MEF cells were infected with *Chlamydia trachomatis* at the indicated MOI for 24 hours. Some uninfected cells were treated with UV light and cultured overnight as a positive control. Following the indicated infection or treatment, cells were harvested and lysed in NP40 lysis buffer, and DEVD-cleaving activity was measured in cell extracts. (B) The same treatment was performed as shown in A, before cells were fixed and stained with an antibody specifically recognizing active caspase-3. Cells expressing active caspase-3 were detected by flow cytometry. (C) At the time of infection with *Chlamydia trachomatis* at MOI of 5, or UV irradiation, the broad caspase inhibitor zVAD was added in some conditions to block caspase activation. After Hoechst staining, cells displaying nuclear morphological changes were determined by fluorescence microscopy and at least 300 nuclei were counted twice for each experimental condition. Standard deviation was calculated from values of triplicates (A) or 2 times counting (C) in a single sample. All experiments were performed at least 3 times, showing similar results.

3.3.5 Involvement of the pro-apoptotic proteins Bax and Bak in Chlamydia-induced cell death

Bax and Bak are pro-apoptotic proteins belonging to the Bcl-2 family, controlling cytochrome *c* release from mitochondria to the cytosol. Combined loss of Bax and Bak has been shown to block most or all cases of apoptosis. A previous study using Bax knock-out mice demonstrated that Bax played a role in *Chlamydia muridarum* propagation *in vivo* (Perfettini et al., 2003). Although no evidence of caspase activation has been found in Chlamydia infection-triggered cell death, it is principly conceivable that Bax and/or Bak may cause caspase-independent apoptosis. Thus, analysis of the roles of Bax and Bak in Chlamydia-induced cell death is relevant. We therefore compared Chlamydia-induced cell death in wild type MEF cells, as well as MEF cells lacking Bax, Bak or both (Fig. 3.17). Similar infection susceptibility was found in all four cell lines (Fig. 3.18). Again, hoechst staining and TUNEL assay were performed to determine the extent of cell death induction in these cells. As expected, UV-induced nuclear morphological changes and TUNEL-positive signals were diminished to a background level in Bax/Bak double deficient cells. However, no reduction in TUNEL-positive signals and only a mild decrease of nuclear morphological changes were observed in these cells infected with *Chlamydia trachmatis* compared to that induced in wild type cells. Even more surprisingly, cells lacking Bak alone were more resistant to Chlamydia-induced nuclear changes than Bax/Bak double deficient cells. The roles played by Bax and Bak during Chlamydia-induced cell death thus are unclear. Cytochrome *c* release was tested in the following experiments.

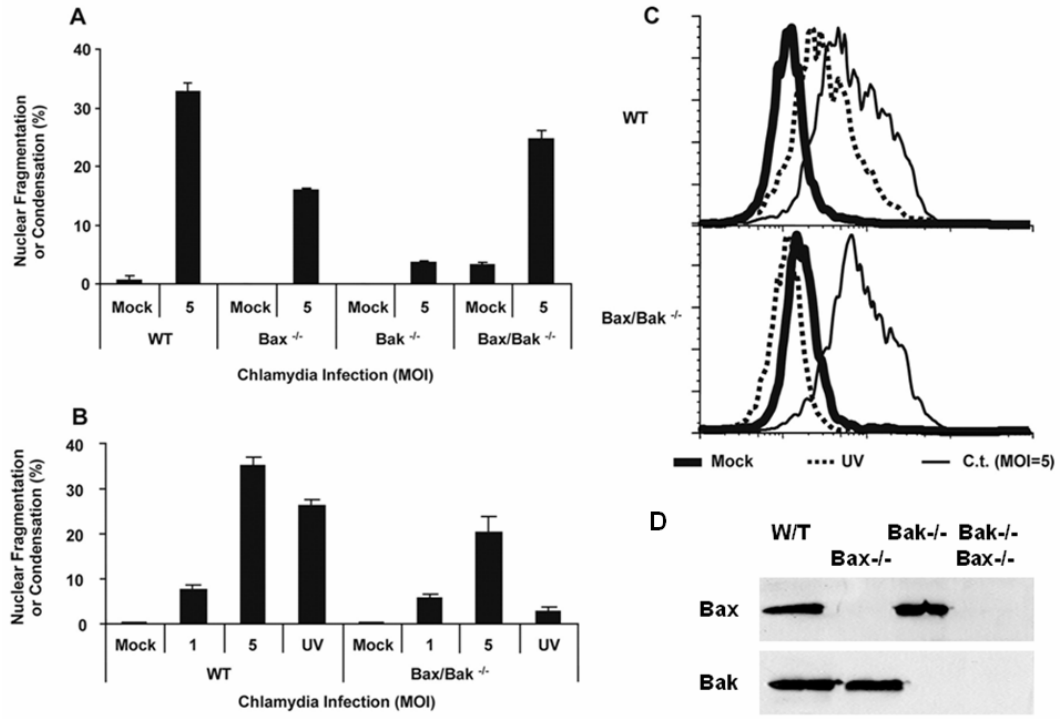

Fig. 3.17 Roles of pro-apoptotic proteins Bax and Bak during Chlamydia induced cell death

Wild type MEF cells as well as cells lacking Bax and/or Bak (genotypes were confirmed by Western blot in D) were infected with *Chlamydia trachomatis* at the indicated MOI for 24 hours. Some wells containing uninfected cells were exposed to UV irradiation 16 hours before analysis. (A, B) Following infection or treatment, cells were stained with Hoechst and analysed by fluorescence microscopy. For each experimental condition, at least 300 nuclei were counted twice to calculate the average and standard deviation that was shown in the pictures. (C) The same infection or treatment was performed, before cells were fixed and analysed by FACS following TUNEL staining. Each experiment was performed at least 2 times with similar results.

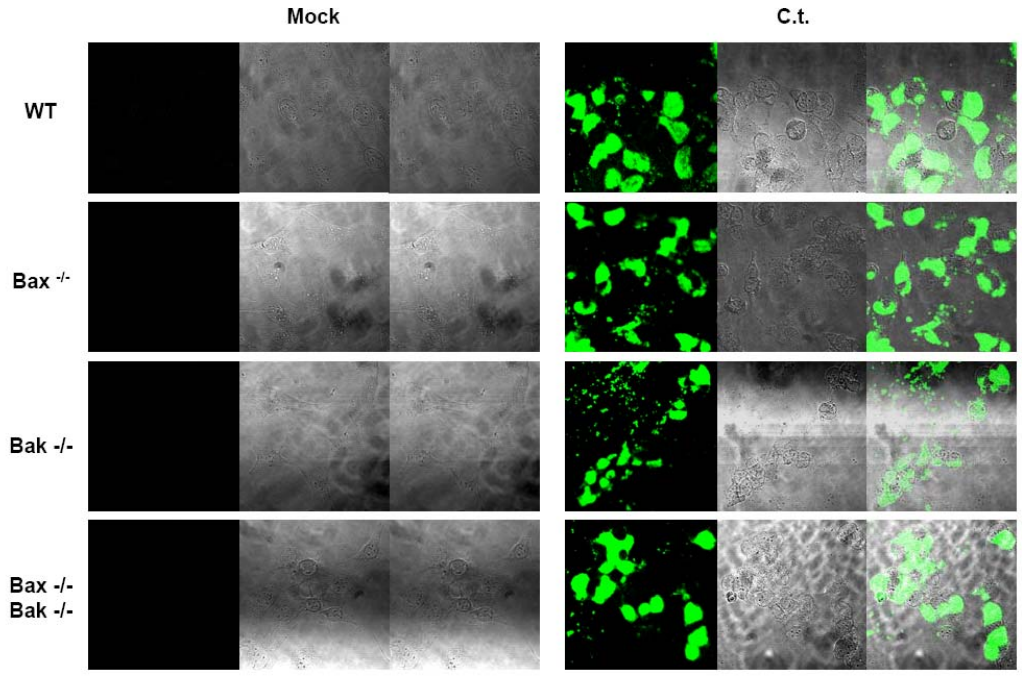

Fig. 3.18 Comparison of Chlamydia infection in wild type MEF cells and MEF cells lacking Bax and/or Bak

Wild type MEF cells or cells lacking Bax, Bak or both, were infected with *Chlamydia trachomatis* at an MOI of 1 for 24 hours. Cells were fixed and stained with an antibody against Chlamydia LPS. Similar ratios of Chlamydia inclusions were observed by confocal microscopy.

3.3.6 Cytochrome *c* is retained in mitochondria during Chlamydia infection

Cells lacking Bax and Bak are resistant to apoptosis because of a deficiency to release cytochrome *c* from mitochondria into the cytosol when an apoptotic stimulus is applied. To test for cytochrome *c* release, we co-stained wild type cells with Mitotracker-red (a mitochondrial marker) and an antibody against cytochrome *c* during Chlamydia-induced cell death. Interestingly, even after 24 hours of infection with *Chlamydia trachomatis* at an MOI of 5 (cells were dramatically enlarged by a big inclusion in the cytosol), at which point nuclear morphological changes and TUNEL-positive signals were detectable, most of cytochrome *c* was still localized in mitochondria as revealed by co-localization of cytochrome *c* with mitotracker (Fig. 3.19). This lack of cytochrome *c* release

suggests two conclusions. First, no typical apoptosis pathway signals upstream of mitochondria were activated during Chlamydia-induced cell death. Second, the role played by Bax and Bak during cell death induced by Chlamydia infection is not the induction of cytochrome *c* release.

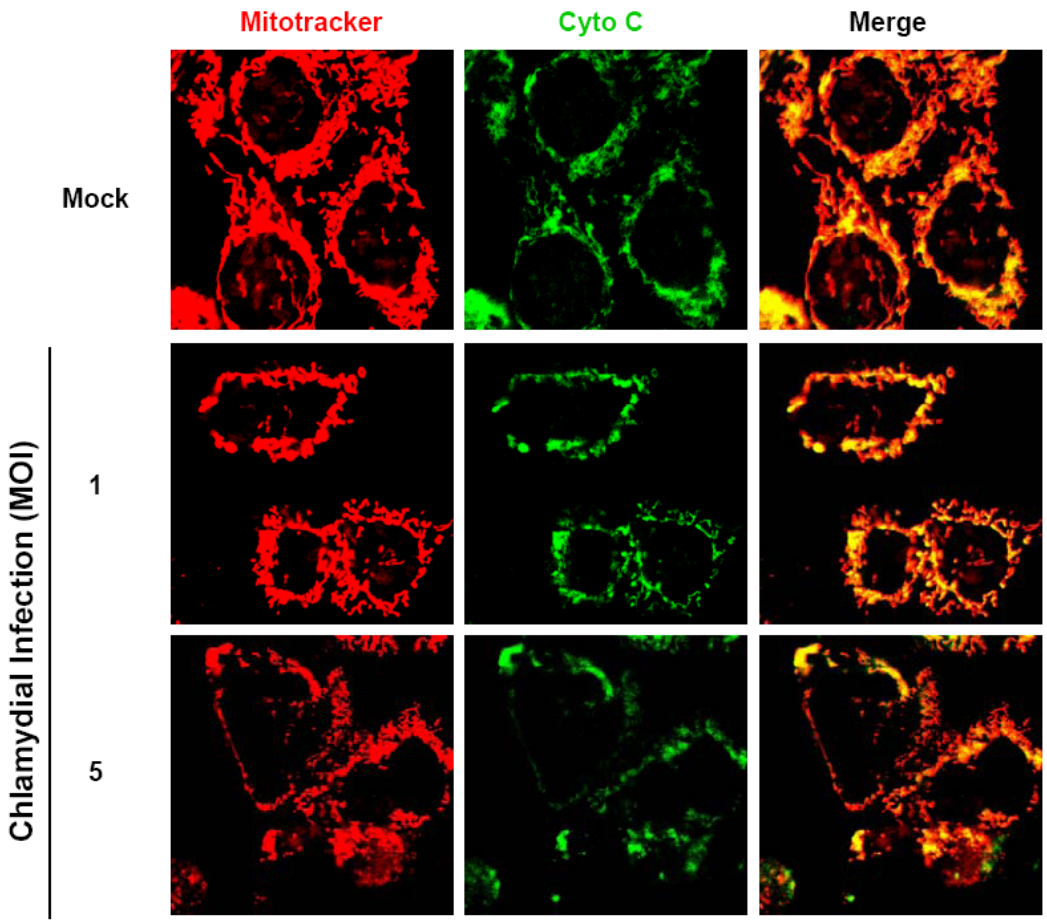

Fig. 3.19 Cytochrome *c* is not released during Chlamydia-induced cell death

Wild type MEF cells were infected with *Chlamydia trachomatis* at the indicated MOI for 24 hours. Cells were stained with Mitotracker-red before fixation. Cells were then harvested and stained with an anti-cytochrome *c* antibody, followed by a secondary FITC-conjugated antibody. Confocal microscopy analysis was performed to reveal the extent of co-localization between mitochondria and cytochrome *c*. Two independent experiments generated similar results with most of cells showing the same pattern.

3.3.7 Uptake of Chlamydia-infected cells by professional phagocytes

A common fate of apoptotic or necrotic cells *in vivo* is the internalization by phagocytes. In fact, infection of macrophages by Chlamydia *in vivo* has been described for many years (Beutler et al., 1995), despite a very low susceptibility of macrophages to Chlamydia infection *in vitro*. Thus, cell death induced by Chlamydia may provide a way that leads to infection of immune cells *in vivo*. An *in vitro* phagocytosis experiment as described previously (Kirschnek et al., 2004) was thus performed to test the hypothesis of ingestion of cells infected and killed by Chlamydia by phagocytes. Macrophages and dendritic cells were used for this experiment. Interestingly, following labelling of the cell membrane and indicated infection, the red-labelled MEF cells infected with Chlamydia after 24 hours (when cell death was observed) were clearly ingested by co-cultured green-labelled dendritic cells (Fig. 3.20). Shorter infection (6 hours) or infection with inactivated Chlamydia did not lead to the uptake. Ingestion of infected cells by macrophages was observed in a similar manner (not shown).

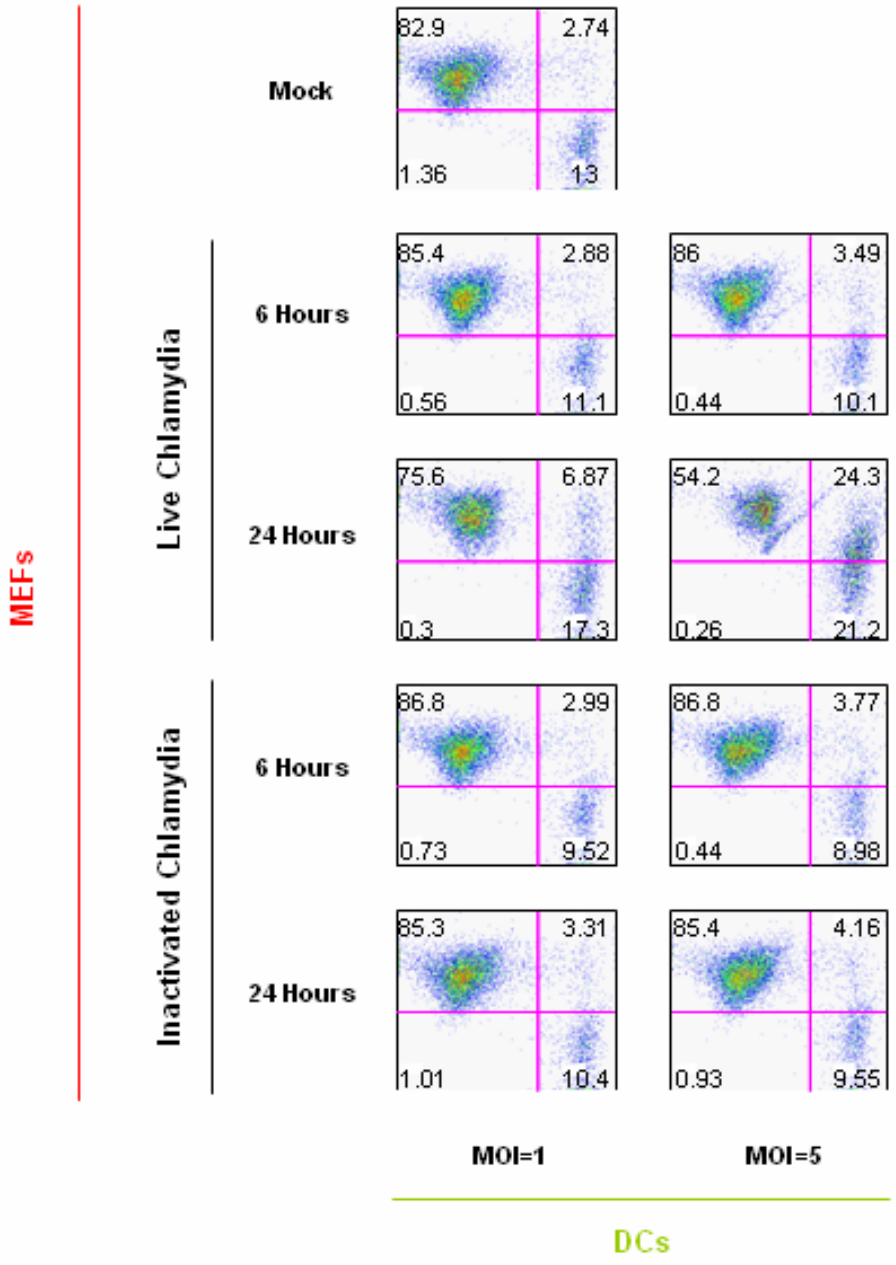

Fig. 3.20 Chlamydia-killed cells are ingested by professional phagocytes

MEF cells (stained with PKH26) were infected with infectious or heat-inactivated C. trachomatis at the indicated infectious dose. After the indicated period of infection, PKH-67-stained DCs were added to MEF cells for an additional 2 hours. Following the co-incubation, all cells were harvested and analyzed by flow cytometry. Percentages of single- and double-positive events are shown.

4. Discussion

4.1 BH3-only proteins are broadly targeted for destruction during Chlamydia infection

After nearly a decade of detailed research on the mechanisms of chlamydial anti-apoptotic effects, following the first publication in 1998 (Fan et al., 1998), it has been well established and consistently shown that Chlamydia-infected cells are potently resistant to a wide range of apoptotic stimuli, including UV, staurosporine, CD95 and to spontaneous apoptosis. It has been previously shown that the appearance of a series of apoptosis markers is blocked during Chlamydia infection. Briefly, caspase activation and cytochrome *c* release from mitochondria are not observed upon apoptotic stimulation in infected cells (Fan et al., 1998; Fischer et al., 2001). While caspase activation is a major event that takes place in apoptosis, cytochrome *c* only plays a role in mitochondria-mediated apoptosis. Cytochrome *c* is released from mitochondria into the cytosol to further bind and activate Apaf-1, which leads to caspase activation. The release of cytochrome *c* is tightly controlled by Bcl-2 family proteins (see introduction). Since no anti-apoptotic effects of Chlamydia have been observed in CD95-stimulated type I cells (where mitochondria do not participate in apoptosis signal transduction) during Chlamydia infection (Fischer et al., 2004a), attention was focused on the mitochondrial step to elucidate the potent anti-apoptotic effects exerted by Chlamydia. We and others have previously reported that UV- or staurosporine- induced activation of Bax and Bak is inhibited in Chlamydia-infected cells (Fischer et al., 2004b; Xiao et al., 2004). Although the exact molecular actions leading to Bax/Bak activation are yet to be determined, BH3-only proteins undoubtedly serve as upstream players that promote Bax/Bak-mediated cytochrome *c* release.

We demonstrated in this project that all BH3-only proteins that would be tested are degraded during Chlamydia infection. BH3-only proteins belong to the greater Bcl-2 family proteins, sharing only a short region, the BH3 domain. Point mutation studies in the BH3 domain have revealed that the BH3 domain

is required for the degradation of Bim (not shown). Eight members (Bim, Puma, Noxa, Bid, Bad, Bmf, Hrk, and Bik/Nbk) of the group of BH3-only proteins have been identified. In most cases of apoptosis initiation, one or several BH3-only proteins are involved through transcriptional up-regulation and/or post-translational modification. Bim, for example, is required for cell death induced by taxol, etoposide and γ-rays (Bouillet et al., 1999). Bim, Puma and maybe also Noxa trigger induction of activated T cell death (Bauer et al., 2006; Bouillet et al., 1999; Hildeman et al., 2002). Puma and Noxa are described to be responsible for DNA damage-induced and p53- dependent apoptosis (Villunger et al., 2003), while Noxa is also found active in cell death that is induced during viral infection and by interferons (Sun and Leaman, 2005) as well as by inhibition of proteasome activity (Fernandez et al., 2005; Qin et al., 2005). The roles of most other BH3-only proteins are less clear. Although the killing activity differs between various BH3-only proteins, loss of this class of proteins in Chlamydia-infected cells could be expected to explain the potent anti-apoptotic phenotype. Knock-out mice that are deficient in one or two BH3-only proteins have been generated to evaluate the roles of BH3-only proteins in apoptosis and relevance in biological diseases. It has been previously shown that CD95-stimulated apoptosis is only blocked in infected type II cells, but not in infected type I cells (Fischer et al., 2004a). In type II cells, apoptosis signals are transmitted from the death receptor pathway to the mitochondrial pathway by Bid cleavage. Intact Bid is inactive because of the hidden BH3 domain revealed by two structural studies (Chou et al., 1999; McDonnell et al., 1999). After activation, the BH3 domain is exposed on the surface of truncated Bid that translocates to mitochondria and activates Bax/Bak. Interestingly, we found that truncated Bid is degraded during Chlamydia infection while intact Bid remains unaffected. This is likely an explanation for the mechanism that mediates the resistance of CD95-induced apoptosis in type II cells. Broad degradation of BH3-only proteins is relevant and may explain almost all cases of apoptosis inhibition in Chlamydia-infected cells.

Time course experiments have shown that disappearance of Bim, Puma and Bad starts around the same time. Since no significant changes in RNA levels

of BH3-only proteins were observed by microarray analysis, we proposed that destruction of all BH3-only proteins is mediated by the same mechanism. Regulatory proteins or misfolded proteins are constantly degraded by the proteasome. We used the proteasome inhibitors MG-132 and Lactacystin in Chlamydia-infected cells to assess the role of the proteasome in the degradation of BH3-only proteins. Interestingly, while Bim, Puma, Bad and tBid are fully degraded by Chlamydia in the absence of MG-132, degradation is almost completely blocked by the addition of MG-132. Protection of Puma requires higher concentrations of MG-132 (40 μM) while Bim and Bad are protected even with low doses of MG-132 (20 μM). Another group reproduced our results by providing *in vitro* data, showing degradation of BH3-only proteins in Chlamydia-infected cell lysates, which was also preventable by a proteasome inhibitor (Dong et al., 2005). Proteins to be degraded by the proteasome are normally modified by small molecules called ubiquitin ligases (although exceptions exists) (Matsuzawa et al., 2005), followed by recognition of the ubiquitinated protein by the proteasome. Mono-ubiquitination or poly-ubiquitination can be detected by Western blotting because modified proteins run at higher molecular weight and often show a ‘smear’ due to modification with various repeats of ubiquitin. Smears of higher molecular weight BH3-only proteins have never been detectable in Western blots, even when the proteasome inhibitor MG-132 was included to prevent degradation. In addition, one of the targeted BH3-only proteins, Puma, contains no lysine, which is usually the amino acid that is recognized and modified by ubiquitin. Therefore, ubiquitination does not seem to be involved in this case of BH3-only proteins degradation. One possibility is that BH3-only proteins are targeted by a Chlamydia-secreted factor and the complex then is degraded by the cellular proteasome.

A chlamydial protease called chlamydial protease-like activity factor (CPAF) has been identified and characterized to be responsible for degradation of several cellular substrates, including transcription factor RFX5 (Zhong et al., 2001) and host keratin 8 (Dong et al., 2004). Surprisingly, the activity of CPAF can also be blocked by proteasome inhibitors. CPAF is obviously another possible candidate as the BH3-only protein degrading factor. In fact, Pirbhai *et*

al observed cleavage of several BH3-only proteins (i.e. Puma and Bik) (Pirbhai et al., 2006) following *in vitro* co-incubation with recombinant CPAF (Pirbhai et al., 2006). Until now, at least 5 proteins have been demonstrated to be targets of CPAF. CPAF is a protease that is secreted by Chlamydia into the cytosol, and may be important for completion of the entire chlamydial intracellular lifespan. However, the specificity and exact roles of CPAF *in vivo* still remain unclear.

Although the major part of BH3-only proteins is degraded during Chlamydia infection, remaining traces of BH3-only proteins may still play a role. However, the Akt survival pathway has been reported to be activated in infected cells, which leads to phosphorylation of the BH3-only protein Bad. Bad is therefore inactivated and recruited to the chlamydial inclusion membrane via interaction with 14-3-3β (Verbeke et al., 2006). The physical sequestration of Bad away from its site of function may be an additional mechanism that aids Chlamydia to protect the host cells from apoptosis. Besides Bad, another pro-apoptotic molecule, PKCδ, is also sequestered by the chlamydial inclusion due to the accumulated concentration of diacylglycerol at the vacuole (Tse et al., 2005). Both degradation of BH3-only proteins and modification of pro-apoptotic effectors during Chlamydia infection may be important, and at least in part contribute to the host cell's resistance to spontaneous apoptosis and cell death promoted by a wide range of apoptotic stimuli.

Apart from post-translational modifications of pro-apoptotic proteins, transcriptional up-regulation of anti-apoptotic proteins has also been reported. Conclusions are still conflicting for NF-κB activated anti-apoptotic gene regulation in Chlamydia-infected cells. Besides its unclear anti-apoptotic role, NF-κB activation has been observed a number of times in immune cells infected with Chlamydia (Wahl et al., 2003; Wahl et al., 2001). Although Paland *et al*. reported NF-κB activation in HeLa cells (Paland et al., 2006), this activity was not observed by us and others in epithelial cells like HeLa where most of the potent chlamydial anti-apoptotic role is observed (Fischer et al., 2001; Xiao et al., 2005). It may be reasonable to speculate that activation of NF-κB is a result of the recognition of chlamydial components by the innate

immune system, especially through TLRs (Prebeck et al., 2001). Deletion of the NF-κB p65 gene does not prevent Chlamydia from inhibiting host cell apoptosis (Xiao et al., 2005). NF-κB may thus be activated in some situations by Chlamydia, but NF-κB activation is certainly not the factor that always leads to protection of Chlamydia-infected cells. Although NF-κB does not seem to be strongly involved in chlamydial anti-apoptotic actions, a number of NF-κB regulated anti-apoptotic genes are induced during Chlamydia infection.

Up-regulation of the IAP family protein cIAP-2 but not the other IAP family members, by NF-κB, has been described a number of times in Chlamydia-infected cells (Paland et al., 2006; Rajalingam et al., 2006; Wahl et al., 2003). Unexpectedly, RNA interference experiments suggested that not only cIAP-2 but also XIAP and cIAP-1 are required for protection of infected cells against apoptosis. Rajalingam *et al.* proposed that various IAP members may form a complex they called the IAPsome, within which different IAPs interact and stabilize each other (Rajalingam et al., 2006). If this is true, deletion of one may lead to destruction of others, and therefore they are all required for Chlamydia to prevent host cell death by molecularly uncertain means. As discussed above, IAPs are described to be inhibitors against caspases. More recent experimental results indicate that only XIAP is able to bind and inhibit caspases via its BIR domains, but all the others do not have this activity (Eckelman et al., 2006). Therefore the role of IAP members in Chlamydia anti-apoptotic effects has yet to be clearly established.

Several changes of apoptosis-regulating proteins (e.g. IAPs and Mcl-1 etc.) in infected cells have thus been demonstrated. In order to understand the relevance of those changes, we analysed the apoptosis inhibition of infected cells where pro-apoptotic Bim_S is over-expressed. The molecular events leading to apoptosis following Bim_S induction will be discussed later in more detail. In this model, it is clear that Chlamydia-infected cells are resistant to UV- induced apoptosis, but remain susceptible to Bim_S-induced apoptosis. UV irradiation is well known to engage the mitochondrial pathway to induce apoptosis, while Bim_S is an activator of the mitochondrial effector proteins Bax and Bak. This indicates that the signaling pathways downstream of BH3-only

proteins remain fully functional during Chlamydia infection. IAP family members probably function at the caspase level, downstream of mitochondrial events. Hence, sensitivity of Chlamydia infected cells to Bim_S argues against the involvement of IAPs in apoptosis inhibition by Chlamydia.

4.2 Over-expression of Bim_S induces mitochondria-mediated apoptosis

In the T-REx Bim_S cell line, Bim_S expression is under control of a Tet-repressor that can be removed by tetracycline, or its derivatives (e.g. anhydrotetracycline and doxcycline) (Gossen et al., 1995). In this project, experiments were performed and Bim_S induction was achieved by using either tetracycline or anhydrotetracycline to activate Bim_S transcription in human cells. Anhydrotetracycline was used because of its low antibiotic effects (Gossen and Bujard, 1993).

Bim_S is the shortest isoform of the BH3-only protein Bim, which appears to be a more potent killer compared to the other two isoforms (O'Connor et al., 1998). In this system, Bim_S over-expression induced by tetracycline can kill cells very quickly (up to 80-90 % after 6 hours). Release of cytochrome *c* and Smac/Diablo was observed, which indicates the expected involvement of mitochondria in apoptosis induced by Bim_S. Caspase activation was monitored following Bim_S induction. As expected, caspases that are involved downstream of mitochondria, i.e. caspase-9, caspase-7 and caspase-3, were all activated by Bim_S. Comparison between Bim_S and staurosporine did not show any significant differences in the above mentioned markers. We conclude that Bim_S induces typical mitochondria-dependent apoptosis, and therefore the Bim_S inducible cell line is suitable to test the integrity of the mitochondrial pathway in Chlamydia infection.

4.3 Apoptosis induction at the middle stage of infection can block chlamydial propagation

Although apoptosis inhibition has been described for nearly a decade, there is so far no evidence for the relevance *in vivo*. An obvious hypothesis is that, based on the unique intracellular lifestyle, Chlamydia needs substantial space and time for the complete process of invasion (EB), conversion (EB-RB), replication (RB-RB), re-conversion (RB-EB) and release (EB). Disruption by apoptosis induction between the steps, especially before re-conversion, may interrupt bacterial replication. No clear evidence for this has been presented until now because of the strong apoptosis inhibition in Chlamydia-infected cells. It has not been possible to induce apoptosis in Chlamydia-infected cells before the T-REx Bim_S cell line had been generated. In this cell line, Bim_S can be induced by addition of tetracycline/anhydrotetracycline (to avoid side-effects of tetracycline against Chlamydia). After 24 hours of infection, although UV-induced apoptosis is significantly blocked by Chlamydia, Bim_S induces a similar rate of apoptosis in either uninfected or infected cells. After both 24 and 48 hours of infection, Chlamydia-infected cells remain sensitive to Bim_S. This indicates the integrity of downstream pathway and also indicates the biological relevance of BH3-only proteins degradation.

To test the hypothesis that host cell survival is required for Chlamydia growth, *Chlamydia trachomatis* infected T-REx Bim_S cells were killed at different time points by anhydrotetracycline via Bim_S induction, followed by co-culture of the infected cells together with a HEp2 monolayer. Chlamydia were found to be still active and infective when the host cells were killed after 48 hours of infection, but no infectivity was achieved when the infected cells were killed earlier after 24 hours of infection. Electron microscopy analysis revealed that no or very few EBs were produced when apoptosis in infected cells was induced 24 hours post infection. EB is the only chlamydial form that is infective. Loss of infectivity may thus be caused by blockage of conversation from RB to EB. But the factors that contribute to the blockage have to be determined. In apoptotic cells, mitochondria are damaged, which leads to

dramatic decrease in ATP level (Richter et al., 1996). Lack of sufficient ATP in infected apoptotic cells may be a possible factor that reduces nutrient supply required for the intracellular bacteria.

The caspase inhibitor zVAD blocked Bim_S-triggered apoptosis in Chlamydia-infected cells as expected. EB production was also observed in Bim_S-induced cells in the presence of zVAD. However, it was very surprising to note that inhibition of caspase activity did not rescue chlamydial infectivity. Coincident with the lack of infectivity, a drop in ATP levels following Bim_S induction was not prevented by caspase inhibition. Hence, conversion from RB to EB is necessary but maybe not sufficient to keep Chlamydia infective. ATP levels in the host cells may correlate with Chlamydia infectivity. It is also conceivable that an undefined factor may be required for EB infectivity, which is eliminated following mitochondrial apoptotic events and is not the result of caspase activation.

4.4 Chlamydia leads to caspase-independent cell death

To characterize cell death induced by Chlamydia, we performed a set of standard assays to test molecular and biochemical changes during infection with *Chlamydia trachomatis* or *Chlamydia muridarum* (Ying et al., 2006). In agreement with previous findings, we observed in infected cells nuclear morphological changes that were indistinguishable from typical apoptosis induced by UV irradiation, as revealed by Hoechst staining as well as TUNEL labelling. However, the key apoptosis markers caspase activation or activity were not detected during infection. Some DNA fragmentation was observed following DNA separation by agarose gel electrophoresis, the pattern of which was, however, different from the DNA fragment composition generated in cells treated with UV irradiation. Studies using cells deficient in Bax, Bak or both revealed that these pro-apoptotic molecules may play a role in Chlamydia-induced cell death, but not because of their conventional function in apoptosis regulation.

Several questions may be raised regarding cell death induction by Chlamydia. Does Chlamydia infection induce host cell death? Which form of cell death does Chlamydia infection promote? What is the physiological role of cell death induction by Chlamydia infection *in vivo*?

There are numerous pieces of evidence that Chlamydia-infected host cells die at the end of the bacterial intracellular life. Massive changes of intracellular organelles have been reported, such as dilation and vacuolation of the endoplasmic reticulum (ER), distortion of mitochondria, and nuclear condensation (Ojcius et al., 1998; Todd et al., 1976; Todd and Storz, 1975). All of those events happen during apoptosis, but the order of events is not typical as seen in apoptosis. In apoptotic cells, nuclear morphological changes occur relatively early followed by massive but late damage of intracellular organelles. In this study, we showed that apoptotic features of nuclear morphological changes and TUNEL positive staining. Nuclear morphological changes are

normally the result of apoptotic signaling pathway activation, represented as DNA fragmentation or DNA condensation.

Some small DNA fragments were detected following Chlamydia infection, although the pattern was atypical compared to the one induced by UV irradiation. In apoptotic cells, DNAses are activated and cause DNA digestion, which generates first larger and then smaller DNA fragments that form a ladder-like appearance on agarose gels (Sakahira et al., 1999). In Chlamydia-infected cells, only small fragments are detected, however, without evidence of step-by-step cleavage of DNA. This indicates that only a small portion of DNA is degraded almost completely, which does not correlate with the ratio of nuclear morphological changes or positive TUNEL staining. Apoptotic DNA degradation is normally achieved by CAD (caspase-activated DNase) activation, a result of caspase-mediated ICAD (inhibitor of CAD) inactivation. CAD activation is very likely not achieved in infected cells, since caspase activation does not occur. The results therefore suggest that DNA fragmentation induced in Chlamydia-infected cells is not the result of CAD activation as it would be during apoptosis. Positive TUNEL staining represents another common marker for nuclear damage during apoptosis. The TUNEL assay also recognizes DNA single strand breaks which give a positive signal, but this will not be seen by gel electrophoresis. DNA damage has been known to trigger apoptosis via p53-dependent activation of the BH3-only proteins Puma and Noxa. No involvement of p53 has ever been described in Chlamydia infection. Even if the p53 pathway was activated following damage of DNA, the downstream Puma and Noxa are not present in Chlamydia-infected cells due to their specific destruction (Fischer et al., 2004b; Ying et al., 2005), and typical apoptotic signaling activation could be blocked at this step. Hence, DNA damage may occur prior to or independently of cell death initiation in Chlamydia-infected cells.

Bax and Bak are key regulators that control release of mitochondrial proteins. N-terminal conformational changes of Bax were observed during Chlamydia infection, and Bax-deficient mice showed increased inflammatory cytokine secretion (Jungas et al., 2004; Perfettini et al., 2003). In uninfected cells, Bax

is normally activated by activation of BH3-only proteins, although the precise molecular action of Bax activation remains unclear. It is unclear how this activation would work since BH3-only proteins are degraded. However, since Bak may be alternatively activated by release from its anti-apoptotic sequestration partners Mcl-1 and Bcl-xL (Willis et al., 2005), Bax may also be activated by this way without participation of BH3-only proteins. Other than the conventional pathways, Bax could also be activated by physical stress, for instance oxidative stress, or heat (Jungas et al., 2002; Pagliari et al., 2005). It has to be noted that completion of Bax activation requires not only the conformational changes, but also oligomerization and insertion into the mitochondrial outer membrane, which has yet to be investigated during infection.

It was surprising to find that Chlamydia-infected MEF cells lacking Bax or Bak show a lower rate of cell death, with a higher decrease in Bak knock out cells. However, combined loss of Bax and Bak resulted in an increase of nuclear apoptosis, comparable to the level of wild type cells. The paradoxical results are very difficult to explain. A complementary molecular pathway leading to Chlamydia-induced cell death may be activated when both Bax and Bak are lost. It is also possible that a second mutation has occurred in cells lacking both Bax and Bak. But such a complementary mechanism would not be involved in apoptosis induction, since typical apoptosis induced by UV irradiation is completely blocked in $Bax^{-/-}Bak^{-/-}$ cells. Since during Chlamydia-induced cell death, cytochrome *c* is retained in mitochondria, it can be concluded that Bax and Bak in Chlamydia-infected cells do not play the role that they have in uninfected cells, for example to release cytochrome *c*. Recent studies suggest that Bax may contribute to mitochondrial fission (Karbowski et al., 2002). It is then also possible to speculate that Bax and Bak participate in Chlamydia-induced cell death by a role that is unrelated to that in apoptosis induction, but instead by for example a role in mitochondrial metabolism. Bax and Bak have also been suggested to be involved in the response to unfolded proteins in the ER (Hetz et al., 2006). It is conceivable that Bax and Bak participate in ER-dependent cell death induced by Chlamydia infection.

Apoptotic or necrotic cells are known to be taken up by professional phagocytes, where dead cells are further degraded and thus removed (Bondanza et al., 2004; Fan et al., 2004; Jehle et al., 2006; Kim et al., 2005). It is intriguing that dying infected cells are efficiently ingested by co-incubated dendritic cells *in vitro*, which may implicate a possible mechanism triggering or enhancing adaptive immune responses against chlamydial infection *in vivo*. In fact, a recent study suggested that apoptotic bodies could deliver intracellular bacteria-derived antigens, to crossprime CD8 T cells against mycobacterial infection (Winau et al., 2006). The exact physiological role of Chlamydia-induced cell death *in vivo* has yet to be determined. However, it is an attractive model that dying host cells express 'eat me' signals to facilitate phagocytosis of infected cells. Ingestion of infected cells may have several effects. First, Chlamydia infection-induced cell death may cause the activation of the immune system that in turn could eliminate the infection. Phagocytosis of infected cells, however, cannot only benefit the host. Chlamydia may, by this way, spread infection into phagocytes *in vivo*.

5. Summary

Chlamydia-infected cells are resistant to a wide range of experimental apoptotic stimuli. However, apoptosis induction has been also described in Chlamydia infection. Interference with the cell death program is a common finding during microbial infection, which may favor both host and microbes. In this study, we first identified the group of pro-apoptotic BH3-only proteins as a target for destruction during Chlamydia infection. Proteasome-mediated degradation of BH3-only proteins was found to be a major mechanism that leads to the inhibition of apoptosis in Chlamydia-infected cells. Chlamydia-infected cells remain sensitive to killing through the BH3-only protein Bim_S, which not only indicates that the downstream signaling is unaffected, but also indicates the biological relevance of BH3-only proteins degradation in apoptosis inhibition. Apoptosis inhibition is probably necessary and important in Chlamydia infection, especially before the end of the infection cycle. This is suggested by the finding that the induction of apoptosis by Bim_S at 24 hours post-infection can block reconstitution of infectious bacteria and therefore block chlamydial propagation. In contrast, at the end of an infection cycle, infected cells undergo cell death with apoptotic features in terms of nuclear morphology (nuclear fragmentation or condensation, as well as positive TUNEL staining). Chlamydia-induced cell death was shown not to be a result of apoptosis signaling, as cytochrome *c* was retained at mitochondria and caspase-3 activation was not detectable. The dying cells were, however, efficiently ingested by phagocytes *in vitro*, indicating possible roles of Chlamydia-induced cell death *in vivo*, by initiating an immune response or by transmission of the infection into immune cells. In conclusion, Chlamydia infection strongly inhibits experimentally induced apoptosis, and apoptosis inhibition is likely required for the completion of the developmental cycle. Cell death induction is also present at the end of the developmental cycle, which may play a role in the release of the bacteria, in immune stimulation and in the spreading of the infection.

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8. Publications

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