Cellular response to double-stranded RNA in *Chlamydia trachomatis*-infected human host cells

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II. ABBREVIATIONS

µ	Micro
Apaf-1	Apoptosis activating factor 1
APS	Ammonium persulfate
Bcl-2	B cell lymphoma 2
BH	Bcl-2 homology
CED	Cell death abnormal
cFlip	Cellular FLICE like inhibitory protein
CHX	Cycloheximide
CIN	Cervical intraepithelial neoplasia
DD	Death domain
DED	Death effector domain
DEPC	Diethylpyrocarbonate
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EB	Elementary body
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
e.g.	*Exempli gratia*, for example
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FADD	Fas associated protein with a death domain
FAM	Fluorescein
FBS	Fetal bovine serum
GDP	Guanosine diphosphate
h	Hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV	Human papilloma virus
Hsp	Heat shock protein
IAP	Inhibitor of apoptosis protein
IB	Immunoblotting
ICE	IL-1 converting enzyme
i.e.	*id est*, that is
IF	Immunofluorescence
IFU	Inclusion forming unit
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
IRF-3	IFN regulatory factor 3
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
KO	Knock out

l	Liter
LGV	Lymphogranuloma venereum
M	Molar
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell lymphoma 1
MDA5	Melanoma differentiation-associated gene 5
MEK	MAP ERK kinase
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
ms	Mouse
NF-kB	Nuclear factor kappa B
OAS	Oligoadenylate synthetase
ORF	Open reading frame
PARP	Poly (ADP- ribose) polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
p.i.	*Post infectionem*, post infection
PKR	Protein kinase RNA-dependent
polyI:C	Polyinosinic:polycytidylic acid
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time PCR
rb	Rabbit
RB	Reticulate body
RIG-I	Retinoic acid inducible gene 1
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamid gel electrophoresis
SE	Standard error
sec	Seconds
siRNA	Small interfering RNA
SPG	Sucrose phosphate glutamate
STS	Staurosporine
tBid	Truncated Bid
TBS	Tris buffered saline
TdT	Terminal deoxinucleotidyl transferase
TLR	Toll like receptor
TUNEL	TdT dUTP nick end labelling
UTP	Uridine triphosphate
v/v	Volume per volume
w/v	Weight per volume

III. ZUSAMMENFASSUNG

Chlamydien sind Gram-negative, obligat-intrazelluläre Bakterien, die für ein weites Spektrum an relevanten Krankheiten verantwortlich sind. Auf Grund ihres zweiphasigen Entwicklungszyklusses sind Chlamydien von einer intakten Wirtszelle abhängig, um sich erfolgreich vermehren und im Organismus ausbreiten zu können. Daher haben Chlamydien anspruchsvolle Strategien entwickelt, um das Immunsystem des Wirtes auszuschalten oder den programmierten Zelltod ihrer Wirtszelle zu verhindern. In der vorliegenden Arbeit wurde untersucht, ob eine Infektion mit *C. trachomatis* einen Einfluss auf die zelluläre Antwort auf dsRNA nehmen kann. Die Synthese von dsRNA ist ein charakteristisches Merkmal der Replikation von Viren, welche sowohl die Apoptose induzieren als auch das Immunsystem aktivieren kann. Um eine chlamydiale und virale Co-Infektion zu simulieren, wurden Chlamydien-infizierte Epithelzellen mit der synthetischen dsRNA Polyinosin-Polycytidinsäure (polyI:C) transfiziert. Im ersten Teil der Arbeit wurde untersucht, ob Chlamydien die durch dsRNA eingeleitete Apoptose verhindern können. Eine signifikante Reduktion der dsRNA-induzierten Apoptose konnte in infizierten Zellen beobachtet werden. Es zeigte sich, dass die Prozessierung der Initiator-Caspase-8 in infizierten Zellen unterblieb. Dies war von der frühen bakteriellen Proteinsynthese abhängig und für die dsRNA-vermittelte Apoptose spezifisch, da der durch TNFα bewirkte Zelltod nicht auf der Ebene der Caspase-8 verhindert werden konnte. Die Aktivierung von zellulären Faktoren, die bei der Apoptoseinduzierung eine wichtige Rolle spielen, beispielsweise PKR und RNase L, war in infizierten Zellen jedoch unverändert. Stattdessen konnte durch RNA Interferenz-vermittelte Depletion gezeigt werden, dass der zelluläre Caspase-8-Inhibitor cFlip eine entscheidende Rolle bei der chlamydialen Blockierung der dsRNA-vermittelten Apoptose spielt. Mittels Co-Immunopräzipitation konnte ein erster Hinweis darauf gefunden werden, dass *C. trachomatis* eine Anreicherung von cFlip im dsRNA-induzierten Komplex von Caspase-8 und FADD bewirkt. Im zweiten Teil der Arbeit wurde untersucht, ob Chlamydien die Immunantwort auf virale Infektionen beeinflussen, welche vor allem die Expression von Interferonen und Interleukinen beinhaltet. Es stellte sich heraus, dass die Aktivierung des *Interferon regulatory factor 3* (IRF-3) und des zur Familie von NF-κB Trankriptionsfaktoren gehörenden p65, zwei zentralen Regulatoren der Immunantwort auf dsRNA, in infizierten Epithelzellen verändert war. Die Degradation von IκB-α, des Inhibitors von NF-κB, war in infizierten Zellen beschleunigt, begleitet von einer Veränderung der Translokation des Transkriptionsfaktors in den Zellkern. Im Gegensatz dazu wurde die nukleäre Translokation von IRF-3 durch die Infektion signifikant verhindert. Die hier vorgestellten Daten zeigen erstmals, dass eine Infektion mit *C. trachomatis* die zelluläre Antwort auf dsRNA signifikant verändern kann und implizieren einen Einfluss von chlamydialen Infektionen auf den Ausgang von viralen Superinfektionen.

IV. ABSTRACT

Chlamydia are Gram-negative obligate intracellular bacteria responsible for a wide spectrum of relevant diseases. Due to their biphasic developmental cycle *Chlamydia* depend on an intact host cell for replication and establishment of an acute infection. *Chlamydia* have therefore evolved sophisticated strategies to inhibit programmed cell death (PCD) induced by a variety of stimuli and to subvert the host immune system. This work aimed at elucidating whether an infection with *C. trachomatis* can influence the cellular response to double-stranded RNA (dsRNA). The synthesis of dsRNA is a prominent feature of viral replication inside infected cells that can induce both PCD and the activation of a cellular innate immune response. In order to mimic chlamydial and viral co-infections, *Chlamydia*-infected cells were transfected with polyinosinic:polycytidylic acid (polyI:C), a synthetic dsRNA. In the first part of this work it was investigated whether *C. trachomatis*-infected host cells could resist apoptosis induced by polyI:C. A significant reduction in apoptosis, determined by PARP cleavage and DNA fragmentation, could be observed in infected cells. It could be shown that processing of the initiator caspase-8 was inhibited in infected host cells. This process was dependent on early bacterial protein synthesis and was specific for dsRNA because apoptosis induced by TNFα was not blocked at the level of caspase-8. Interestingly, the activation of cellular factors involved in apoptosis induction by dsRNA, most importantly PKR and RNase L, was not abrogated in infected cells. Instead, RNA interference experiments revealed the crucial role of cFlip, a cellular caspase-8 inhibitor, for chlamydial inhibition of dsRNA-induced apoptosis. First data acquired by co-immunoprecipitation experiments pointed to an infection-induced concentration of cFlip in the dsRNA-induced death complex of caspase-8 and FADD. In the second part of this work, the chlamydial influence on the first line of defense against viral infections, involving expression of interferons and interleukins, was examined. Activation of the interferon regulatory factor 3 (IRF-3) and the NF-κB transcription factor family member p65, both central regulators of the innate immune response to dsRNA, was altered in *Chlamydia*-infected epithelial cells. polyI:C-induced degradation of IκB-α, the inhibitor of NF-κB, was accelerated in infected cells which was accompanied by a change in nuclear translocation of the transcription factor. Translocation of IRF-3, in contrast, was significantly blocked upon infection. Together the data presented here demonstrate that infection with *C. trachomatis* can drastically alter the cellular response to dsRNA and imply an impact of chlamydial infections on the outcome of viral super-infections.

1. INTRODUCTION

1.1 *Chlamydia*

Chlamydiaceae constitute a family of obligate intracellular human and animal pathogenic bacteria characterized by a unique biphasic life cycle that are responsible for a variety of relevant diseases. The discovery of *Chlamydia* dates back to the very beginning of the last century, when Halberstadter and von Prowazek (1907) described tiny particles inside an intracytoplasmatic vacuole of conjunctival epithelial cells which they identified as the causative agent of the trachoma, a chronic kerato-conjunctivitis. Few decades later, the same particles were found in the blood of humans that suffered from a ghastly pneumonia which they had acquired from birds, called psittacosis (Bedson, 1932). At that time, *Chlamydia* were suspected to be intermediate organisms between viruses and bacteria and, therefore, named *Chlamydozoa* (mantle viruses) after the Greek term *khlamus* (mantle). In the 1960es, biological, biochemical, and molecular studies finally revealed the prokaryotic nature of *Chlamydia*. With the advent of electron microscopy and other molecular techniques, the bacterial cell wall, ribosomes, and RNA were identified demonstrating that the agent of the trachoma and psittacosis were Gram-negative bacteria (Moulder, 1966). The misnomer *Chlamydia* persisted though.

1.1.1 Taxonomy of *Chlamydia*

According to their divergence in the glycogen accumulation inside the inclusion (Gordon *et al.*, 1965) and resistance towards sulfadiazine (Lin *et al.*, 1966), the family of *Chlamydiaceae* initially contained the two species *Chlamydia trachomatis* and *Chlamydia psittaci*. The development of DNA-based classification methods led to the identification of the two additional species *C. pneumoniae* (Grayston *et al.*, 1986) and *C. pecorum* (Fukushi *et al.*, 1992). In 1999, Everett and colleagues suggested a rearrangement of the taxonomic classifications. Based on 16S and 23S ribosomal RNA sequence analyses and taking into account recently identified obligate intracellular species with *Chlamydia*-like developmental cycles, including *Simkania negevensis* (Kahane *et al.*, 1995) and the amobae isolate *Parachlamydia acanthamoebae* (Amann *et al.*, 1997), the *Chlamydiaceae* were grouped into the phylogenetically heterogeneous order of *Chlamydiales* (Everett *et al.*, 1999). They comprise the two genera *Chlamydia* and *Chlamydophil*a, each of them harbouring one of the two relevant human pathogens, *Chlamydia trachomatis* and *Chlamydophila pneumoniae* (see figure 1.1).

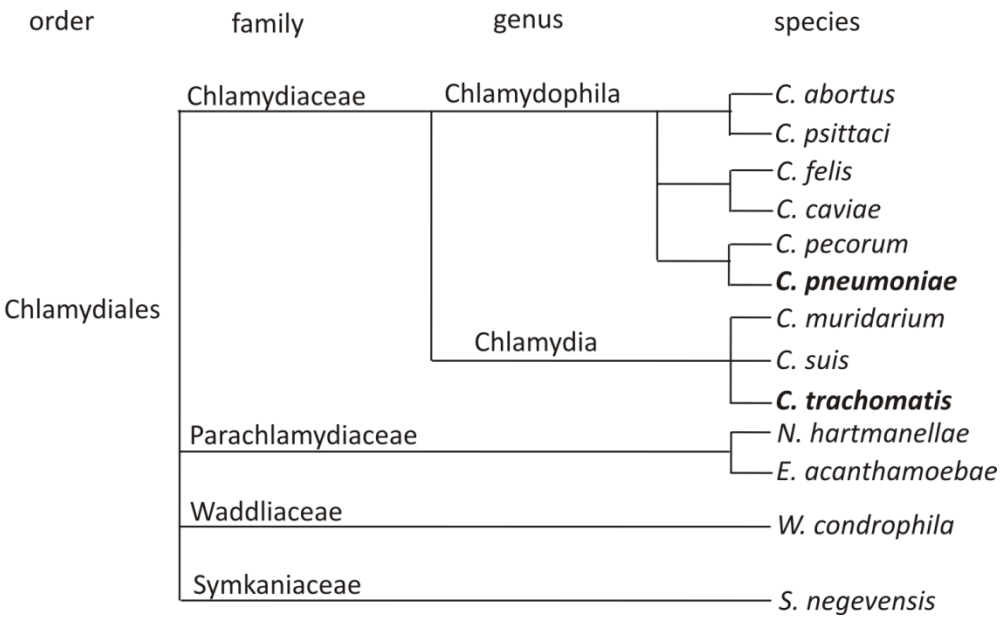

Fig. 1.1: Taxonomy of *Chlamydiales*.
According to 16S and 23S rRNA analyses, the order *Chlamydiales* comprises four families including *Chlamydiaceae* with the two genera *Chlamydia* and *Chlamydophila*. The two major human-specific pathogens *C. trachomatis* and *C. pneumoniae* (marked in bold) each belong to a different genus. The lengths of the lines do not represent the phylogenetic distance. Modified after Everett *et al.*, 1999.

1.1.2 Medical relevance of *Chlamydia* infections

Owing to their involvement in a variety of relevant acute and chronic diseases and their high prevalence rate, the two most important human chlamydial pathogens, *C. trachomatis* and *C. pneumoniae*, are of utmost scientific interest and subject of a plethora of clinical studies. *C. pneumoniae* is a common cause of respiratory tract infections and is directly transmitted via aerosols. It is estimated that virtually everybody is infected with *C. pneumoniae* at least once in a lifetime. While the prevalence of younger adults, as demonstrated by respective antibody titres, is about 50 %, it increases over adulthood reaching a prevalence of 80 % in elder people, most probably due to re-infections (Grayston, 2000). Although most of the infections remain asymptomatic, they can cause acute respiratory diseases, such as pharyngytis (Komaroff *et al.*, 1983), acute bronchitis, sinusitis, and persistent cough (Grayston, 1992). Clinical studies implied the association of chronic *C. pneumoniae* infections with asthma (Hahn *et al.*, 1991) and lung cancer (Littman *et al.*, 2005). Accumulating evidence further suggests their involvement in diverse non-respiratory diseases, including reactive arthritis (Gran *et al.*, 1993, Saario *et al.*, 1993), Alzheimer's disease (Balin *et al.*, 1998, Little *et al.*, 2004), and multiple sclerosis (Munger *et al.*, 2003).

C. trachomatis is the causative agent of the trachoma, the most common infectious source of blindness worldwide and is among the most frequently sexually acquired pathogens with an estimated 90 million new cases worldwide each year (Gerbase *et al.*, 1998). Genital tract infections with *C. trachomatis* account for urethritis (Smith *et al.*, 1975), prostatitis (Mardh *et al.*, 1972), and salpingitis, the pelvic inflammatory disease (Mardh *et al.*, 1977). In the industrialized world, infection-

associated scarring of the fallopian tubes is a major source for ectopic pregnancy and infertility (Wilkowska-Trojniel *et al.*, 2009). In the developing countries, virtually all infants and young children suffer from infections with *Chlamydia*. While an estimated 500 million people are affected by the trachoma, 1.3 million of them have developed blindness (Resnikoff et al., 2004). Ocular infections are mostly due to acquisition of the *C. trachomatis* biovariant *Trachoma* serovars A to C. Although the serovars D to K primarily infect the urogenital tract, they occasionally cause ocular infections upon horizontal transfer during birth passage. The second biovariant of *C. trachomatis, Lymphogranuloma venereum* (LGV), contains the three serovars L1, L2, and L3 that were isolated from lymphoid tissue. They are transmitted sexually and can provoke systemic infections. Importantly, evidence is accumulating that point to a role of *C. trachomatis* in cancer development and progression. Clinical studies on cervical intraepithelial neoplasia (CIN), a premalignant transformation of the squamous cells of the cervix surface, revealed their significant correlation with *C. trachomatis* infections (Guaschino *et al.*, 1988, Hare *et al.*, 1982).

1.1.3 Developmental cycle of *Chlamydia*

A very unique feature of the *Chlamydiaceae* is the biphasic developmental cycle. The term was introduced by Bedson and colleagues who first described the series of events they observed during the reproduction of the psittacosis agent in cell tissue (Bedson, 1932). Electron microscopical studies soon confirmed and extended Bedson´s observations, suggesting that two morphologically and structurally distinct developmental forms exist (Constable, 1959, Gaylord, 1954). The infectious form, termed elementary body (EB), is metabolically inactive, thus, it requires a differentiation into the replicative form, the so-called reticulate body (RB), for multiplication inside the host cell. Because RBs are not infectious, the re-differentiation into EBs is an essential step in the chlamydial developmental cycle for the establishment of an acute infection. Due to their extremely compact structure with a size of 0.3 µm in diameter, their highly condensed DNA, and their potential to survive in the extracellular environment, the chlamydial EBs resemble spore-like particles (Eb *et al.*, 1976). With a diameter of approximately 1 µm, RBs are bigger in size, rich in ribosomes (Chi *et al.*, 1987), and possess the typical membrane structure of Gram-negative bacteria (Matsumoto *et al.*, 1970).

The developmental cycle, depicted in figure 1.2, is initiated by the internalization of the EB by a susceptible eukaryotic host cell. Although a growing number of cellular and bacterial factors that mediate chlamydial uptake are being discovered, our understanding of the invasion process is still rather incomplete. Heparan sulphate on the cellular side (Zhang *et al.*, 1992) and the major outer membrane protein on the pathogen side (Swanson *et al.*, 1994) were the first factors identified to mediate the attachment of the chlamydial EB. Recent studies further revealed the crucial role of the

cellular PDGF-β receptor, the host kinase c-Abl, and the chlamydial protein Tarp (translocated actin recruiting phosphoprotein) in pathogen uptake (Clifton *et al.*, 2004, Elwell *et al.*, 2008). Together with other chlamydial effector proteins Tarp is translocated into the host cell via a type three secretion system (T3SS), a needle-like protein complex that spans the bacterial membranes (Subtil *et al.*, 2000). Via cytoskeleton rearrangements uptake of the EB is induced, which can also be seen in non-phagocytic cells (Jewett *et al.*, 2006). However, whether the chlamydial internalization is mediated by a phagycytosis-like process (Finlay *et al.*, 1997) or by receptor-mediated endocytosis (Hodinka *et al.*, 1988, Ward *et al.*, 1984) still needs to be elucidated.

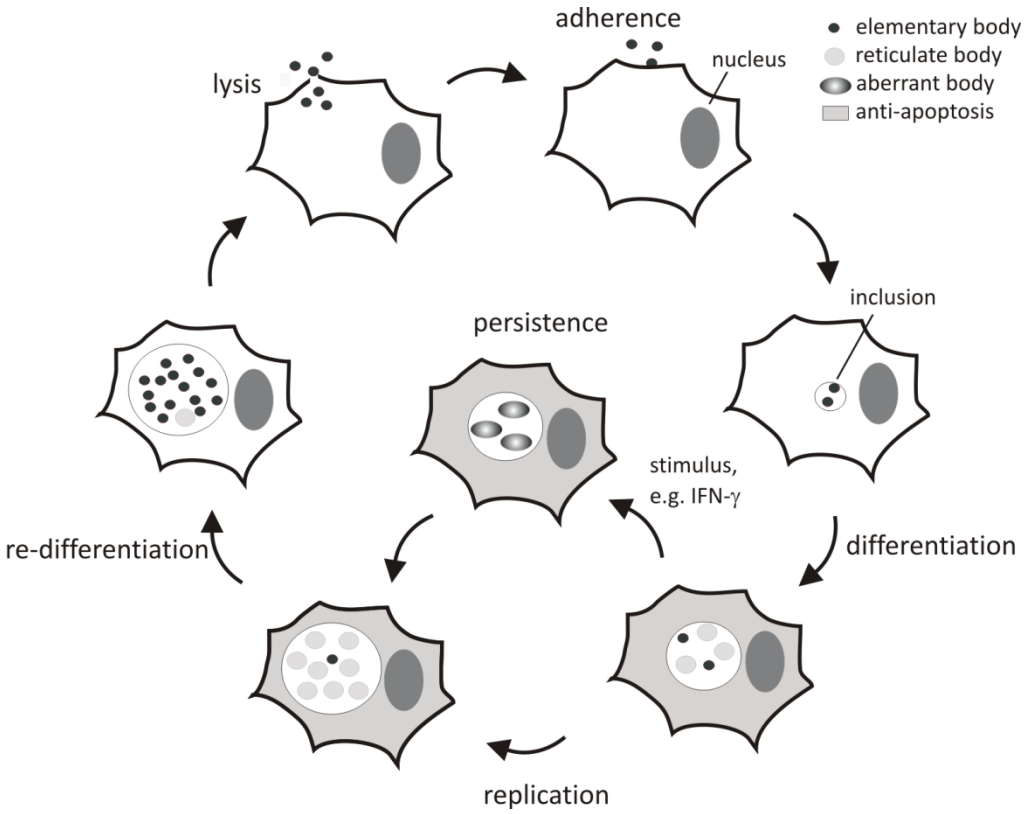

Fig. 1.2: The chlamydial developmental cycle.
Infectious elementary bodies (EBs) adhere to the host cell. Upon internalization, the chlamydial inclusion is formed where EBs differentiate into reticulate bodies (RBs). RBs replicate by binary fission to produce several hundred particles that re-differentiate into infectious EBs which leave the cell in order to start a new round of infection. Under certain conditions, such as sublethal doses of antibiotics or IFN-γ stimulation, aberrant bodies can be formed to persist inside the host cell. Depending on the species, the developmental cycle is completed after 48-72 h in tissue culture cell lines.

Once internalized, EBs are enclosed in an endosome-like vesicle (Zeichner, 1982), the so-called chlamydial inclusion. Within a few hours they develop into the metabolically active RB form. Temporary interactions with early endosomes have been described based on respective markers on the chlamydial inclusion (Wissel *et al.*, 2003). Nevertheless, modifications of the chlamydial inclusion subsequently prevent its interaction with late endosomes and lysosomes, thereby circumventing the cellular degradation machinery (Al-Younes *et al.*, 1999), whereas the fusion to sphingomyelin-rich

vesicles is enhanced (Hackstadt *et al.*, 1995). During the replicative phase, several hundred new RBs are produced by binary fission. At the end of the developmental cycle, a significant proportion of RBs re-differentiates into infectious EBs. Up to date, the signals that induce this re-differentiation process have not been identified. It was suggested that the physical detachment of the RB from the inner inclusion membrane, due to the growing number of RBs and space limitations account for the EB-formation (Bavoil *et al.*, 2000, Hackstadt *et al.*, 1997). The engagement of the chlamydial T3SS in RB to EB re-differentiation was recently discussed by Peters *et al.* (2007). Depending on the species and the amount of bacteria that entered the cell, the developmental cycle of *C. trachomatis* and *C. pneumoniae* in cultured cell lines is completed after 48 h and 72 h, respectively. After the release of EBs by cell lysis, a new round of infection is started (Campbell *et al.*, 1989).

Apart from the two life stages that dominate the chlamydial developmental cycle, a third distinct form has been shown to occur *in-vitro*. Following treatment of infected host cells with certain stimuli, such as interferon gamma (IFN-γ) (de la Maza *et al.*, 1987), or due to tryptophan starvation (Beatty *et al.*, 1994), chlamydial RBs can develop into aberrant bodies, also called persistent bodies (Fig. 1.2). This life stage is phenotypically characterized by an enlarged and multinucleated morphology of the aberrant bodies and by the reduced generation of infectious particles. Up to date, the underlying mechanisms have not been identified. Although the *in-vitro* data is often referred to as persistence, whether this form of aberrant bodies is related to the clinically persistent infection remains to be proven.

1.2 Programmed cell death

Programmed cell death (PCD) is a highly ancestral and pivotal mechanism for the elimination of unwanted cells that is tightly regulated by a coordinated series of molecular events. For multicellular organisms, PCD is an indispensable means for tissue development during embryogenesis (Saunders, 1966), to maintain tissue homeostasis, and to remove dangerous cells, such as autoreactive B cells, cancer cells, or infected cells from the body (Cohen, 1991). Both the lack and the excess of apoptosis is implicated in several relevant human diseases, including Alzheimer´s disease (Loo *et al.*, 1993) and Huntington disease (Portera-Cailliau *et al.*, 1995), autoimmune disorders, and cancer (Fukuhara *et al.*, 1979, Tsujimoto *et al.*, 1985).

Although the importance of PCD for the physiological processes of multicellular organisms has already been recognized in the 19th century, reviewed by Clarke and Clarke (1996), the term was introduced by Lockshin and Williams in the 1960es. They postulated that cell death was controlled by a series of molecular processes rather than being accidental (Lockshin *et al.*, 1965). After decades of intensive research where PCD has solely been attributed to multicellular organisms, accumulating evidence suggests its crucial role for unicellular eukaryotic organisms, including the yeast

Saccharomyces cerevisiae (Madeo *et al.*, 1997) and *Trypanosoma cruzi* (Ameisen *et al.*, 1995) as well as for prokaryotic organisms, reviewed by (Lewis, 2000). This emphasizes the tremendous role of PCD as a significant part of life and an evolutionary concept.

1.2.1 Types of PCD

According to their morphological and biochemical features, at least four different types of PCD can be distinguished (Fig. 1.3). Apoptosis, probably the best-characterized form of PCD, was coined by Kerr *et al.* (1972) who termed this cellular phenomenon after the Greek saying "falling off leaves" (apo=off and ptosis=fall). Apoptosis is morphologically characterized by rounding and shrinkage of the cell (pyknosis), by chromatin condensation and nuclear fragmentation (karyorrhexis), accompanied by the loss of tissue adherence and the formation of membrane blebs. A cell dying by apoptosis maintains its integrity until the final process of breaking-up into so-called apoptotic bodies (Kerr *et al.*, 1972, Wyllie *et al.*, 1980). These are composed of cytosol, organelles, and chromatin and, *in-vivo*, are specifically recognized and subsequently engulfed by macrophages (Hedgecock *et al.*, 1983). Thereby, the release of cellular content into the environment and subsequent inflammatory responses of the surrounding tissue is prevented. Another form of orchestrated collapse is necrosis. It is characterized by swelling of the cytoplasma (oncosis) and of cytoplasmic organelles, and rupture of the plasma membrane (Kerr, 1971, Grooten *et al.*, 1993). Because necrosis terminates with the release of inflammatory cell content, it has long been considered accidental rather than being the result of a regulated program. Only recently, the complex underlying program gave rise to its reckoning as another form of PCD (Holler *et al.*, 2000). Autophagy, the "self digestion" of the cell, is characterized by the sequestration of cellular material by autophagosomes that, upon fusion with lysosomes, results in bulk protein degradation (Schin *et al.*, 1965, Schweichel *et al.*, 1973). Although a massive accumulation of autophagic vacuoles (Takeshige *et al.*, 1992) and the lack of chromatin condensation can help to diagnose autophagic cell death, a set of molecular markers are required for its definite identification. A comparatively young type of PCD is pyroptosis. It can be described as a combination of apoptosis and necrosis as it both displays apoptotic characteristics and provokes inflammation (Cookson *et al.*, 2001). Due to its features resembling parts of necrosis, a fifth type of PCD has lately been proposed, termed pyronecrosis (Willingham *et al.*, 2007). In fact, several other death-related terminations exist in the literature and a yet rising number of distinct types of PCD with mixed phenotypic characteristics might exist. A recent recommendation for the death-related terminology has been stated by the Nomenclature Committee on Cell death (Kroemer *et al.*, 2009).

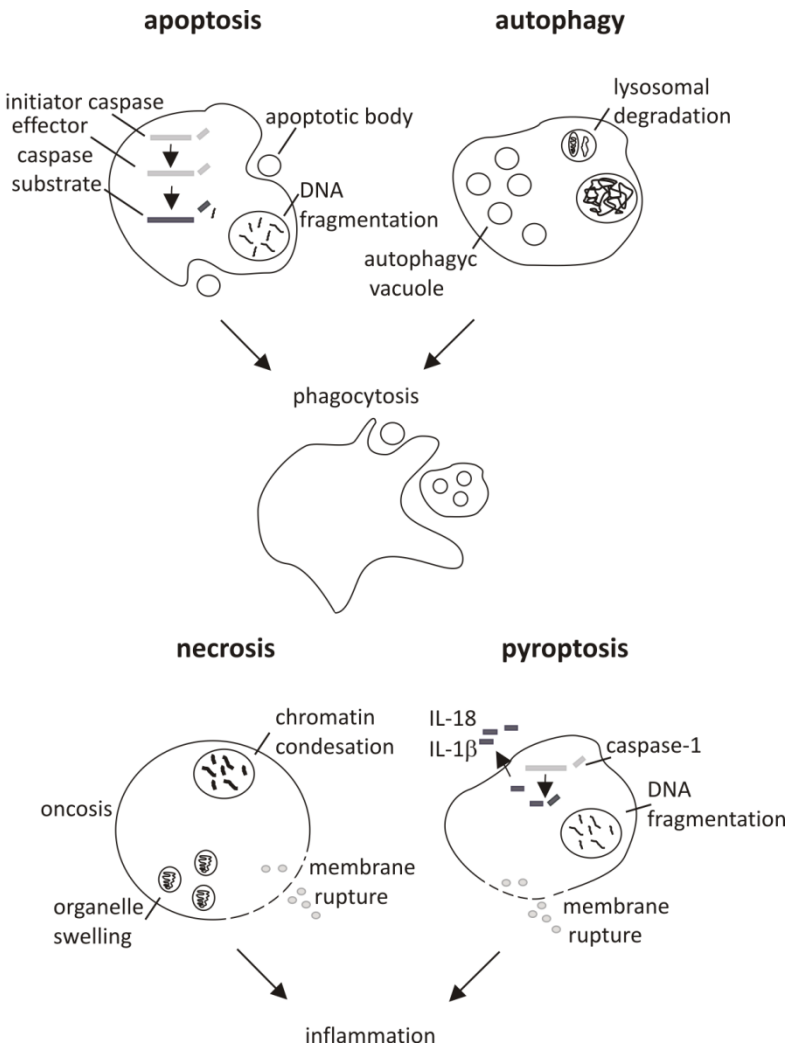

Fig. 1.3: Characteristic features of different known PCD types.
Depending on the kind and strength of the death stimulus, a cell can undergo different types of PCD. Caspases are the major executioners of apoptosis, responsible for its morphological traits by cleavage of a set of substrates. The apoptotic cell displays DNA fragmentation and disrupts into apoptotic bodies which are engulfed by macrophages. Cells dying by autophagy are morphologically characterized by autophagic vacuoles. A necrotic cell displays swelling of the cytosol (oncosis) and of organelles and, due to membrane rupture, induces inflammation. Pyroptosis is characterized by DNA fragmentation accompanied by cleavage and release of pro-inflammatory cytokines (IL-1β and IL-18). Modified after Fink *et al.*, 2005.

1.2.2 Biochemical features of apoptosis

Most of the morphological traits displayed by an apoptotic cell are the consequence of a complex and delicate interplay of cellular pro- and anti-apoptotic factors. Under conditions favouring the survival of the cell, a multitude of anti-apoptotic factors repress their specific cellular counterparts, thereby constantly preventing the activation of central pro-apoptotic pathways. Due to external stimuli or intracellular danger signals, such as DNA damage or infection, a subtle shift towards the side of pro-apoptotic players induces PCD.

The most central role during apoptosis execution plays a family of evolutionary conserved cystein-rich aspartate specific proteinases, called caspases. Caspases are synthesized as inactive zymogens and their activation depends on the cleavage of their pro-domain either by self processing or by the action of upstream located caspases. This results in a highly controlled cascade of activation which enables the amplification of the death signals. The first caspase to be identified was CED-3, a positive regulator of apoptosis in the nematode *Caenorhabditis elegans* (Ellis *et al.*, 1986). In fact, this identification paved the way for the discovery of the genetic regulation of PCD in that model organism, which was finally honoured with the Nobel Prize in Medicine awarded to Sydney Brenner, Robert Horvitz, and John Sulston in 2002. To date, at least 14 human caspases have been identified. Caspases-8, -9, and -10 own the most apical position in the protease cascade, therefore termed initiator caspases. The cascade terminates with the effector caspases-2, -3, -6, and -7 which, upon activation, cleave a set of diverse cellular proteins responsible for the morphological characteristics of apoptosis. Caspase-1 or ICE (IL-1 converting enzyme), the first identified human caspase (Thornberry *et al.*, 1992), can be distinguished from the classical apoptosis proteases. By processing the pro-inflammatory cytokines IL-1β and IL-18 to promote their release, caspase-1 is a critical mediator of inflammation and thereby a central component of the pyroptosis phenotype (Monack *et al.*, 2001) (Fig. 1.3). Most of the other mammalian caspases have been described to function in cytokine maturation, though their definite physiological role has still to be defined.

Following caspase activation, a plethora of cellular substrates is enzymatically processed, some of which have gained interest as biological markers both in research and in the clinics. Cleavage of the cytoskeletal proteins gelsolin and fodrin, for instance, is responsible for cell shrinkage (Kothakota *et al.*, 1997), cleavage of nuclear lamines induces nuclear remodelling (Rao *et al.*, 1996), and processing of PAK2 mediates formation of apoptotic bodies (Rudel *et al.*, 1997). Another well-studied substrate of caspase-3 is CAD (caspase activated DNase) which, upon cleavage of the inhibitory domain (ICAD), mediates fragmentation of the DNA (Enari *et al.*, 1998, Sakahira *et al.*, 1998).

Because the activation of the caspase cascade displays a step of “no return” during the induction of apoptosis, it is tightly regulated in healthy and growing cells. Key regulators of the caspase cascade are the cellular Inhibitor of Apoptosis Proteins (IAP) cIAP-1, cIAP-2, XIAP, and survivin (Ambrosini *et al.*, 1997). Due to the presence of a zinc binding domain, the BIR (baculovirus IAP repeat) domain, the IAPs were initially identified as mammalian homologues to the baculovirus IAP, an inhibitor of apoptosis (Crook *et al.*, 1993). While the role of XIAP for a direct inhibition of caspase-3 and -7 has been demonstrated repeatedly (Deveraux *et al.*, 1997, Duckett *et al.*, 1998, Liston *et al.*, 1996), the importance of cIAP-1 and cIAP-2 for direct caspase inhibition, though demonstrated (Roy *et al.*, 1997), is under dispute. Interestingly, it was recently shown that cIAP-1 is a crucial component of the innate immune response to *C. pneumoniae* infections (Prakash *et al.*, 2009).

1.2.3 Intrinsic pathway of apoptosis

Activation of the caspase cascade as an essential step of apoptosis induction is mediated via two different pathways, termed intrinsic and extrinsic pathways of apoptosis. While the extrinsic pathway is triggered upon death receptor ligation by external stimuli, the intrinsic or mitochondria-mediated pathway is initiated by intracellular stresses like cytotoxic agents, UV light, and growth factor depletion. Upon stress induction, the integrity of the mitochondria is disrupted leading to mitochondrial outer membrane permeabilization (MOMP). As a consequence, a number of death-inducing factors is released into the cytosol, most importantly the electron transport chain component cytochrome *c* (Liu *et al.*, 1996). Once released, cytochrome *c* together with the apoptosis activating factor (Apaf-1) and caspase-9 assembles into a heterogenous complex called apoptosome (Fig. 1.4) (Li *et al.*, 1997). This protein platform then mediates the activation of effector caspases leading to substrate cleavage and the typical morphological features of apoptosis. The apoptogenic factors normally sequestered in the mitochondria and released upon MOMP include the apoptosis inducing factor (AIF) (Susin *et al.*, 1999), Smac/Diablo (Du *et al.*, 2000, Verhagen *et al.*, 2000), and Endonuclease G (EndoG) (Li *et al.*, 2001). Key regulators of the mitochondrial integrity are the members of the B-cell lymphoma 2 (Bcl-2) like family (Vaux *et al.*, 1988). This family comprises both pro- and anti-apoptotic proteins characterized by the existence of one or more conserved Bcl-2 homology (BH) domains. While the pro-survival factors, including Bcl-2, Bcl-X_L, Bfl-1/A1 and Mcl-1 (myeloid cell lymphoma 1), contain all four BH domains, the pro-apoptotic proteins are subdivided into two groups. As indicated by their terminology, the BH-3 only proteins like Bid, Bad, Bim, Bmf, Bik, and Puma possess only one BH domain. This BH-3 domain mediates the interaction with other Bcl-2 like proteins and is sufficient for their pro-apoptotic function (Boyd *et al.*, 1995, Chittenden *et al.*, 1995a). The second group of apoptogenic factors constitutes proteins that possess the first three BH domains, most importantly Bax (Oltvai *et al.*, 1993) and Bak (Chittenden *et al.*, 1995b). The integration of Bak and Bax into the mitochondrial outer membrane facilitates the release of pro-apoptotic factors and amplification of the death signal, thus represents the most crucial step of the intrinsic apoptosis pathway (Narita *et al.*, 1998, Rosse *et al.*, 1998, Wolter *et al.*, 1997). Accordingly, this process is tightly regulated either directly (Oltvai *et al.*, 1993, Sedlak *et al.*, 1995) or via sequestration of activators or inhibitors of Bak and Bax (Puthalakath *et al.*, 2001, Wang *et al.*, 1996). In fact, the significance of the mitochondria as a central component of the apoptosis pathway is underlined by its involvement in the execution of the second, namely extrinsic pathway.

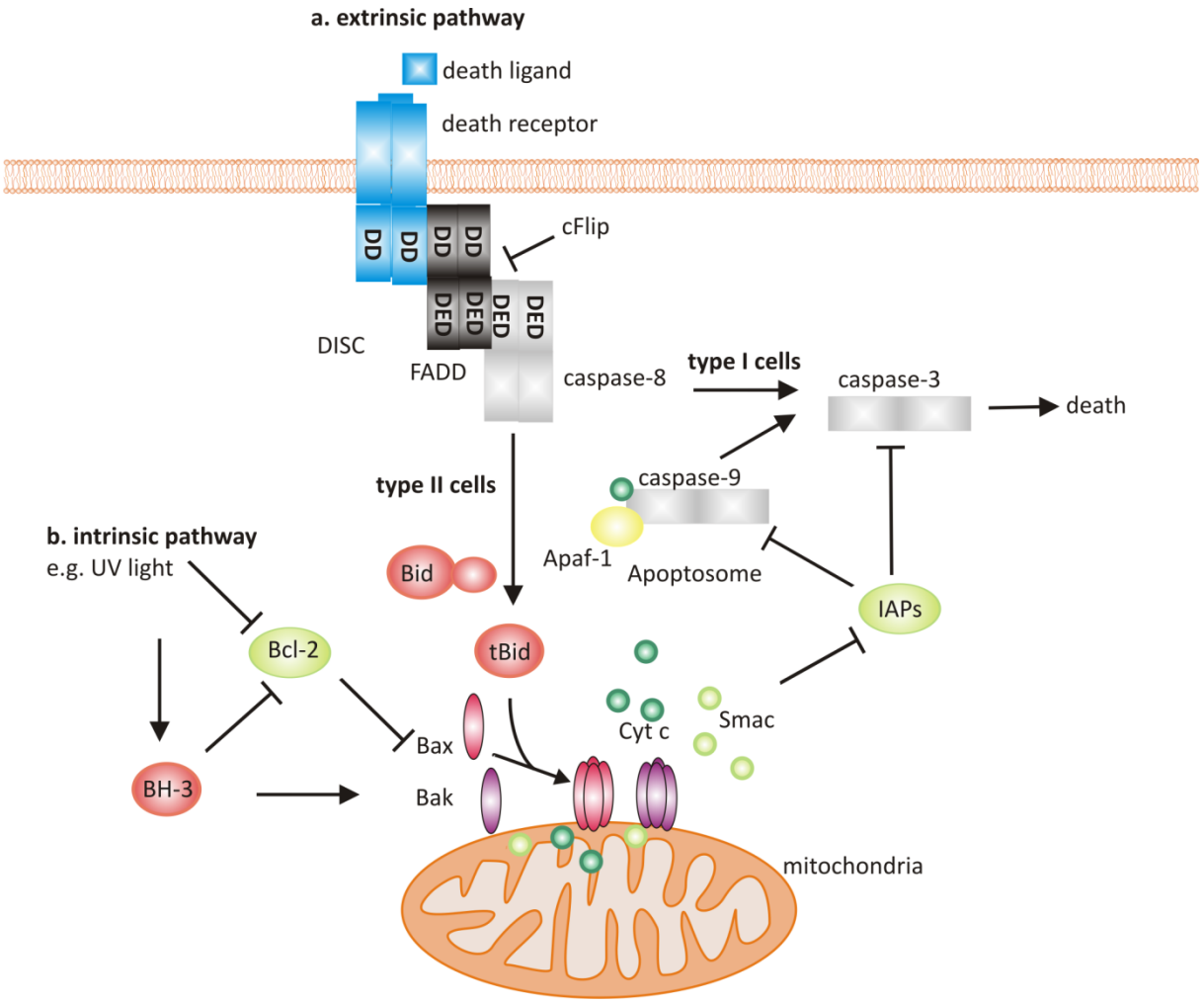

Fig. 1.4: Extrinsic and intrinsic pathways of apoptosis.
(a.) The extrinsic pathway is initiated by death receptor ligation (e.g. Fas ligand to Fas) which induces recruitment of FADD and procaspase-8 to the death inducing signalling complex (DISC). Autocleavage of procaspase-8 into the active form, which can be blocked by the caspase-8 inhibitor cFlip, allows for direct activation of effector caspase-3 in type I cells. In type II cells, amplification of the death signal via the mitochondria is required which is mediated by truncation of Bid into tBid. Bak and Bax translocation to the mitochondria induces mitochondrial outer membrane permabilization (MOMP) and release of apoptogenic factors, including Smac and cytochrome *c* (Cyt c) into the cytosol. The latter, together with Apaf-1 and caspase-9 then forms the apoptosome that induces cleavage of caspase-3. Inhibitor of Apoptosis Proteins (IAPs) can interfere with caspase activation. (b.) During intrinsic activation of apoptosis, death-inducing stimuli such as DNA damaging agents lead to activation of pro-apoptotic BH-3 only proteins accompanied by downregulation of anti-apoptotic Bcl-2 like proteins. The shift of pro- and anti-apoptotic proteins induces the activation of Bak and Bax followed by MOMP.

1.2.4 Extrinsic pathway of apoptosis

Initiation of the extrinsic apoptosis pathway requires binding of death ligands to their respective death receptors which belong to the family of tumor necrosis factor receptor (TNFR) super family. Depending on the cell type, several of these receptors can be found on the cell surface. The Fas, also referred to as CD95 or Apo-1 (Itoh *et al.*, 1991, Trauth *et al.*, 1989), and TNFR1 (Brockhaus *et al.*, 1990, Loetscher *et al.*, 1990, Schall *et al.*, 1990) are most likely the best-studied mammalian TNFR family members. They are composed of an external domain with five cystein rich repeats, a transmembrane domain, and an intracellular tail containing a death domain (DD) (Tartaglia *et al.*,

1993). Following ligation, a conformational change in the intracellular domain of the receptor induces the assembly to a homotrimeric complex (Fields *et al.*, 1989). Driven by the DD, adaptor proteins like FADD (Fas associated protein with a death domain) are recruited to the receptors (Boldin *et al.*, 1995, Chinnaiyan *et al.*, 1995). Because of its specific protein-protein interaction module, the so-called death effector domain (DED), FADD belongs to the family of DED-containing proteins that orchestrate cell death from the death receptors. In addition to FADD, seven mammalian proteins have been identified that comprise this family, including DEDD, DEDD2, PEA-15, caspase-8, caspase-10, and the two small and one large isoform of cellular Flice inhibitory protein, $cFlip_{S,/R}$ and $cFlip_L$, respectively (Fig. 1.5).

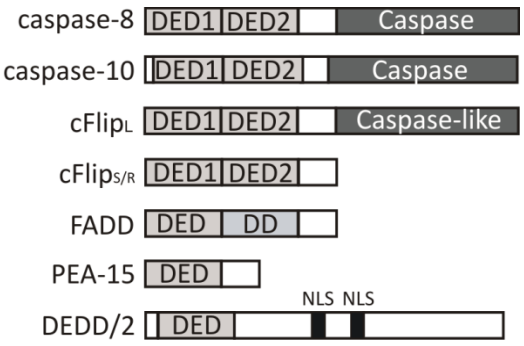

Fig. 1.5: DED-containing proteins.
The human death effector domain (DED) family comprises caspase-8 and -10, the pro-and anti-apoptotic proteins $cFlip_L$ and $cFlip_S$, respectively, and the linker FADD with an additional death domain (DD) that drives interactions with death receptors. Besides these factors that are part of the DISC and discussed in detail in the text, PEA-15 has been implicated in death inhibition via interaction with FADD (Condorelli *et al.*, 1999), while DEDD and DEDD2 have pro-apoptotic functions that are executed partly in the nucleus (Roth *et al.*, 2002, Stegh *et al.*, 1998). NLS: nuclear translocation signal. Modified after Yu *et al.*, 2008.

Homotypic interactions via the DED of FADD and procaspase-8 mediate the recruitment of the initiator caspases to the receptor (Medema *et al.*, 1997) where they cluster in a large oligomeric complex that is called the death inducing signalling complex (DISC) (Kischkel *et al.*, 1995). Due to dimerization of the C-terminal protease domains of the caspases, proximity-induced autocleavage releases a mature and highly active dimeric form from the DISC (Muzio *et al.*, 1998) (Fig. 1.6). Active caspase-8 then potentiates the death signal via two routes: first, caspase-8 cleaves the BH-3 only protein Bid (Li *et al.*, 1998). Truncated Bid (tBid) becomes myristoylated at its N-terminus (Zha *et al.*, 2000) and subsequently translocates to the mitochondria where it induces pore formation in concert with Bak and Bax (Wang *et al.*, 1996). This is followed, as described above, by formation of the apoptosome and effector caspase activation. In addition, processed caspase-8 can directly cleave the downstream effector caspase-3 (Scaffidi *et al.*, 1998) (Fig.1.4). In certain cells, called type I cells, DISC formation and procaspase-8 processing is sufficient for apoptosis induction. However, in most cell

types, the so-called type II cells, signal amplification via MOMP and apoptosome formation is obligatory for induction of cell death (Scaffidi *et al.*, 1998).

As a prime regulator of the extrinsic apoptosis pathway, cFlip can prevent DISC formation. Via its DED, cFlip can interact with procaspase-8 thereby avoiding its recruitment by FADD and subsequent dimerization and autocleavage (Irmler *et al.*, 1997) (Fig. 1.6). At least three transcript variants of cFlip exist yet only two of them have been found on protein level. While the two short forms, $cFlip_S$ and $cFlip_R$, possess two N-terminal DED domains, the longer version, $cFlip_L$, contains an additional caspase-like domain at the C-terminus that is catalytically inactive due to the lack of active side residues (Fig. 1.5). Although cFlip was initially established as a cellular analogue to viral caspase-8 inhibitor proteins, vFlips (Thome *et al.*, 1997), and as a potent anti-apoptotic protein, the role of $cFlip_L$ in cell death is currently under discussion. Several studies suggest it to have anti-apoptotic functions (Hu *et al.*, 1997, Irmler *et al.*, 1997, Rasper *et al.*, 1998, Srinivasula *et al.*, 1997), while an increasing number of publications pointed to its role as a pro-apoptotic factor (Goltsev *et al.*, 1997, Hahn *et al.*, 1991, Inohara *et al.*, 1997, Shu *et al.*, 1997). Chang *et al.* (2002) finally clarified this controversial issue and demonstrated the dual function of $cFlip_L$. While expression of physiologically relevant levels of $cFlip_L$ facilitates activation of caspase-8 and apoptosis, a decrease in expression leads to the inhibition of cell death. Recently, crystal structure analysis of the caspase-like domain of $cFlip_L$ confirmed its ability to promote either death or survival depending on the cellular context (Yu *et al.*, 2009).

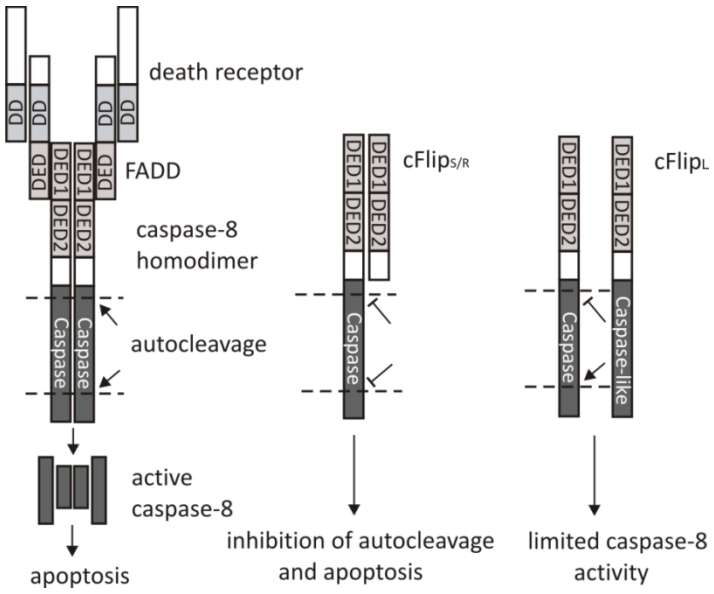

Fig. 1.6: Regulation of caspase-8 by DED-containing proteins.
Schematic representation of the interactions of caspase-8 with FADD or the cFlip isoforms and their impact on cell death induction. Proximity-induced caspase-8 autocleavage is mediated by FADD due to recruitment to the DISC. Homotypic interaction of caspase-8 with $cFlip_S$ prevents autocleavage, while the regulation of caspase-8 by $cFlip_L$ can result either in apoptosis or in its inhibition. Modified after Budd *et al.*, 2006.

1.2.5 PCD and infection

Apart from its indispensible role in tissue shaping and homeostasis, PCD is a powerful tool for pathogen clearance. By eliminating infected cells from the organism, reproduction and spread of the infectious agents can be limited. Many intracellular pathogens, including viruses, a number of bacteria, and parasites depend on an intact host cell for replication. Hence, it is not surprising that a plethora of pathogens have evolved strategies to modulate host cell signalling in order to inhibit PCD. The cellular factors that compose the death machinery and are prone to manipulation by bacterial pathogens were recently reviewed (Böhme *et al.*, 2009). A commonly utilized mechanism is to shift the balance of anti- and pro-apoptotic factors in favor of cell survival. Upregulation of the Bcl-2 like proteins Bfl-1/A1 and Mcl-1, for instance, has been demonstrated for apoptosis resistance of *Anaplasma phagocytophilum*-infected neutrophils (Ge *et al.*, 2005) and of monocytes infected with *Coxiella burnettii*, the causative agent of human Q fever (Voth *et al.*, 2007). In contrast, during infection with *Neisseria gonorrhoeae* downmodulation of Bim and Bad accounts for inhibition of host PCD (Howie *et al.*, 2008). Another successful strategy to promote host cell survival is the exploitation of the cellular survival pathways. Phosphorylation of the extracellular signal regulated kinase (ERK) and AKT is induced by a multitude of bacteria, including *Neisseria gonorrhoeae* (Howie *et al.*, 2008), *Coxiella burnettii* (Voth *et al.*, 2009), and *Salmonella enterica* serovar Typhimurium (Knodler *et al.*, 2005). While the prevention of host PCD might allow for replication, certain circumstances favor the induction of PCD. In order to overcome the natural barrier of the host and to gain access to deeper tissues of the organism, epithelial cells are often targeted by pathogens, such as *Neisseria* (Muller *et al.*, 1999). Additionally, the elimination of immune cells, e.g. macrophages and neutrophils, via induced PCD protects from their anti-microbial actions. Besides its ability to prevent cell death of infected epithelial cells, *Salmonella* can induce PCD of macrophages, a dual trait that is shared with *Shigella*. The effector proteins SipB (Hersh *et al.*, 1999) and IpaB (Hilbi *et al.*, 1998) of *Salmonella* Thyphimurium and *Shigella flexnerii*, respectively, were among the first bacterial factors identified to induce host cell death. They directly trigger the cleavage and activation of caspase-1 to induce pyroptosis. Via their T3SS, *Yersinia enterolitica* and *Yersinia pseudotuberculosis* secrete their effector proteins YopP and YopJ, respectively, into macrophages to promote cell death (Mills *et al.*, 1997, Monack *et al.*, 1997). Figure 1.7 provides an overview of the currently known bacterial strategies to influence cell death signalling for the benefit of the pathogens.

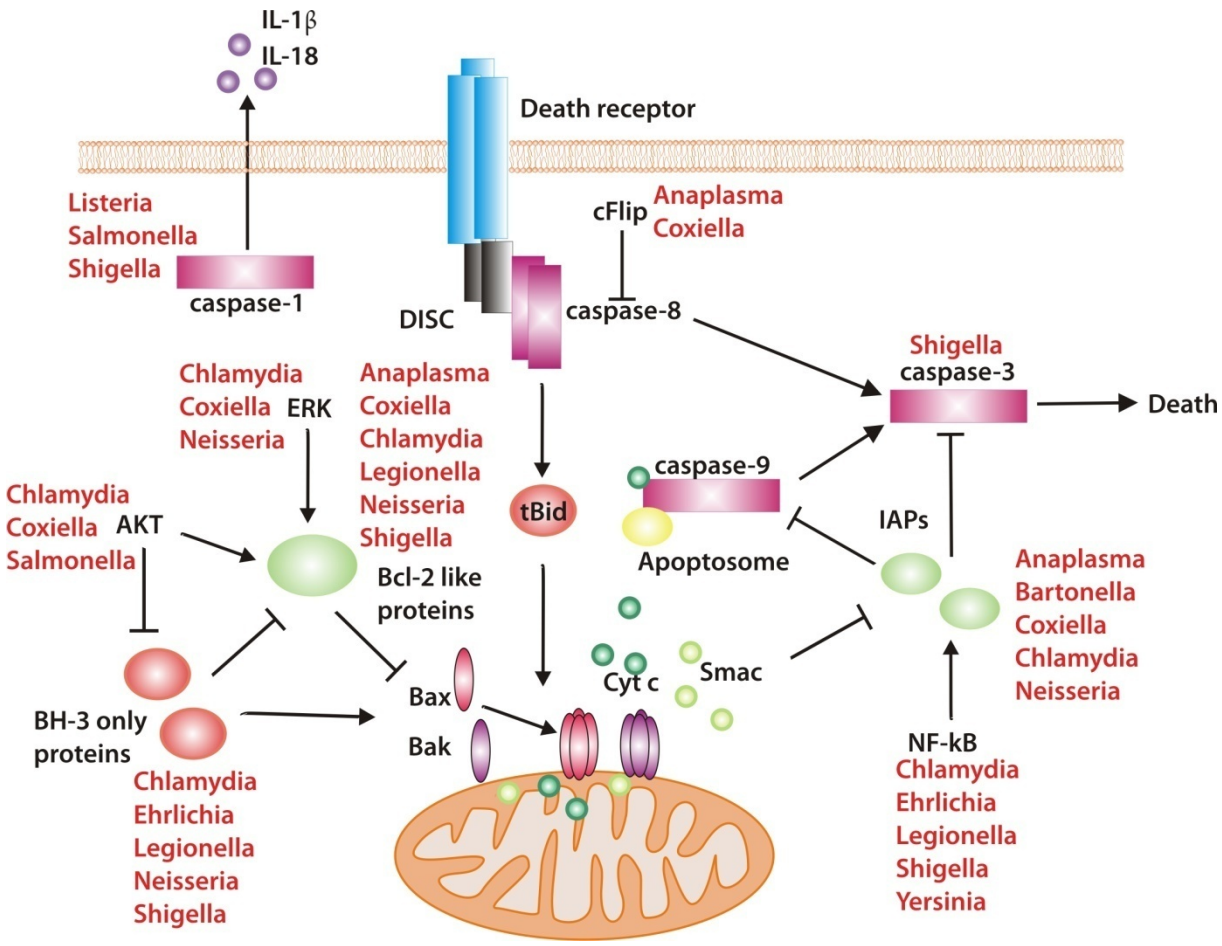

Fig. 1.7: Modulation of the host cell death machinery by bacterial pathogens.
Pro- and anti-apoptotic factors are up- or downregulated by diverse bacteria (genera indicated in red) at multiple levels of the signaling cascades. Modified after Böhme *et al.*, 2009.

1.2.6 *Chlamydia* and apoptosis

Due to their obligate intracellular life style, *Chlamydia* depend on an intact host cell until the completion of the developmental cycle. To maintain the replicative niche, *Chlamydia* have evolved sophisticated mechanisms to escape the immune response of the host, such as downregulation of major histocompatibility complex (MHC) class I and II molecules (Zhong *et al.*, 1999, Zhong *et al.*, 2000) or of TNFR1 (Paland *et al.*, 2008) from the surface of infected cells. Moreover, *Chlamydia* can inhibit host cell death induced by a variety of extrinsic and intrinsic stimuli, including the cytotoxic actions of T cells (Sharma *et al.*, 2009). Therefore, it interferes with the cellular apoptosis machinery at multiple levels (Fig. 1.7). Initial work revealed that *C. trachomatis* inhibits cytochrome *c* release from staurosporine (STS), TNFα, and Fas-treated cells, demonstrating a block of apoptosis upstream of mitochondria (Fan *et al.*, 1998). Soon afterwards, the anti-apoptotic qualities of *C. pneumoniae* infections were described (Fischer *et al.*, 2001, Rajalingam *et al.*, 2001). The block of mitochondria permeabilization was further confirmed by the finding that infected cells displayed a lack of Bak and Bax activation (Fischer *et al.*, 2004a, Xiao *et al.*, 2004). A number of following studies indicated that bulk degradation of pro-apoptotic BH-3 only proteins was responsible for the block of MOMP (Dong

et al., 2005, Fischer *et al.*, 2004b, Ying *et al.*, 2005) though others failed to show this phenomenon (Rajalingam *et al.*, 2008). *In-vitro* studies identified the chlamydial protease-like activity factor (CPAF) as the bacterial factor responsible for the destruction of BH-3 only proteins (Pirbhai *et al.*, 2006, Zhong *et al.*, 2001). Another general anti-apoptotic mechanism is the upregulation and stabilization of pro-survival factors. Transcription profiles of *Chlamydia*-infected host cells pointed to the upregulation of certain anti-apoptotic genes, most importantly cIAP-2 (Hess *et al.*, 2001, Xia *et al.*, 2003). The significance of cIAP-2 during infection-induced anti-apoptosis was later demonstrated for both *C. trachomatis* (Rajalingam *et al.*, 2006) and *C. pneumoniae* infections (Paland *et al.*, 2006). Depletion of the IAPs by RNAi further revealed that also cIAP-1 and XIAP, though transcriptionally not regulated, were required to inhibit host cell apoptosis (Rajalingam *et al.*, 2006), indicating the concerted actions of all three proteins to prevent caspase activation. A crucial role for the Bcl-2 like family member Mcl-1 and the pro-survival kinases ERK and AKT was demonstrated during infection with *C. trachomatis* (Rajalingam *et al.*, 2008). Silencing of Mcl-1 abrogated the chlamydial block of apoptosis, as did inhibition of the survival pathways. Interestingly, despite the requirement for upregulation of anti-apoptotic proteins, the utilization of the translation inhibitor cycloheximide (CHX) in diverse studies suggests the chlamydial ability to block apoptosis is independent of host protein synthesis. Antibiotic treatment revealed that bacterial protein synthesis, in contrast, is necessary to prevent host cell death (Fan *et al.*, 1998).

Although most studies on *Chlamydia* and apoptosis report on an inhibition of host cell death, contradictory data has been published. It was reported that the infection with *C. psittaci* led to caspase-independent death of surrounding non-infected epithelial cells and macrophages, suggesting the secretion of a pro-apoptotic factor from infected cells (Ojcius *et al.*, 1998). Others reported that production and release of TNFα from *Chlamydia*-infected macrophages induced death of neighbouring cells (Jendro *et al.*, 2004). It seems likely that protection against apoptosis is subject to the phase within the chamydial life cycle. Thus, at the end of the developmental cycle disruption of the host cell would permit the spread of infectious EBs, as proposed by Jungas *et al.* (2004). They showed that inhibition of apoptosis in *C. trachomatis*- and *C. muridarium*-infected epithelial cells and fibroblasts was followed by induction of cell death at later time points after infection.

1.3 The cellular response to dsRNA

A variety of cell death stimuli have been investigated with respect to their impact on chlamydial infection demonstrating a successful inhibition of host cell death by the intracellular pathogen. A very relevant stimulus, however, has so-far been missing attention: polyinosinic:polycytidylic acid (polyI:C), a synthetic double-stranded RNA (dsRNA), is a well established and potent inductor of innate immunity both *in-vitro* (Lampson *et al.*, 1967) and *in-vivo* (Djeu *et al.*, 1979, Gidlund *et al.*,

1978) and of apoptosis (Iordanov *et al.*, 2005a, Iordanov *et al.*, 2005b, Kibler *et al.*, 1997). Because dsRNA is a by-product of viral replication inside the infected host cell (Colby *et al.*, 1969), polyI:C is commonly used to mimic viral infections and, by activating cellular dsRNA sensors, it efficiently induces innate immune signalling and apoptosis pathways.

1.3.1 Innate immunity signalling in response to dsRNA

A key component of the cellular response to dsRNA is the induction of interferons (IFN) (Isaacs *et al.*, 1961). These potent innate immune modulators were the first family of cytokines identified and were demonstrated to be released by virus infected animal cells (Isaacs *et al.*, 1957). Classified as type I (IFN-α and IFN-β) and type II (IFN-γ), they govern the transcriptional regulation of several hundred gene products (Der *et al.*, 1998). Although most of these gene products contribute to the anti-viral activities, IFNs regulate diverse fundamental cellular processes, including proliferation (Sikora *et al.*, 1980, Taetle *et al.*, 1980) and differentiation (Parker *et al.*, 1981). The discovery of the link between polyI:C and INF dates back to the 1970es (De Clercq *et al.*, 1970), yet just recently, the factors involved in mediating this response have been identified. The pathways activated upon dsRNA recognition by the different cellular sensors are illustrated in figure 1.8.

1.3.1.1 PKR

The first identified and hitherto best-studied IFN-inducible protein involved in the cellular anti-viral response is the RNA-dependent protein kinase (PKR). It is a serine/threonine kinase of 551 amino acid residues and is composed of two N-terminal dsRNA-binding domains (DRBD) and a C-terminal kinase domain (Meurs *et al.*, 1990). Via its DRBD, PKR binds long dsRNA without sequence specificity, while affinity for dsDNA or ssDNA is missing (Manche *et al.*, 1992). This leads to homodimerization and autophosphorylation and, subsequently, phosphorylation of a set of substrates. The α-subunit of the eukaryotic translation initiation factor (eIF2α) is the best characterized substrate of PKR (Farrell *et al.*, 1977). Functioning as a kinase of the guanine nucleotide exchange factor eIF2B, eIF2α can control the constant reactivation of the translation initiation factor eIF2. Once phosphorylated by PKR at serine residue 57, eIF2α prevents the dissociation of eIF2B-GDP from the initiation complex, thereby inhibiting the proceeding of translation (Levin *et al.*, 1978). Although the most prevalent role of PKR can be considered to be the inhibition of translation as a means of conferring an cellular anti-viral state, PKR has been described to be involved in a plethora of signalling pathways mediating diverse cellular processes, such as cell growth (Chong *et al.*, 1992), differentiation (Salzberg *et al.*, 2000), antitumor activities (Koromilas *et al.*, 1992, Meurs *et al.*, 1993), and cell cycle regulation (Zamanian-Daryoush *et al.*, 1999). Several knock out studies demonstrated a role of PKR in

transcriptional control via the transcription factor family NF-κB (Gil *et al.*, 2000a) and stress-signalling via mitogen activated protein (MAP) kinase pathways of p38 (Goh *et al.*, 2000). However, others reported on an activation of p38 upon dsRNA in PKR deficient cells, suggesting the involvement of at least one other kinase (Iordanov *et al.*, 2000). With the identification of other dsRNA sensors, light was shed on this controversial matter.

1.3.1.2 RNase L

The second major contributer to the anti-viral effect derived from IFN and activated in response to dsRNA is the RNase L (Zhou *et al.*, 1993), which is located in the 2'-5'-oligoadenylate synthetase (2-5 OAS) pathway. It has been shown to interact with the dsRNA of the encephalomyelitis virus (ECMV) (Gribaudo *et al.*, 1991). Upon binding of dsRNA, a conformational change in the 2-5 OAS induces its activation (Hartmann *et al.*, 2003), resulting in the conversion of ATP into PP_i and 2'-5'-linked oligoadenylates (Clemens *et al.*, 1978, Kerr *et al.*, 1978). The increased levels of 2'-5'-linked oligoadenylates subsequently induce the dimerization of RNase L which catalyzes the degradation of the 28S ribosomal RNA (rRNA), thereby causing a block of protein synthesis (Hassel *et al.*, 1993, Silverman *et al.*, 1983) and suppressing viral replication (Zhou *et al.*, 1998).

1.3.1.3 TLR3

Despite the prominent role of PKR and RNase L in signal transduction in response to dsRNA, knock out (KO) studies soon urged the existence of further cytoplasmic sensors involved in anti-viral actions and led to the identification of toll-like receptor 3 (TLR3) as a receptor for dsRNA (Alexopoulou *et al.*, 2001). TLR3 is a member of the TLR family which comprises proteins that specifically recognize molecular patterns associated with pathogens and trigger the elimination of pathogens (Medzhitov *et al.*, 1997, Rock *et al.*, 1998). While TLR4, the receptor for lipopolysaccharide (LPS), is exclusively found on the cell surface, TLR3 is localized intracellularly in endosomes in mononuclear cells and monocyte-derived and immature dendritic cells (Matsumoto *et al.*, 2003). In fibroblasts, in contrast, TLR3 is mainly localized at the cell surface (Matsumoto *et al.*, 2002). Although TLR3 was concurrently believed to be absent in epithelial cells, recent publications demonstrate its expression in epithelial cells (Cario *et al.*, 2000) and argue for an endosomally localized form of the receptor (Funami *et al.*, 2004, Funami *et al.*, 2007). Recognition of polyI:C by TLR3 was shown to require the extracellular domain of the receptor (Bell *et al.*, 2005, Choe *et al.*, 2005) where the nucleic acid can bind to a glycan-free stretch within the otherwise glycosylated surface of TLR3 (Bell *et al.*, 2006).

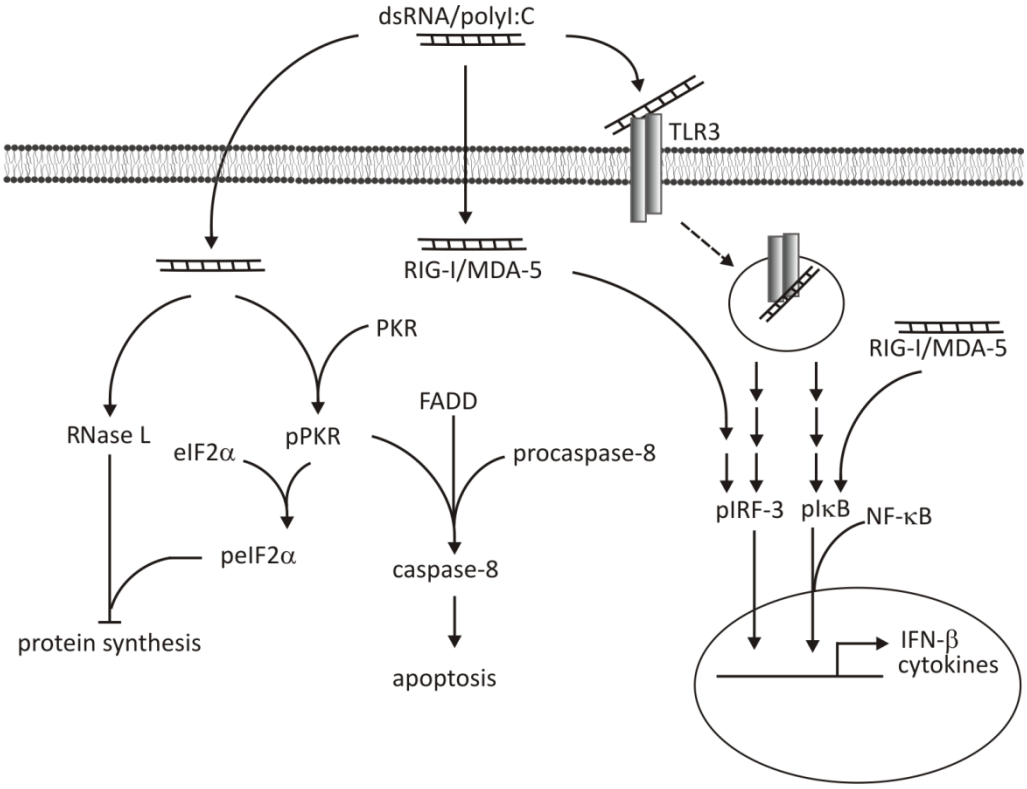

Fig. 1.8: Signalling pathways induced by dsRNA.
dsRNA or polyI:C can bind to surface localized TLR3 which is subsequently internalized, or is directly produced inside the host cell during viral replication. Cellular dsRNA sensors include PKR which phosphorylates the eukaryotic initiation factor 2α, and the 2-5 OAS pathway including RNase L, both resulting in translational block. PKR induces dsRNA-DISC formation via FADD leading to the activation of the caspase cascade and apoptosis. RIG-I/MDA-5 and endosomal localized TLR3 provoke an innate immunological response to dsRNA/polyI:C mediated by activation of IRF-3 and NF-κB. Nuclear translocation of the transcription factors leads to expression of IFN-β and inflammatory cytokines.

1.3.1.4 RIG-I and MDA5

Few years after the discovery of TLR3 as dsRNA receptor, the helicases RIG-I (retinoic acid inducible gene 1) (Yoneyama *et al.*, 2004) and MDA-5 (melanoma differentiation-associated gene 5) (Andrejeva *et al.*, 2004, Kang *et al.*, 2002) were identified as crucial mediators of viral clearance by inducing the transcription of inflammatory cytokines and type I IFNs. Both proteins are related members of the helicase family and contain a C-terminal helicase and two N-terminal caspase recruitment domains (CARD) and display ATPase activity in reponse to dsRNA (Yoneyama *et al.*, 2004, Kang *et al.*, 2002). Although they were first believed to function redundantly, Kato *et al.* (2006) demonstrated that these two RNA sensors possess different roles in recognizing viral RNA. While RIG-I mediates IFN-induction in response to paramyxovirus and influenza virus (Pichlmair *et al.*, 2006), MDA-5 is required for detection of picornavirus and, moreover, is the primary sensor for polyI:C (Gitlin *et al.*, 2006, Kato *et al.*, 2006). However, recognition of polyI:C was recently demonstrated to be length-dependent, as shortening by dsRNA-specific RNAseIII converted polyI:C to a RIG-I substrate (Kato *et al.*, 2008).

1.3.1.5 IRF-3 and NF-κB

Together with TLR3, MDA-5 and RIG-I trigger the induction of type I IFN expression. These signalling pathways majorly depend on two transcription factors which are therefore crucial mediators of the anti-viral response: the interferon regulatory factor 3 (IRF-3) and NF-κB. The prominent role of IRF-3 in triggering virus-induced innate immunity was demonstrated by knock out studies (Sato *et al.*, 2000). The constitutively expressed IRF-3 is a phosphoprotein with 427 amino acids (Au *et al.*, 1995) and belongs to the IRF family, the nine members of which share extensive homology in their N-terminal DNA-binding domain (DBD). Inactive IRF-3 remains in the cytosol until the phosphorylation of specific C-terminal serine residues by viral infection or polyI:C treatment induces its translocation to the nucleus and subsequent induction of type I IFN genes (Lin *et al.*, 1998, Yoneyama *et al.*, 1998). This process is mediated by the kinases TBK1 and IKKε (Fitzgerald *et al.*, 2003, Sharma *et al.*, 2003). The molecular regulation of the NF-κB family of transcription factors is very well established. Sequestration of the family members NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel in the cytoplasm is mediated by the inhibitor IκB (Baeuerle *et al.*, 1988) until phosphorylation and ubiquitination induce the degradation of IkB (Chen *et al.*, 1995). Subsequently, NF-κB is released to translocate into the nucleus. Besides the known upstream kinases, IKKα (Regnier *et al.*, 1997) and IKKβ (Mercurio *et al.*, 1997), the scaffold protein NEMO was recently identified to be required for both NF-κB and IRF-3 activation in response to dsRNA (Zhao *et al.*, 2007).

1.3.2 dsRNA-induced apoptosis signalling

Whereas the first line of defense against dsRNA is the induction of the expression of anti-viral genes and the inhibition of viral translation and spread, the ultimate answer might be the elimination of the infected cell by PCD. A multitude of studies revealed the inevitable ability of dsRNA to induce apoptosis. By overexpressing PKR with a vaccinia virus recombinant construct, Lee and Esteban (1993) were the first to demonstrate the role of this kinase in apoptosis induction. By a yet not fully resolved mechanism, PKR induces processing of initiator caspase-8 and it was demonstrated by independent studies that this process requires the linker FADD, however, it is independent of death receptors (Balachandran *et al.*, 1998, Gil *et al.*, 2000b). Whereas a set of intrinsic death stimuli are known to induce MOMP leading to activation of caspase-9, dsRNA-induced apoptosis was demonstrated to provoke caspase-9 activation only as a consequence of caspase-8-mediated truncation of Bid or following cleavage by activated caspase-3 (Iordanov *et al.*, 2005b). Although dsRNA-induced apoptosis has majorly been attributed to PKR, KO studies revealed that RNase L is in part responsible for the cytotoxic effect of viral infections (Diaz-Guerra *et al.*, 1997). These findings could be extended to other death stimuli. With the help of dominant negative mutants, it was shown that in the absence of active RNase L STS-induced apoptosis was abrogated (Castelli *et al.*, 1998)

while overexpression of the enzyme even increased apoptosis (Zhou *et al.*, 1998). The pathways responsible for RNase L mediated apoptosis are to date not completely disclosed. Among the caspases involved, caspase-8 was shown to posses the most relevant role (Domingo-Gil *et al.*, 2006). Another factor required in this process is the stress-activated kinase JNK (c-Jun N-terminal kinase). Phosphorylation of JNK upon treatment with 2'-5'-linked oligoadenylates was shown to be abrogated in RNase L-/- mouse cells and chemical inhibition of the kinase suppressed apoptosis (Li *et al.*, 2004). Additionally, recent work indicated a role of TLR3 signalling in apoptosis induction (Salaun *et al.*, 2006).

1.4 Aim of this work

The present study aims at elucidating the influence of infections with *C. trachomatis* on the outcome of the cellular response to dsRNA and, vice versa, the impact of dsRNA on the fate of *C. trachomatis* inside the host cell. The synthetic double-stranded RNA (dsRNA) polyinosinic:polycytidylic acid (polyI:C) will be used to activate the cell death machinery and the innate immune regulatory pathways in *Chlamydia*-infected host cells. In a first part of the study, it shall be revealed whether, and if so, how *Chlamydia* can interfere with the cellular apoptotic response to dsRNA. Activation of the central apoptosis pathway, the caspase cascade, and of upstream located dsRNA sensors, such as PKR will be investigated. In a second part, activation of the crucial pathways that mediate the anti-viral innate immune response, i.e. NF-κB and IRF-3, will be analyzed in infected cells upon polyI:C transfection. This work shall give a first idea on the interplay of bacterial and viral factors in a co-infected cell and shall thereby contribute to reveal the outcome of chlamydial and viral co-infections, which are highly prevalent in nature.

2. MATERIALS AND METHODS

2. 1 Materials

2.1.1 Cell lines

Table 2.1: Human tissue culture cell lines used in this study.

Name	Origin	ATCC number
Hela229	Cervix carcinoma	ATCC CCL-2.1
HEp-2	Hela contaminant, larynx epithelium	ATCC CCL23
HepG2	Hepatic carcinoma	HB-8065
Jurkat	T cell leukemia	TIB-152

2.1.2 Bacterial strains

Table 2.2: Chlamydial strains used in this study.

Species	Serovar	ATCC number
C. trachomatis	LGV L2	ATCC-VR-902B
C. pneumoniae	CWL-029	ATCC-VR-1310

2.1.3 Oligonucleotides

Table 2.3: Quantitative RT-PCR primers.

Target	Sequence
cFlip	forward 5'-ACTCGCAGTGTTTACTCCTAACG-3' reverse 5'-AAGCTCGCTGTTTCCTGCTA-3´
IFN-β	forward 5´-ACGCCGCATTGACCATCTATG-3´ reverse 5´-CGGAGGTAACCTGTAAGTCTGT-3´
IL-6	forward 5´-AAGCACACTTTCCCCTTCC-3´ reverse 5´-CTATCGTTCTTGGTGGGCTC-3´
IL-8	forward 5´-GGGCCATCAGTTGCAAATC-3´ reverse 5´-TTCCTTCCGGTGGTTTCTTC-3´
GAPDH	forward 5´-GGTATCGTGGAAGGACTCATG-3´ reverse 5´-AGTCTTCTGGGTGGCAGTGA-3´
5S rRNA	forward 5'-GTCTACGGCCATACCACCC-3' reverse 5'-AAAGCCTACAGCACCCGGT-3'

Table 2.4: siRNAs for RNA interference.

Target	Sequence	Company
cFlip-1	pool of different sequences	Dharmacon
cFlip-2	pool of different sequences	Santa Cruz
Mcl-1	5´-CGGGACTGGCTAGTTAAACAA-3´	Qiagen
Luciferase	5´-AACUUACGCUGAGUACUUCGA-3´	Qiagen

2.1.4 Antibodies

Table 2.5: Primary antibodies for immunoblotting (IB), immunofluorescence (IF), or immunoprecipitation (IP).

Name	Source	Company	Product #	Dilution	Application
β-Actin	ms	Santa Cruz	8432	1:3000	IB
β-Tubulin	rb	Santa Cruz	9104	1:1000	IB
Bid	rb	Cell Signaling	2002	1:500	IB
Caspase-8	ms	Cell Signaling	9746	1:500	IB
Caspase-8	ms	Alexis	804-429	1:50, 2µg/ml	IF, IP
Caspase-9	ms	Cell Signaling	9502	1:500	IB
cFlip	ms	Cell Signaling	3210	1:500, 1:50	IB, IF
cHsp60	ms	Alexis	804-027	1:3000, 1:500	IB, IF
eIF2α	rb	Cell Signaling	9722	1:1000	IB
ERK-2	rb	Cell Signaling	9108	1:1000	IB
FADD	ms	BD	610399	1:500	IB
IκB-α	rb	Cell Signaling	9242	1:100	IB
IRF-3	rb	Santa Cruz	9082	1:1000, 1:50	IB, IF
Mcl-1	rb	Epitomics	ab2163	1:500	IB
MEK	rb	Cell Signaling	9122	1:1000	IB
NF-κB (p65)	rb	Santa Cruz	372	1:50	IF
PARP	rb	Santa Cruz	7150	1:3000	IB
PKR	ms	Santa Cruz	6282	1:500	IB
P38	rb	Cell Signaling	9212	1:1000	IB
Phospho-eIF2α	rb	Cell Signaling	9721	1:1000	IB
Phospho-ERK-1/2	ms	Cell Signaling	9106	1:1000	IB
Phospho-JNK	rb	Cell Signaling	4668	1:500	IB
Phospho-MEK1/2	rb	Cell Signaling	9121	1:1000	IB
Phospho-PKR	rb	Cell Signaling	3075	1:500	IB
Phospho-p38	rb	Cell Signaling	9215	1:1000	IB

Table 2.6: Secondary antibodies utilized in this study.

Name	Source	Company	Product #	Dilution	Application
ECL™ Anti-mouse IgG HRP-linked	sheep	GE Healthcare	NA931V	1:2500	IB
ECL™ Anti-rabbit IgG HRP-linked	donkey	GE Healthcare	NA934V	1:2500	IB
Anti-mouse-Cy2™-linked	goat	dianova	115-225-146	1:200	IF
Anti-rabbit Cy2™-linked	goat	dianova	111-225-144	1:200	IF

2.1.5 Chemicals

Table 2.7: Inhibitors, inductors, and fine chemicals.

Compound	Manufacturer
Complete protease inhibitor	Roche
DRAQ5	Alexis
ECL immunoblotting substrate	Thermo
Hoechst 33342	Sigma
Lipofectamine™ 2000	Invitrogen
PhosSTOP phosphatase inhibitor	Roche
polyI:C, γ-irradiated	Sigma
Protein A Sepharose beads	Amersham
TNFα	BD Pharmingen
U0126 MEK1/2 inhibitor	Cell Signalling

All other chemicals were purchased from Sigma or Roth if not stated otherwise.

2.1.6 Kits

Table 2.8: Commercial kits

Product name	Manufacturer
Active Caspase-3 PE MAb Apoptosis Kit	BD Pharmingen
Caspase-Glo 8 Assay	Promega
DeadEnd™Fluorometric TUNEL Kit	Promega
ProteoExtract Subcellular Proteome Extraction Kit	Calbiochem
RNA 6000 Nano Chip Kit	Agilent
Silencer siRNALabeling Kit	Ambion
SuperScript VILO™ cDNA synthesis Kit	Invitrogen
TURBO DNA-free™ Kit	Ambion

2.1.7 Buffers, solutions, and media

Table 2.9: Buffers for immunoblotting.

Buffer	Ingredients
10 x SDS buffer (1l)	30.275 g Tris, 144 g glycine, 10 g SDS
Native PAGE anode buffer	25 mM Tris, 192 mM glycine, adjust to pH 8.4
Native PAGE cathode buffer	Anode buffer + 1% deoxycholate
10x Semi dry buffer (1l)	24 g Tris, 113 g glycine, 2 g SDS
Semi dry transfer buffer	1x semi dry buffer + 20% (v/v) methanol
10x TBS (1l)	60.5 g Tris, 87.6 g NaCl, adjust to pH 7.5 with HCl
$TBST_{20}$	1 x TBS + 0.5% (v/v) Tween20
Blocking solution	$TBST_{20}$ + 5% (w/v) dry milk powder or BSA
Stripping buffer (500 ml)	7.5 g glycine, 0.5 g SDS, 1.54 g DTT, 5 ml $Tween_{20}$, adjust to pH 2.2
2x Laemmli buffer for lysis	100 mM Tris/HCl [pH 6.8], 20% (v/v) glycerol, 4% (w/v) SDS, 1.5% (v/v) 2-mercaptoethanol, dash bromophenol blue
Lysis buffer for phosphor proteins	20 mM HEPES, 400 mM NaCl, 10 mM KCl, 20% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 1x Complete, 1x PhosSTOP
Lysis buffer for native PAGE	50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1x Complete, 1x PhosSTOP
2x Sample buffer for native PAGE	75 mM Tris/HCl [pH 6.8], 30% (v/v) glycerol, 2% (w/v) deoxycholate
DISC-IP buffer	30 mM Tris/HCl [pH 7.5], 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton-X 100, 1x Complete
12% SDS lower gel solution	For 10 ml: 3.3 ml H_2O, 4 ml 30% (v/v) acrylamid-bisacrylamid mix (37.5:1), 0.1 ml 10% (w/v) SDS, 2.5 ml 1.5 M Tris/HCl [pH 8.8], 0.1 ml 10% APS, 4 µl TEMED
SDS upper gel solution	For 5 ml: 3.4 ml H_2O, 0.83 ml 30% (v/v) acrylamid-bisacrylamid mix (37.5:1), 0.63 ml 1.0 M Tris/HCl [pH 6.8], 0.1 ml 10% (w/v) SDS, 0.05 ml 10% APS, 5 µl TEMED
7.5% native gel solution	For 10 ml: 4.8 ml H_2O, 2.5 ml 30% (v/v) acrylamid-bisacrylamid mix (37.5:1), 2.5 ml 1.5 M Tris/HCl [pH 8.8], 0.1 ml 10% APS, 4 µl TEMED

Table 2. 10: Buffers for immunofluorescence.

Buffer	Ingredients
10 x PBS (1l)	80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 (1xH_2O), 2.4 g KH_2PO_4, pH 7.4
PFA solution (1l)	1x PBS + 4% (w/v) PFA, adjust to pH 7.2
Permeabilization buffer	1x PBS + 0.2% (v/v) Triton-X 100
Blocking buffer	1x PBS + 10% (v/v) FCS
Mowiol mounting medium	2.4 g Mowiol 4-88, 6 g glycerol, 6 ml H_2O, 12 ml 0.2 M Tris/HCl [pH 8.5]

Table 2. 11: Cell culture media used in this study.

Medium	Ingredients
Cell culture and infection medium	RPMI 1640 + 25 mM HEPES + L-Glutamax (GIBCO) + 5 % (v/v) heat-inactivated FBS (Biochrome)
Stocking medium	FBS + 10% (v/v) DMSO
Transfection medium	Cell culture medium + 20% (v/v)Optimem (GIBCO)
SPG buffer	0.22 M sucrose, 10 mM Na_2HPO_4, 3.8 mM KH_2PO_4, 5 mM glutamate

2.1.8 Technical equipment

The following technical devices were used in this study: Hera Cell 150 incubator (Thermo), Hera Cell sterile bench (Thermo), Rotanta 460R centrifuge (Hettich), Avanti™ J-25I centrifuge (Beckman Coulter), cold centrifuge 5417R (Eppendorf), Owl Hep semidry electroblotting system (Thermo), PerfectBlue™ Dual Gel Twin PAGE chambers (Peqlab Biotechnology), DM1500 confocal microscope (Leica), DMR epifluorescent microscope (Leica), DMIL light microscope (Leica), plate reader infinite 200 (TECAN), Step One Plus RT PCR system (Applied Biosystems), G-Storm GS1 thermal cycler (GRI), Scanjet G4010 (HP), Thermomixer comfort (Eppendorf), NanoDrop 1000 spectrophotometer (Peqlab Biotechnology), 2100 Bioanalyzer (Agilent Technologies), C6 Flow Cytometer (Accuri).

2.1.9 Software

Windows XP, Microsoft Office 2007, EndNote, Adobe Photoshop CS3, Corel Draw X3, ABI StepOne v2.1, LAS AF confocal microscopy software, ImageJ.

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Cell cultivation

All cell lines that were used in this study (see table 2.1) were maintained in cell culture medium RPMI1640 buffered with 25 mM HEPES (GIBCO, Invitrogen) and L-Glutamax, supplemented with 5% heat-inactivated fetal bovine serum (FBS, Biochrome) in 75 cm_2 cell culture flasks (Greiner). Cells were incubated at 37°C and 5% CO_2 in a cell incubator (Thermo). Every two to three days, cells were passaged for a total of ten passages. Therefore, adherent epithelial cells were washed with sterile PBS (GIBCO, Invitrogen) once and detached by incubation with 1.5 ml Trypsin/EDTA (GIBCO, Invitrogen) per flask. Fresh cell culture medium was added to the cell suspension and one part was transferred to a new culture flask containing culture medium. Jurkat suspension cells were diluted without Trypsin treatment by transferring one part of the cell suspension to a new cell culture flask and addition of fresh culture medium.

2.2.1.2 Cryo stocking of cell lines

Because cell lines were kept in culture for no more than ten passages, cell stocks had to be prepared and thawed if needed. Epithelial cells in a 75 cm^2 flask were washed with PBS, detached by incubation with Trypsin/EDTA for 5 min at 37°C, and transferred to a 15 ml tube (Greiner) with 5 ml cell RPMI1640 + 10% FBS. After centrifugation at 800 rpm (Hettich) for 5 min, the cell pellet was resolved in 3 ml stocking medium and 1 ml per cryo tube was gradually cooled to -80°C in isopropanol. For long-term storage, cryo stocks were transferred to a liquid nitrogen tank. For thawing of cell stocks, they were heated to 37°C until almost completely solved and transferred to 4 ml ice cold FBS in a 15 ml tube. Cells were centrifuged at 800 rpm (Hettich) for 5 min at 4°C and the pellet was resolved in RPMI + 10% FBS. After transfer to a 75 cm^2 flask, cells were incubated at 37°C and 5% CO_2. After the first passage, cells were kept in cell culture medium containing 5% FBS.

2.2.1.3 Infection with *C. trachomatis*

One day before infection, epithelial cells were seeded in 6- or 12-well plates (Greiner) to reach subconfluency the next day. Cell media was replaced by fresh infection medium containing an appropriate amount of *C. trachomatis* LGV L2 to reach the desired multiplicity of infection (MOI). Infected cells were incubated at 35°C and 5% CO_2 for 2.5 h before the infection medium was replaced by fresh cell culture medium for further incubation. For infection of suspension cells, Jurkat cells were counted in a light microscope (Leica) with the help of a Fuchs-Rosenthal counting chamber and transferred to fresh medium in sterile 15 ml tubes (Greiner). After addition of *C. trachomatis* to the

cells, they were transferred to 12- or 6-well plates and centrifuged for 1 h at 35°C and 920 x g (Hettich). The cells were incubated at 35°C and 5% CO_2 until further experiments were performed. For a non-infected control, also termed mock-infected, cells were treated as described without *C. trachomatis*.

2.2.1.4 Infection with *C. pneumoniae*

Due to their highly negative charge, the infection with *C. pneumoniae* EBs was performed as described in 2.2.1.3 with an additional centrifugation step for 1 h at 35°C and 920 x g (Hettich) to facilitate the uptake by epithelial cells. 2.5 h after infection, the infection medium was replaced by fresh culture medium containing 1 µg/ml CHX to block protein synthesis and prevent overgrowing of non-infected cells. Cells were incubated at 35°C and 5% CO_2 for 40 h until apoptosis was induced.

2.2.1.5 Preparation of *Chlamydia* stocks

Owing to their obligate intracellular life style, *Chlamydia* cannot be cultivated *in-vitro* on solid media. Therefore, infectious EBs had to be prepared from infected cell cultures and stored as frozen stocks for later usage. In order to prepare a stock of *C. trachomatis* LGV serovar L2 or *C. pneumoniae* CWL-029, HEp-2 cells were seeded in a 75 cm^2 cell culture flask, infected at MOI 1, and incubated at 35°C and 5% CO_2 for 48 h and 72 h, respectively. Infected cells were detached with a rubber policeman (Sarstedt) and transferred to a falcon tube with sterile glass beads. Cells were ruptured by vortexing for 3 min and the supernatant containing chlamydial EBs was transferred to twelve 150 cm^2 culture flasks of HEp-2 cells for propagation of infection in a dilution of 1:100. After 48 h (*C. trachomatis*) or 72 h (*C. pneumoniae*) of incubation, HEp-2 cells were lysed as above. To remove cell debris, the suspension was centrifuged at 4,000 rpm for 10 min at 4°C (Hettich) followed by a centrifugation step at 25,000 x g for 1 h at 4°C (Beckman Coulter). The chlamydial pellet was washed once with SPG (sucrose phosphate glucose) buffer, centrifuged as above, and resolved in 5 ml SPG buffer. In order to separate clumps of chlamydial EBs, the bacterial suspension was passed through a 20 gauge needle for several times. The chlamydial suspension was aliquoted and stored at -80°C. For each infection experiment, aliquots were freshly thawed and diluted in RPMI1640 + 5% FBS to reach the desired MOI.

2.2.1.6 Titration of chlamydial stocks

To determine the appropriate amount of chlamydial suspension to reach a multiplicity of infection (MOI) of 1, that is one inclusion forming unit (IFU) per cell, freshly prepared stocks of *C. trachomatis* and *C. pneumoniae* were titrated. Therefore, HEp2 and Hela cells were seeded in 24-well plates on

glass cover slips and, the next day, were infected with increasing volumes of *C. pneumoniae* and *C. trachomatis* suspension, respectively, as described before. 20 h post infection (p.i.), cells were fixed with 4% PFA in PBS for 20 min at 35°C, washed twice with PBS and incubated in 10% FBS in PBS for 1 h at RT before incubation with a mouse anti-chlamydial heat shock protein 60 (cHsp60) antibody in 2% FBS/PBS for 1 h at RT. Cells were washed thrice with PBS and incubated with a secondary Cy2™-linked sheep anti-mouse antibody in 2% FBS/PBS. To counterstain nuclei, cells were incubated with Hoechst 33342 dye (Sigma) for 5 min at RT, washed twice with PBS, and mounted with Mowiol mounting medium. Chlamydial inclusions and cellular nuclei were counted in an epifluorescence microscope (Leica) with 400-fold magnification to calculate the concentration of the chlamydial stocks.

2.2.1.7 Infectivity assay

In order to investigate whether the production of chlamydial progeny is altered by dsRNA and/or pathways activated in response to dsRNA, an infectivity assay was carried out. Therefore, chlamydial inclusions were ruptured at the end of the developmental cycle and chlamydial particles were transferred to uninfected cells for a second round of infection. The primary infection and transfection with polyI:C were performed as described in section 2.2.1.3 and 2.2.1.10, respectively. 24 h post transfection with polyI:C, that was 44 h p.i., cells and inclusions were lysed by a freeze-thaw cycle: cells were incubated at -80°C for 1 h followed by an incubation at 35°C and ruptured by pipetting the cells up and down. Supernatant containing EBs was transferred to Hela cells on glass cover slips in a dilution of 1:100. Cells were incubated for 24 h at 35°C and fixed in 4% PFA. Cells were stained for chlamydial Hsp60 and counterstained with Hoechst 33324 for DNA as described in section 2.2.1.6.

2.2.1.8 Treatment with inhibitors or antibiotics

For inhibition of ERK activation, cells were treated with 10 µM U0126 (Cell Signaling) in DMSO at the time point of infection. Control cells were treated with solvent alone. The medium was aspirated after 2.5 h to remove unbound EBs and was replaced by cell culture medium containing 10 µM U0126. 20 h p.i., before transfection with polyI:C, the medium was replaced one more time by fresh medium containing the chemical ERK inhibitor. Bacterial protein translation was inhibited with chloramphenicol (CAM). For inhibition of early bacterial protein synthesis, cells were treated 2.5 h after infection with 2 µg/ml CAM in cell culture medium. When added 20 h p. i., 4 µg/ml CAM was used.

2.2.1.9 siRNA transfection

With the advent of RNA interference, the function of cellular proteins can be investigated by the delivery of small interfering RNAs (siRNAs) into the cells which then leads to the degradation of respective complementary mRNA transcripts (Elbashir *et al.*, 2001). In order to deplete host cells of anti-apoptotic proteins, siRNA transfection was performed with Lipofectamine™2000 (Invitrogen). One day before transfection, cells were seeded into 6-well plates to gain 70% confluency. 120 nM siRNAs were incubated in 200 µl Optimem transfection medium (GIBCO) containing 4 µl Lipofectamine™ 2000 for 25 min at RT before dropwise addition to 800 µl fresh RPMI medium containing 5% FBS. The next day, cells were split and transferred to 12- or 96-well plates. At day two post transfection, cells were infected with *Chlamydia* and experiments were performed as described before.

2.2.1.10 Application of polyI:C

Because activation of cytosolic RNA sensors is induced by intracellular dsRNA, the synthetic dsRNA polyinosinic:polycytidylic acid (polyI:C) had to be delivered into the host cells. Therefore, epithelial cells were transfected with 1 µg/ml γ-irradiated polyI:C (Sigma) in Optimem medium and Lipofectamine™ 2000 reagent as described before (see 2.2.1.9). For one 12-well, 100 µl Optimem (GIBCO) containing 2 µl Lipofectamin™ 2000 and 0.5 µg polyI:C or siRNA against firefly Luciferase (200 nM) as a negative control was carefully added to 400 µl fresh RPMI1640 + 5% FBS. For external application of polyI:C, 1 µg/ml or 100 µg/ml was added to culture medium in the absence of lipofectamine. Cells were incubated at 35°C for the indicated time points.

2.2.1.11 Apoptosis induction

In order to compare the anti-apoptotic mechanisms of *C. trachomatis* during polyI:C-induced apoptosis with a second death stimulus, tumor necrosis factor α (TNFα) was applied together with cycloheximide (CHX). The addition of CHX was required to prevent translation of anti-apoptotic proteins and to provoke apoptosis. 20 h after infection, the culture medium of epithelial cells was replaced by medium containing 10 ng/ml TNFα + 5 µg/ml CHX. Cells were incubated at 35°C for the indicated time points.

2.2.1.12 TUNEL assay

A morphological feature of an apoptotic cell is the fragmentation of the DNA by endonucleases. In order to detect a significant difference in apoptosis of infected versus non-infected cells, DNA fragmentation was determined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-

nick end-labeling (TUNEL) method. This method allows for detection of apoptotic cells on single cell level (Gavrieli *et al.*, 1992). It is based on the integration of FITC-12-dUTP molecules to the 3-OH' end of fragmented DNA by TdT and was performed with the DeadEnd™Fluorometric TUNEL Kit (Promega). Therefore, cells were seeded on glass cover slips, infected or mock-infected, and treated with polyI:C or siLuciferase for 4 h. Cells were fixed with 4% PFA for 20 min at 35°C and labelled according to the manufacturer`s instructions. Briefly, cells were permeabilized with 0.2% [v/v] Triton X-100 in PBS for 5 min, washed once with PBS, and stained with 1 µg/ml Hoechst 33324 dye in PBS for 5 min. Cells were washed and permeabilized as above and equilibrated with equilibration buffer before incubation with the nucleotide mix and TdT enzyme for 1 h. The reaction was terminated with 2x SSC (saline sodium citrate), the cells were washed thrice and mounted with Mowiol. The ratio of TUNEL-positive cells was evaluated by counting five random fields in an epifluorescence microscope (Leica) with 400-fold magnification.

2.2.1.13 Luminescent caspase-8 activity assay

Despite the fact that autoprocessing of procaspase-8 into its active form is a prerequisite for the apoptotic actions of the initiator caspase (Kang *et al.*, 2008), cleavage of caspase-8 does not necessarily reflect its enzymatic activity. In order to determine the activity of caspase-8 in infected polyI:C-treated cells, a luminescence-based Caspase-Glo 8 Assay (Promega) was performed. This assay makes use of a substrate with a LETD sequence that was proposed to be specifically cleaved by caspase-8 (Garcia-Calvo *et al.*, 1999, Thornberry *et al.*, 1997). According to the instructions, 12,000 HeLa cells were seeded in a white flat bottom 96-well plate (Nunc) to reach about 70% confluency at the day of infection. Cells were treated as described above in a volume of 100 µl and the luminescent caspase-8 substrate was added 7 h after polyI:C transfection to reach a total of 200 µl. The plate was shaken for 30 sec at 450 rpm with a Thermomixer (Eppendorf) and incubated for 75 min at RT in the dark. Subsequently, luminescence was measured in a plate reader (Tecan) with a light exposure duration of 1 sec/well.

2.2.1.14 Fluorescence-activated cell sorting (FACS)

In order to quantify apoptotic cells, fluorescence-activated cell sorting (FACS) analysis was performed. Hela cells were infected and apoptosis was induced with polyI:C for 8 h as described before. Staining was performed with the Active Caspase-3 PE Apoptosis Kit (BD Pharmingen) according to the manufacturer´s instructions. $1x10^6$ cells were detached with Trypsin/EDTA and washed with ice cold PBS once. Cells were pelleted by centrifugation at 350 x g for 5 min at 4°C and incubated in Cytofix/Cytoperm™ solution for 20 min on ice to allow for fixation and permeabilization.

Cells were washed twice with Perm/Wash™ buffer and resuspended in 100 µl of the same buffer containing 20 µl phycoerythrin (PE)-labelled rabbit anti-active caspase-3 antibody. After an incubation of 30 min at RT, the samples were washed once, resuspended in 500 µl Perm/Wash™ buffer, and subjected to FACS analysis in a C6 flow cytometer (Accuri). 10,000 cells per sample were detected with an exitation wavelength of 488 nm and an emission wavelength of 575 nm.

2.2.1.15 Confocal microscopy

For confocal fluorescent microscopy, cells were seeded on glass cover slips the day before infection. Cells were infected as described above and treated with polyI:C or control siRNA against Luciferase or with FAM-labeled polyI:C (see 2.2.2.5) for 2.5 h. After fixation in 4% PFA for 20 min at 35°C, cells were washed three times with ice-cold PBS, were permeabilized with 0.2% [v/v] Triton X-100 in PBS for 20 min, and were washed again before blocking with 10% FBS in PBS for 1 h at RT. Samples were incubated with the primary antibody (table 2.5) in PBS + 2% FBS at 4 C overnight, washed thrice with PBS, and incubated with a goat anti-mouse or anti-rabbit Cy2™-labelled secondary antibody and the DNA stain DRAQ5 (1:500, Alexis) in PBS + 2% FBS for 1 h in the dark. Slides were washed twice in PBS and once in distilled water to remove the PBS and were mounted on glass slides with Mowiol solution. Cells were inspected in a laser scanning confocal microscope (Leica) with 630-fold magnification and an additional 1.5 x software magnification. Images were further processed with the software Adobe Photoshop CS3.

2.2.1.16 Statistical analysis

Statistical significance of the acquired data was calculated with the two-sided Student´s t-test.

2.2.2 Biochemical methods

2.2.2.1 SDS-PAGE and immunoblotting

Proteins were separated for detection of altered protein levels or of posttranslational modifications such as phosphorylation using the sodium-dodecylsulfate polyacrylamide electrophoresis (SDS-PAGE). Here, proteins migrate in an electric field according to their size which is mediated by SDS providing a uniform negative charge to the proteins. Samples were treated as follows: After infection and polyI:C transfection, cells from a 12 well plate were transferred to ice, the medium with detached cells was collected, and the adherent cells were directly lysed in 100 µl 2x Laemmli buffer. The lysates were then combined with the pellet of the respective detached cells after centrifugation at 4°C for 5 min at 350 x g and were heated to 95°C for 5 min. For phospho-proteins, a lysis buffer containing phosphatase inhibitors was used (see table 2.9). Cells were scraped in lysis buffer, torn by

pipetting up and down, and transferred to a reaction tube (Eppendorf). Cell debris was removed by centrifugation for 5 min at 13,000 x g and 4°C. The supernatant was transferred to a fresh tube and mixed with an equal volume of 2x Laemmli buffer. Lysates were heated to 95° for 5 min. Acrylamid concentrations of 10% to 12% were used depending on the size of the protein of interest.Voltages applied were 70 V for 30 min and 140 V for 1.5 h for stacking (upper) and running (lower) gel, respectively.

Subsequently, proteins were transferred to a methanol-activated PVDF membrane (GE Healthcare) in a semidry electroblotting system (Thermo) with a current of 1.5 mA per 1 cm^2 membrane for 2 h. Membranes were rinsed in $TBST_{20}$ for 1 min before blocking the membrane for 1 h in blocking buffer to reduce unspecific binding of the antibody. The membrane was incubated with the primary antibodies listed in table 2.5 overnight at 4°C. After washing the membrane thrice for 10 min in $TBST_{20}$, antibody-antigen complexes were detected by HRP-linked donkey anti-rabbit or sheep anti-mouse secondary antibodies (GE Healthcare). For reprobing with other antibodies, membranes were re-activated with 100% methanol and incubated in stripping buffer for 30 min at RT before processing as described above.

2.2.2.2 Native PAGE

In order to identify dimerization of IRF-3, native PAGE was performed. In contrast to SDS-PAGE, native PAGE is performed under non-reducing conditions, thereby the tertiary structure of proteins is preserved and dimerized forms can be detected. During native PAGE, the protein separation is mainly based on their charge while their size plays a minor role. Due to its negative charge (isoelectric point: 5.7), IRF-3 will migrate in an electric field towards the anode. Because protein dimers will migrate more slowly, a band shift will allow for detection of dimerization. Hela cells were seeded in 6-well plates, infected with *C. trachomatis* at MOI 2 and transfected with polyI:C as described before. 30 min and 1 h after transfection, cells were transferred to ice, washed with ice cold PBS once, and scraped in PBS containing 1x protease inhibitior (Roche). Cells were centrifuged at 350 x g for 5 min at 4°C and the pellet was resuspended in 100 µl native PAGE lysis buffer (table 2.9). Cells were broken by passing through a 20 gauge needle and cleared by centrifugation at 10,000 x g for 10 min. 100 µl 2x sample buffer was added to the lysates. The 7.5% native gel was pre-run for 30 min at 40 mA before 20 µl of each sample was applied to the gel and electrophoresed at 25 mA for 1.5 h. Protein transfer to a PVDF membrane and antibody incubation was performed as described in 2.2.2.1.

2.2.2.3 Co-Immunoprecipitation

Isolation of a single protein from total cell lysates can be achieved with immunoprecipitation (IP). This method is based on the interaction of the antibody IgG chain to the protein A of *Staphyloccocus aureus* (Forsgren *et al.*, 1966) which is covalently linked to Sepharose beads. In order to purify a protein of interest, the respective antibody can be linked to Sepharose A beads. Moreover, because proteins are purified under non-denaturing conditions, IP allows for detection of protein-protein interactions, therefore also named co-IP. Here, the interaction of caspase-8 with FADD and/or cFlip was investigated. Hela cells were seeded in 6-well plates and 2 wells were combined for each sample. Infected and non-infected Hela cells were transfected with polyI:C for 2 h and transferred to ice. All subsequent steps were performed on ice or at 4°C in a cold room. Cells were washed once with PBS and detached with a rubber policeman in PBS containing protease inhibitor (Roche). Samples were centrifuged at 350 x g for 5 min at 4°C and pellets were resolved in 400 µl DISC IP buffer containing protease inhibitor. Lysates were passed through a 20 gauge needle for ten times, incubated for 5 min and cleared by centrifugation at 10,000 x g for 10 min. The supernatants were transferred to fresh reaction tubes and caspase-8 antibody (C15, Alexis) was added to reach a concentration of 2 µg/ml antibody. Samples were incubated for 2 h at 4°C with constant rotation. Subsequently, 100 µl pre-washed (DISC IP buffer) Sepharose A beads were added and the samples were incubated for 2 h on a rotator. To pellet the beads, samples were centrifuged at 2000 rpm (Eppendorf) and the beads were washed twice with DISC IP buffer and twice with PBS. 30 µl 2x Laemmli buffer was added to the beads, vortexed, and heated to 95°C for 10 min. Samples were subjected to SDS PAGE as describe in 2.2.2.1.

2.2.2.4 Subcellular fractionation

In order to identify whether a chlamydial infection influences the distribution of cellular proteins, they were extracted according to their subcellular localization with the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) following the manufacturer´s instructions. Cells were seeded in 6-well plates, infected, and treated with polyI:C as described above. Cells were washed twice with the wash buffer provided in the kit for 5 min at 4°C. Buffer was removed and cells were incubated with extraction buffer I containing protease inhibitor for 5 min. Fraction one containing cytosolic proteins was carefully transferred to a fresh tube. Cells were then incubated with buffer for extraction of membranes and organelles for 30 min (fraction 2). During the next extraction step, fraction 3 containing nuclear proteins was extracted, followed by the cytoskeleton fraction (fraction 4). Fractions were mixed with the same volume of 2x Laemmli buffer and heated to 95°C before samples were electrophoresed as described in 2.2.2.1.

2.2.2.5 Fluorescent labelling of polyI:C

In order to control uptake of polyI:C into epithelial cells via detection by fluorescence microscopy, the dsRNA was labelled with the Silencer siRNA Labeling Kit (Ambion) according to the manufacturer`s instructions. 5 µg of polyI:C was incubated with fluorescein (FAM) at 37° C for 1 h, precipitated with 60% isopropanol by incubation at -20°C for 20 min followed by centrifugation at 13,000 x g for 30 min at 4°C. The RNA pellet was washed once with 75% ethanol, was air-dried, and resuspended in sterile DEPC-treated water. Transfection of labelled polyI:C was performed as described in 2.2.1.10.

2.2.3 Molecular biological methods

2.2.3.1 RNA-Isolation

RNA isolation was performed either for quantification of specific transcripts by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (2.2.3.5) or for detection of degraded 28S rRNA (2.2.3.6). Hela cells in 6-well plates were infected and treated as described above. Cells were lysed in 1 ml TRIzol reagent (Invitrogen) per 6-well, transferred to reaction tubes (Eppendorf) and incubated for 5 min at RT. After addition of 0.2 ml chloroform, the samples were shaken, incubated for 5 min at RT, and centrifuged at 12,000 x g for 15 min at 4°C to separate RNA from DNA and proteins. The upper, aqueous phase containing RNA was transferred to a fresh tube and treated with 0.5 ml isopropyl alcohol to precipitate the RNA. Samples were incubated for 10 min at RT and centrifuged for 10 min as above. The RNA pellet was washed once with 1 ml 75% ethanol and centrifuged at 7,500 x g for 5 min. The RNA pellet was air-dried, dissolved in 50 µl DEPC-H_2O, and stored at -80°C until further use.

2.2.3.2 DNA digestion

In order to remove contaminating DNA from isolated RNA, DNA was digested with the TURBO DNA-free™ Kit (Ambion). 5 µl TURBO DNase buffer and 1 µl DNase were added to 50 µl RNA sample and incubated at 37°C for 20 min. After addition of 5 µl Inactivation reagent, samples were mixed, incubated at RT for 5 min, and centrifuged at 10,000 x g for 1.5 min. The supernatant was transferred to a fresh reaction tube.

2.2.3.3 Determination of RNA concentration

The concentration of the isolated RNA was determined with the spectrophotometer NanoDrop 1000 (Peqlab) by measuring the absorbance at 260 nm.

2.2.3.4 Copy (c)DNA synthesis

For qRT-PCR, isolated mRNA was reverse transcribed into copy DNA (cDNA) with the Superscript VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer´s instructions. 4 µl 5 x VILO Reaction Mix and 2 µl Enzyme Mix were added to 2 µg RNA and DEPC-H_2O in a total volume of 20 µl. Reverse transcription into cDNA was performed in a thermocycler (GRI) with the following temperature profile: 10 min at 25°C, 60 min at 42°C, and 5 min at 85°C. Samples were stored at -20°C until further use.

2.2.3.5 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To quantify the amount of specific mRNA populations, e.g. after siRNA-mediated knock down of target mRNA, qRT-PCR was performed. Amplification of cDNA and quantification of amplificates were performed in a Step One Plus RT PCR system (Applied Biosystems) using ABSolute Blue QPCR SYBR low ROX mix (Thermo) according to the manufacturer`s protocol: 0.1 µl of cDNA was mixed with 10 µl 2x ABSolute Blue QPCR SYBR low ROX mix, 125 nM of each primer (table 2.3), and ddH_2O was added to a total volume of 20 µl. Each sample was analysed in triplicates.

2.2.3.6 Measurement of 28S rRNA

This assay served to detect activity of the RNase L. Upon activation by 2-5 oligoadenylates, the enzyme cleaves 28S ribosomal RNA (rRNA) into fragments (Hassel *et al.*, 1993). After isolation of total RNA (see 2.2.3.1), quantification of RNA species was performed with a 2100 Bioanalyzer (Agilent) micro fluidic device using the RNA 6000 Nano kit (Agilent).

3. RESULTS

3.1 Influence of *Chlamydia trachomatis* infection on dsRNA-induced apoptosis

3.1.1 *C. trachomatis* infected host cells resist polyI:C-induced apoptosis

In order to investigate whether an existing infection with human pathogenic *Chlamydia trachomatis* can influence the outcome of viral co-infections, the human epithelial cell lines Hela229 and HEp-2 were infected with *C. trachomatis* and treated with the synthetic double-stranded RNA polyinosinic:polycytidylic acid (polyI:C), a potent activator of cytoplasmic RNA sensors such as the RNA-dependent protein kinase (PKR). To detect and quantify apoptosis, cleavage of the caspase-3 substrate poly (ADP-ribose) polymerase (PARP) was analyzed by immunoblotting. Whereas transfection with a short siRNA against Luciferase did not influence cell viability at all, polyI:C transfection caused a strong cleavage of PARP, indicated by the reduction of the upper band (full-length PARP, 120 kDa) and the appearance of a lower band (cleaved PARP, 89 kDa). In contrast, host cells infected with *C. trachomatis* did not display PARP cleavage after treatment with either Luciferase siRNA or polyI:C (Fig. 3.1 A and B). This indicated a pathogen-mediated block of caspase activation. The intensitity of the lower and upper bands of PARP were determined by densitometry.

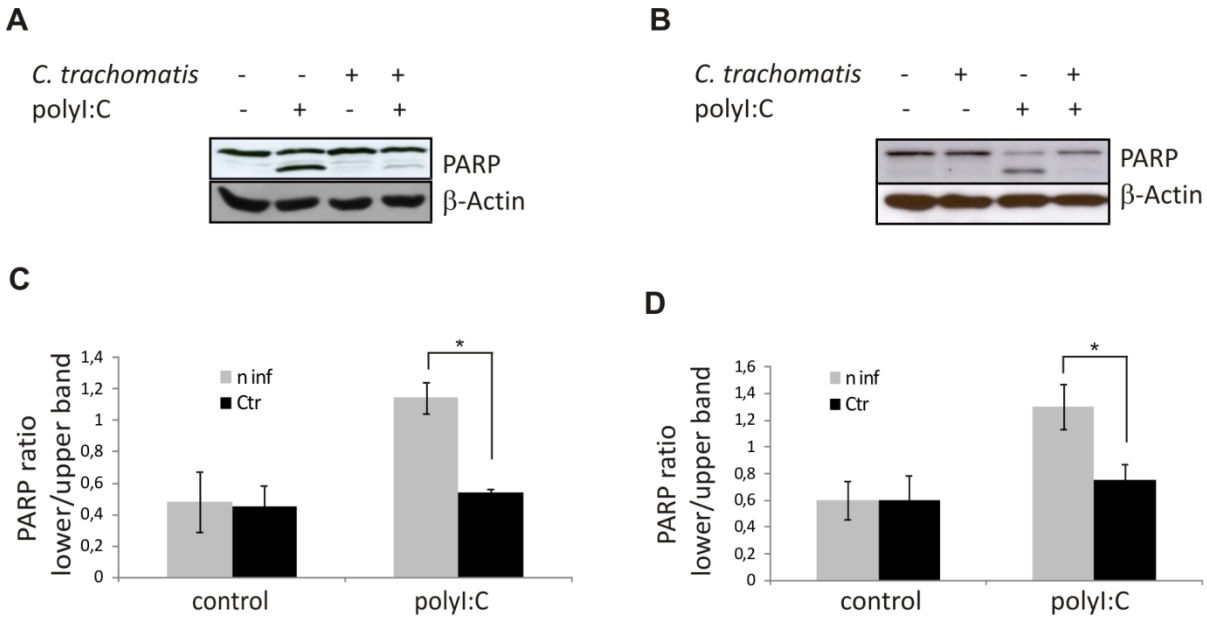

Fig. 3.1: polyI:C-induced PARP cleavage is inhibited in *C. trachomatis*-infected host cells.
Hela [A, C] and HEp-2 cells [B, D] were infected with *C. trachomatis* at MOI 4 and, 20 h post infection, cells were transfected with 1 µg/ml polyI:C (+) or with siRNA directed against Luciferase (-). [A, B] Cells were lysed and PARP cleavage was detected by immunoblotting. β-Actin served as a loading control. [C, D] PARP cleavage was quantified by densitometry. Shown are the mean values of the lower to upper PARP band ratios from three independent experiments -/+ SD. Significance was determined with the two-sided Student`s t-test. *= $p < 0.05$.

The calculation of the ratios of cleaved to non-cleaved PARP from three independent experiments demonstrated that inhibition of polyI:C-induced apoptosis by *C. trachomatis* infection was statistically significant (Fig. 3.1 C and D). For a deeper insight into the cellular response to polyI:C and the influence of *Chlamydia* on apoptosis induced by the synthetic dsRNA, a time course experiment was performed, depicted in figure 3.2. Apoptosis could be detected as early as 4 h post transfection with polyI:C, indicated by PARP cleavage, and further increased at 6 h post transfection. PARP cleavage was blocked by infection at all time points investigated and was, moreover, comparable to the inhibition of TNFα-induced apoptosis (Fig. 3.2).

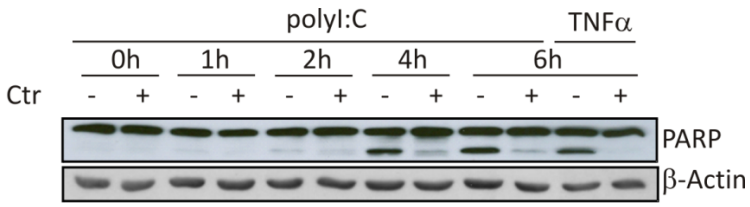

Fig. 3.2: Time course experiment for polyI:C-induced apoptosis.
Hela cells were infected with *C. trachomatis* at MOI 4 and, 20 h post infection, were transfected with 1 µg/ml polyI:C or treated with 10 ng/ml TNFα and 5 µg/ml CHX for the indicated time. Lysates were analyzed for PARP cleavage by immunoblotting. Equal loading was controlled with an antibody specific for β-Actin.

3.1.2 Apoptosis inhibition is MOI-dependent and requires early bacterial protein synthesis

It has been demonstrated that apoptosis resistance by *Chlamydia* infection is strongly dependent on the infection conditions, such as the time point after infection and the multiplicity of infection (MOI) (Rajalingam *et al.*, 2001). To determine whether the MOI influenced inhibition of polyI:C-induced apoptosis, epithelial cells were infected with *C. trachomatis* at different MOIs or remained uninfected and were transfected with polyI:C. As shown in figure 3.3 A, cleavage of PARP decreased in Hela cells with rising MOI. Although equal loading, indicated by β-Actin, was not achieved for HEp-2 cells, a clear reduction in the ratio of the lower to upper PARP band was obvious (Fig. 3.3 B). This indicated that inhibition of dsRNA-induced apoptosis was bacterial dose dependent.

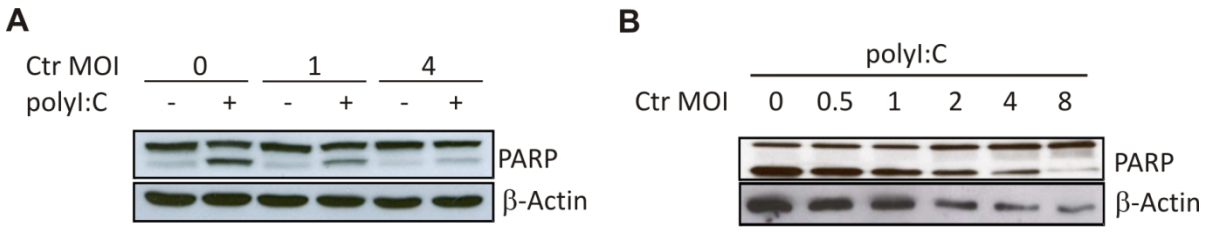

Fig. 3.3: Inhibition of dsRNA-induced apoptosis is MOI-dependent.
[A] Hela cells were infected with rising MOIs of *C. trachomatis* and transfected 20 hours later with 1 µg/ml polyI:C (+) or with siRNA against Luciferase (-). 8 h post transfection, cells were lysed and analyzed for PARP cleavage by immunoblotting. Equal loading was controlled by β-Actin levels. [B] HEp-2 cells were treated as in (A). Lysates were prepared 16 h post transfection with polyI:C.

Because an involvement of chlamydial protein synthesis in the inhibition of host cell death has been demonstrated (Fan *et al.*, 1998), it was next tested whether this was also essential during dsRNA-induced apoptosis. The role of early and late bacterial protein synthesis was investigated by application of chloramphenicol (CAM) to infected Hela cells at 2.5 h and 20 h post infection, respectively. While a reduction in PARP cleavage could still be detected in *Chlamydia*-infected cells that were treated with CAM at 20 h post infection, inhibition of bacterial protein synthesis at an early time point led to an increase in PARP cleavage (Fig. 3.4 A and B).

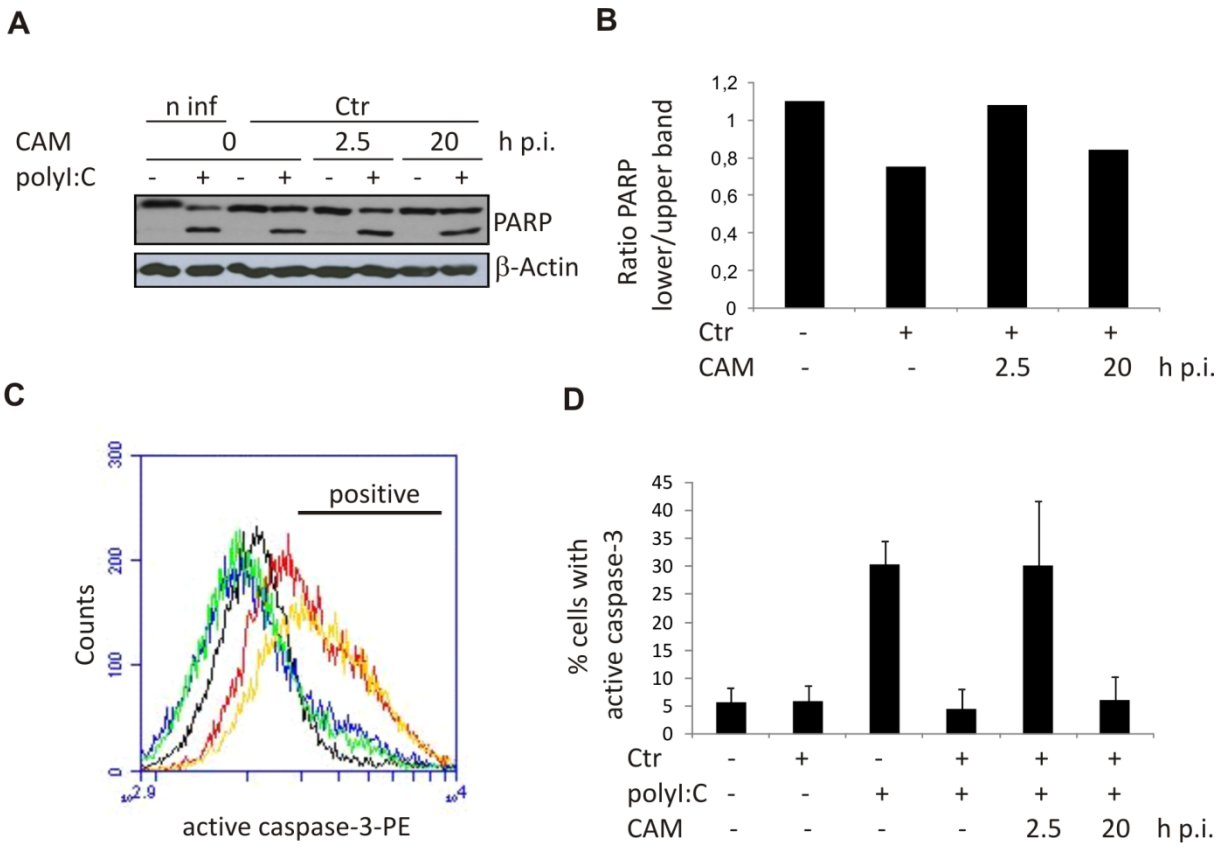

Fig. 3.4: Apoptosis inhibition requires early bacterial protein synthesis.
[A] Hela cells were infected at MOI 1 and treated with 2 µg/ml or 4 µg/ml chloramphenicol (CAM) at 2.5 h and 20 h p.i., respectively. Cells were lysed at 8 h post transfection with 1 µg/ml polyI:C and subjected to immunoblotting for PARP with β-Actin as loading control. [B] The ratios of the upper to lower PARP bands from (A) were analyzed by densitometry. [C] FACS histogram overlays of cells with active caspase-3 PE that were infected at MOI 4 and treated with polyI:C and CAM as described in (A). The colored lines represent the following samples: black: non-treated, red: polyI:C, blue: infected + polyI:C, yellow: CAM 2.5 h, green: CAM 20 h [D] Quantification of the FACS analysis from (C). Shown are the mean values of active caspase-3 from two independent experiments with SD.

To further confirm these findings, the percentage of cells with active caspase-3 was determined by FACS analysis (Fig. 3.4 C, see marker). As shown in figure 3.4 D, 5.8% and 5.9% of non-infected and *Chlamydia*-infected cells, respectively, displayed positive staining for active caspase-3 after control transfection. In contrast, polyI:C transfection led to an increase in active caspase-3 positive cells to 30.4% in the non-infected sample, while a clear reduction to 4.6% was found upon infection with

C. trachomatis. As expected, inhibition of early bacterial protein synthesis by addition of CAM at 2.5 h p.i. resulted in a loss of apoptosis inhibition as 30.2% of cells were positive for active caspase-3, whereas only 6.2% of cells displayed cleaved caspase-3 when CAM was added at 20 h after infection (Fig. 3.4 D). This implied a prerequisite of early bacterial protein synthesis for the chlamydial ability to prevent polyI:C-induced apoptosis.

3.1.3 DNA fragmentation is reduced in infected host cells

Apoptosis is characterized by a set of morphological and biochemical features, among which DNA fragmentation is one of the most important phenotypes. Upon activation of caspases, cleavage of the Inhibitor of Caspase Activated DNase (ICAD) releases the Caspase Activated DNase (CAD) which subsequently fragments choromosomal DNA (Enari *et al.*, 1998, Liu *et al.*, 1997, Sakahira *et al.*, 1998). To investigate inhibition of DNA fragmentation in *C. trachomatis*-infected host cell, the terminal dUTP nick-end labelling (TUNEL) assay was performed. Figure 3.5 reveals that upon polyI:C treatment the amount of TUNEL positive cells, i.e. cells with fragmented DNA, was reduced by infection in an MOI-dependent manner. Whereas 13.5% of non-infected Hela cells displayed fragmented DNA 4 h post transfection with polyI:C, only 4.9% and 1.1% of host cells infected with *C. trachomatis* at MOI 1 and MOI 4, respectively, showed positive TUNEL staining. As expected from previous PARP cleavage quantification experiments, the level of inhibition was comparable to the level of apoptosis inhibition during TNFα-induced apoptosis. Here, the percentage of TUNEL-positive Hela cells was reduced from 12.8% in non-infected to 5.3% and 1.5% in cells infected at MOI 1 and MOI 4, respectively (Fig. 3.5). These data emphasize the finding that *C. trachomatis* infection reduces the host cell apoptotic response to dsRNA in an MOI-dependent manner.

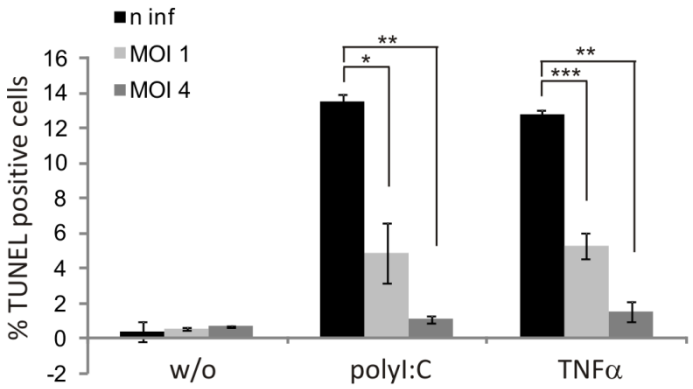

Fig. 3.5: DNA fragmentation is inhibited by *Chlamydia* infection.
Hela cells were infected at different MOI and at 20 h p.i.apoptosis was induced for 4 h with 1 µg/ml polyI:C or 10 ng/ml TNFα + 5 µg/ml CHX or cells remained untreated (w/o). Cells were fixed and stained for DNA fragmentation with the TUNEL method. The percentage of TUNEL positive cells was calculated by counting five random fields in an epifluorescence microscope with 400-fold magnification. Shown are the mean values of two independent experiments -/+ SD. Statistical significance was evaluated with the two-sided Student`s t-test, where *= $p < 0.05$, **= $p < 0.01$, and ***= $p < 0.001$.

3.1.4 Infection with *C. trachomatis* inhibits polyI:C-induced activation of caspase-8

Several chlamydial strategies have been described for manipulation of the host cell death signalling pathways, previously reviewed by Sharma *et al.* (2009). In order to identify how an infection with *C. trachomatis* can inhibit polyI:C-induced apoptosis, the cellular pathways known to be involved in the death response to dsRNA were investigated. A crucial step for apoptosis induction by polyI:C is the processing of caspase-8 into its active fragments (Iordanov *et al.*, 2005a), which was shown to be mediated by the dsRNA-dependent protein kinase (PKR) (Balachandran *et al.*, 1998, Gil *et al.*, 2000b). Active caspase-8 subsequently induces truncation of the BH-3 only protein Bid leading to the release of apoptogenic factors from the mitochondria and processing of caspase-9 (Iordanov *et al.*, 2005b). Therefore, activation of the initiator caspase-8 and downstream signalling was evaluated in *C. trachomatis* infected host cells transfected with polyI:C.

3.1.4.1 Truncation of Bid is reduced in infected cells

In order to identify whether infected cells undergo activation of caspase-8 upon polyI:C transfection, processing of procaspase-8, truncation of the caspase-8 substrate Bid, and processing of downstream caspase-9 were examined by immunoblotting. Hela cells were infected with *C. trachomatis* for 20 h and transfected with 1 µg/ml polyI:C or control transfected with siRNA against Luciferase. Immunoblotting revealed that infection with *C. trachomatis* could prevent cleavage not only of caspase-9, but interestingly also processing of procaspase-8 was blocked. As a consequence, truncation of the caspase-8 substrate Bid was inhibited in an MOI-dependent fashion (Fig. 3.6 A). The block of caspase-8 activation, demonstrated by the rescue of Bid truncation and caspase-9 processing, could also be detected in infected HEp-2 cells (Fig. 3.6 B). These results suggested that infection induced a block of apoptosis upstream of caspase-8.

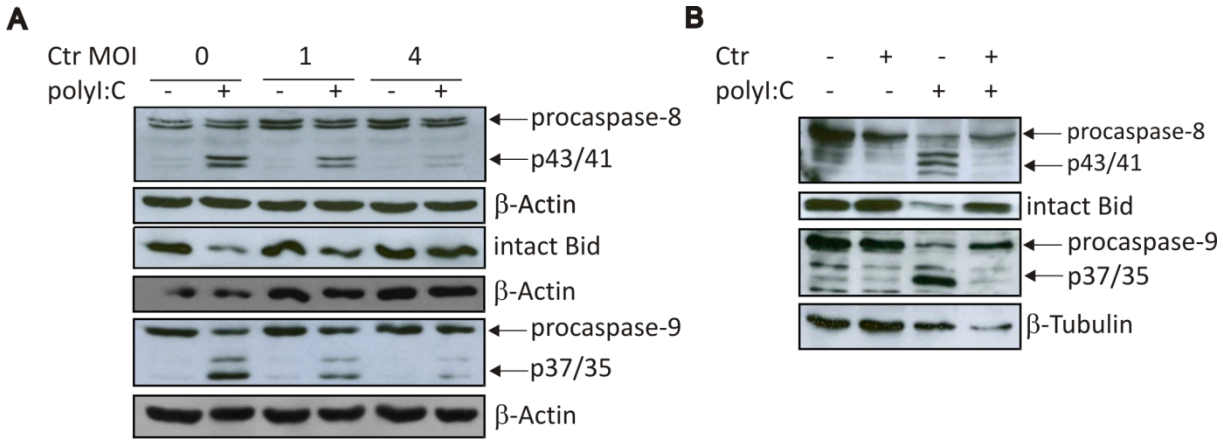

Fig. 3.6: Bid truncation is inhibited in infected host cells.
20 h after infection with *C. trachomatis* (Ctr), Hela [A] and HEp-2 cells [B] were transfected with 1 µg/ml polyI:C for 8 h and 16 h, respectively, and lysed in 2x Laemmli buffer. Lysates were analyzed for the caspase-8 and caspase-9 cleavage products p43/41 and p37/35, respectively, and for truncation of intact Bid. β-Actin served as a loading control.

3.1.4.2 polyI:C-induced caspase-8 activity is inhibited in an MOI-dependent manner

To confirm the observation that infections with *C. trachomatis* can inhibit polyI:C-induced activation of caspase-8, a chemiluminescence-based activity assay specific for caspase-8 was performed. Transfection with polyI:C provoked a strong signal for active caspase-8 in non-infected cells that was significantly reduced in infected cells in an MOI-dependent manner. Compared to uninfected cells, host cells infected with *C. trachomatis* displayed only 80%, 68%, 45%, and 42% of caspase-8 activity at MOI 1, 2, 4, and 8, respectively, while the basal level detected in control treated cells was 29% (Fig. 3.7 A). Thus, a block of caspase-8 activity by the pathogen could be proofed. Next, it was tested whether chlamydial factors, most likely secreted effector proteins, play a role in caspase-8 inhibition. Therefore, bacterial protein synthesis was inhibited by CAM addition. Infected Hela cells were treated with CAM at different time points after infection to differentiate between early and late bacterial protein synthesis. Whereas the application of CAM at 20 h post infection did not influence chlamydial inhibition of caspase-8, activity of caspase-8 was comparable in uninfected cells and infected cells treated with CAM at 2.5 h p.i. (Fig. 3.7 B). This demonstrated that early bacterial protein synthesis was required for preventing caspase-8 activity and was consistent with previous observations concerning PARP cleavage (Fig. 3.4).

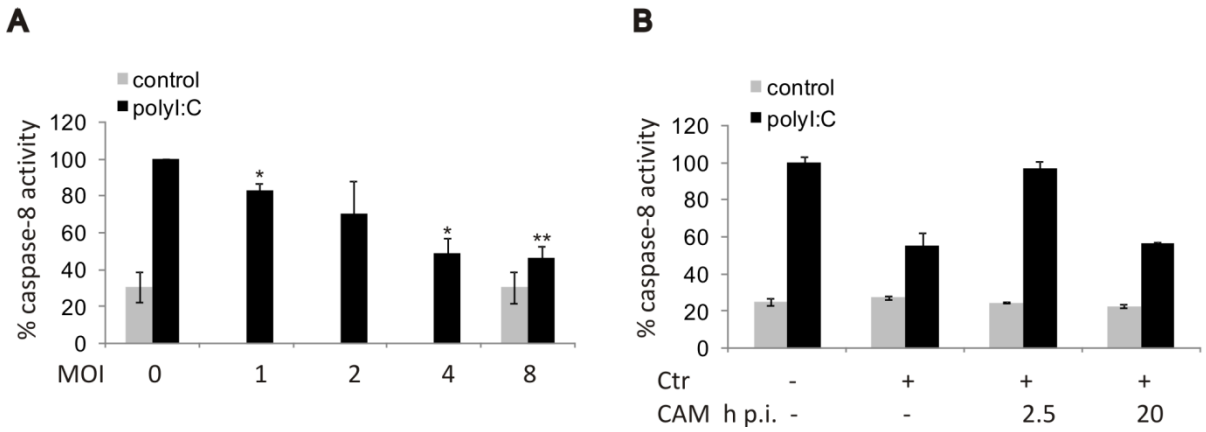

Fig. 3.7: Caspase-8 activity is reduced in an MOI-dependent manner and depends on early bacterial protein synthesis. [A] Hela cells were infected with *C. trachomatis* (Ctr) at different MOIs and were transfected with 1 µg/ml polyI:C for 7 h or were control transfected using a Luciferase siRNA (control). Caspase-8 specific substrate was added to the cells for an incubation at RT for 75 min before caspase-8 activity was detected by luminescence. Shown are the mean values of two independent experiments -/+ SD. *= $p < 0.05$, **= $p < 0.01$. [B] Hela cells were infected with *C. trachomatis* (Ctr) at MOI 4 and treated with chloramphenicol (CAM) at the indicated time points post infection (p.i.). 7 h after transfection with 1 µg/ml polyI:C, caspase-8 substrate was added to the cells as described in (A). Shown is the mean value of duplicates from one representative experiment -/+ SD.

3.1.4.3 The chlamydial block of caspase-8 activity is stimulus-specific

Because inhibition of polyI:C-induced apoptosis by *C. trachomatis* was comparable to the level of TNFα-induced apoptosis, as revealed by PARP cleavage (Fig. 3.2) and by TUNEL staining (Fig. 3.5), it was investigated whether caspase-8 inhibition could also be observed after treatment with either

stimulus. Infected Hela cells treated either with polyI:C or with TNFα were analyzed for cleavage of the luminescent caspase-8 substrate. The relative caspase-8 activity is depicted in figure 3.8, demonstrating that, in contrast to dsRNA-induced apoptosis, TNFα-induced activation of caspase-8 could not be blocked by infection, irrespective of the MOI used. Taken together, it becomes obvious that *C. trachomatis* specifically inhibits polyI:C- but not TNF-α-induced apoptosis upstream of caspase-8 which is dependent on one or several bacterial effector proteins.

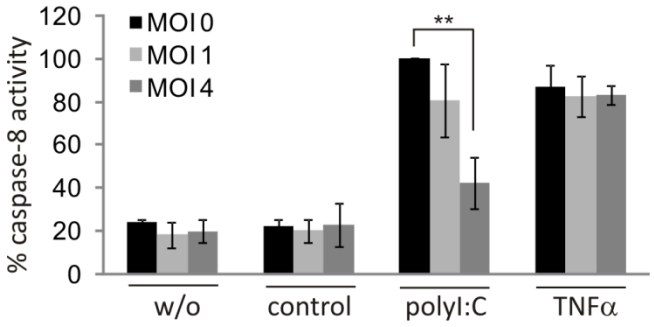

Fig. 3.8: Infected Hela cells display caspase-8 activity after treatment with TNFα.
Hela cells were infected with different MOIs of *C. trachomatis* and, 20 h later, were transfected with siRNA against Luciferase (control), transfected with 1 µg/ml polyI:C, were treated with 10 ng/ml TNFα + 5 µg/ml CHX, or remained untreated (w/o). 7 h later, luminescent caspase-8 substrate was added to the cells to detect caspase-8 activity. Shown are the mean values of two independent experiments -/+ SD. **= $p < 0.01$.

3.1.4.4 polyI:C-induced caspase-8 activity and apoptosis are inhibited by *C. pneumoniae* infection

Successful inhibition of apoptosis is a trait that is shared by a plethora of obligate intracellular bacteria as a mechanism to maintain the replicative niche. Apart from diverse studies that reveal the ability of *C. trachomatis* to subvert host cell death, the related bacterium *C. pneumoniae* has been shown to possess likewise strategies (Fischer *et al.*, 2001, Paland *et al.*, 2006, Rajalingam *et al.*, 2001). In order to reveal a possible species specificity for the inhibition of dsRNA-induced apoptosis, the impact of an infection with *C. pneumoniae* on the cellular response to polyI:C was investigated. HEp-2 cells were infected for 50 h, transfected with polyI:C, and the cleavage of PARP and caspases was investigated by immunoblotting. A mild reduction in PARP processing could be detected in host cells infected at MOI 0.5 which was more prominent at MOI 2 (Fig. 3.9). Moreover, a weak decrease in the caspase-8 and -9 fragments p43/p41 and p37/p35, respectively, could be observed after infection, accompanied by a mild increase of intact Bid (Fig. 3.9). Thus, although not as prominent as observed in *C. trachomatis*-infected host cells, the ability to downmodulate the apoptotic response to dsRNA at the level of caspase-8 can also be attributed to *C. pneumoniae* and might therefore be a general mechanism of *Chlamydiaceae*.

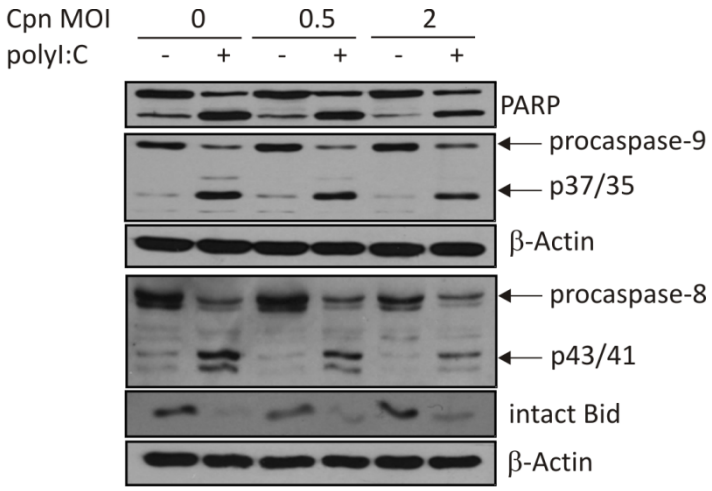

Fig. 3.9: *C. pneumoniae*-infected host cells resist dsRNA-induced apoptosis.
HEp-2 cells infected with different MOIs of *C. pneumoniae* for 50 h were transfected with 1 µg/ml polyI:C (+) or with siRNA against Luciferase (-). 15 h later, cells were lysed and subjected to immunoblotting. Processing of PARP, procaspase-8 and-9, and truncation of Bid was investigated with specific antibodies, with β-Actin as loading control. Cleaved caspase-8 (p43/41) and caspase-9 fragments (p37/35) are indicated.

3.1.5 Uptake of polyI:C is not prevented by infection

Infections with *Chlamydia* have been demonstrated to cause a rearrangement of the host cell membrane, e.g. externalization of phosphatidylserine to the outer leaflet of the plasma membrane (Goth *et al.*, 2001). It was therefore possible that infected cells were altered in their ability to take up nucleic acids via lipofectamine transfection.

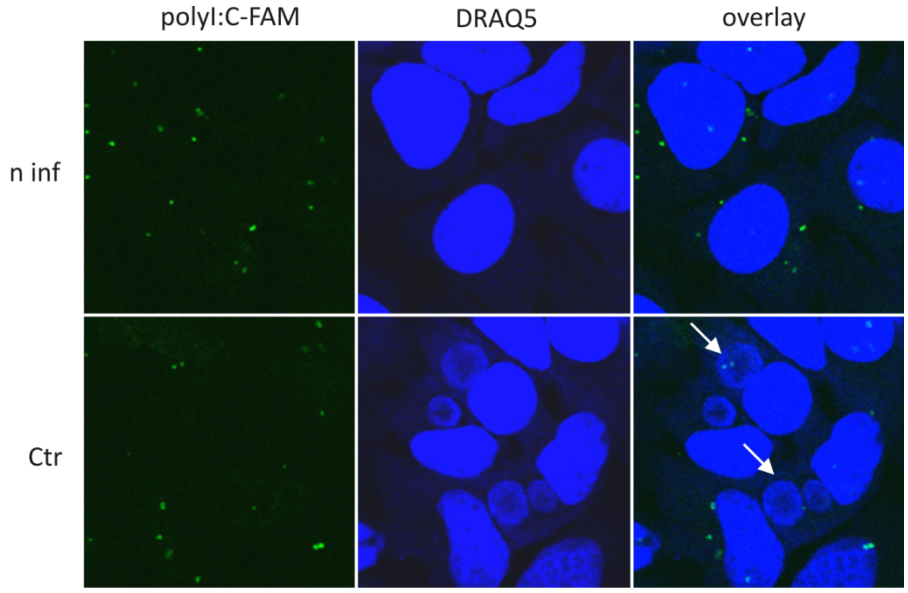

Fig. 3.10: FAM-labeled polyI:C can be detected in *C. trachomatis*-infected host cells.
One day before infection with *C. trachmatis* (Ctr) at MOI 2, Hela cells were seeded on glass cover slips. 20 h p.i., cells were transfected with fluorescently-labelled polyI:C (polyI:C-FAM) for 2 h. Samples were stained with DRAQ5 to monitor DNA and were subjected to confocal microscopy to detect intracellular polyI:C-FAM in non-infected (n inf) and *Chlamydia*-infected cells (Ctr) carrying inclusions (marked with an arrow).

In order to exclude that infection-mediated inhibition of polyI:C-induced apoptosis was due to a decrease in the uptake of polyI:C into infected cells, the presence of intracellular polyI:C was controlled. Hela cells were infected with *C. trachomatis* for 20 h or left uninfected and subsequently the samples were transfected with fluorescently-labelled polyI:C. Confocal microscopic analysis revealed that both non-infected cells and cells carrying chlamydial inclusions displayed comparable fluorescent patterns of incorporated polyI:C (Fig. 3.10). From this, it could be concluded that the uptake of polyI:C into the cytosol via transfection with lipofectamine was not inhibited in infected cells.

3.1.6 Impact of chlamydial infection on cellular dsRNA sensors

It could be demonstrated that uptake of polyI:C via transfection was successful in infected cells. Consequently, the presence of intracellular polyI:C could lead to an activation of cytosolic RNA sensors. Among the cytosolic sensors for dsRNA, PKR, 2-5 OAS/RNase L, and TLR3 present the most prominent inductors of apoptosis whereas MDA5 and RIG-I rather contribute to IFN-induction in response to dsRNA. In order to identify the mechanisms underlying chlamydial inhibition of caspase-8 activity the cellular signalling response upon activation of the different dsRNA sensors was scrutinized in infected cells.

3.1.6.1 PKR signalling is not impaired in infected cells

Upon binding of dsRNA, the RNA-dependent protein kinase PKR can induce caspase-8 activation by a so-far not completely resolved pathway that depends on the DED-linker FADD but was described to be death receptor independent (Balachandran *et al.*, 1998, Gil *et al.*, 2000b).

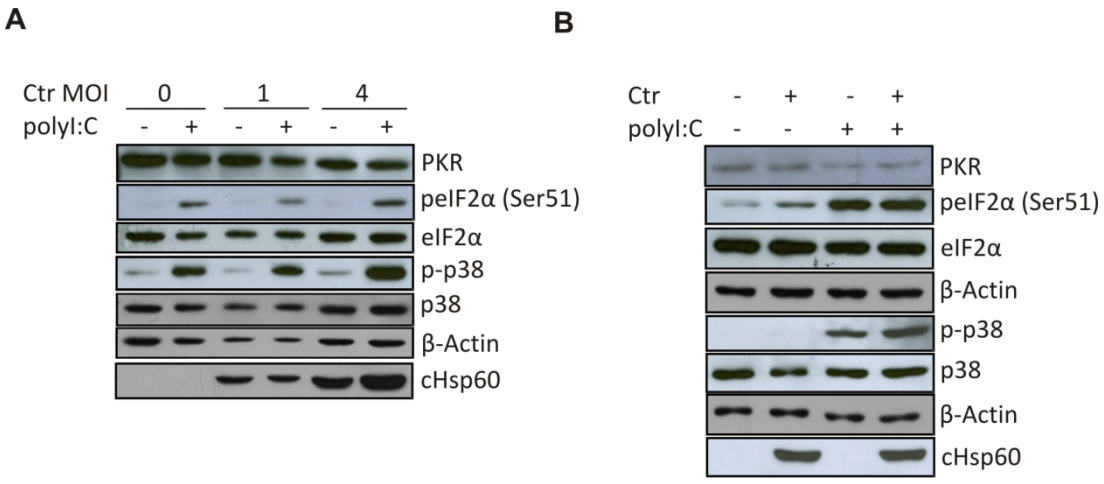

Fig. 3.11: PKR activity is not impaired in infected cells upon dsRNA treatment.
Hela [A] and HEp-2 cells [B] were infected (Ctr) at different MOIs and at MOI 4, respectively, transfected with 1 µg/ml polyI:C and, 2.5 h later, were lysed in lysis buffer containing phosphatase inhibitors. The phosphorylation status of the PKR substrates eIF2α and p38 was detected by immunoblotting with specific antibodies. Infection was controlled with a chlamydial Hsp60 (cHsp60) antibody, while β-Actin served as a loading control.

For detection of PKR activity, the phosphorylation status of two of its cellular substrates, i.e. the translation initiation factor eIF2α and the mitogen-activated protein (MAP) kinase p38, was investigated. Immunoblotting of Hela and HEp2 cell lysates demonstrated that polyI:C-induced phosphorylation of eIF2α and p38 could be detected 2.5 h post transfection both in non-infected and in *Chlamydia*-infected cells (Fig. 3.11). This indicated that the principal activity of PKR was not inhibited by infection.

3.1.6.2 RNase L activity is not altered by *Chlamydia*

Activation of the 2-5 OAS pathway by dsRNA leads to dimerization of RNase L and subsequent degradation of 28S rRNA (Hassel *et al.*, 1993, Silverman *et al.*, 1983). The consequence is a block of protein synthesis and the induction of apoptosis via the JNK pathway (Li *et al.*, 2004). Thus, to detect and compare the activity of RNase L in non-infected and infected cells treated with polyI:C, degradation of 28S rRNA was determined using a Bioanalyzer device. The amount of intact 28S rRNA was reduced in polyI:C treated cells accompanied by the appearance of degraded RNA products (Fig. 3.12 A, marked with asterisks). The same pattern could be observed in infected host cells after treatment with polyI:C, with some additional bands for bacterial 23S rRNA and 16S rRNA. Moreover, phosphorylation of JNK occurred in both non-infected and infected HEp-2 (Fig. 3.12 B) and Hela cells (Fig. 3.12 C) after transfection with polyI:C. Together the data demonstrate that the 2-5 OAS pathway is not impaired by infection and, instead, suggest that *C. trachomatis* interferes with caspase-8 activation downstream of PKR and JNK.

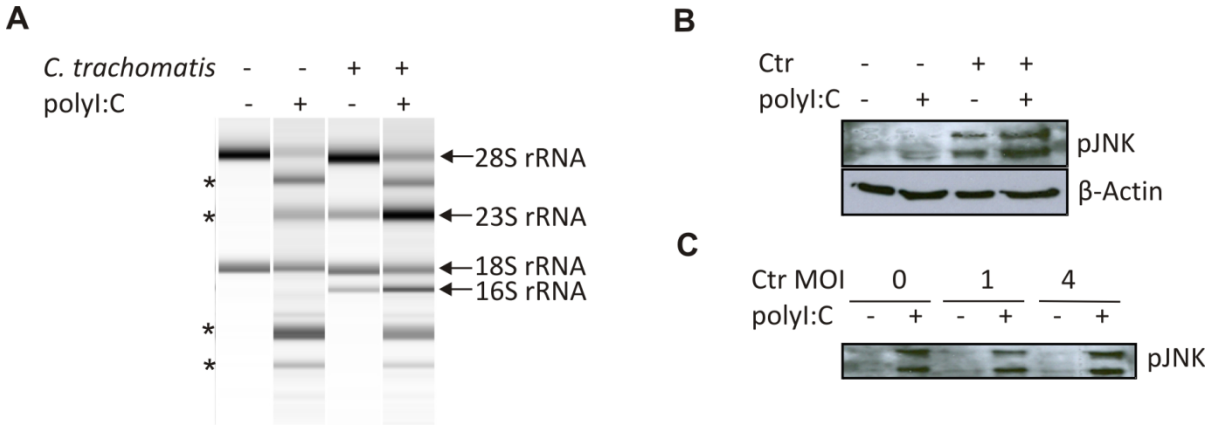

Fig. 3.12: Degradation of 28S rRNA and JNK activation is comparable in non-infected and infected cells treated with polyI:C.
[A] Hela cells were infected with *C. trachomatis* at MOI 4, transfected with 1 µg/ml polyI:C, and total RNA was isolated 4 h post transfection. rRNA species were analyzed by a Bioanalyzer device. [B] The same lysates of infected and mock-infected, polyI:C transfected HEp-2 cells and [C] Hela cells from figure 3.11 were analyzed for phosphorylated JNK by immunoblotting with a phospho-specific antibody.

3.1.6.3 polyI:C-induced apoptosis is independent of surface TLR3

It was demonstrated that in human breast cancer cells external application of synthetic dsRNA induces apoptosis in a toll-like receptor 3 (TLR3)-dependent manner (Salaun *et al.*, 2006). While TLR3 is localized in endosomes in DCs and monocytic cells (Matsumoto *et al.*, 2003), in epithelial cells it was believed to be localized only at the cell surface although it was recently reported to be found in intracellular compartments (Guillot *et al.*, 2005). In order to elucidate whether surface TLR3 accounted for apoptosis induction in the experimental system of this study, the outcome of external polyI:C application was tested. Therefore, Hela cells were treated with 1 µg/ml or 100 µg/ml polyI:C in the absence of lipofectamine. As revealed by immunoblotting, treatment with polyI:C at concentrations of up to 100 µg/ml in the absence of the transfection reagent did induce neither PARP cleavage nor procaspase-8 processing which both was clearly detectable in the lipofectamine positive control (Fig. 3.13). Thus, the involvement of surface TLR3 in polyI:C-induced apoptosis of Hela cells seems very unlikely.

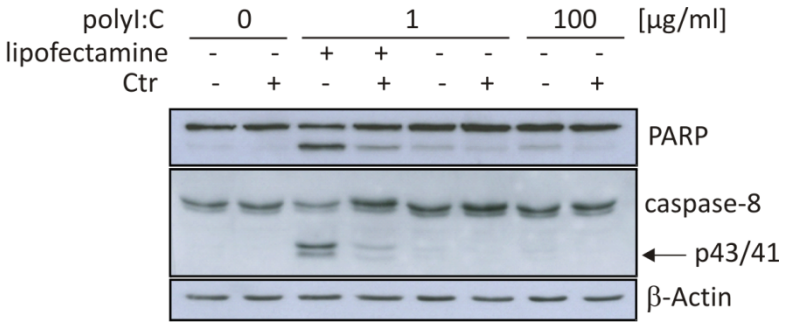

Fig. 3.13: Transfection of polyI:C is required for apoptosis induction.
Hela cells were infected with *C. trachomatis* (Ctr) at MOI 4 and treated with different concentrations of polyI:C in the presence (+) or absence (-) of lipofectamine reagent. After 8 h, cells were lysed with 2x Laemmli buffer and analyzed for cleavage of PARP and caspase-8. Cleaved caspase-8 fragments (p43/41) are indicated. Equal loading was controlled by β-Actin.

3.1.7 cFlip is required for caspase-8 inhibition

Because it could be demonstrated that PKR activity was not prevented in *C. trachomatis*-infected host cells, it was concluded that a different mechanism accounted for the inhibition of caspase-8 by the pathogen. A known modulator of caspase-8 activation is the cellular Flice like inhibitory protein (cFlip) (Irmler *et al.*, 1997). Upregulation of cFlip expression has been described for infections with several pathogens that possess anti-apoptotic properties, e.g. *Legionella* (Bartfeld *et al.*, 2009) and *Anaplasma* (Lee *et al.*, 2006). Thus, it was investigated whether cFlip is involved in chlamydial caspase-8 inhibition after polyI:C-treatment. Hela cells were transfected with a pool of siRNAs against cFlip or with a control siRNA against Luciferase, were treated as described above and caspase-8 activity was measured using the chemiluminescent assay. Whereas caspase-8 inhibition could be observed in cells transfected with siRNA against Luciferase, depletion of cFlip resulted in a prominent

increase in polyI:C-induced caspase-8 activation in both non-infected and infected cells (Fig. 3.14 A). Because no inhibition could be detected after infection, this demonstrated a role of cFlip in *Chlamydia*-induced caspase inhibition. To confirm these observations, Hela cells were transfected with a second pool of siRNAs directed against cFlip (cFlip-2) and the relative caspase-8 inhibition was calculated for both pools. Inhibition of caspase-8 by *C. trachomatis* was reduced to 13% and 27% in host cells transfected with the pool cFlip-1 and cFlip-2, respectively, compared to control cells (Fig. 3.14 B). This further verified the crucial function of cFlip in chlamydial inhibition of caspase-8. Successful silencing of cFlip could be demonstrated with quantitative RT-PCR for both siRNAs employed, yielding a decrease of cFlip mRNA levels to 22% and 36% for cFlip-1 and cFlip-2, respectively, as compared to control transfected cells (Fig. 3.14 C).

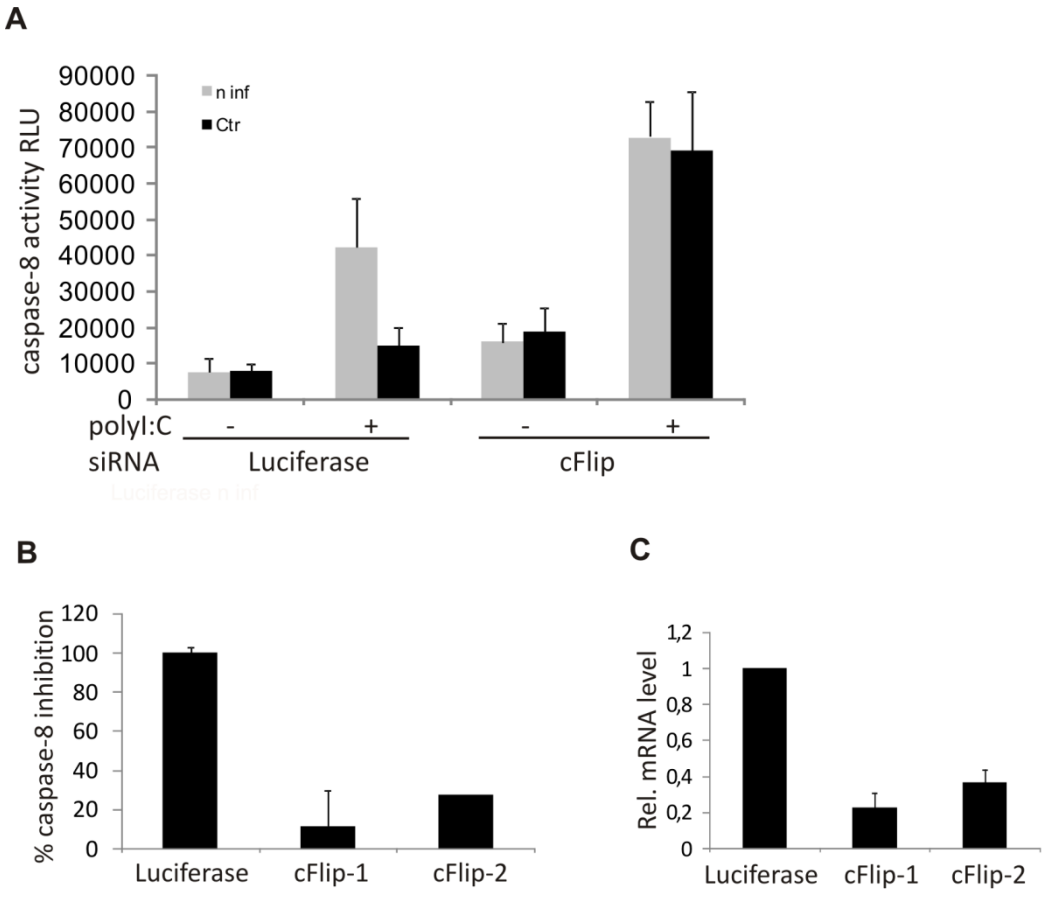

Fig. 3.14: Caspase-8 inhibition is cFlip-dependent.
[A] Hela cells were transfected with a pool of siRNAs against cFlip or a control siRNA targeting Luciferase. One day later, cells were seeded into 96 well plates and infected with *C. trachomatis* (Ctr) at MOI 4 or remained uninfected (n inf). 20 h post infection, Hela cells were transfected with 1 µg/ml polyI:C or were mock-transfected. Caspase-8 activity was determined by luminescence 7 h later. The mean of three independent experiments with SE is shown. [B] The experiment described in (A) was repeated with a second siRNA pool directed against cFlip (cFlip-2). Shown is the relative inhibition of caspase-8 in *C.trachomatis*-infected polyI:C-treated cells transfected with the pool of siRNAs from (A) (cFlip-1) or with the second siRNA pool against cFlip (cFlip-2). The mean with SD for cFlip-1 and single experiment data for cFlip-2 is depicted. [C] Relative cFlip mRNA levels in Hela cells transfected with Luciferase siRNA and the two pools of siRNAs against cFlip (cFlip-1 and cFlip-2). Shown are the mean values of two independent experiments with SD.

3.1.8 cFlip knock down sensitizes infected cells to polyI:C-induced apoptosis

Because knock down of cFlip resulted in an increase in caspase-8 activity in infected host cells to the level of non-infected cells, apoptosis inhibition in cells depleted of cFlip should be investigated. Hela cells were transfected with siRNAs against cFlip and 48 h later, were infected with *C. trachomatis* and treated with polyI:C. Cells with fragmented DNA were detected using the TUNEL assay (Fig. 3.15 A). Whereas the amount of TUNEL positive cells upon polyI:C application was reduced from 18% in non-infected cells to 9% in infected cells after transfection with Luciferase siRNA, there was only a minor decrease in the percentage of cells with fragmented DNA from 20% in non-infected cells to 18% in infected cells after cFlip depletion (Fig. 3.15 B). To further verify the finding that cFlip depletion sensitizes infected cells to apoptosis, PARP cleavage was examined by immunoblotting. Consistent with the results gained before (see figures 3.1 to 3.3) polyI:C-induced PARP cleavage was reduced in control transfected cells. However, in cFlip knock down cells, the level of PARP cleavage was comparable in non-infected and *Chlamydia*-infected host cells (Fig. 3.15 C). Thus, the conclusion could be drawn that *C. trachomatis* is impaired in blocking host cell apoptosis induced by dsRNA in the absence of the cellular caspase-8 inhibitor cFlip.

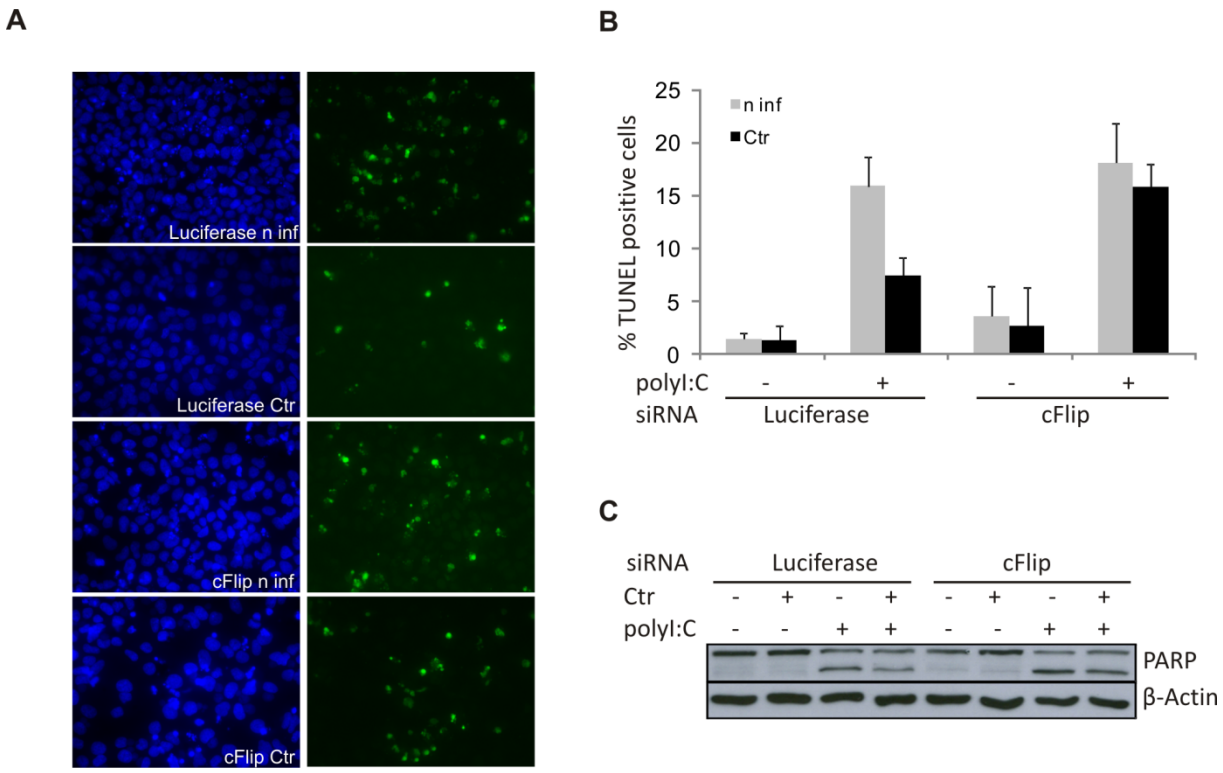

Fig. 3.15: cFlip is required for bacterial apoptosis inhibition upon dsRNA treatment.
[A] cFlip or control knock down cells (Luciferase) were infected with *C. trachomatis* (Ctr) at MOI 1 for 20 h or remained uninfected (n inf) before transfection with 1 µg/ml polyI:C for 4 h. Cells were subjected to TUNEL and analyzed by fluorescence microscopy with a 40-fold objective. [B] The percentage of TUNEL positive cells from (A) was calculated and the mean values of two independent experiments -/+ SD are shown. [C] Immunoblot of PARP processing in cFlip and control (Luciferase) knock down cells. Hela cells were treated as in (A), lysed after 8 h and subjected to immunoblotting to analyze PARP cleavage. β-Actin served as a loading control.

3.1.9 Regulation of cFlip by *C. trachomatis*-infection

RNA interference revealed that cFlip was as a crucial factor for chlamydial inhibition of polyI:C-induced caspase-8 activation and apoptosis. Next, the question was addressed of how *Chlamydia* utilizes cFlip during infection. Because infection-induced upregulation of anti-apoptotic proteins, such as IAPs (Paland *et al.*, 2006, Rajalingam *et al.*, 2006), has been shown to account for apoptosis inhibition by *Chlamydia*, it was assumed that cFlip was upregulated in *C. trachomatis*-infected host cells. Moreover, it had to be tested whether the cellular localization of the anti-apoptotic protein was altered upon infection. Therefore, both expression and distribution of cFlip were investigated in infected host cells.

3.1.9.1 cFlip levels are mildly altered by *C. trachomatis*-infection

To reveal whether *C. trachomatis*-infection had an influence on cFlip expression both mRNA and protein levels were investigated. Transcription levels of cFlip were determined by quantitative RT-PCR revealing only a very mild, but significant infection-induced upregulation. Hela cells infected with *C. trachomatis* for 24 h displayed a 1.6-fold increase of cFlip mRNA compared to non-infected cells (Fig. 3.16 A).

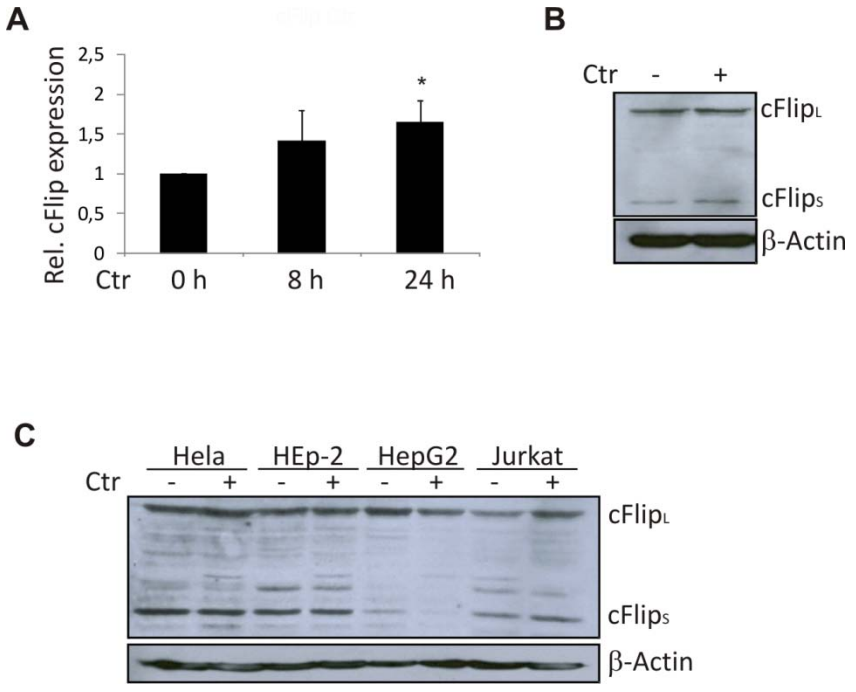

Fig. 3.16: cFlip levels are mildly altered in infected Hela cells.
[A] At the indicated time point after infection with *C. trachomatis* (Ctr), total RNA was isolated and subjected to quantitative RT-PCR. GAPDH served as internal control. * = $p < 0.05$. [B] Hela cells were infected with *C. trachomatis* (Ctr) for 24 h, lysed with DISC buffer and protein levels of the long ($cFlip_L$) and short ($cFlip_S$) isoforms of cFlip were analyzed by immunoblotting. Loading was controlled by β-Actin detection. [C] In addition to Hela cells, the larynx epithelial cell line HEp-2, the hepatic carcinoma cell line HepG2, and the T cell leukemia cell line Jurkat were treated as in (B).

However, analysis of the protein levels of cFlip by immunoblotting suggested a somewhat variable phenotype of cFlip regulation during *Chlamydia* infection. A slight infection-induced upregulation of the small Flip isoform ($cFlip_S$) accompanied by unaltered protein levels of the large isoform ($cFlip_L$) could be observed in Hela cells under certain conditions (Fig. 3.16 B). But when several other human cell lines were investigated for their cFlip expression upon *Chlamydia* infection, different regulation patterns could be found. Whereas an obvious pathogen-induced upregulation of cFlip could not be detected neither in Hela nor HEp-2 cells, the hepatic cell line HepG2, frequently used for cFlip analyses (Ganten *et al.*, 2004), and the T cell leukemia line Jurkat displayed down- and upregulation of cFlip, respectively. This suggested a complex, cell line dependent regulation of cFlip.

3.1.9.2 Confocal microscopic analysis of cFlip during infection

The cellular localization of cFlip during infection with *C. trachomatis* was investigated by confocal microscopy. Therefore, 24 h post infection with *C. trachomatis*, Hela cells were fixed and stained with a rabbit cFlip-specific antibody. Nuclei of host cells and bacteria were counterstained with DRAQ5. Figure 3.17 illustrates confocal images of non-infected (upper panel) and infected cells (lower panel). A diffuse staining of cFlip in non-infected Hela cells indicated it to be localized in the cytosol. In infected cells, the antibody clearly and robustly co-stained with chlamydial inclusions (marked with arrows).

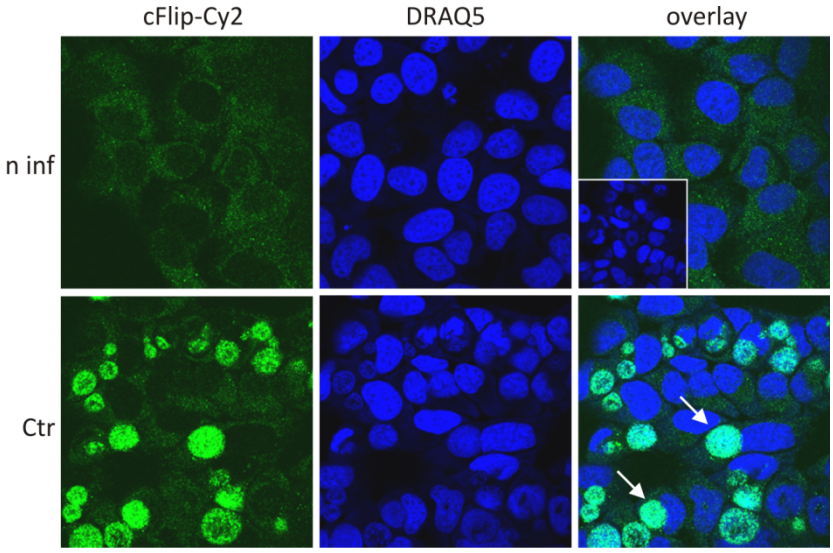

Fig. 3.17: cFlip can be detected in chlamydial inclusions.
Hela cells were infected with *C. trachomatis* (Ctr) at MOI 2 for 24 h, fixed with PFA and stained with a rabbit polyclonal antibody against cFlip and a Cy2™-linked goat anti-rabbit antibody. DRAQ5 was used for counterstaining of chlamydial (marked with arrows) and cellular DNA. Background staining of the secondary antibody was controlled in the absence of the primary antibody against cFlip (inlay).

This suggested not only a translocation of cFlip into the chlamydial vacuole but also a strong infection-induced upregulation of the protein, inconsistent with the data described above (Fig. 3.16). Moreover, it seemed rather unlikely that cFlip mediates its anti-apoptotic actions from within the chlamydial inclusion. Therefore, it had to be tested whether the observed co-staining was an artefact and, consequently, cFlip staining was performed for Hela cells depleted of cFlip. Two days after transfection with two pools of siRNAs against cFlip (cFlip-1 and cFlip-2), or against Luciferase as a control, cells were infected for 24 h and stained for confocal microscopy. Both in Luciferase (upper panel) and in cFlip knock down cells (middle and lower panels), staining of the chlamydial inclusions with the cFlip antibody could be detected (Fig. 3.18). This revealed that the observed staining of cFlip inside the chlamydial inclusions was not specific and indicated that confocal staining was not a proper method to identify regulation of cFlip. Next, caspase-8 localization was investigated by confocal microscopy. Uninfected cells displayed staining of caspase-8 both in the cytosol and the nucleus which was comparable to the staining of infected cells (Fig. 3.19). Thus, an infection-induced translocation of the enzyme could not be observed.

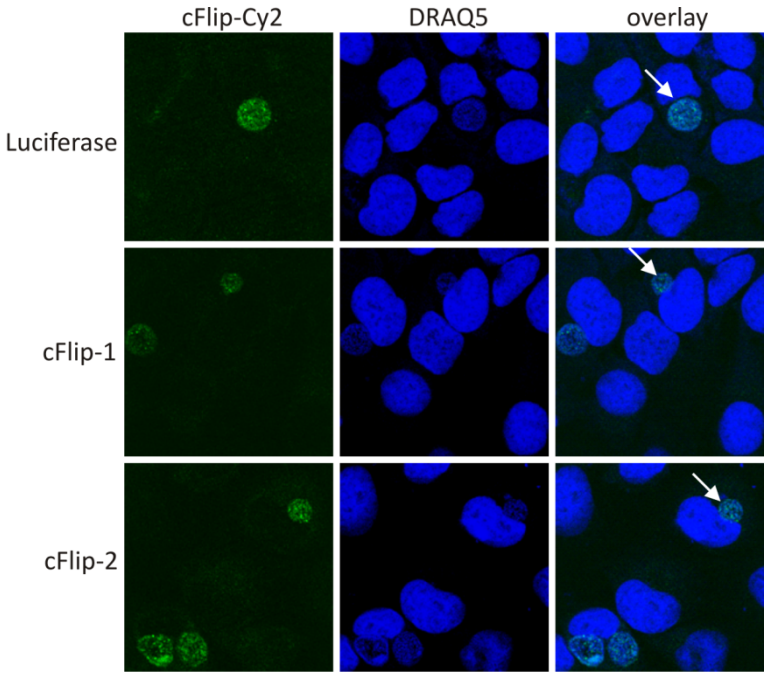

Fig. 3.18: Positive cFlip staining in cFlip depleted cells.
Hela cells were transfected with two pools of siRNA against cFlip (cFlip-1 and cFlip-2) or a single siRNA against Luciferase and, at day two post transfection, were infected with *C. trachomatis* for 24 h. Cells were fixed with PFA, stained with an antibody raised against cFlip and a Cy2™-linked secondary antibody. DNA was counterstained with DRAQ5. Chlamydial inclusions are marked with an arrow.

3.1.10 Interaction of caspase-8 and cFlip in infected host cells

The fact that cFlip depletion sensitized infected host cells to caspase-8 activation pointed to an infection-induced interaction of cFlip with the initiator caspase-8 to prevent its interaction and

activation with FADD. In contrast, activation of caspase-8 could not be blocked by infection during TNFα-induced apoptosis. Because TNFα induces DISC recruitment at the TNFR1 while dsRNA-induced caspase-8 activation is independent of death receptors (Balachandran *et al.*, 1998, Gil *et al.*, 2000b), it was hypothesized that upon polyI:C transfection a *Chlamydia* infection induced the interaction of caspase-8 and its inhibitor at a specific compartment of the cell. To elucidate whether *C. trachomatis* could influence a localization specific interaction between cFlip and caspase-8, confocal microscopic analysis of caspase-8, subcellular fractionation, and co-immunoprecipitation (co-IP) experiments were performed.

3.1.10.1 Caspase-8 localization is not altered in infected cells

The cellular distribution of caspase-8 was investigated by confocal microscopy. *C. trachomatis*-infected and mock infected Hela cells were transfected with polyI:C or control transfected with Luciferase siRNA for 2 h.

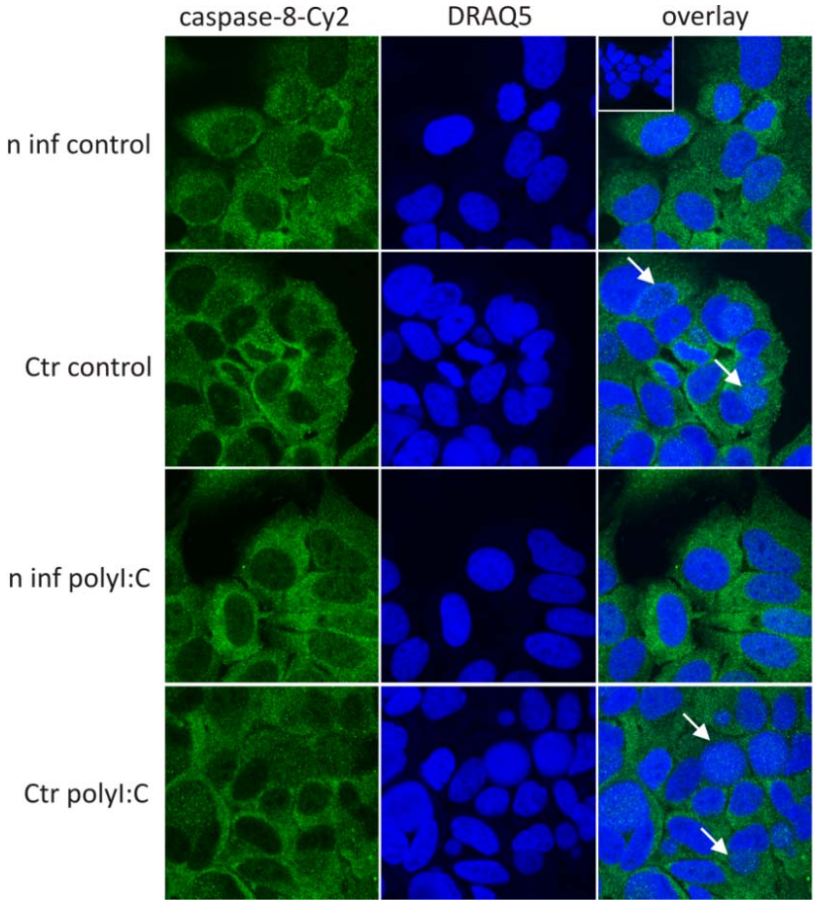

Fig. 3.19: Localization of caspase-8 is not influenced by infection.
Hela cells were infected with *C. trachomatis* (Ctr) at MOI 2 for 2 h or remained uninfected (n inf). Cells were transfected with 1 µg/ml polyI:C or control transfected with Luciferase siRNA for 2 h, fixed with PFA, and stained with a primary mouse monoclonal caspase-8 antibody and a secondary sheep anti-mouse Cy2™-linked antibody. Nuclei were stained with DRAQ5. Chlamydial inclusions are marked with an arrow. Background staining of the secondary antibody in the absence of the primary antibody is demonstrated by the inlay.

Figure 3.19 depicts that caspase-8 can be detected in the cytosol of all samples tested, with an additional, though minor staining in the nucleus of the cells. No obvious alteration in the staining pattern of caspase-8 could be observed upon infection with *C. trachomatis* and/or polyI:C transfection. A regulation by the pathogen could thus not be detected with this method.

3.1.10.2 Subcellular fractionation

Next, the localization of cFlip, caspase-8, and FADD was investigated with the help of subcellular fractionation. Therefore, the cellular fractions containing cytosol, the membranes and organelles, or the cytoskeleton were isolated using a commercial purification kit and analyzed by immunoblotting. As revealed by figure 3.20, FADD and cFlip could exclusively be found in the cytosol, while some minor signals for caspase-8 could also be detected in the membrane fraction. Because the cytosolic protein 14-3-3, which was used as a marker here, could also be found in the membrane fraction this suggested that part of the cytosol was isolated together with the membranes. Again, no obvious difference in the localization of the DISC components, such as detection in the membrane fraction, could be found upon polyI:C-transfection of *Chlamydia*-infected compared to non-infected cells.

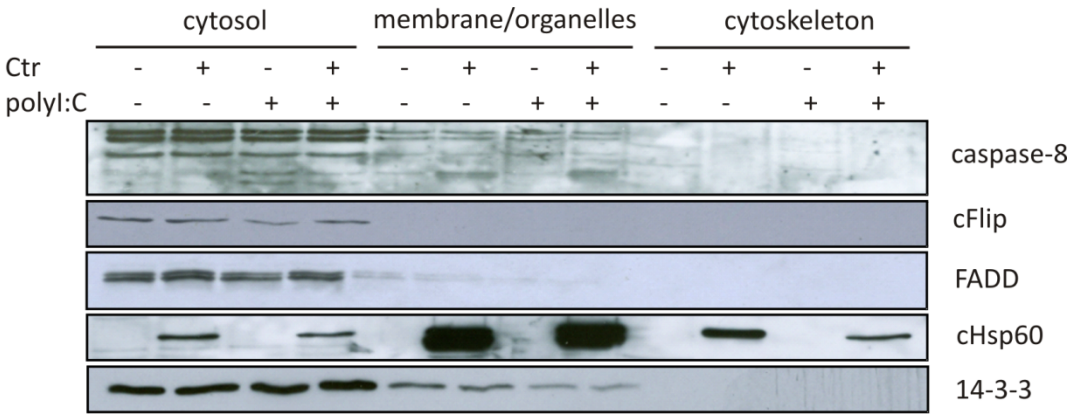

Fig. 3.20: Subcellular fractionation of infected polyI:C-transfected host cells.
Hela cells were infected with *C. trachomatis* (Ctr) at MOI 2 for 20 h and transfected with 1 µg/ml polyI:C for 2 h. Cells were fractionated and purified fractions were subjected to immunoblotting with respective antibodies.

3.1.10.3 Co-IP in *C. trachomatis* infected cells after polyI:C treatment

Another approach to reveal whether an interaction of caspase-8 and cFlip was enhanced in *Chlamydia*-infected host cells was the immunoprecipitation of caspase-8 and subsequent detection of cFlip by immunoblotting. Indeed, an increase in cFlip protein levels could be observed in Hela cells that were infected with *C. trachomatis* and treated with polyI:C (Fig. 3.21 A) suggesting an infection-induced elevated interaction with caspase-8. Moreover, it could be found that the amount of FADD that co-immunoprecipitated with caspase-8 was higher in polyI:C transfected cells compared to control treated cells, implying the activation of caspase-8 by this pro-apoptotic protein. However, co-

immunoprecipitated FADD could also be detected upon polyI:C transfection in infected host cells. Therefore, the ratio of cFlip and FADD that interacted with caspase-8 was determined by densitometry. Figure 3.21 B reveals that in *Chlamydia*-infected cells transfected with polyI:C the amount of caspase-8 bound cFlip was higher than that of FADD. This is an indication that *C. trachomatis* can reduce the homotypic interaction of caspase-8 and FADD required for activation of the caspase in response to dsRNA by promoting the contact between caspase-8 and cFlip.

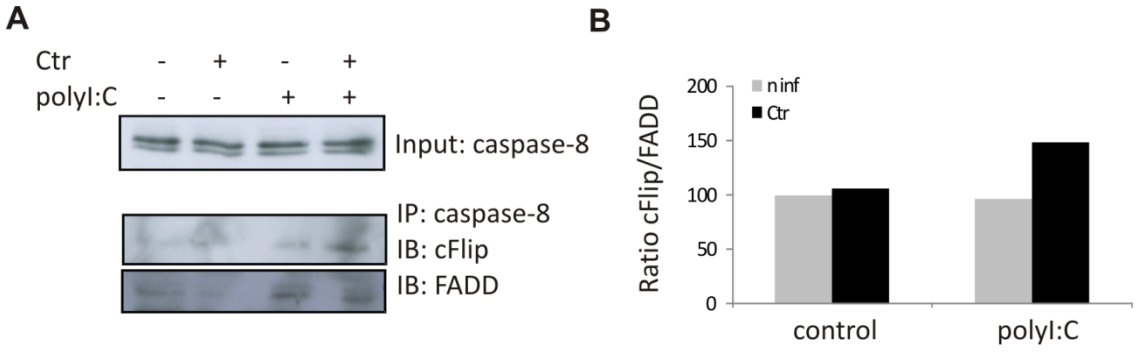

Fig. 3.21: cFlip$_S$ and caspase-8 interaction upon infection.
[A] Hela cells were infected with *C. trachomatis* (Ctr) at MOI 1 for 20 h and transfected with 1 µg/ml polyI:C for 2 h. Cells were lysed in DISC IP buffer and incubated with Sepharose A beads linked to a caspase-8 antibody. Lysates were subjected to immunoblotting and protein levels of cFlip and FADD were detected with specific antibodies. [B] The bands from (A) were analyzed by densitometry and the relative ratios of cFlip to FADD compared to non-treated and non-infected cells were calculated.

3.1.11 Role of Mcl-1 for chlamydial inhibition of polyI:C-induced apoptosis

Upregulation of anti-apoptotic proteins of the Bcl-2 like family is a widespread and successful mechanism of intracellular pathogens to prevent mitochondrial permeabilization and induction of host cell death (Böhme *et al.*, 2009). The crucial role of Mcl-1 upregulation and stabilization during anti-apoptosis of *C. trachomatis*-infected host cells has been demonstrated for TNFα- and Granzyme B-induced apoptosis (Rajalingam *et al.*, 2008). Moreover, it was shown that phosphorylation of eIF2α preceded and was required for degradation of Mcl-1, induced by a variety of stresses, such as UV light (Fritsch *et al.*, 2007). Therefore, it was investigated whether polyI:C could induce degradation of the anti-apoptotic protein and, in addition, whether Mcl-1 played a role in the chlamydial inhibition of apoptosis induced by dsRNA.

3.1.11.1 dsRNA-induced downregulation of Mcl-1 is inhibited in infected host cells

In order to reveal the possible degradation of Mcl-1 upon dsRNA transfection, HEp-2 cells were treated with polyI:C in the presence of the transfection reagent lipofectamine and analyzed by immunoblotting. As expected, polyI:C induced the phosphorylation of PKR and downstream phosphorylation of the PKR substrate eIF2α (Fig. 3.22). Moreover, a clear decrease in Mcl-1 protein

amount could be detected in polyI:C transfected cells. This implied that Mcl-1 was degraded as a consequence of or a necessary step during apoptosis induction. Next, it should be elucidated whether infection had an impact on polyI:C-induced Mcl-1 downregulation. Therefore, HEp-2 cells (Fig. 3.23 A) and Hela cells (Fig. 3.23 B) were infected with *C. trachomatis* and, 20 h later, were transfected with polyI:C.

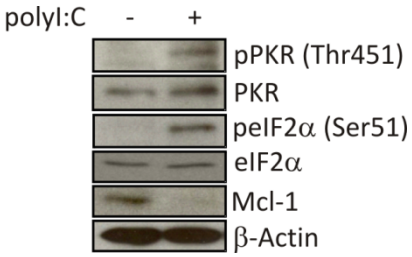

Fig. 3.22: polyI:C induces downregulation of Mcl-1.
HEp-2 cells were transfected with 1 µg/ml polyI:C for 2.5 h and lysates were analyzed for phosphorylated PKR and eIF2α as well as for Mcl-1 protein levels by immunoblotting**.** Loading was controlled by β-Actin levels.

Again, in polyI:C transfected cells, Mcl-1 protein levels disappeared. Infection, however, could rescue the downregulation of Mcl-1 in an MOI-dependent manner. Host cells infected at MOI 4 displayed comparable amounts of Mcl-1 upon both control and polyI:C transfection. Due to the fact that phosphorylation of eIF2α was not altered during infection (Fig. 3.11), it seems reasonable to speculate that proteasomal degradation of Mcl-1 induced by polyI:C can be blocked by *Chlamydia* infection.

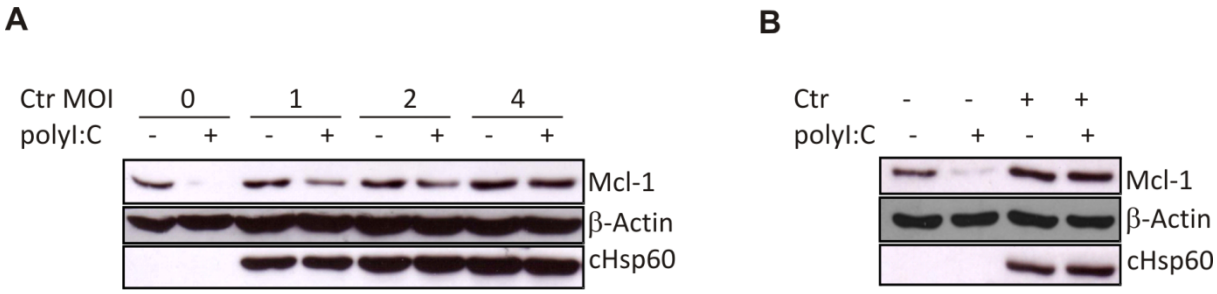

Fig. 3.23: Mcl-1 downregulation is prevented by infection.
[A] HEp-2 and [B] Hela cells were infected with *C. trachomatis* at rising MOIs and at MOI 4, respectively, and were treated with 1 µg/ml polyI:C for 2.5 h. Lysates were subjected to immunoblotting to detect the amount of Mcl-1. β-Actin levels demonstrate equal loading, chlamydial Hsp60 (cHsp60) served as infection control.

3.1.11.2 *Chlamydia*-infected cells resist dsRNA-induced apoptosis in the absence of Mcl-1

In order to test whether Mcl-1 was required for inhibition of polyI:C-induced apoptosis in *Chlamydia*-infected cells, HEp-2 cells were depleted of Mcl-1 using specific siRNAs and PARP cleavage was investigated by immunoblotting following polyI:C treatment. As demonstrated by figure 3.24 A, Mcl-1 was successfully depleted after siRNA application. Although a portion of Mcl-1 could still be detected

in infected control treated cells, the remaining protein level after polyI:C treatment was negligible. Nevertheless, PARP cleavage was blocked in respective infected, polyI:C-treated cells as demonstrated by densitometric analysis of the upper and lower PARP bands (Fig. 3.24 B). Together these findings indicate that polyI:C-induced downregulation of Mcl-1 is prevented by infection with *C. trachomatis* albeit it is not required for the chlamydial inhibition of apoptosis .

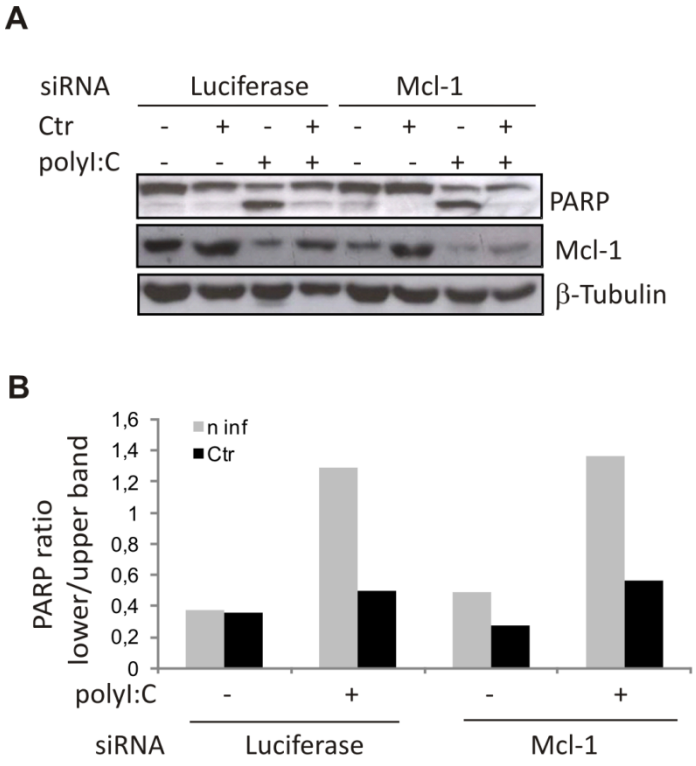

Fig. 3.24: Chlamydial inhibition of polyI:C-induced apoptosis in the absence of Mcl-1.
HEp-2 cells were transfected with siRNA directed against Mcl-1. Two days later, cells were infected with *C. trachomatis* (Ctr) at MOI 4 or remained uninfected (n inf) and apoptosis was induced with 1 µg/ml polyI:C for 16 h. [A] PARP cleavage and knock down of Mcl-1 was detected by immunoblotting with specific antibodies and with β-Tubulin as a loading control. [B] The ratios of the lower and the upper PARP band from (A) were determined by densitometry.

3.1.12 ERK is not required for inhibition of polyI:C-induced apoptosis during chlamydial infection

One of the best-studied proteins in *C. trachomatis* infections of mammalian cells is the MAP kinase Extracellular signal-regulated kinase (ERK). Our laboratory found that the infection with *C. trachomatis* can induce ERK-dependent shedding of TFNR1 (Paland *et al.*, 2008) and that activation of ERK was required for upregulation of the anti-apoptotic Bcl-2 like protein Mcl-1 (Rajalingam *et al.*, 2008). Since it was observed that HEp-2 cells infected with *C. trachomatis* displayed a time dependent increase in ERK phosphorylation (Fig. 3.25 A), it should be examined whether infection-induced actvation of ERK was required for inhibition of polyI:C-induced apoptosis. Therefore, the chemical inhibitor U0126 was utilized to interfere with MEK-1 activation, which is the upstream kinase of ERK and responsible for its activation. ERK inhibition led to a significant increase in caspase-

8 activity both in non-infected and infected cells compared to control cells treated with the solvent DMSO only upon transfection with polyI:C (Fig. 3.25 B). However, a significant reduction of caspase-8 activity by *C. trachomatis* could still be detected, indicating that ERK activation was not required for the chlamydial block of caspase-8. To further elucidate the role of ERK in anti-apoptosis to polyI:C, PARP cleavage was investigated in U0126 treated Hela cells. As demonstrated in figure 3.25 C, PARP cleavage could be blocked by infection even in the absence of phosphorylated ERK. Despite an increase in apoptosis following U0126 treatment, demonstrated by enhanced levels of cleaved PARP, infection with *C. trachomatis* induced a significant block of PARP cleavage (Fig. 3.25 D). From this it could be concluded that the MEK/ERK pathway is not involved in inhibition of caspase-8 and subsequent apoptosis inhibition in response to polyI:C. Further, it supports the finding that the underlying mechanism differs from the so-far identified strategies for inhibition of TNFα-induced cell death.

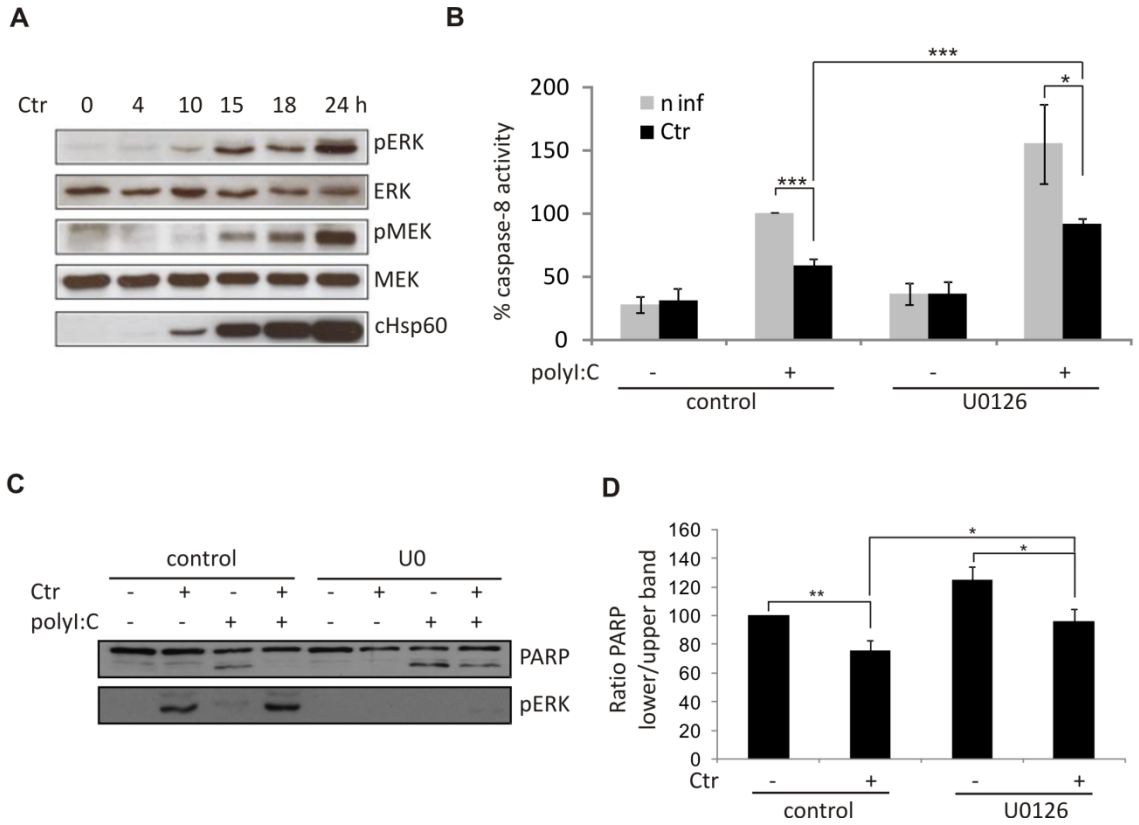

Fig. 3.25: Activation of ERK is not required for apoptosis inhibition.
[A] HEp-2 cells were infected with *C. trachomatis* (Ctr) at MOI 2 and lysed at the indicated time point after infection. Lysates were subjected to immunoblotting and analyzed for phosphorylation of MEK and ERK with phosphorylation-specific antibodies, while protein levels of total MEK and ERK were detected for control purpose. Chlamydial Hsp60 (cHsp60) served as infection control. [B] Hela cells were infected with *C. trachomatis* in the presence or absence of the MEK-1 inhibitor U0126 (10 µM) and transfected with 1 µg/ml polyI:C for 7 h. Caspase-8 activity was determined in DMSO (control) or U0126 treated infected (Ctr) or mock-infected (n inf) cells. The mean of three independent experiments is shown with -/+ SD. * = $p < 0.05$, *** = $p < 0.001$. [C] Hela cells were treated as in (B) and analyzed for PARP cleavage by immunoblotting. Inhibition of ERK phosphorylation by U0126 was controlled with a phosphorylation-specific antibody. Shown is a representative of three independent experiments. [D] The relative ratios of the PARP lower to upper bands from (C) were determined by densitometry. Shown are the mean values of three independent experiments with SD. * = $p < 0.05$, ** = $p < 0.01$.

3.2. Influence of *C. trachomatis* on the cellular immune response to dsRNA

Apart from programmed cell death (PCD), the cellular response to a viral infection can result in defeating the infectious agent by induction of innate immune pathways while the cell remains alive. Among the most potent anti-viral proteins with a crucial role in the cellular defense against dsRNA are the interferons (IFN) and inflammatory cytokines, such as interleukins (IL) that can induce the expression of several hundred anti-viral genes. Basically two signalling pathways are key mediators of this process, i.e. NF-κB and IRF-3 pathway. Thus, it was of interest to reveal whether an infection with *C. trachomatis* could regulate polyI:C-induced activation and subsequent nuclear translocation of NF-κB and IRF-3 thereby undermining the cellular innate immune response to dsRNA and supporting the establishment of a viral infection.

3.2.1 IκB-α degradation is enhanced in host cells infected with *C. trachomatis*

polyI:C induces the activation of several members of the NF-κB family, namely p65, p50, and Rel-c (Alexopoulou *et al.*, 2001, Matsumoto *et al.*, 2002, Oshiumi *et al.*, 2003), and the translocation of these factors into the nucleus is dependent on IκB-α degradation (Baeuerle *et al.*, 1988, Chen *et al.*, 1995). To investigate whether infection with *C. trachomatis* could interfere with NF-κB activation, IκB-α degradation was analyzed. While control cells treated with 25 ng/ml TNFα displayed degradation of IκB-α, transfection with 1 µg/ml polyI:C did not lead to degradation of the kinase at neither time point investigated (Fig. 3.26 A).

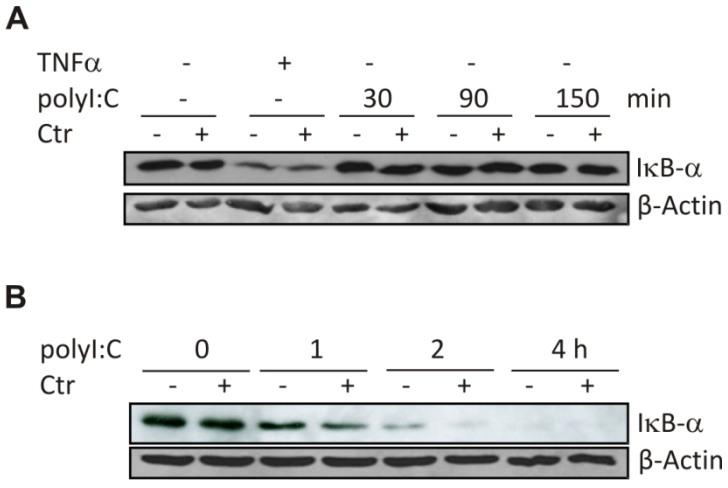

Fig. 3.26: IκB-α degradation upon polyI:C transfection.
[A] 20 h after infection with *C. trachomatis* at MOI 4 (Ctr +) or mock-infection (-) Hela cells were transfected with 1 µg/ml polyI:C for the indicated time, were treated with 25 ng/ml TNFα for 30 min, or remained untreated. Cell lysates were analyzed for protein levels of IκB-α by immunoblotting with β-Actin serving as a loading control. [B] The experiment from (A) was performed with 10 µg/ml polyI:C in the presence of 5 µg/ml CHX. Lysates were collected at the indicated time point post transfection and analyzed by immunoblotting.

Therefore, a concentration of 10 µg/ml polyI:C was used and, in addition, the cells were treated with the mRNA translation inhibitor cycloheximid (CHX) to prevent a rapid production of newly synthesized IκB-α protein. A slight decrease of IκB-α could be observed as soon as 1 h after transfection with polyI:C both in infected and non-infected cells (Fig. 3.26 B). One hour later, only marginal levels of IκB-α were detectable in non-infected cells whereas in infected cells, IκB-α was totally absent. This suggested that the infection with *C. trachomatis* rather enhanced than inhibited the degradation of IκB-α in polyI:C-treated host cells.

3.2.2 Nuclear translocation of p65 is altered in infected cells

To further reveal whether the enhanced IκB-α degradation in host cells both infected with *C. trachomatis* and treated with dsRNA had an influence on nuclear translocation of p65, confocal microscopic analysis was performed. Consistent with the observation that 1 µg/ml of polyI:C did not induce detectable degradation of IκB-α, only few cells displayed nuclear p65 after transfection with this concentration of polyI:C (Fig. 3.27 A) Therefore, again, the concentration was increased to 10 µg/ml. Figure 3.27 B depicts mock-infected and *Chlamydia*-infected Hela cells 4 h after transfection. Whereas p65 can be detected predominantly in the cytoplasm in non-treated cells (upper panels), localization within the nucleus can be found in the majority of cells transfected with polyI:C. Also in infected cells, nuclear p65 can be detected; however, the nuclear signal appears much weaker suggesting a reduced amount of p65 in the nucleus (lower panel). A quantification of cells with nuclear p65 is shown in figure 3.27 C. While 89.1% of non-infected cells were positive for nuclear p65 upon polyI:C transfection, only 66.9% of cells infected with *C. trachomatis* displayed nuclear translocation of the transcription factor. This was consistent with the results obtained by immunoblotting of IκB-α, suggesting an influence of *Chlamydia* infection on the activation of the NF-κB pathway by dsRNA.

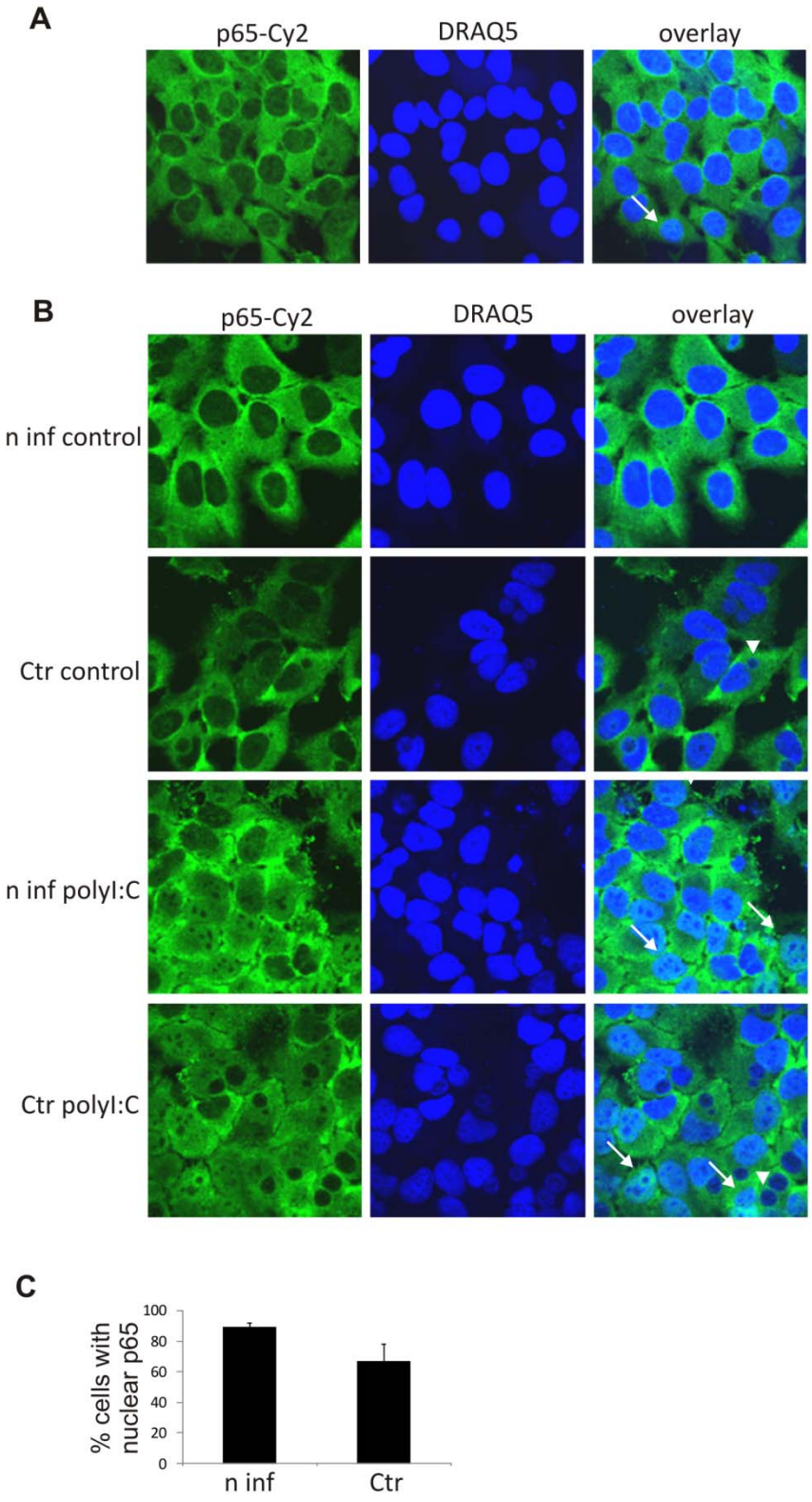

Fig. 3.27: polyI:C-induced p65 translocation in *Chlamydia*-infected host cells.
[A] Hela cells were transfected with 1 µg/ml polyI:C for 4 h and stained for p65 using a specific primary antibody and a Cy2-labelled secondary antibody. DNA was counterstained with DRAQ5. Cells were analyzed by confocal microscopy with 630-fold magnification. Nuclear p65 is indicated by an arrow. [B] Hela cells were infected with *C. trachomatis* (Ctr) at MOI 2 or remained uninfected (n inf) and were transfected with 10 µg/ml polyI:C in the presence of 5 µg/ml CHX for 4 h or were treated with lipofectamine alone (control). Cells were fixed and stained as described in (A). Nuclear p65 is marked with an arrow and chlamydial inclusions are marked with arrow heads. [C] Quantification of cells with nuclear p65 from the experiment depicted in (B). Shown is the mean of two independent experiments -/+ SD.

3.2.3 polyI:C-induced IRF-3 translocation is reduced in infected cells

Next, it was investigated whether nuclear translocation of the interferon regulatory factor 3 (IRF-3) in response to dsRNA was altered upon infection. The amount of epithelial cells with nuclear IRF-3 was determined 4 h post transfection with polyI:C using a specific antibody and confocal microscopy.

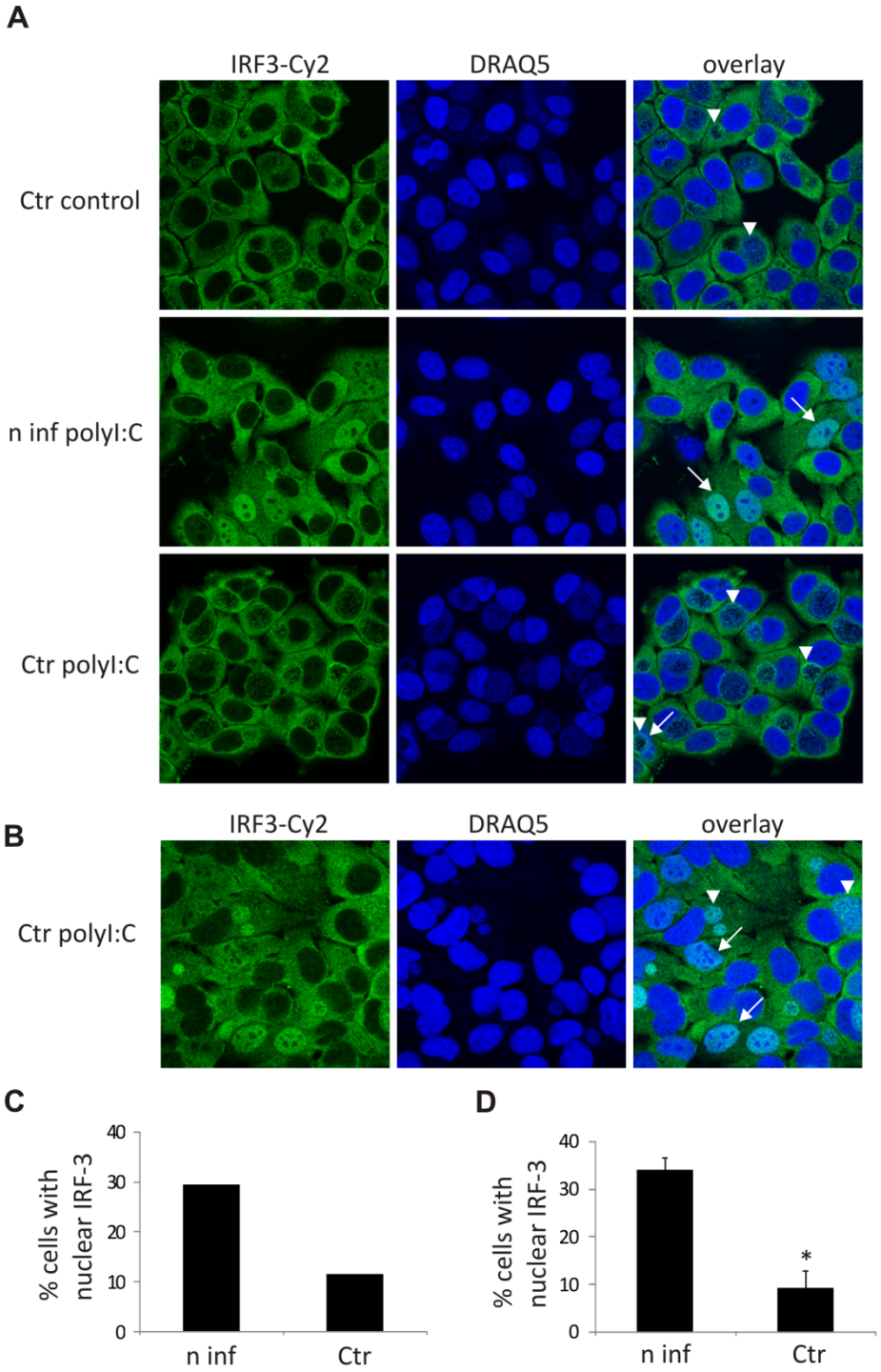

Fig. 3.28: Nuclear translocation of IRF-3 upon dsRNA transfection decreases in infected cells.
[A, C] HEp-2 and [B, D] Hela cells were infected with *C. trachomatis* at MOI 2 and transfected with 1 µg/ml polyI:C. [A, B] Confocal staining of IRF-3 with a Cy2™-labelled secondary antibody, DNA is stained with DRAQ5. Images were taken with 630-fold magnification. Arrows indicate nuclear IRF-3, arrow heads indicate chlamydial inclusions. [C, D] Quantification of cells that display nuclear IRF-3 from the experiments from (A) and (B). Five random fields were analyzed. [D] The mean percentage of cells with nuclear IRF-3 of two independent experiments is shown -/+ SD. * = $p < 0.05$.

While polyI:C transfection caused an obvious translocation of IRF-3 into the nucleus of non-infected HEp-2 (Fig. 3.28 A) and Hela cells (Fig. 3.28 B), inclusion carrying cells only rarely displayed nuclear signals for IRF-3. It was calculated that IRF-3 was present in the nucleus of 29.5% and 31% of non-infected HEp-2 (Fig. 3.28 C) and Hela cells (Fig. 3.28 D), respectively, while only 11.6% and 9%, respectively, of *Chlamydia*-infected cells were positive for nuclear IRF-3, suggesting an infection-induced repression of IRF-3 translocation into the nucleus. Because translocation of IRF-3 into the nucleus requires its phosphorylation at several amino acid residues at the C-terminus (Lin *et al.*, 1998, Yoneyama *et al.*, 1998), it was tested whether infection could prevent IRF-3 phosphorylation. Immunoblotting revealed an increase in the upper band of the transcription factor one hour after transfection with polyI:C, suggested to be the N-terminal phosphorylated form of IRF-3, while a second shift of IRF-3, which was suggested to represent the C-terminal phosphorylated protein, could be observed at 4 h after transfection (Fig. 3.29 A, marked with arrows). However, the same pattern could be detected in *Chlamydia*-infected cells, arguing against a block of IRF-3 phosphorylation by the pathogen. Interestingly, *C. trachomatis*-infected host cells displayed an increase in the first upper band also in the absence of polyI:C, suggesting a possible involvement of the transcription factor during *Chlamydia* infection.

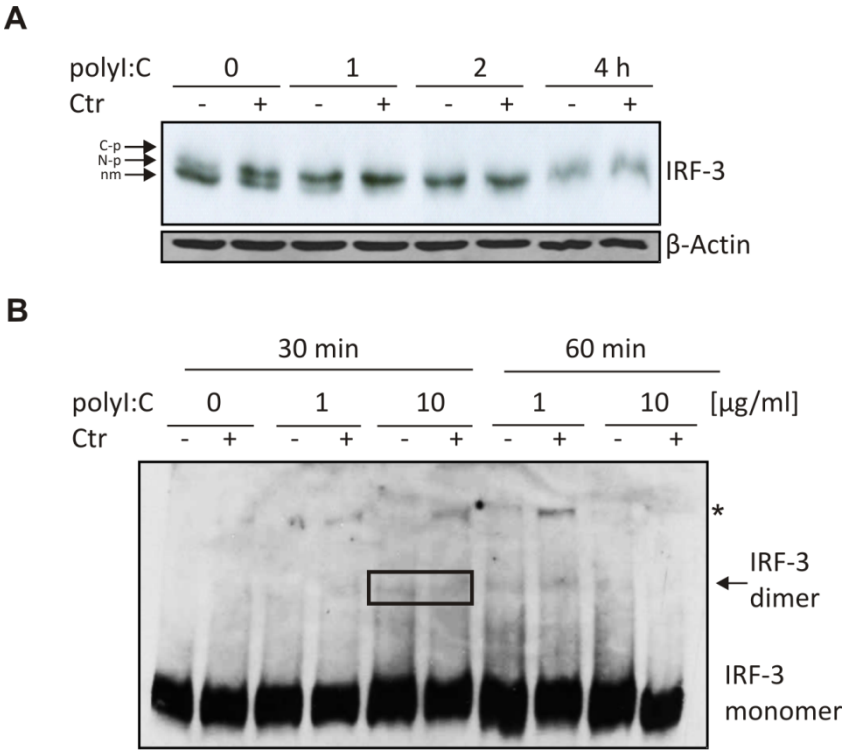

Fig. 3.29: IRF-3 modification by *C. trachomatis* infection.
[A] The lysates from figure 3.26 B were analyzed for IRF-3 by immunoblotting. The arrows indicate non-modified (nm), N-terminal phosphorylated (N-p), and C-terminal phosphorylated (C-p) IRF-3 [B] Hela cells were infected with *C. trachomatis* at MOI 2 (Ctr +) or mock infected (-)and after 20 h were transfected with the indicated concentrations of polyI:C for the indicated time and were subjected to native PAGE. Possible IRF-3 dimers are indicated with an arrow. The box highlights one example of each non-infected and infected samples with respect to IRF-3 dimerization. The star indicates a potential chlamydial protein.

SInce no inhibition of IRF-3 phosphorylation could be monitored, it was assumed that infection can prevent the nuclear translocation of IRF-3 at a different level. Because dimerization of IRF-3 preceeds its translocation, it was investigated whether *C. trachomatis* could subvert dimerization of the transcription factor. Therefore, a native PAGE experiment was performed. In polyI:C-transfected Hela cells, only a very mild band could be detected that might represent dimerized IRF-3 (Fig. 3.29 B, marked with an arrow). However, an infection-induced decrease in this band could only vaguely be observed in the samples that were treated with 10 µg/ml polyI:C (highlighted by the box) while others did not clearly show a decrease. Instead, a prominent band could be found in infected samples. Together these results propose a modification of IRF-3 by *C. trachomatis*.

3.2.4 Expression of inflammatory cytokines is not impaired in infected host cells

Due to the fact that nuclear translocation of both NF-κB and IRF-3 are required for induction of anti-viral type I IFN and inflammatory cytokines (Wathelet *et al.*, 1998), the data presented above suggested that chlamydial infection would interfere with the expression of these anti-viral proteins. In order to elucidate whether infection with *C. trachomatis* could downmodulate their cellular expression, the relative expression levels of IFN-β, IL-6, and IL-8 mRNA were investigated by quantitative RT-PCR.

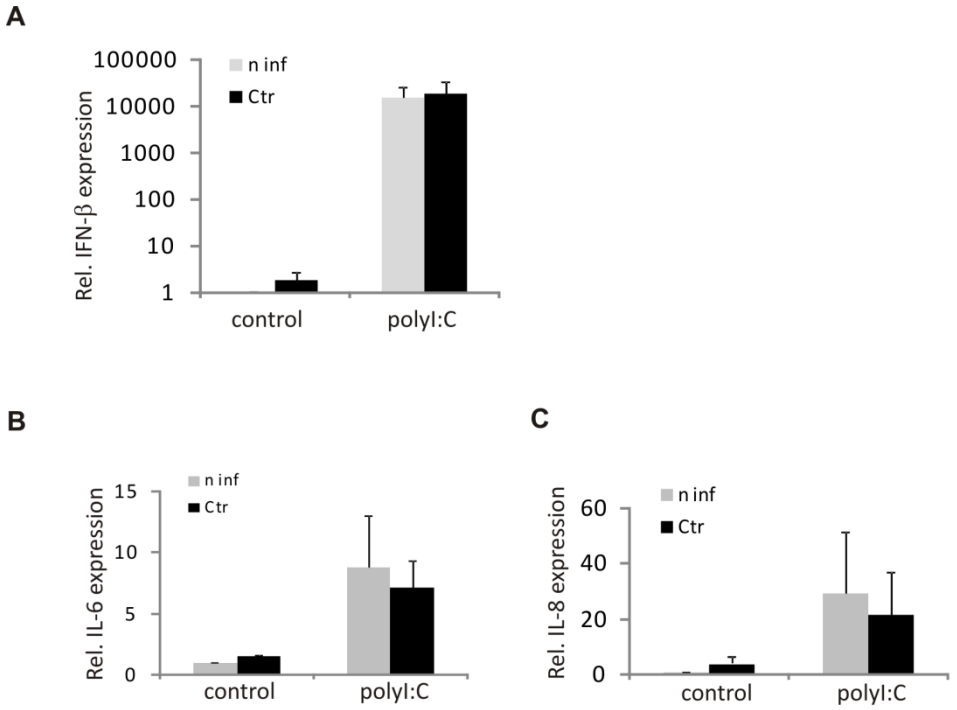

Fig. 3.30: polyI:C-induced expression of pro-inflammatory cytokines is intact in infected host cells.
Infected and non infected cells were transfected with 1 µg/ml polyI:C at 20 h post infection with *C. trachomatis* (Ctr) at MOI 4. After 4 h, total RNA was isolated, subjected to cDNA synthesis and qRT-PCR with a set of primers specific for IFN-β, IL-6, or IL-8. GAPDH and 5S rRNA served as internal controls. Shown are the mean values of two independent experiments with SE.

As expected, a dramatic increase in IFN-β could be detected upon transfection with polyI:C which was approximately 14,000-fold compared to untreated cells (Fig. 3.30 A). However, infected host cells showed a likewise expression of IFN-β if transfected with polyI:C while infection alone provoked a minor, 1.8-fold upregulation of the interferon. Also polyI:C-induced expression of IL-6 and IL-8 was not significantly reduced by infection. mRNA levels of IL-6 increased 8.8-fold and 7.2-fold upon transfection with polyI:C in non-infected and *C. trachomatis*-infected host cells, respectively (Fig. 3.30 B). After polyI:C transfection, the mean transcript expression of IL-8 increased 29-fold in non-infected cells and 22-fold in infected host cells. Infection alone induced an increase of IL-8 that was 4-fold compared to non-infected control cells (Fig. 3.30 C), which is consistent with the published data demonstrating an increase in IL-8 secretion in *Chlamydia*-infected cells (Rasmussen *et al.*, 1997). These results together imply that although the infection with *C. trachomatis* interferes with the activation of NF-κB and IRF-3 signalling, it does not block transcriptional upregulation of cytokines induced by dsRNA.

3.2.5 Production of chlamydial progeny is only mildly affected in cells treated with dsRNA

It could be demonstrated that several cellular defense pathways aiming at initiating viral clearance could still be activated by dsRNA in *C. trachomatis* infected host cells. It was therefore interesting to reveal whether the block of protein translation, indicated by activation of PKR and RNase L, influenced the ability of *Chlamydia* to produce infectious EBs. Hela cells were infected at MOI 2, were treated with polyI:C, with lipofectamine alone, or with CAM, and 24 h later, cells were lysed and EBs were transferred to new, uninfected Hela cells for a second round of infection. The next day, cells were fixed, stained for chlamydial Hsp60, and inclusions were counted to determine the infectivity of each sample. Both EBs from control and polyI:C-treated cells were infectious in the re-infection, whereas from cells treated with CAM no infectious particles could be recovered, indicated by a lack of cHsp60 staining (Fig. 3.31 A). The infectivity of EBs from polyI:C-treated cells was reduced to about 75% compared to control treated cells (Fig. 3.31 B). To confirm these results, the amount of chlamydial Hsp60 after re-infection was investigated by immunoblotting. Figure 3.31 C illustrates that the bacterial infection was only mildly reduced in cells infected with the supernatant from polyI:C-treated cells compared to control treated cells. These results demonstrate the ability of *C. trachomatis* to successfully replicate in the presence of dsRNA.

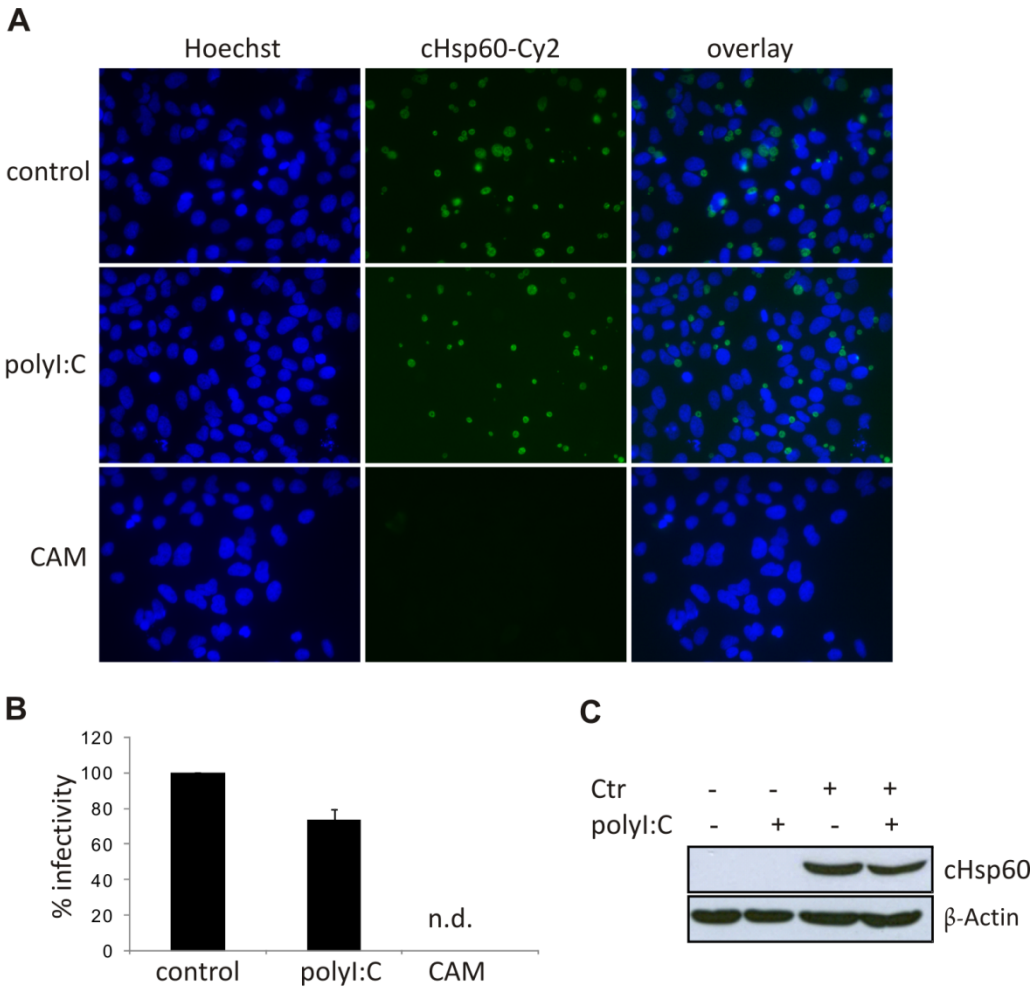

Fig. 3.31: Production of chlamydial progeny is mildly affected by polyI:C.
Hela cells were infected with *C. trachomatis* at MOI 2 for 20 h and were treated with 1 µg/ml polyI:C or with lipofectamine alone (control). After 24 h, cells were lysed by a freeze/thaw cycle and the supernatant was transferred to uninfected Hela cells. [A] 24 h after transfer of the supernatant, Hela cells were fixed with PFA and stained for chlamydial Hsp60 (cHsp60), DNA was stained with Hoechst 33342. [B] The number of inclusions per cell from the experiment from (A) was counted in five random fields in an epifluorescence microscope. The mean percentage of infectivity of two independent experiments normalized to control treated cells is shown -/+ SD. n.d. = not detected. [C] The experiment was performed as in (A) and cells were lysed with 2x Laemmli buffer 24 h after transfer of the supernatant. Lysates were analyzed for cHsp60 and β-Actin by immunoblotting.

4. DISCUSSION

The accumulating number of studies dealing with the complex interactions between pathogens and their host cells has emerged the comparatively young biological discipline termed cellular microbiology (Cossart *et al.*, 1996). Indeed, the investigations within this fascinating field of research provided us with an increasing understanding of the pathogenesis of infectious diseases and allow for the development of efficient therapeutic therapies. Although there is still a long way to go for a complete picture, unravelling the mechanisms by which pathogens manipulate the host cell signalling pathways further deepens our understanding on the intricate cellular processes and the impact of their dysregulation, such as resistance to programmed cell death (PCD). In a time when cancer ranks among the most frequently causes of death, research on PCD becomes of utmost importance. According to Hanahan and Weinberg (2000), a cell must undergo several physiological alterations in order to become cancerous. These hallmarks include the unlimited potential to replicate, the ability to grow in the absence of growth factors and in the presence of anti-growth signals, a sustained angiogenesis, the ability to invade tissues and to metastasize. Last but not least: the capability of evading PCD.

Although not accepted for many decades, it is now well established that viral infections can induce cancer formation. Infections with the human papilloma virus (HPV), for instance, can become a source of cell malignancy (Gissmann *et al.*, 1984) - a discovery that was recently awarded with the Nobel Prize in Medice 2008 to Harald zur Hausen. By integrating into the genome of the host cell and synthesizing viral proteins that promote cell growth, infected cells undergo constant replication thereby increasing the infected population (Syrjanen, 1986). While this leads to the development of warts in most cases, the sexually transmitted high risk types like HPV-18 and HPV-33 eventually induce cervical intraepithelial neoplasia (CIN) (Reid, 1983, Reid *et al.*, 1980). Remarkably, it was estimated that HPV is prevalent in 99% of cervical cancers (Walboomers *et al.*, 1999). Recent clinical studies revealed a significant correlation between infections with high risk HPV and *Chlamydia trachomatis*, suggesting that the bacterial infection is an important co-factor for persistent HPV infections (Paba *et al.*, 2008, Silins *et al.*, 2005, Smith *et al.*, 2002). Because *Chlamydia trachomatis* is among the most prevalent sexually transmitted pathogens in the Western world, the investigation of the molecular processes provoked by a co-infection of *C. trachomatis* and HPV would be of enormous interest. However, hitherto no model for *in-vitro* or *in-vivo* studies of HPV infections has been established. Therefore, examining the impact of chlamydial infections on the cellular response to double-stranded RNA (dsRNA) might help to draw a first picture of this scenario and pave the way for further studies.

The aim of the present study was to reveal whether *C. trachomatis* can influence the cellular response to dsRNA. This response comprises two concomitant routes of defense. First, the activation of cellular signalling pathways which terminates in the transcription of anti-viral cytokines, most importantly type I interferons (IFN-α and IFN-β), and subsequent expression of several hundred anti-viral genes is designed to prime neighbouring cells for the viral attack by paracrine signalling and to strengthen the infected cell itself via autocrine loops. In addition, the induction of PCD functions to limit viral loads and to protect the organism from a systemic viral infection by eliminating the infected cells. A predominant feature of viral infections is the production of dsRNA molecules inside the infected host cell which are recognized by several cellular sensor proteins. While the genome of dsRNA viruses can directly be detected, single stranded RNA viruses, both negative-sense and positive-sense, produce dsRNA during their replication (Lee *et al.*, 1994, Majde *et al.*, 1991). Due to their very compact genomes, the synthesis of dsRNA can also be found upon infection with DNA viruses, for instance vaccinia virus (Colby *et al.*, 1969, Duesberg *et al.*, 1969) or herpes simplex virus (Jacquemont *et al.*, 1975). There, the presence of several overlapping open reading frames leads to the production of transcripts with complementary RNA sequences that can form duplexes. For the investigations performed in this work, the well established synthetic long dsRNA polyinosinic:polycytidylic acid (polyI:C) served as a model to mimic viral super-infections of *Chlamydia*-infected host cells. polyI:C has repeatedly been demonstrated to be a potent activator of the cellular apoptosis signalling pathways (Iordanov *et al.*, 2005a, Iordanov *et al.*, 2005b, Kibler *et al.*, 1997). It was observed in this work that epithelial cells do not efficiently undergo apoptosis in response to polyI:C which is externally applied to the cells (see Fig. 3.13). Therefore, lipofectamine transfection was used to facilitate uptake and to expose infected host cells to intracellular dsRNA. Consistent with the published data, transfection with 1 µg/ml polyI:C was sufficient to induce apoptosis in human epithelial cells, as demonstrated by PARP cleavage, caspase-3 activation, and DNA fragmentation (Fig. 3.1 to 3.5). In contrast, small interfering RNA (siRNA) with a size of approximately 21 nucleotides targeting the Luciferase mRNA did not provoke any noticeable response, arguing for a specific response to long dsRNA species, which is in agreement with the published data (Elbashir *et al.*, 2001). The amount of cells displaying the characteristic features of apoptosis was significantly reduced upon infection with *Chlamydia*, demonstrating the ability of *C. trachomatis* to interfere with the cellular anti-viral response to prevent apoptosis of its host. This observation was not due to the inability of *Chlamydia*-infected cells to take up polyI:C as demonstrated by using fluorescently labelled dsRNA (Fig. 3.10). Interestingly, a correlation to the multiplicity of infection (MOI) could be found, indicating that higher numbers of intracellular bacteria are needed to suppress the apoptotic response to dsRNA (Fig. 3.3) which is consistent with earlier studies concerning inhibition of TNFα- or STS-induced apoptosis in *C. pneumoniae*-infected epithelial

cells (Rajalingam *et al.*, 2001). Thus, for the first time, dsRNA was identified in this study as an inducer of PCD whose pro-apoptotic actions can be adequately subverted by *Chlamydia*. This suggested that the pathogen impairs the host cell death machinery by abrogating the death signals at the level of or downstream of mitochondria as it was demonstrated before (Fan *et al.*, 1998, Fischer *et al.*, 2004a). However, the underlying mechanism for resistency to dsRNA-induced apoptosis could be identified to significantly differ from the mechanisms described so far. Immunoblot analysis and a specific luminescent substrate assay revealed a block of dsRNA-induced apoptosis upstream of caspase-8, which, in contrast, was not the case during TNFα-induced apoptosis (Fig. 3.8). This pointed to a different, yet unidentified mechanism which was then examined in more detail. Thus, the pathways activated upon recognition of dsRNA were investigated to evaluate conceivable routes of chlamydial inhibition. The IFN-inducible RNA-dependent protein kinase (PKR) is a key component of the host response to viral infections and a primary target for many viruses. For instance, the non-structural protein 1 (NS1) of influenzavirus A can bind to dsRNA thereby blocking the activation of PKR (Lu *et al.*, 1995), while the vIRF-2 protein of the human herpesvirus-8, often referred to as Karposi-sarcoma herpesvirus, directly binds to and inhibits autophosphorylation of the kinase (Burysek *et al.*, 2001). Thereby, phosphorylation of the α-subunit of the translation initiation factor 2 (eIF2α) is efficiently prevented by these viruses rescuing the translational block. Moreover, processing of caspase-8 upon dsRNA transfection is mediated by PKR (Balachandran *et al.*, 1998). The kinase was therefore a promising candidate to be manipulated by *Chlamydia*. However, polyI:C transfection-induced phosphorylation of the PKR substrates eIF2α and p38 was not abrogated in infected epithelial cells, indicating that PKR was still active in the presence of *Chlamydia* (Fig. 3.11). Although a bacteria-mediated inhibition of PKR activities towards caspase-8 could not be ruled out, it was proposed to be unlikely. Instead, these findings prompted to investigate the involvement of other cellular modulators of caspase-8 activity. In this context, the cellular caspase-8 inhibitor cFlip (cellular Flice like inhibitory protein) was scrutinized.The powerful tool of RNA interference (RNAi) was applied to study the role of this anti-apoptotic factor during *Chlamydia* infection. In fact, depletion of cFlip resulted in an increase in DNA fragmentation and PARP cleavage in the infected population, strongly arguing for its role in chlamydial caspase-8 inhibition and anti-apoptosis (Fig. 3. 14 and Fig. 3.15). Interestingly, the importance of cFlip for pathogen-induced anti-apoptosis has been proposed for other intracellular bacterial pathogens, though clear experimental evidence was missing (Bartfeld *et al.*, 2009, Lee *et al.*, 2006, Voth *et al.*, 2007). Instead, the authors showed a trancriptional upregulation of cFlip mRNA upon infection. However, strong induction of cFlip expression could not be detected in *Chlamydia*-infected cells, neither on mRNA nor on protein level (Fig. 3.16). Therefore, the question had to be addressed how *Chlamydia* utilizes cFlip specifically for the inhibition of procaspase-8 upon dsRNA transfection. It was speculated that *Chlamydia* can

sequester the anti-apoptotic protein to the site of greatest benefit which would be the location of caspase-8 activation. In fact, confocal microscopic analyses of cFlip suggested a translocation of the anti-apoptotic protein into the chlamydial inclusions (Fig. 3.17). However, because this staining pattern was also present in cFlip depleted cells (Fig. 3.18), it was assumed to be an artefact although it has to be mentioned that a clear siRNA-mediated knock down of cFlip on protein level could not be demonstrated here. Also, subcellular fractionation did not reveal translocation of cFlip to the *Chlamydia*-containing membrane fraction (Fig. 3.20). Yet, co-immunoprecipitation (co-IP) of caspase-8 demonstrated an infection-induced enhanced interaction with cFlip (Fig. 3.21). Based on the increased ratio of cFlip to FADD, as determined by densitometry, a higher proportion of the anti-apoptotic protein in the presumed caspase-8/FADD/cFlip complex seemed likely. This argued for the capability of *Chlamydia* to prevent autocleavage of caspase-8 which is mediated by homotypic interaction with FADD (Medema *et al.*, 1997, Muzio *et al.*, 1998) via promoting its interactions with cFlip. An enforced anti-apoptotic interpaly of cFlip and caspase-8 but not cFlip protein upregulation is therefore proposed as the mechanism responsible for inhibition of dsRNA-induced apoptosis. Still, it is puzzling how *C. trachomatis* exerts its manipulating actions to prevent caspase-8 activation and counteract apoptosis in response to dsRNA, while TNFα-induced apoptosis, as shown in this and other work (Rajalingam *et al.*, 2006) as well as Fas-induced cell death (Fischer *et al.*, 2004a) remains unaffected at the level of the initiator caspase. This intriguing finding requires further elucidation.

Most interestingly, in addition to their importance for orchestrating cell death, the DISC components caspase-8, FADD, and cFlip promote cell survival and proliferation, in particular in lymphocytes. First evidence for their dual function came from *in-vivo* studies with FADD-deficient mice that demonstrated abnormalities in T cell development (Newton *et al.*, 1998, Walsh *et al.*, 1998, Zhang *et al.*, 1998). While KO mice died *in utero*, FADD -/- chimaeras derived from FADD -/- embryonic stem cells in a background devoid of the recombination activating gene 1 were viable but displayed reduced thymocytes due to a lack in T cell proliferation (Zhang *et al.*, 1998). Besides an increase in apoptosis, dominant negative mutant versions of FADD enhanced the deletion of autoreactive T cells in transgenic mice (Newton *et al.*, 1998). These findings could soon be extended to caspase-8. Using chemical inhibitors, the protease activity was shown to be required for proliferation of T cells (Kennedy *et al.*, 1999). Importantly, a clinical study with patients suffering from autoimmune lymphoproliferative syndrome, a human disorder characterized among other symptoms by a defect in lymphocyte apoptosis and by autoimmunity, led to the identification of kindred that additionally displayed defective activation of T lymphocytes, B lymphocytes, and natural killer cells (Chun *et al.*, 2002). This demonstrated that, in contrast to lethal caspase-8 KO mice, humans deficient in the protease develop normally but suffer from immunodeficiency. This defect was later suggested to be due to impaired activation of the transcription factor NF-κB in the absence of caspase-8 (Su *et al.*,

2005). Not surprisingly, the developmental defects observed in mice deficient in cFlip resemble those of caspase-8-/- and FADD-/- mice (Yeh *et al.*, 2000). Overexpression of cFlip, consistently, led to an increase in T cell proliferation in transgenic mice (Lens *et al.*, 2002). Thus, taking into account that caspase-8 and cFlip are assigned crucial functions in development and immunity, it is particularly interesting to further reveal the influence of *Chlamydia* on these host cell factors. It seems likely that, by manipulating the activation of caspase-8, *Chlamydia* might also interfere with the adaptive immune system of the host.

Another important factor that is linked to dsRNA-induced apoptosis is RNase L (Castelli *et al.*, 1998, Zhou *et al.*, 1998) which was therefore investigated in this study. Upon production of 2´-5´-linked adenylates by the 2-5 oligoadenylate synthetase (2-5 OAS) in response to dsRNA (Clemens *et al.*, 1978, Kerr *et al.*, 1978), dimerization and activation of RNase L is induced. However, a manipulation of RNase L activity could not be detected upon infection with *C. trachomatis* as exemplified by the fact that the degradation of 28S rRNA, a widely accepted read-out for RNase L activity, also occurred in infected host cells (Fig. 3.12). Furthermore, because it has been demonstrated that stress-induced degradation of 28S rRNA leads to the activation of the c-jun N-terminal kinase (JNK) (Iordanov *et al.*, 1997, Iordanov *et al.*, 1998), activation of the kinase was examined. Indeed, phosphorylation of JNK upon transfection with polyI:C could be observed both in infected and non-infected cells which confirmed that RNase L is not inhibited by *C. trachomatis* and, moreover, suggested that *Chlamydia* can inhibit PCD downstream of JNK . Because JNK exerts its pro-apoptotic functions at the level of mitochondria (Tournier *et al.*, 2000) and requires Bak and Bax (Lei *et al.*, 2002) it is very likely that other, already described mechanisms account for a block of apoptosis here. Astonishingly, these mechanisms obviously did not account for apoptosis resistence in a cFlip knock down background as infected cells were sensitized to dsRNA-induced apoptosis. Because the expected inhibition of MOMP due to other chlamydial anti-apoptotic strategies was not apparent in the absence of cFlip, an additional role of cFlip in the regulation of other cellular pathways or factors required for chlamydial MOMP inhibition seems likely. These possible interactions should be matter of further elucidation.

When studying the cellular response to dsRNA, the toll-like receptor 3 (TLR3) has to be taken into consideration due to its function as a dsRNA receptor (Alexopoulou *et al.*, 2001). In fact, TLR3 has been suggested to play a role in apoptosis induction (Jiang *et al.*, 2008, Salaun *et al.*, 2006). Astonishingly, Jiang and colleagues demonstrated that external application of polyI:C induced apoptosis in Hela cells, however, only in the context of simulataneous application of cycloheximide and polyI:C. In order to test the engagement of TLR3 in apoptosis induction in this model, external application of high concentrations of polyI:C (100 µg/ml) was performed. In the absence of a transfection reagent, PCD could not be induced which is in agreement with the published data (Jiang *et al.*, 2008) and which implies that intracellular dsRNA was required to provoke cell death. From this

it was concluded that surface TLR3 was not part of the polyI:C-induced apoptosis induction in epithelial cells although it cannot be ruled out that intracellular located TLR3 contributes to the described observations.

Confocal microscopic analyses of cFip indicated that the antibody might cross-react with a chlamydial protein (Fig. 3.17 and Fig. 3.18). This suggested the existence of a bacterial analogue to cFlip which might be involved in the inhibition of caspase-8 activation. However, BLAST anaylsis of the two protein isoforms of cFlip did not reveal any bacterial factor with respective amino acid sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This led to the assumption of a rather unspecific binding of the antibody to the chlamydial inclusions as it was reported by Pollack *et al.* (2008). Nevertheless, as demonstrated by the application of the antibiotic chloramphenicol which specifically inhibits bacterial protein synthesis, one or several effector proteins, synthesized early during infection, are required for chlamydial anti-apoptosis (Fig. 3.4) and caspase-8 inhibition (Fig. 3.7), although it was not possible to further determine their nature. Interestingly, the chlamydial protein associated with death domain (CADD), localized in the inclusion membrane, can interact with cellular death receptors via their DD, including Fas and TNFR1 (Stenner-Liewen *et al.*, 2002). The authors showed a co-localization of Fas and CADD at the vicinity of the inclusion and it was speculated that this bacterial factor might participate in the inhibition of Fas ligand-induced apoptosis via translocation and inhibition of the receptor. Employing immunoflurescent staining techniques, translocation of the TNFR1 to the chlamydial inclusion could also be shown in another study performed in our laboratory (Paland *et al.*, 2008). However, an interaction of CADD with neither cFlip nor FADD was detected (Stenner-Liewen *et al.*, 2002). An involvement of CADD in the complex formation of caspase-8, cFlip, and FADD therefore seems rather unlikely. Nevertheless, it would be very interesting and necessary to investigate other chlamydial effector proteins with respect to their anti-apoptotic qualities and suspected manipulation of cFlip. However, the unfortunate inability to genetically modify the obligate intracellular pathogen has hitherto hampered any functional studies with *Chlamydia*. The biphasic obligate intracellular life style and the feature to undergo persistence rather than die in the presence of antibiotics makes the generation, selection and isolation of specific mutants extremely difficult. Nevertheless, other obligate intracellular bacteria were successfully modified in the 1990es. For example, *Rickettsia* (Rachek *et al.*, 1998) and *Coxiella* (Suhan *et al.*, 1996) were isolated and electroporated before reinfection. Although the same attempts were taken for *Chlamydia* no stable transformation could be achieved (Tam *et al.*, 1994). However, in addition to these first efforts, the existence of a chlamydial plasmid raises hope for the establishment of robust protocols to genetically engineer *Chlamydia* in the future which would allow for detailed genetic studies (Lusher *et al.*, 1989, Palmer *et al.*, 1986).

Several anti-apoptotic factors have been reported to be involved in the resistence towards host cell death by *Chlamydia*, including the Bcl-2 like protein Mcl-1 (Rajalingam *et al.*, 2008) which is an efficient inhibitor of PCD (Reynolds *et al.*, 1994). It was demonstrated that *C. trachomatis* requires Mcl-1 in order to subvert TNFα- and Granzyme B-induced apoptosis, although another study proposed the opposite (Ying *et al.*, 2008). These controversial results might be due to the utilization of different cell lines and experimental set ups. In order to elucidate the role of Mcl-1 in apoptosis resistence towards polyI:C, regulation of the protein was investigated. In fact, it could be observed that Mcl-1 downregulation by dsRNA was abrogated in infected cells (Fig. 3.23). Hence, this protein constituted an interesting candidate for the underlying anti-apoptotic mechanism. However, RNAi experiments revealed that Mcl-1 was not essential for *Chlamydia* to prevent host cell death induced by polyI:C (Fig. 3.24). This was divergent to a study by Fritsch and colleagues who demonstrated that overexpression of Mcl-1 could rescue stress-induced apoptosis that was mediated by Mcl-1 downregulation following eIF2α phosphorylation (Fritsch *et al.*, 2007). Nevertheless, the infection-induced rescue of Mcl-1 might be of interest in a more complex scenario, where virus infected cells, upon recognition by cytotoxic T cells, are exposed to Granzyme B or perforin. It is thus conceivable that co-infected cells might not undergo PCD as efficiently as viral infected cells in the absence of *Chlamydia* because of the alterations in Mcl-1 protein levels. Mcl-1 is a very short-lived protein with a half-life of approxamitely 3 h (Akgul *et al.*, 2000, Moulding *et al.*, 1998) before it is degraded via the proteasome (Zhang *et al.*, 2002). The 26S proteasome is a large protein complex (Ciechanover *et al.*, 1978) that recognizes proteins targeted for destruction through a covalently bound ubiquitin polypeptide chain (Ciechanover *et al.*, 1980). Ubiquitination is a sequential process catalyzed by a family of ubiquitin-activating enzymes E1, E2, and E3 (Ciechanover *et al.*, 1981, Handley *et al.*, 1991). A possible mechanism of how *C. trachomatis* prevents the degradation of Mcl-1 is the deubiquitination of the protein. Indeed, a chlamydial deubiquitinating protein, called ChlaDub1, has been identified that can catalyze the removal of ubiquitin-moeities from the α-subunit of the inhibitor of NF-κB (IκBα) (Le Negrate *et al.*, 2008). Overexpression studies revealed that ChlaDub1 could thereby prevent the nuclear translocation of NF-κB in response to TNFα. It has to be mentioned though that within this study, it could not be shown that degradation of IκBα following TNFα treatment was absent in infected cells (Fig. 3.26). Nevertheless, prevention of preoteasomal degradation via deubiquitination might be a successful chlamydial strategy to subvert the destruction of pro-surival proteins. Also other anti-apoptotic factors might be stabilized by chlamydial deubiquitinating proteins. In an elegant study, scientists around Ashkenazi demonstrated that polyubiquitination of caspase-8 was an essential step during the induction of apoptosis by extrinsic stimuli (Jin *et al.*, 2009). It might therefore be possible that, in addition to controlling cFlip, deubiquitination of caspase-8 plays a role in *Chlamydia*-mediated apoptosis inhibition. Interestingly,

ubiquitin has been found to act as a signal for the internalization and sorting of membrane proteins (Hicke *et al.*, 1996). The first mammalian receptor identified to be regulated via ubiquitination was the epidermail growth factor receptor (Galcheva-Gargova *et al.*, 1995) and several other receptor tyrosine kinases followed (Haglund *et al.*, 2003, Marchese *et al.*, 2001, Peschard *et al.*, 2001). Therefore, to examine the influence of the chlamydial deubiquitinases on host cell factors and processes might reveal new strategies by which the pathogens manipulate their host cells.

A well established mechanism by which *Chlamydia* influence the fate of the cell and facilitate their reproduction is the activation of cellular survival pathways, most importantly the phosphorylation of ERK (Paland *et al.*, 2008, Rajalingam *et al.*, 2008, Su *et al.*, 2004). The importance of the Ras/Raf/MEK/ERK pathway in cell survival (Yujiri *et al.*, 1998), proliferation (Boulton *et al.*, 1990), and tumor progression (Cowley *et al.*, 1994) was demonstrated by an enormous number of studies. Mutations in the Ras protein have been demonstrated to account for a vast number of tumors (Khleif *et al.*, 1999), making this pathway an attractive object to study in terms of infection-induced cancers. Activation of the Raf/MEK/ERK pathway by *C. trachomatis* was initially shown by Su *et al*. (2004) who demonstrated that acquisition of phospholipids into the chlamydial inclusion was dependent on ERK phosphorylation. Later, the crucial role of ERK for the shedding of TNFR1 from the surface of infected cells, a process that is mediated by the metalloprotease TACE (tumor necrosis factor α converting enzyme) (Paland *et al.*, 2008), and for apoptosis inhibition (Rajalingam *et al.*, 2008) could be shown in our laboratory. Hence, it was tested whether pathogen-induced activation of ERK was required to prevent PCD induced by dsRNA. Despite the finding that caspase-8 activity increased after inhibition of ERK activation in both non-infected and infected samples treated with polyI:C, a significant block by *Chlamydia* could still be detected (Fig. 3.25). Consequently, apoptosis inhibition was not impaired in the absence of active ERK. A significant reduction of PARP cleavage in *Chlamydia*-infected cells compared to non-infected cells could be detected, although the overall level of apoptosis was elevated. Thus, *C. trachomatis* seems to possess a limited capability to counteract the cytotoxic effect of dsRNA which, upon increase of the apoptotic stress, for instance by ERK inhibition, does not allow for an absolute block of host cell death. Astonishingly, inhibition of ERK activation by the chemical compound U0126 was demonstrated to downregulate cFlip expression in Hodkin cells (Zheng *et al.*, 2003) indicating an interaction between the Raf/MEK/ERK pathway and cFlip regulation. This is contradictory to the results gained in this work, which might be due to the utilization of different cell lines.

Taken together, the data gained in this work suggest the inhition of dsRNA-induced apoptosis in *C. trachomatis*-infected epithelial cells at the level of caspase-8 which is mediated by the utilization of cFlip through one or several chlamydial effector proteins. A proposed model is illustrated in figure 4.1.

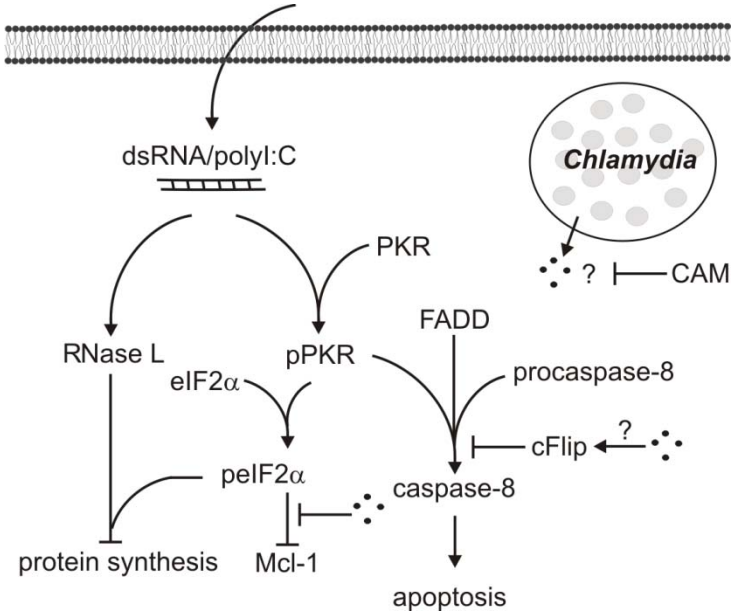

Fig. 4.1: Model for the interference of *C. trachomatis* with polyI:C-induced apoptosis signalling pathways.
Intracellular dsRNA or polyI:C activates several signalling pathways, of which PKR and 2-5 OAS/RNase L most potently orchestrate apoptosis. PKR can recruit FADD thereby mediating autocleavage and action of procaspase-8. Via secretion of one or several effector proteins, presented by black dots, *Chlamydia* can prevent caspase-8 activation by promoting interactions between cFlip and procaspase-8. In addition, degradation of Mcl-1 can be inhibited by infection.

The present study revealed that infection with *Chlamydia trachomatis* can actively block PCD induced by dsRNA. Self-sacrifice of the infected cell represents only one possible, though very drastic route to promote virus clearance. Alternatively, infected cells can respond to the invader by inducing a potent anti-viral program. The synthesis of a battery of peptides which can be secreted from the infected cell is able to prime the environment for the elimination of the virus by induction of proteins that confer the anti-viral state. The most prominent factors involved in this process are type I interferons (IFN-α and IFN-β) and interleukines (IL). Upon recognition of dsRNA by the cellular sensors TLR3, MDA5, and RIG-I, signalling cascades are activated that lead to the translocation of the transcription factors NF-κB and the interferon regulatory factor 3 (IRF-3) into the nucleus where they bind to respective promoter regions. Because it is a crucial mediator of the anti-viral response, IRF-3 activation can be inhibited by a number of viral proteins, including the vaccinia virus factor E3L (Zhang *et al.*, 2009). Here, it was scrutinized whether *C. trachomatis* can interfere with the response to polyI:C by inhibiting IRF-3 activation. Indeed, confocal microscopy revealed a significant decrease in nuclear IRF-3 translocation upon polyI:C transfection in *Chlamydia*-infected host cells (Fig. 3.28). Hence, to further elucidate the impact of an infection with *C. trachomatis* on the transcription factor, phosphorylation and dimerization of IRF-3 was investigated by immunoblotting and native polyacrylamid gel electrophoresis (PAGE), respectively. Activation of IRF-3 requires its phosphorylation at several amino acid residues located at the C-terminus of the protein (Lin *et al.*,

1998, Yoneyama *et al.*, 1998) mediated by TBK-1 and IKKε (Fitzgerald *et al.*, 2003, Sharma *et al.*, 2003), and NEMO (Zhao *et al.*, 2007) in a two-step manner. A first-step phosphorylation of IRF-3 at residues 385 and 386 alleviates its autoinhibition, whereas the second-step phosphorylation of residues 396 and 405 induces its dimerization (Panne *et al.*, 2007). It was reported that a reduction in gel mobility on SDS PAGE allowed for detection of both unphosphorylated and phosphorylated protein (Lin *et al.*, 1998). While the major band corresponds to unphosphorylated IRF-3, a second band with slower migration speed corresponds to IRF-3 phosphorylated at the N-terminus. The most slowly migrating form of IRF-3 in SDS PAGE then represents the activated protein that is phosphorylated at the N- and C-terminus and thereafter translocates to the nucleus (Lin *et al.*, 1998). Interestingly, both polyI:C transfection and *C. trachomatis* induced N-terminal phosphorylation of IRF-3, demonstrated by the appearance of an upper band and the disappearance of the lower band (Fig. 3.29). At 4 h after transfection, a further shift in the IRF-3 band could be detected both in non-infected and infected host cells, most likely representing C-terminal phosphorylated IRF-3. This suggested that phosphorylation could not be blocked by infection and implied that nuclear translocation was prevented, as observed by confocal microscopy, by a different mechanism. Thus, dimerization of the transcription factor was examined which was shown to occur in response to polyI:C (Iwamura *et al.*, 2001). Although a very faint second band could be detected in polyI:C-transfected samples in native PAGE gels, which was partly less prominent in infected samples, no clear dimerization of IRF-3 could be stated. Instead, a third band became obvious in infected samples which might either be due to cross-reactions with a chlamydial protein or due to complex formation of IRF-3 with a chlamydial protein. Thus, the microscopic finding that IRF-3 nuclear translocation is inhibited in *Chlamydia*-infected cells should be unequivocally demonstrated with additional experimental methods. Interestingly, IRF3 was reported to be required for IFN-β induction during *C. muridarium* infections (Derbigny *et al.*, 2007) while in *C. pneumoniae*-infection of macrophages a role for IRF-3 could not be detected (Trumstedt *et al.*, 2007). Rodel et al (1999) showed IRF-1 upregulation by *C. trachomatis* serovar D in synovial fibroblasts. It seems therefore likely that IRF-3 that can also be activated via other TLRs and bacterial products plays a distinct, so-far unidentified role during *C. trachomatis* infections. Interestingly, inhibition of IRF-3 by the vaccinia virus protein E3L was shown to be responsible for apoptosis inhibition as E3L deletion mutants failed to repress cell death upon infection (Kibler *et al.*, 1997). Thus, manipulation of IRF-3 by *Chlamydia* could be an additional mechanism to prevent dsRNA-induced apoptosis and should be further investigated.

It could further be revealed in this study that infection with *C. trachomatis* had an influence on the cytosol to nucleus translocation of p65, a member of the NF-κB family, upon polyI:C transfection (Fig. 3.27). Interestingly, it was found that infection with *Chlamydia* rather enhanced that prevented degradation of IκB-α (Fig. 3.26). Hence, the decrease in nuclear p65, which was detected by confocal

microscopy, could be the result of a more rapid nuclear shuttling of p65 in infected cells compared to non-infected cells. Another explanation might be the pathogen-mediated degradation of p65 leading to a less prominent staining in infected cells. Indeed, the chlamydial tail specific protease (Tsp) was demonstrated to potently cleave p65 in Hela cells and other cell lines (Lad *et al.*, 2007a, Lad *et al.*, 2007b). Thus, taken together, p65 degradation and prevention of IRF-3 translocation appeared to be efficient mechanisms to subvert inflammatory responses in virally superinfected host cells. Consequently, a down-modulation of IFN- and IL-expression in infected cells was expected. However, analysis of the mRNA levels of IL-6, IL-8, and IFN-β by quantitative RT-PCR revealed a polyI:C-induced increase both in non-infected and infected epithelial cells (Fig. 3.30). Astonishingly, the increase in IFN-β expression was as drastic as 14,000-fold. Such an extreme upregulation seems rather unlikely which is supported by publications showing expression levels in the range of 100-fold induction (McAllister *et al.*, 2009). An explanation might be a saturation of detectable mRNA levels which would suggest that, albeit considered a quantitative detection method, this result should rather be regarded as a qualitative one.

It was shown in this work that polyI:C-induced activation of several pathways that constitute the anti-viral state by inhibiting protein translation was not abrogated in *Chlamydia*-infected cells. Hence, it was of interest to examine whether this had an influence on chlamydial reproduction. Interestingly, sufficient bacterial reproduction was observed despite the presence of dsRNA, as quantified by an infectivity assay. There, the amount of infectious particles derived from polyI:C-transfected host cells was only mildly reduced compared to the amount of infectious EBs from mock-treated cells (Fig. 3.31). Thus, it seems plausible that *Chlamydia* can complete its developmental cycle and establish an acute infection in the presence of dsRNA. This is consistent with early *in-vivo* studies on a rabbit trachoma model where treatment with polyI:C did reduce the extent of eye tissue damage while production of *Chlamydia* was unaffected (Oh *et al.*, 1970).

Together the data acquired in this work suggest a dramatic influence of the infection with *C. trachomatis* on the cellular response to dsRNA by preventing dsRNA-induced apoptosis and by manipulating the signalling pathways that play a fundamental role in conferring an anti-viral state.

4.1. Outlook

The present study was an attempt to mimic chlamydial and viral co-infections by utilizing the synthetic dsRNA polyI:C transfected into human epithelial cells. It was aimed at giving a first idea of the complex interactions between cellular and bacterial factors upon stimulation of host cell defense mechanisms by virally produced dsRNA. It has to be considered though that during viral and chlamydial co-infections, additional viral factors than dsRNA are of importance. Thus, in order to obtain a more complete picture of how eukaryotic cells respond to viral infections in the presence of intracellular *Chlamydia*, an obvious increment of this scientific scope is the performance of co-

infection experiments with viruses. Revealing the molecular processes that occur during respective co-infections might help to understand the role of *C. trachomatis* as a relevant co-factor for instance in HPV infections and CIN development. Of course, these studies should not be restricted to *C. trachomatis* infections. Instead, the influence of *C. pneumoniae* on the outcome of influenza virus infections would also be of high relevance. The questions should be addressed whether an acute and also persistent infection with *Chlamydia* can promote viral reproduction or facilitate the integration of viral DNA into the host cell's genome. Special attention should be placed on the issue whether the chlamydial manipulation of the host immune system can abrogate viral clearance. Because it was described that infections with *Chlamydia* can downregulate the expression of MHC-I molecules (Zhong *et al.*, 2000), it is conceivable that the recognition of virus-infected cells by cytotoxic T cells is impaired in co-infected cells. For this issue, co-culture models would give respective information.

The pathogen-induced interaction of cFlip and caspase-8 was identified as a mechanism responsible for the inhibition of apoptosis induced by dsRNA. However, an issue that was not covered in detail by this work was the role of TLR3 in chlamydial apoptosis resistance. Although the involvement of surface TLR3 could be excluded in this work, it should be revealed whether *C. trachomatis* can manipulate the actions of intracellular forms of this dsRNA sensor. Also, the exact mechanisms by which PKR can induce FADD-mediated activation of caspase-8 deserve further elucidation. Another factor that appears to be prone to chlamydial manipulation is IRF-3. It was indicated in this work that *C. trachomatis* can modulate dsRNA-induced IRF-3 activation. Therefore, the function of this important immunoregulatory transcription factor during chlamydial infections should be further investigated. Here, tissue-like cultures, e.g. the fallopian tube model system, would be of great interest. Owing to the prominent role of ubiquitination in fundamental cellular processes, future studies should also take a deeper look into the function of chlamydial deubiquitinating proteins, e.g. the ChlaDubs, in the host pathogen interactions.

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7.2 List of publications

Böhme L, Albrecht M, Rudel T. C*hlamydia trachomatis*-infected host cells resist dsRNA-induced apoptosis. (In revision: *Cellular Microbiology*)

Böhme L and Rudel T (2009) Host cell death machinery as a target for bacterial pathogens. *Microbes Infect*, 11(13): 1063-70

Prakash H, Becker D, Böhme L, Albert L, Witzenrath M, Rosseau S, Meyer TF, Rudel T (2009) cIAP-1 controls innate immunity to *C. pneumoniae* pulmonary infection. *PLoS One*, 4(8)

Paland N, Böhme L, Kumar Gurumurthy R, Mäurer A, Szczepek AJ, Rudel T (2008) Reduced display of tumor necrosis factor receptor I at the host cell surface supports infection with *Chlamydia trachomatis*. *J Biol Chem*, 283(10): 6438-48

7.3 Oral presentations and poster presentations

Böhme L, Albrecht M, Rudel T. *Chlamydia trachomatis*-infected host cells resist dsRNA-induced apoptosis. 17th Euroconference on Apoptosis and 5th Course on Concepts and Methods in Programmed Cell Death 2009, Paris. (Poster)

Böhme L, Albrecht M, Rudel T. *Chlamydia trachomatis*-infected host cells resist dsRNA-induced apoptosis. Leopoldina Symposium for Evolution of Programmed Cell Death in Infection and Immunity 2009, Würzburg. (Oral presentation)

Paland N, Böhme L, Kumar Gurumurthy R, Mäurer A, Szczepek AJ, Rudel T. Ectodomain shedding of tumor necrosis factor receptor I in *Chlamydia trachomatis*-infected host cells. 60th Annual Conference of the DGHM 2008, Dresden. (Oral presentation)

Böhme L and Rudel T. Influence of *C. trachomatis* infection on the cellular reponse to dsRNA. 15th Euroconference on Apoptosis and 3rd Course on Concepts and Methods in Programmed Cell Death 2007, Portoroz, Slowenia. (Poster)

Paland N, Böhme L, Kumar Gurumurthy R, Mäurer A, Szczepek AJ, Rudel T. Reduced display of tumor necrosis factor receptor I at the host cell surface supports infection with *Chlamydia trachomatis*. 5th German Chlamydia workshop 2007, Hannover. (Poster)

8. SELBSTÄNDIGKEITSERKLÄRUNG

Ich erkläre ehrenwörtlich, dass die vorliegende Arbeit von mir selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe früher, außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden, keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg,

Linda Böhme