
# Mechanisms of apoptosis regulation in human cells infected with *Simkania negevensis.*

# Mechanismen der Apoptoseregulation in *Simkania negevensis* infizierten Humanzellen.

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg
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Würzburg 2012

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## Affidavit

I hereby confirm that the thesis entitled ‘Mechanisms of apoptosis regulation in human cells infected with *Simkania negevensis*’ is the result of my own work. I did not receive any help or support from any commercial consultants. All sources and/or materials applied are specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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## Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation `Mechanismen der Apoptoseregulation in *Simkania negevensis* infizierten Humanzellen` eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Würzburg,

*Dedicated to my beloved father*

## Acknowledgements

*I bow before Lord, the almighty for his bountiful blessings*

I feel elated to express my deep sense of gratitude to Prof. Dr. Thomas Rudel, my principle investigator, for giving me an opportunity to work under his guidance. I extend my deep indebtedness for the freedom of experimentation, wholehearted interest, constant motivation, ever willing help and insightful ideas throughout the research. I owe to him for the moral support and affection, which helped me to complete this endeavor successfully. His enthusiasm for science had been infectious, that had ignited my self-confidence, which enables me to persistently move towards my goal. I would also thank him for the critical scrutiny of the thesis.

I express my sincere thanks to Dr. Adrian Mehlitz for his timely help and suggestions and valuable guidance at all stages of study and critical scrutiny of my theisis. I owe him for he had taught me to be competitive and focused. I extend my heartfelt gratitude to Dr. Knut Ohlsen for the critical evaluation of my thesis and candid suggestions throughout the conduct of the work. I record my sincere gratitude to my colleagues Nadine, Claudia, Prema, Annette (especially for the Zussamenfassung!), Christines for the cozy environment in the lab and for all the help and support they gave me through out. My heartfelt thanks are due to Bhupesh for helping me with EMSA and the tasty mango lassis. My sincere gratitude to Vera and Monika for their timely help.

Words fail to express my love and gratitude to my mother, brother; husband and son for their care and selfless help, understanding that had made this attempt a

reality. I would like to express my sincere gratitude to Dr. Vettom, Dr. Wegener and Dr. Elsamma Wegener for care and constant support. Finally I would thank Prof. Eric Buchner and Dr. Stephan Schröder-Köhne, Dr. Liane Lichtlein and Dr. Gabriele Blum-Oehler for their constant help and DFG for offering the GSLS fellowship, without which this achievement would have been, just a dream.

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## III. Abbreviations

### A

| | |
|---|---|
| AIF | Apoptosis-inducing factor |
| Apaf-1 | Apoptotic protease activating factor 1 |
| APS | Ammonium per sulfate |
| ATCC | American Type Culture Collection |

### B

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

### C

| | |
|---|---|
| CED | Cell Death Abnormal |
| cFLIP | Cellular FLICE like inhibitory protein |
| Cpn | *Chlamydia pneumonia* |
| Ctr | *Chlamydia trachomatis* |

### D

| | |
|---|---|
| dpi | Days post infection |
| DED | Death effector domain |
| DEPC | Diethylpyrocarbonate |
| DMSO | Dimethyl sulphoxide |

**E**

| | |
|---|---|
| EB | Elementary body |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylene diamine tetra acetic acid |
| e.g. | Exempli gratia (for example) |
| ERK | Extracellular signal regulated kinase |

**F**

| | |
|---|---|
| FADD | Fas associated death domain |
| FBS | Fetal bovine serum |

**H**

| | |
|---|---|
| hpi | Hours post infection |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HIV | Human immunodeficiency virus |
| HMBG1 | High mobility Box group 1 |
| Hsp | Heat Shock Protein |

**I**

| | |
|---|---|
| IAPs | Inhibitor of apoptosis proteins |
| IB | Immunoblotting |
| ICE | Interleukin-1ß-converting enzyme |
| IL-1 | Interleukin 1 |
| i.e. | *id est*, that is |
| IF | Immuno fluorescence |
| IFU | Inclusion forming unit |
| IκB | inhibitor of kappa |
| Ig | Immunoglobulin |
| IPAF | ICE protease activating factor |

**K**

| | |
|---|---|
| kDa | Kilo Dalton |

**L**

| | |
|---|---|
| l | Liter |

| | |
|---|---|
| **M** | |
| M | Molar |
| mM | Milli molar |
| µM | Micro molar |
| nM | Nano molar |
| MAPK | Mitogen activated protein kinase |
| Mcl-1 | Myeloid cell lymphoma-1 |
| MEK | MAP ERK kinase |
| MOI | Multiplicity of infection |
| ms | Mouse |
| MMP | Mitochondria membrane potential |
| min | Minutes |
| **N** | |
| NF-κB | Nuclear Factor kappa B |
| **P** | |
| PARP | Poly ADP ribose polymerase |
| PBS | Phosphate buffer saline |
| PCD | Programmed cell death |
| PCR | Polymerase chain reaction |
| PFA | Paraformaldehyde |
| PVDF | Polyvinylidene fluoride |
| **Q** | |
| qRT PCR | Quantitative Real Time PCR |
| **R** | |
| rb | Rabbit |
| RB | Reticulate body |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RT | Room temperature |
| **S** | |
| SD | Standard deviation |

| | |
|---|---|
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | SDS polyacrylamidegel electrophoresis |
| s | Second |
| Sn | *Simkania negevensis* |
| siRNA | Small interfering RNA |
| SPG | Sucrose phosphate glutamate |
| STS | Staurosporine |
| SSC | Saline sodium citrate |
| **T** | |
| TNF-α | Tumor necrosis factor alpha |
| tBid | Truncated BID |
| TBS | Tris buffer saline |
| TLR | Toll like receptor |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labelling |
| **V** | |
| v/v | Volume per volume |
| **W** | |
| w/v | Weight per volume |

## IV. Summary

*Chlamydiales* are obligate intracellular gram-negative bacteria that have gained high medical relevance. These important human pathogens cause diverse diseases including trachoma and wide spread sexually transmitted diseases. *Chlamydia* establishes membrane bound inclusions in the host cell and loots the host for nutritional requirements. Infections are usually recognized by the host immune system and eliminated systematically, by triggering apoptosis. However, the pathogen *Chlamydia* has evolved various strategies to prevent the detection as well as protect the invaded cell against apoptosis or any other form of cell death. The evolutionary conservation of cell death regulation has not been investigated in the order *Chlamydiales*, which also includes *Chlamydia*-like organisms with a broader host spectrum. The present study was aimed at investigating the apoptotic response of human cells infected with the *Chlamydia*-like organism *Simkania negevensis* (Sn).

*Simkania* infected cells exhibited strong resistance to apoptosis induced by intrinsic stress or by the activation of cell death receptors. Apoptotic signaling was blocked upstream of mitochondria since Bax translocation, Bax and Bak oligomerisation and cytochrome c release were absent in these cells. Caspases were differentially regulated upon Sn infection. Caspase-3 and -9 were not activated upon Sn infection and apoptosis induction; whereas caspases-8 was activated in Sn infected cells even without apoptosis induction. This indicates that, Sn utilizes death receptor association independent caspase activation for thriving in the host environment. Infected cells turned on pro-survival pathways like cellular Inhibitor of Apoptosis Proteins (IAP-1/2 and XIAP) and the Akt/PI3K pathway. Sn infection also

activated the pro-survival transcription factor NF-κB. Blocking any of these survival pathways sensitized the infected host cell towards apoptosis induction, demonstrating their role in infection-induced apoptosis resistance. The NF-κB mutant cells also showed reduced infectivity of Sn, which indicated an essential role of NF-κB in Sn infection.

It was interesting to observe that, *Acanthamoeba castellanii*, a natural host of Sn, survived maintaining its trophozoite forms after infection with Sn upon starvation. The metacaspases, responsible for encystment could be regulated by Sn upon infection. This suggests an early level of gene regulation indicating how the pathogen evolved its ability to inhibit apoptosis in higher organisms.

The resistance to apoptosis pathways subverted in Sn-infected cells was similar but not identical to those modulated by *Chlamydia*. Together, the data supports the hypothesis of evolutionary conserved signaling pathways to apoptosis resistance as common denominators in the order *Chlamydiales*.

# V. Zusammenfassung

Vertreter der Ordnung *Chlamydiales* sind obligat intrazelluläre gram-negative Bakterien mit einer zunehmenden medizinischen Relevanz. Diese Humanpathogene lösen verschiedene Krankheiten aus, unter anderem Blindheit und sexuell übertragbare Krankheiten. *Chlamydia* führt zur Ausbildung einer Membran-umschlossenen Inklusion innerhalb der Wirtszelle und entzieht dem Wirt die nötigen Nährstoffe. Infektionen können vom Immunsystem des Wirts erkannt und systematisch mittels Apoptose bekämpft werden. *Chlamydia* hat jedoch verschiedene Strategien entwickelt um der Erkennung durch das Immunsystem zu entgehen und um ihre Wirtszelle vor dem Zelltod zu schützen. Ob die Fähigkeit den Zelltod der Wirtszelle zu regulieren evolutionär innerhalb der Ordnung der *Chlamydiales*, welche auch Chlamydia-ähnliche Organismen mit einem breiteren Wirtsspektrum beinhalten, konserviert ist wurde bislang noch nicht untersucht. In dieser Arbeit wurde das apoptotische Verhalten humaner mit dem Chlamydia-ähnlichen Organismus *Simkania negevensis* (Sn) infizierter Zellen untersucht.

Mit Simkanien infizierte Zellen zeigen eine starke Apoptose-Resistenz, unabhängig ob diese über intrinsischen Stress oder durch die Aktivierung von Todesrezeptoren ausgelöst wurde. Die Apoptose-Signalkaskade wurde vor den Mitochondrien blockiert, da es weder zu einer Bax Translokation, Bax und Bak Oligomerisierung noch zur Cytochrom c Freisetzung in den infizierten Zellen kam. Auch Caspasen zeigten eine veränderte Regulation. Die Caspase-3 und -9 wurden während einer Sn-Infektion und Apoptose-Induktion nicht aktiviert. Die Caspase 8 jedoch ist während der Sn-Infektion unabhängig von einer Apoptose-Induktion aktiv. Diese Tatsache lässt vermuten, dass Sn sich eines von den Todesrezeptoren

unabhängigen Mechanismus der Caspaseaktivierung bedienen um in der Wirtzelle zu verbleiben. Infizierte Zellen zeigen eine Aktivierung verschiedener antiapoptotischer Signalwege wie der der Inhibitor of Apoptotis Proteins (IAP1/2 and XIAP)- und den Akt/PI3K Signalweg. Durch eine Sn-Infektion kommt es außerdem zur Aktivierung des Transkriptionsfaktors NF-kB. Wird einer dieser Überlebenssignalwege blockiert zeigen die infizierten Wirtszellen eine erhöhte Sensitivität gegenüber einer Apoptoseinduktion, was wiederum die zentrale Rolle der infektionsvermittelten Apoptoseresistenz aufzeigt. Eine Zelllinie mit mutiertem NF-kB zeigte eine verminderte Infektiosität von Sn, was die zentrale Rolle von NF-kB in der Sn-Infektion unterstreicht.

Infektionsversuche mit Sn und dessen natürlichem Wirt *Acanthamoeba castellanii* zeigten, dass diese im Sn-infizierten Stadium die Aufrechterhaltung ihrer Trophozyten-Form nach Nährstoffmangel überlebten. Die Metacaspasen, welche für die Einkapselung verantwortlich sind konnten durch eine Sn-Infektion reguliert werden. Dies weist auf ein frühes Stadium der Genregulation hin und lässt vermuten wie die Pathogene die Fähigkeit zur Apoptose-Inhibition höherer Organismen erlangt haben könnten.

Die durch eine Sn-Infektion vermittelte Resistenz der Wirtszelle gegenüber Apoptose-Signalwegen ist ähnlich, jedoch nicht identisch zu der durch *Chlamydia* vermittelten Resistenz. Diese Ergebnisse unterstützen die Hypothese eines evolutionär konservierten Signalwegs der Apoptoseresistenz als allgemeiner Nenner in der Ordnung der *Chlamydiales.*

# Introduction

## 1.1. *Chlamydiales*

The bacterial order *Chlamydiales* includes obligate intracellular bacteria that have a *Chlamydia*-like developmental cycle and at least 80% 16S rRNA or 23S rRNA gene sequence identity. They infect cells of vertebrates (causing relevant diseases) and amoebae, while similar particles have been reported in invertebrate species including coelenterates, arthropods and mollusks.

### 1.1.1. History

*Chlamydia*-like disease 'trachoma' has been described in ancient Chinese and Egyptian manuscripts. In 1907, Halberstaedter and von Prowazek took conjunctival scraping from infected individuals and inoculated into the eyes of orang-utans (Fields and Barnes 1999). They named the new organism they found Chlamydozoa. Similar inclusions were subsequently described in the conjunctival cells of babies with non-gonococcal ophthalmia neonatorum in the uterine cervix from their mothers and in the urethral epithelium from male patients with non-gonococcal urethritis. These particles were capable of passing filters that retained most other bacteria. This property, coupled with the inability of these agents to grow on artificial media, led to the erroneous belief that these agents were viruses. Later in 1932 similar particles were reported in birds with acute pneumonia, psittacosis (Rivers & Schwentker, 1932). The finding of a common complement-fixing antigen strengthened the idea that these agents are those of Lymphogranuloma venereum (LGV) and hence was first propagated in monkey brain. The term 'Chlamydia' (Gr. Chlamus, a cloak) appeared in the literature in 1945. In 1965 with the advent of tissue culture

techniques and electron microscopy, evidence for bacterial rRNA, ribosomes and cell wall structures in *Chlamydiae*, it became evident that they were not viruses (Graham, 1965). Thus these particles were set out for further studies and classification under the order *Chlamydiales.*

### 1.1.2. Taxonomy

For years, *Chlamydiales* was the only bacterial order that had just one family and one genus (the *Chlamydiaceae* and *Chlamydia*, respectively). In the 1990s, based on the divergence in the glycogen accumulation inside the inclusion (Gordon & Quan, 1965) and resistance towards sulfadiazine (Lin & Moulder, 1966) the family *Chlamydiaceae* contained two species, *Chlamydia pneumoniae* and *Chlamydia pecorum* [both now placed into the genus *Chlamydophila*]. New molecular diagnostic methods based on nucleic acid amplification led to *Chlamydiae* being discovered in tissues and cells never before reported (joints, atherosclerotic plaques, brains, amoebae) (Table 1.1) and associated with diseases of previously unknown etiology like reactive arthritis, Alzheimer's disease, coronary artery disease, etc led to the deposition of 40 more chlamydial strains in the American Type Culture Collection (ATCC). DNA-DNA re-association, in particular, was established as a tool for distinguishing species (70% homology) and genera (20%homology) (Amann et al, 1995; Schleifer & Stackebrandt, 1983; Wayne & Diaz, 1987). 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 amoebae isolate *Parachlamydia acanthamoebae* (Amann et al, 1997). With all this existing findings, Everett et al

(1999) made the new taxonomy of the order *Chlamydiales* which split the former family *Chlamydiaceae* into two genera, *Chlamydia* and *Chlamydophila* [the name *Chlamydophila*, given by Hans Truper and Johannes Storz, means 'chlamydia-like'], encompassing nine species, and added three new known *Chlamydiaceae* families, the *Parachlamydiaceae, Waddliaceae* and *Simkaniaceae*. Later (Schachter et al, 2001) mentioned that as there is no sufficient sequence difference in the order *Chlamydiales*, a division of genus into *Chlamydophila* and *Chlamydia* is unnecessary. Hence a modified version of the phylogenetic tree of *Chlamydiales* is shown in Figure 1.1.

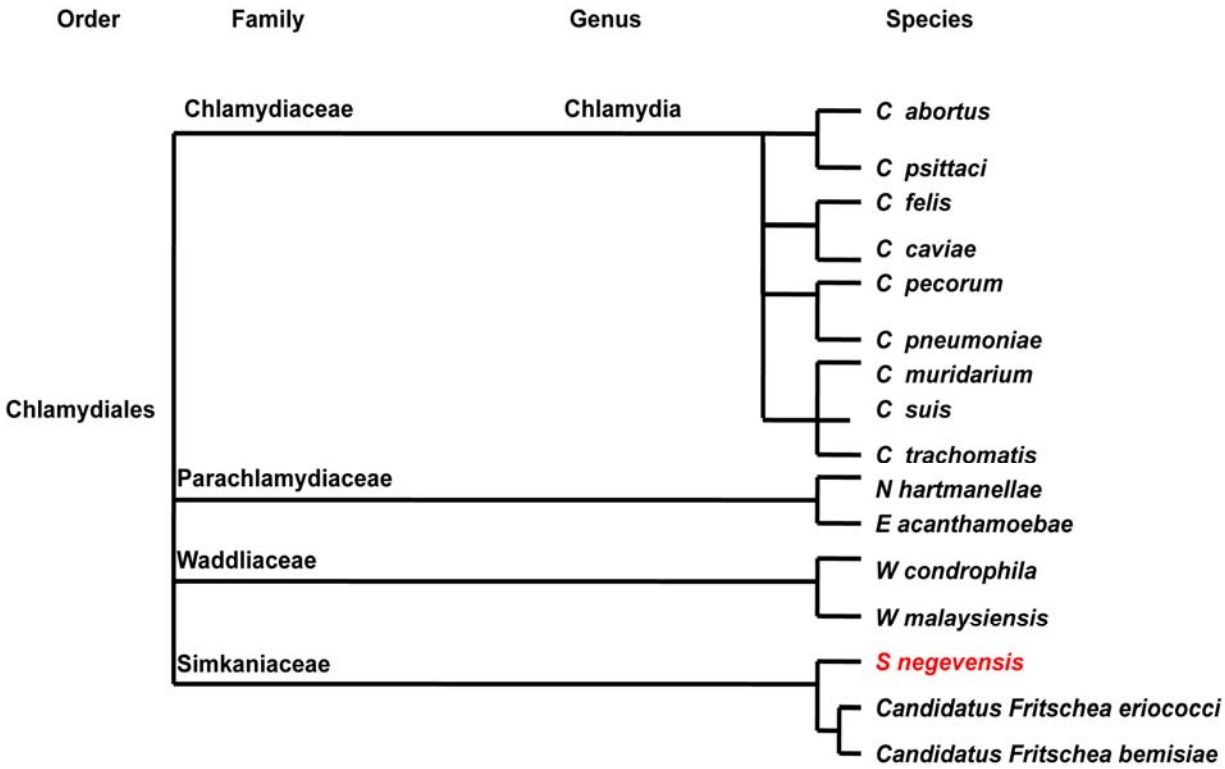


**Figure 1.1:** The phylogenetic tree of *Chlamydiales*. Based on the 16S and 23S ribosomal RNA sequence analysis, four families of *Chlamydiales* were classified with single genera in the family *Chlamydiacea.* Subsequently 16 species (many serovars not mentioned) identified are classified above

with the respective hosts. The length of the lines does not represent the phylogenetic distance (modified from (Everett et al, 1999)). The pathogen under study is depicted in red.

| Species | Host | Route of entry |
|---|---|---|
| *C.muridarum* | Mouse, hamster | Pharyngeal, genital |
| *C.suis* | Swine | Pharyngeal |
| *C. trachomatis* | Human | Pharyngeal, ocular, genital, rectal |
| *C. abortus* | Mammals | Oral, genital |
| *C. caviae* | Guinea pig | Pharyngeal, ocular, genital, urethral |
| *C. felis* | Cat | Pharyngeal, ocular, genital |
| *C. pecorum* | Mammals | Oral |
| *C. pneumoniae* | Human, frog, koala, horse | Pharyngeal, ocular |
| *C. psittaci* | Birds | Pharyngeal, ocular, genital |
| *S.negevensis* | Amoeba, human | Pharyngeal |

(Modified after (Everett, 2000))

**Table 1.1.** List of different species in the genus *Chlamydia*, the specific hosts and the route of entry and site of infection.

### 1.1.3. Pathogenicity and epidemiology of *Simkania negevensis* (Sn)

*Chlamydiae* are obligate intracellular bacteria that cause a variety of acute and chronic diseases with high prevalence rate. The increase in reported chlamydial infections during the last 20 years reflects the expansion of clinical studies to understand better the virulence mechanism and the biology of the pathogen.

Discovered recently, *Simkania negevensis* is another obligate intracellular bacterium, isolated as a cell culture contaminant of unknown origin (Kahane et al, 1993). To date only one strain of *Simkania*, $Z^T$ (ATCC VR-1471$^T$) (Everett et al, 1999) has been recognized. It has been suggested that Sn is an ubiquitous microorganism present in most aquatic habitats because of its relative resistance to commonly used procedures for routine treatment of drinking water supplies and its ability to replicate and exist in amoeba (Kahane et al, 1998). Later *Simkania* was reported as an emerging pathogen associated with several types of respiratory tract infection such as bronchiolitis in infants (Lieberman, Kahane et al. 1997; Kahane, Greenberg et al. 1998; (Greenberg et al, 2003; Greub, 2009), community acquired pneumonia (Guttmacher et al, 1997; Heiskanen-Kosma et al, 2008; Nascimento-Carvalho et al, 2009), chronic obstructive pulmonary disease in adults (Lieberman et al, 2002) and acute rejection in lung transplant recipients (Husain et al, 2007). Sn has also been amplified from an aortic aneurysm (Friedman et al, 2003). Moreover, seroprevalence rates in adults between 46–80% suggest a broad distribution of the organism in human populations (Lieberman et al, 1997). Sn has been suggested to represent a newly emerging pathogen because of its versatile infection capability (Friedman, Dvoskin et al. 2003; Kahane, Fruchter et al. 2007).

*Simkania* replicates in a biphasic life cycle similar to *Chlamydia*. Infection starts with an electron dense elementary body (0.2-0.3 µm), which differentiates into the electron lucent replicative form called the reticulate body (0.3-0.7 µm). The active replication cycle of Sn takes about 3–5 days and extensive long-term relationship with the host cell of about 10–15 days has been reported (Kahane et al, 2002). The strategy that this pathogen takes to keep its host alive for such a long period of time is unknown. Unlike other *Chlamydiales* (Kahane et al, 2002) observed that in Sn the replicative form may also be infective. This assumption was derived from the fact that they could observe similar kinetics of growth curve in less dense (RB) and more dense (EB) urografin fractions.

For decades research progress has been hampered to fully understand the genetics of *Chlamydiales* as they are obligate intracellular pathogens, there is no means to genetically manipulate them. Recently (Wang et al, 2011) had discovered that foreign DNA could be introduced into the chlamydial inclusion by using calcium ions. This will pave way for the chlamydial genetics and could lead to the development of chlamydial vaccines and therapeutic interventions.

### 1.1.4. The chlamydial life style

*Chlamydiales* have a unique biphasic life cycle. Bedson and colleagues first observed these distinct particles when they examined psittacosis-infected tissue (Bedson et al 1932). Later the existence of these developmental forms confirmed under electron microscopic studies (Constable, 1959; Gaylord, 1954). The pathogen exists in two morphological forms namely elementary body (EB), which is infective,

but metabolically inactive and reticulate body (RB), metabolically active and non-infective. The EB is 0.3 μm in diameter and RB around 1 μm. Infection is initiated by adherence of EB to the host cells through an unknown receptor that probably binds with heparan sulfate-like glycosaminoglycan present on the cell surface (Gutierrez-Martin et al, 1997; Kuo et al, 1973). Several other ligands for *Chlamydia* binding have also been proposed, including Hsp70 and Omp2 (Joseph & Bose, 1991; Raulston et al, 1998; Su et al, 1996; Swanson & Kuo, 1994; Ting et al, 1995). 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 III secretion system (T3SS), a needle-like protein complex that spans the bacterial membranes (Subtil et al, 2000). Cytoskeleton rearrangements induce the uptake of the EB, which can also be seen in non-phagocytic cell (Jewett et al, 2008). After binding, the *Chlamydia* is internalized, enveloped within membrane-bound compartments that are subjected to bacteria induced modifications both in their luminal environment and in their membrane composition, and transported to a perinuclear location. These modified *Chlamydia*-containing membrane compartments are termed inclusions. The bacteria appear to survive within the inclusion in host epithelial cells through their ability to inhibit fusion between the inclusions and lysosomes (Eissenberg et al, 1983; Scidmore et al, 1996a; Scidmore et al, 1996b) whereas the fusion to sphingomyelinrich vesicles is enhanced (Hackstadt et al, 1995).

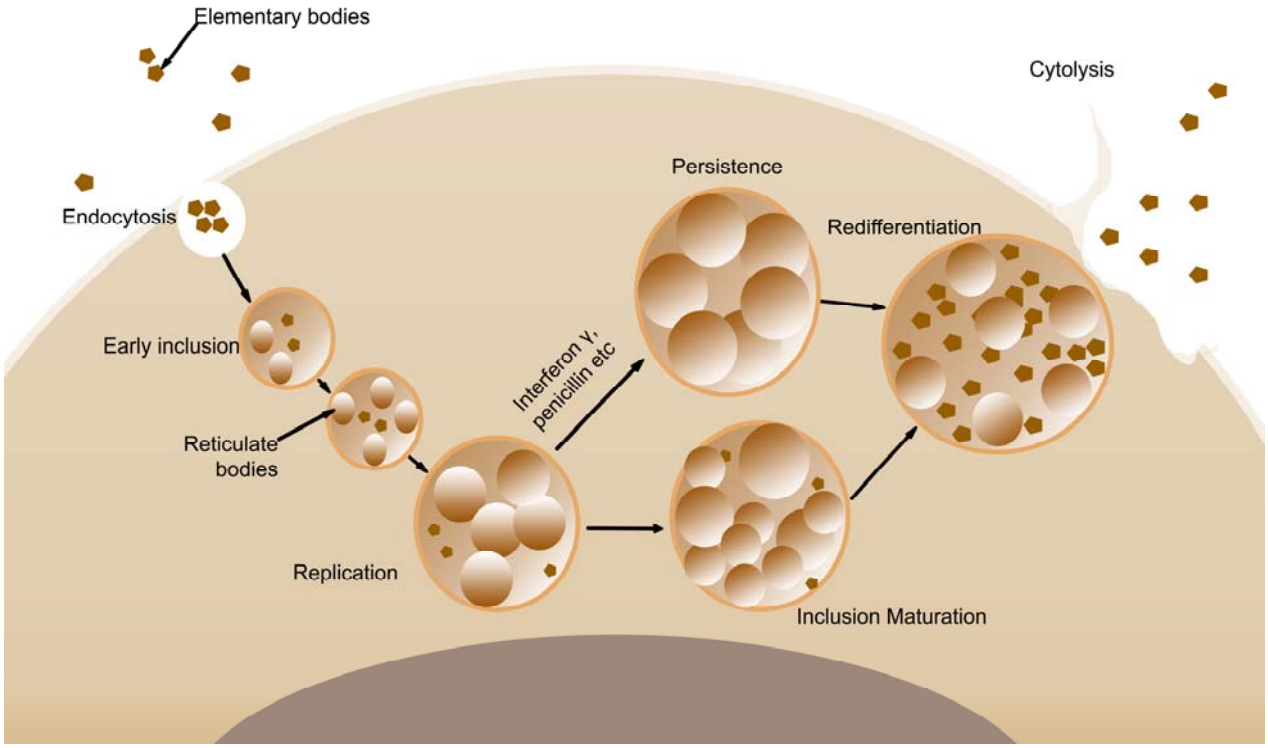


**Figure 1.2.** Infectious EB adhere to the receptors on the host cell. Upon internalization, the chlamydial inclusion is formed where EB differentiate into RB, which replicate by binary fission to produce several hundred particles that re-differentiate into infectious EB which leave the cell in order to start a new round of infection. Under certain conditions, such as sub-lethal doses of antibiotics like penicillin, IFN-γ stimulation or co-infection with virus, aberrant bodies can be formed to persist inside the host cell. Depending on the species, the developmental cycle is completed after 48-72 h or 5-8 days in tissue culture cell lines.

Within the inclusions, the EB differentiates into RB, which synthesize proteins and their own DNA and RNA then replicate by binary fission to form micro-colonies within the inclusion bodies. Differentiation of an EB into RB involves an increase in size, reduction of disulphide links in the outer membrane, unraveling of DNA in the condensed nucleoid and appearance of granular ribosomes (Wang et al, 1994). The initial signal for the differentiation of EB into RB is not known. By 18 hours post

infection (hpi), a proportion of the developed RB begins to transform back into EB while the remainder continues to divide in the growing inclusion (Hackstadt et al, 1999) within 24 h large inclusions are formed. Between 24 and 72 hpi the RB re-differentiate into EB and are released into the extracellular space by rupturing the cells to start a new round of infection (Moulder, 1991) as shown in Figure 1.2. As the RB is not infectious, re-differentiation into EB is an essential step in the chlamydial developmental cycle for the establishment of an acute infection. The engagement of the chlamydial type III secretion system in RB to EB re-differentiation was recently discussed by (Peters et al, 2007). The environmental signal that induces the reorganization from RB to EB is undefined. In the presence of Interferon γ, virus infection or antibiotics like penicillin the chlamydial particles gets stuck reversibly in the persistence stage where only aberrant bodies are observed. Persistence is the viable but non-cultivable growth stage of *Chlamydia* where it maintains long term relation with the host cells (Hogan et al, 2004). Depending on the species and the amount of bacteria that entered the cell, the developmental cycle of *C. trachomatis, C. pneumonia* and *Simkania negevensis* in cultured cell lines is completed after 48 h, 72 h and 5 days, respectively.

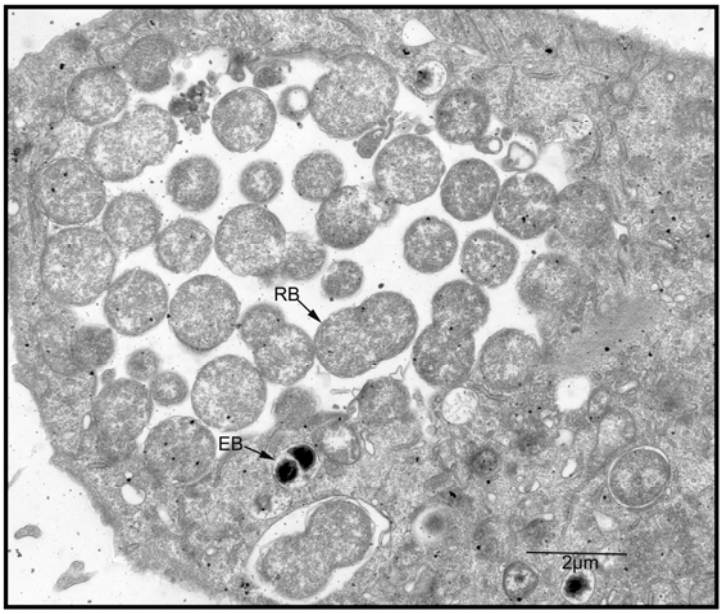


**Figure 1.3.** Transmission electron micrograph of intracellular *Chlamydia* in membranous vacuoles of an infected cervical cancer cell. Black arrowheads indicate the condensed elementary bodies (EB) and multiplying reticulate bodies (RB). Bar-2µm

## 1.2. Cell death: Evolution

Cell death has historically been subdivided into regulated and unregulated mechanisms. It is a fundamental cellular response that has a crucial role in shaping our body during development and in regulating tissue homeostasis by eliminating unwanted cells. The first form of regulated or programmed cell death (PCD) to be characterized was apoptosis, which was described in *Caenorhabitis elegans* in the early 1990s (Horvitz et al, 1994). PCD was first defined as the active physiological process of cell death depending on signaling events in the dying cell (Lockshin & Williams, 1965). Later different types of PCDs were characterized, including the apoptosis (type I PCD) and autophagy (typeII PCD).

Apoptosis reflects a cell's decision to die in response to cues and is executed by intrinsic cellular machinery. Apoptosis involves controlled and well organized

events leading to cell death, in contrast the uncontrolled cell death (necrosis) entails a rapid loss of membrane integrity and release of cellular components into the extracellular space which leads to damage of neighboring cells and elicitation of immune response (Wyllie et al, 1980). 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 & Brennan, 2001). Due to its features resembling parts of necrosis, a fourth type of PCD has lately been proposed, termed pyronecrosis (Willingham, Bergstralh et al. 2007). Both the lack and excess of cell death are 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)

Emerging evidence, however, suggests that the above said types of cell death do not adequately explain the various cell death mechanisms observed. Recent data point to the existence of multiple non-apoptotic, regulated cell death mechanisms, some of which overlap or are mutually exclusive with apoptosis (Degterev & Yuan, 2008). In addition to serving the maintenance of the body, the immune system activates PCD to eliminate the infected cell from the organism. Hence it is not surprising that the microbial pathogens have evolved a variety of strategies to modulate host cell death and subvert normal host defense response (Best, 2008).

### 1.2.1. Morphological and biochemical features of apoptosis

Apoptosis, 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). Morphologically apoptosis is characterized by plasma membrane blebbing, cell body shrinkage (pyknosis), nuclear condensation and fragmentation (karyorrhexis), and formation of membrane bound cell fragments (apoptotic bodies). The apoptotic bodies consist of cytoplasm and tightly packed organelles enclosed within an intact plasma membranethat are rapidly phagocytosed *invivo* by neighboring cells and resident phagocytes. This helps to prevent any inflammatory response, as the cytoplasmic content is not spilled into the extracellular milieu and the engulfment of apoptotic cells may actively prevent the production of inflammatory cytokines by macrophages and suppress dendritic cell maturation and antigen presentation (Brown et al, 2002). Biochemical features of apoptosis include a decrease in mitochondrial inner transmembrane potential, the activation of selective proteases leading to restrictive hydrolysis of more than 400 proteins, the cleavage of chromosomal DNA into internucleosomal fragments, the selective cleavage of various cellular proteins, and the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane (Salvesen and Riedl, 2008; Strasser et al., 2000). The main proteases involved in this extensive hydrolysis are members of caspase family (Chinnaiyan & Dixit, 1996) besides caspases, other proteases like lysosomal cathepsin, calpains and granzymes are also characterized in the apoptotic process (Lord et al, 2003). The specific morphological and biochemical features of apoptosis are manifestations of

evolutionarily conserved cell suicide machinery comprising of many apoptosis mediators described below.

### 1.2.2. Apoptosis-The fundamental mechanism of PCD

A simple pathway controls the primordial paradigm of PCD, described first in *Caenorhabitis elegans*. The initiation of apoptosis is regulated by transcriptional upregulation of Egl-1, a pro-apoptotic Bcl-2 homology-3 (BH3)-only member of the Bcl-2 protein family (Conradt & Xue, 2005). Binding of Egl-1 to anti- apoptotic CED-9 relieves the inhibition that CED-9 exerts on the adaptor protein CED-4, allowing CED-4 to bind and activate the cysteine protease CED-3, which in turn cleaves multiple specific cellular substrates to execute cell death (Metzstein et al, 1998) as described in Figure 1.4. Later, the mammalian homologues for the CED protein were revealed, though more complex, the mechanism seems to be highly conserved.

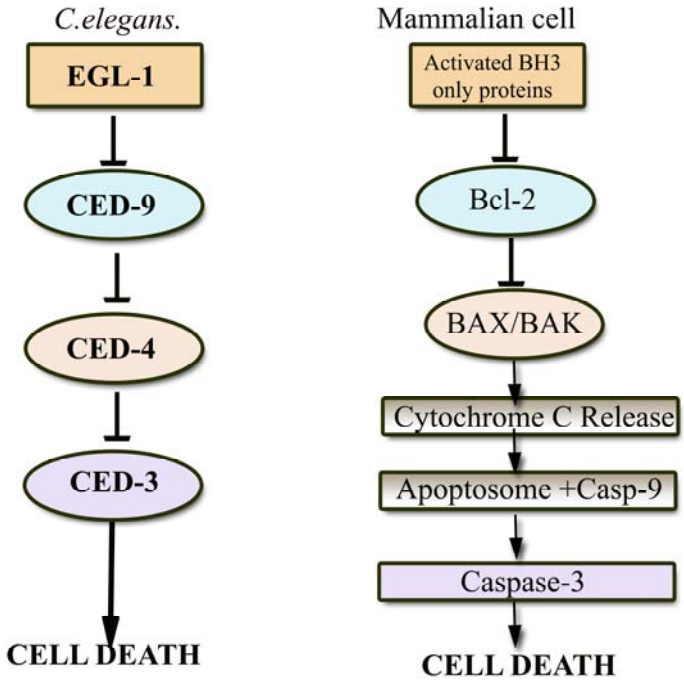

**Figure 1.4**. Comparison of cell death in *C. elegans* and mammalian cells. Extension of the apoptotic machinery can be observed at every step of the pathway, including BH3-only-protein activating signals, complex regulation of the Bcl-2 family and the addition of mitochondrial cytochrome c release, which drives the formation of an apoptosome and activation of the upstream caspases (first caspase-9 and then the executioner caspases, such as caspase-3 and caspase-7). Added complexity is provided by the existence of multiple family members in each class of the apoptotic regulators. These regulators provide 'fail-safe' apoptosis machinery that can generate specialized responses to various upstream stimuli.

### 1.2.3. Caspases-The Major class of apoptosis mediators

Caspases, the interleukin-1β-converting enzyme (ICE) is a family of cysteine proteases (Chinnaiyan & Dixit, 1996). They are highly homologues to CED-3 in *C. elegans*. Fourteen different caspases are identified so far in humans (Alnemri et al, 1996), with three major functions, apoptosis activation, apoptosis execution and inflammatory mediation (Table 1.2). Caspases and their homologues are present in diverse species including nematodes, yeast and dipterans (Kumar & Doumanis, 2000).

The caspase family members share similarities in the amino acid sequence, structure and also the substrate specificity among each other (Nicholson & Thornberry, 1997). Caspases have an absolute requirement for cleavage after an aspartic acid in the substrate, but there is some exception to this rule that some caspases can cleave their substrate after a Glutamate (Hawkins et al, 2000). The protease activity of caspases is highly efficient, but not all proteins containing the tetrapeptide sequence X-Glu-X Asp are cleaved by caspases, implying that the

tertiary structural elements are also important for substrate recognition (Thornberry & Lazebnik, 1998). This strict substrate specificity explains why apoptosis does not entail indiscriminate protein digestion, but instead consists of cleavage of specific proteins in a coordinated fashion.

Caspases are synthesized as inactive zymogens in the cells and are activated either by autocatalytic processing by adaptor-protein mediated aggregation or by proteolysis by other active caspases (Thornberry & Lazebnik, 1998). Caspases are characterized by almost absolute specificity for aspartic acid in the $P_1$ position. Caspases contain three main domains: a pro-domain, large (p20) and small (p10) catalytic subunits (Thornberry & Lazebnik, 1998) as shown in Figure 1.5. The pro-domains of activator and inflammatory caspases contain protein–protein interaction domains (such as the caspase recruitment domain (CARD) and the death effector domain (DED) that link them to apoptosis signaling molecules (Degterev et al, 2003). The large domain contains the active site cysteine residue (Figure 1.5). All the caspases (ICE-like proteases) contain a conserved QACXG (where X is R, Q or G) pentapeptide active site motif. Activation of caspases involves the proteolytic cleavage of zymogens by auto-activation of pro-domain and activating other caspases to produce a heterodimer, two of which forms an enzymatically active heterotetramer (Figure 1.5). The activation of caspases can be initiated by the initiator caspases listed in Table 1.2, this inturn activate the downstream effector caspases. The autocatalytic cleavage of caspases is brought about by the close proximity by the adaptor proteins (Muzio et al, 1998). The CARD and DED domains interact with adaptor proteins to bring the caspases together for autocatalytic activity (Adams &

Cory, 2002). The caspase-8 gets activated by sequestering the adaptor protein Fas-Associated Death Domain (FADD) into the Death Inducing Signaling complex (DISC) (Muzio et al, 1998). Caspase-9 gets activated by forming a heteromeric complex, termed 'Apoptosome' with the adaptor protein Apoptotic Protease Activating Factor (Apaf-1) (Ahmad et al, 1998).

| Subfamily | Role | Members |
|---|---|---|
| I | Apoptosis activator | Caspase-2,-8,-9,-10 |
| II | Apoptosis executioner | Caspase-3,-6,-7 |
| III | Inflammatory mediator | Caspase-1,-4,-5,-11,-12,-13,-14 |

**Table 1.2**. Subfamily of Caspases and their function. Based on the homology of the aminoacids the caspases are differentiated into three subfamilies with specific functions

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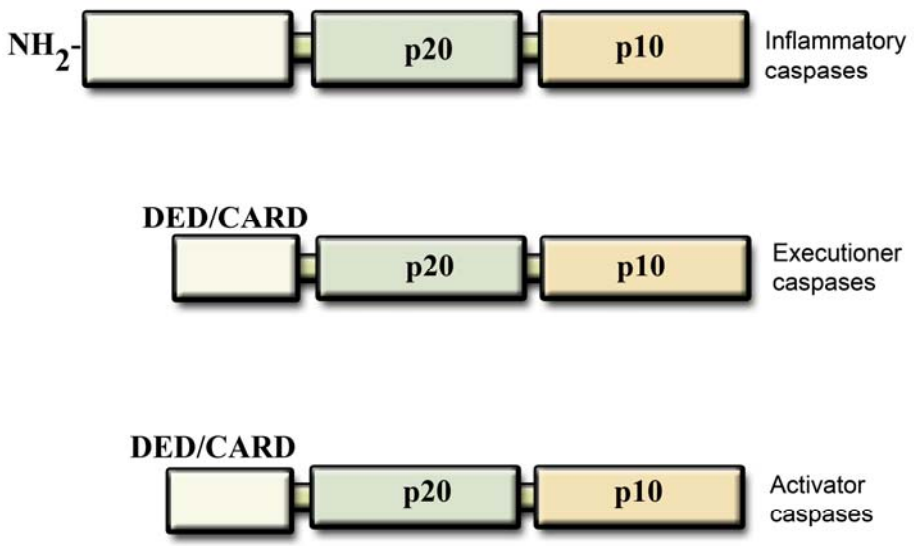

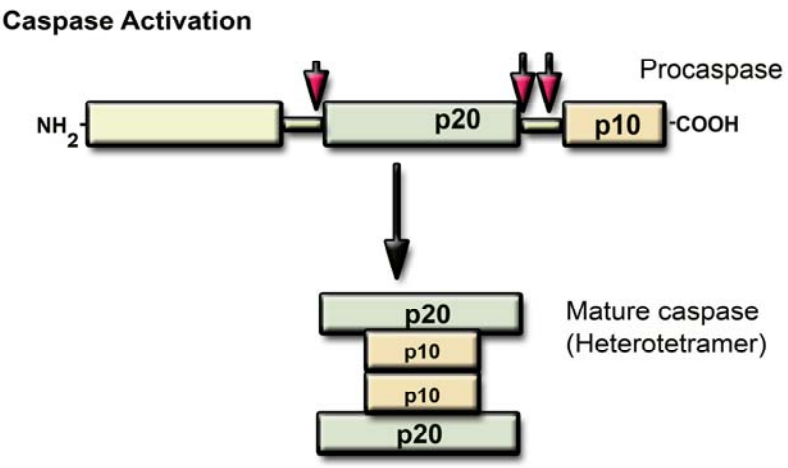


**Figure 1.5.** The sketch of the three major groups of caspases. The executioner and activator caspases have the DED/CARD domain, while the three different classes of caspases have the p20 and p10 domains. Scheme of pro-caspase activation. Cleavage of pro-caspase at the specific Asp-x- bonds leads to the formation of mature caspases, which comprises the heterotetramer $(p20)_2$-$(p10)_2$, and the release of the pro domains.

### 1.2.4. Consequences of caspase activation

During apoptosis, controlled demolition of the major structural components of the cell takes place. The activated executioner caspases, orchestrate the dismantling of the cell structures through cleavage of distinct critical cellular substrates, including poly (ADP-ribose) polymerase (PARP), lamins (Rao et al, 1996), fodrin (Martin et al, 1995), gelsolin, filamin (Kothakota et al, 1997) and DNA PK which are involved in DNA repair, hence this ensues the DNA degradation (Wyllie, 1980). In healthy cells, the caspase activated DNases (CAD) is found in complex with an inhibitor in the form of ICAD (Liu et al, 1997). During apoptosis the ICAD become the substrate of activated caspases and gets degraded liberating the CAD to enter the nucleus and

complete its activity of DNA fragmentation (Enari et al, 1998; Sakahira et al, 1998). Several components of the focal adhesion sites are targets of caspases like, focal adhesion kinases, tensin and components to cell-to-cell adhesions like catenins, cadherins are targets of caspases (Brancolini et al, 1997; Steinhusen et al, 2001). When all kinds of caspase substrates are activated, the cell will go through a series of changes, including the activation of related genes, a decrease in DNA damage repair ability, and the activation of zymogens or inactivation of enzymes, cytoskeleton disassembly, and chromatin fragmentation (Taylor, Cullen et al. 2008). The cell inevitably undergoes apoptosis.

### 1.2.5. Inhibitors of apoptosis (IAPs) the cellular inhibitor of caspases

The IAP proteins, which were originally identified in the genome of Baculovirus on the basis of their ability to suppress apoptosis in infected host cells (Miller, 1999), have also been found in mammals and fruit flies (Salvesen & Duckett, 2002a), but not in nematodes. There are eight mammalian IAPs, which include XIAP (X-linked IAP), c-IAP1, c-IAP2, ML-IAP (melanoma IAP)/Livin, ILP2 (IAP-like protein-2), NAIP (neuronal apoptosis-inhibitory protein), Bruce/Apollon and survivin. The hallmark of IAPs is the Baculoviral IAP repeat (BIR) domain, a ~80-amino-acid zinc-binding domain (Sun et al, 1999). The BIR domains fold as three-stranded ß- sheet surrounded by four α helices that form a hydrophobic core with a Zn ion at the center. The RING domain of IAPs can confer E3 ubiquitin ligase activity to the IAPs conferring to self regulation of the IAP by the degradation by proteasome (Lorick et al, 1999). Other domains in IAPs include the caspase activation and recruitment domain

(CARD), phosphate loop and Ubiquitin Conjugating Domain (UBC) (Figure1.6). The three BIR domains are responsible for mediating protein- protein interaction. Although similar they have specificities for distinct intermediates. BIR2 and BIR3 have been shown to directly bind and inhibit the proteolytic activity of caspases (Eckelman & Salvesen, 2006). BIR2 selectively targets caspase-3 and caspase-7 while BIR3 is specific for caspase-9 and can permanently remove caspases through the ubiquitylation mediated proteasome pathway (Shi, 2002). Interestingly caspase-9 binds to several IAPs but selectively inhibited by XIAP, where as caspase-3 and -7 are inhibited by XIAP, cIAP-1, cIAP-2 and NAIP (Salvesen & Duckett, 2002b). This difference occurs because of the difference in the mechanism of apoptosis inhibition. The cIAP-1 and -2 have a caspase-binding scaffold, while there is no caspase inhibitory activity (Eckelman & Salvesen, 2006). XIAP is hence the most potent among the IAPs, with its BIR2 domain specifically binding to the IAP binding motif of the active site of Caspases (Scott et al, 2005). As the RING domain have the E3 ubiquitin ligase activity, in addition to degrading caspase-3 and -7 they also undergo auto-ubiquitination and cross-ubiquitinate in a RING dependent manner during apoptosis (Yang et al, 2000).

The IAP mediated inhibition of caspases is antagonized by a family of protein that contain IAP binding tetra peptide motif, SMAC/DIABLO, released from the mitochondrial inter-membrane space. SMAC once released into the cytosol can promote the poly-ubiquitination of cIAP-1 and -2 leading to their degradation (Vaux & Silke, 2005a).

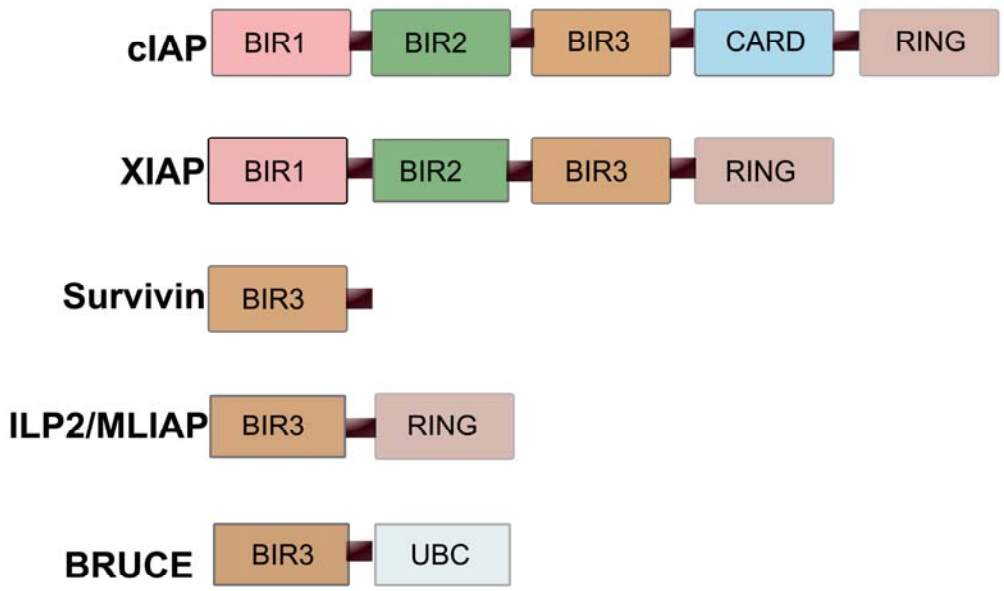


**Figure 1.6.** Molecular sketch of the cellular inhibitor of apoptosis protein. The cIAP-1/2 contains three baculoviral IAP repeat (BIR) domains with a CARD domain, while the CARD domain is absent in XIAP. A single BIR 3 domain makes the IAP, Survivin.

Survivin, which contains a single BIR domain, does not inhibit caspase activity in vitro (Ambrosini et al, 1997). Another single BIR containing IAP, ML-IAP/Livin, was reported to inhibit both caspase-3 and -9 although it does not seem to contain the sequence elements that are required for this inhibition.

### 1.2.6. The Bcl2 family

The first mammalian homolog for *ced-3* was described in 1988 as Bcl-2, which is involved in B-cell lymphomas (Vaux et al, 1988). Bcl-2 transfected B cells were shown to be resistant towards apoptosis normally induced in B cells by IL-3 withdrawal (Cleary et al, 1986; Tsujimoto et al, 1985): for the first time it was shown that the pathway toward tumorogenesis depends not only on the ability to escape

growth control but also depends on the ability to prevent apoptosis (Tsujimoto, 1989). The *bcl-2* gene codes for a 25 kDa protein. The C terminal 21 amino acids encode a stretch of hydrophobic amino acids that are important in membrane docking: Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, the nuclear envelop, and the endoplasmic reticulum. Deletion of the C terminus does not abrogate Bcl-2 survival function (Adams & Cory, 1998). Most Bcl-2 homolog has this hydrophobic C terminal domain, though they not necessarily are located on membranes but are cytosolic (e.g. Bax). While Bcl-2 and its most similar pro-survival homologs Bcl-$_{xl}$ and Bcl-w contain all four BH domains, the other pro-survival members contain at least BH1 and BH2 (Cory & Adams, 2002) (Figure 1.7). Eighteen mammalian B-cell lymphoma protein-2 (Bcl-2) family members were identified and they are subdivided into three sub-classes: anti-apoptotic or pro-survival (Bcl-2, Bcl-$_{xl}$, Bcl-w, Mcl-1 and BFL-1/A1), multi-domain pro-apoptotic (Bax, Bok and Bak) and pro-apoptotic BH3-only (Bad, Bid, Puma, Noxa, BIik, BLK, HRK, BNIP3 and Bim, Bmf).

**BH3-only proteins**

The pro-apoptotic BH3-only proteins are the most apical regulators of this death-signaling cascade, and are activated by multiple stimuli from inside or outside the cell to initiate the apoptotic response. They are regulated transcriptionally, and by post-translational modifications such as phosphorylation, ubiquitination, and proteolytic cleavage. Their BH3 domain is an amphipathic α-helix that serves as a binding motif for interaction with a hydrophobic groove on either multi-domain anti- or pro-apoptotic Bcl-2 family members (Adams & Cory, 1998). This BH3 domain-mediated interaction of BH3-only proteins with multi-domain Bcl-2 family proteins

either antagonizes the survival activity of anti-apoptotic proteins or activates pro-apoptotic Bax and Bak, the antagonism of survival pathway is cooperated by the activation of Bax and Bak (van Delft et al, 2006). The Bax and Bak are kept in check in healthy cells by the specificity of BH3-only proteins.

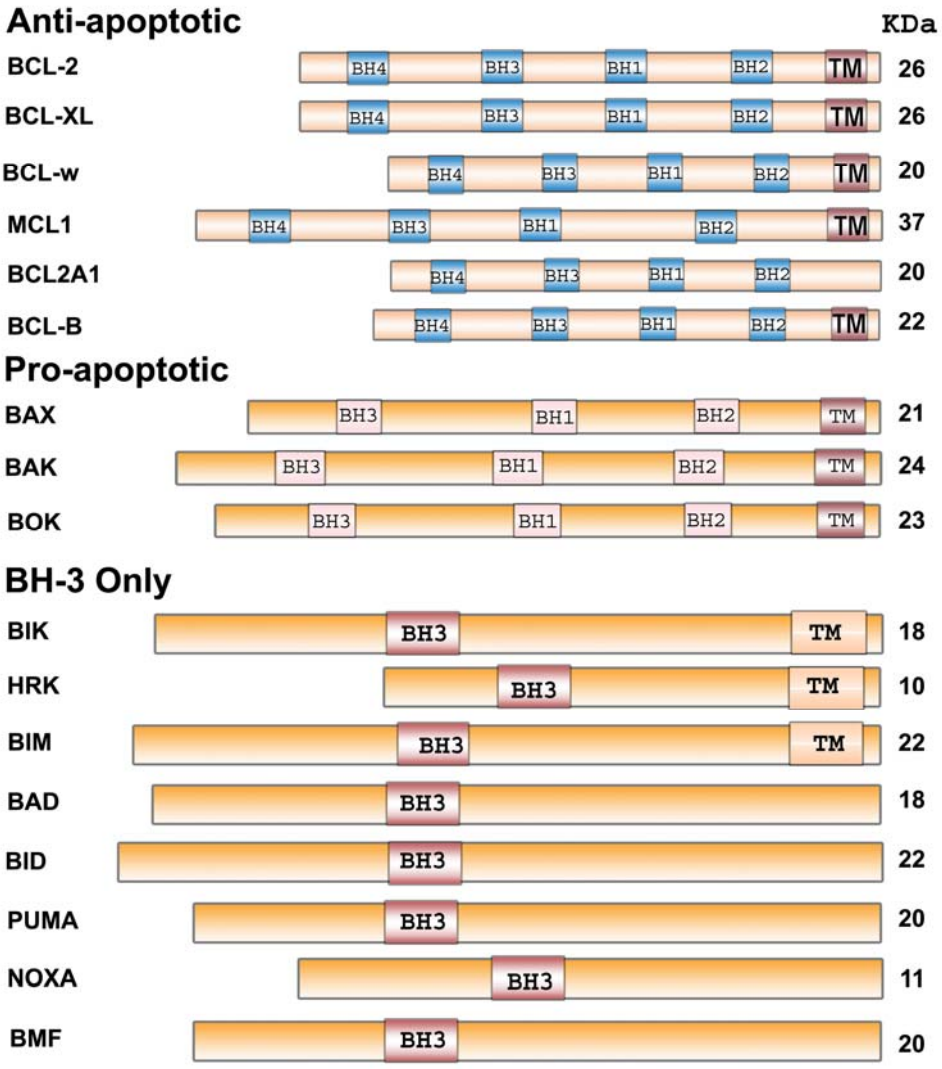


**Figure 1.7.** Pro- and anti-apoptotic family members can heterodimerize: the BH1, BH2 and BH3 domains of an anti-apoptotic member (e.g. Bcl-$_{xl}$) form a hydrophobic cleft to which a BH3 amphipathic alpha helix can bind (Sattler et al, 1997). This BH3 cleft coupling, reminiscent of ligand-receptor

engagement, may account for all dimerization within the family. Heterodimerization is not required for pro-survival function but is essential for the pro-apoptotic activity in the BH3 subfamily.

Bim is required for the removal of auto-reactive B and T cells thus play a role in autoimmunity (Bouillet et al, 2002). It is also induced by growth factor deprivation by class O forkhead box transcription factor-3A (FOXO3A) or by the transcription factors CEPB (CCAAT enhancer binding protein) or CHOP (CEBP homologues protein) in response to endoplasmic reticulum stress (Puthalakath et al, 2007). Bim and Bmf are associated with the microtubules and actin cytoskeleton respectively via interaction with Dynein light chains and their release can induce apoptosis (Puthalakath et al, 2001). It was recently reported that the BH3 peptides could induce mitochondrial fission and cell death in a Bax and Bak negative background (Shroff et al, 2009). Bim, a potent killer have high affinity to pro-survival proteins like Mcl-1 and Bcl-$_{xl}$thereby liberating Bax, which is now unconstrained the default state for cell death (Fletcher & Huang, 2006). Bim, tBid and Puma are known to activate Bax and Bak directly while Bad and Noxa lower the threshold of activation by neutrilising pro-survival Bcl-2 (Cartron et al, 2004; Kuwana et al, 2002; Letai et al, 2002). It is also shown that tBid can induce cell death by release of cytochrome c in an Bax and Bak dependent way, and not in cells lacking one of these (Wei et al, 2001; Zong et al, 2001). This finding indicates that Bax and Bak lie downstream of BH3-only proteins.

**Bax and Bak**

The pro-apoptotic Bax remains in the cytoplasm in healthy cells, as its hydrophobic groove that serves as the receptor for the BH3-only proteins remain occluded by its C terminal trans-membrane domain. During death activation, activated

caspase-8 and tBid signal the conformational change of the protein, which leads to its oligomerisation and mitochondrial membrane localization, which leads to potential loss, and cell death (Annis et al, 2005; Antonsson et al, 2001). In contrast Bak is constitutively associated with the outer mitochondrial membrane where if form large oligomeric complexes in response to apoptotic stimulus (Nechushtan et al, 2001). The Bak in healthy cells are sequestered by Mcl-1 and Bcl-$_{xl}$. While upon induction of apoptosis, Noxa dependent displacement of Bak from Mcl-1 followed by inactivation of Bcl-$_{xl}$ takes place (Willis et al, 2005). Once Bax and Bak oligomers are formed, the mitochondrial permeabilisation is favored, resulting in the release of pro-apoptotic factors into the cytoplasm (Kroemer et al, 2007). Before cytochrome c release Bax and Bak induce the mitochondrial fragmentation (Martinou & Youle, 2006). The BH1 and BH2 domains of Bcl-2 family members (Bcl-2, Bcl-$_{xl}$and Bax) resemble membrane insertion domains of bacterial toxins: hypothetically they can form pores in organelles such as mitochondria, demonstrated in lipid bilayers in vitro. In yeast which lack Bcl-2 like proteins, CED-4 and caspases, Bax and Bak were shown to induce cell death, while Bcl-2 can protect, apparently by preventing mitochondrial disruption (Green & Reed, 1998). Bax and Bax-like proteins might mediate caspase-independent death via channel-forming activity, which would promote the mitochondrial membrane potential lose.

It is known that some of the BH3-only proteins like Bim and Puma, the ‘direct activators of Bax and Bak are more potent apoptosis inducers than the ‘derepressors’ Bad and Noxa (van Delft & Huang, 2006). Studies show that Bim and Puma have high affinity to all pro-survival members, than other members (Chen et al, 2005b).

This suggests that Bcl-2 pro-survival proteins must be neutralized to induce apoptosis. This was confirmed when (Chen et al, 2005b) co-expressed proteins like Noxa (which neutralizes Mcl-1), and Bad (which neutralizes Bcl-2, Bcl-$_{xl}$ and Bcl-w) induced apoptosis in cells.

**The anti-apoptotic Bcl-2 proteins**

The Bcl-2 pro-survival proteins act by directly interacting with Bax and Bak. Bak form complexes with Mcl-1 and Bcl-$_{xl}$ by interacting with BH3 domain (Chen et al, 2005a; Letai et al, 2002). In contrast, the BH3 of Noxa is highly specific for Mcl-1 and BFL-1/A1. The BH3 of Bid and Bim are also capable of binding the grooves of multi-domain Bak and promoting their oligomerization, whereas other BH3 protein likeBad appeared more exclusive for binding to multi-domain of anti-apoptotic proteins such as Bcl-2 and Bcl-$_{xl}$ (Cheng et al, 2001). A central checkpoint of apoptosis is the activation of caspase-9 by mitochondria. The BH4 domain of Bcl-2 and Bcl-$_{xl}$ can bind to the C terminal part of Apaf-1 (Sattler et al, 1997), thus inhibiting the association of caspase-9 with Apaf-1 (Hu et al, 1998; Huang et al, 1998; Pan et al, 1998). This process seems to be conserved from nematodes to vertebrates since in *C. elegans* CED-9 binds to CED-4, preventing it from binding and activating CED-3. This collective type of data leads to the notion of discrete roles for BH3-only protein interactions governed by the binding specificity of their BH3 for multi-domain grooves. The Myeloid Cell Leukemia sequence-1 (Mcl-1) was identified as an early-induced gene during myeloblast cell differentiation. Mcl-1 is one of the most important anti-apoptotic proteins preventing inappropriate activation of Bax and Bak in healthy cells (Cuconati et al, 2003). Mcl-1 have C terminal trans-membrane domain that results in

its localization in the outer mitochondrial membrane (Yang et al, 1995), where it sequester Bak and maintain it in an inactive state. Recently, Sharma et al (2011) showed that the hypoxia-inducing factor (HIF-1α) can stabilize the Mcl-1, which increases its anti-apoptotic effect.

### 1.2.7 NF-κB, the master regulator

NF-κB (Nuclear Factor-KappaB) was initially discovered and characterized as a transcription factor required for B- cell specific gene expression, later it was found to be ubiquitously expressed and required for the inducible expression of many genes (Sen & Baltimore, 1986). NF-κB is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors,which are involved mainly in stress-induced, immune, and inflammatory responses (May & Ghosh, 1998). In addition, these molecules play important roles during thedevelopment of certain hemopoietic cells (Franzoso et al, 1997), keratinocytes, and lymphoid organstructures (Baeuerle & Henkel, 1994; Mattson & Camandola, 2001). More recently, NF-κB family members have been implicated in neoplastic progression and the formation of neuronal synapses (Mattson and Camandola 2001). NF-κB is also an important regulator in cell fate decisions, such as PCD and proliferation control, and is critical in tumorogenesis (Baldwin, 1996b). NF-κB is composed of homo- and hetero-dimers of five members of the Rel family including NF-κB 1(p50), NF-κB 2 (p52), RelA (p65), RelB, and c-Rel (Rel)(Sen & Baltimore, 1986). Hetero and Homo-dimerization of NF-κB proteins which exhibit differential binding specificities includes p50/RelA, p50/c-Rel, p52/c-Rel, p65/c-Rel, RelA/RelA, p50/p50, p52/p52, RelB/p50 and

RelB/p52 (May & Ghosh, 1998). All the Rel proteins contain a conserved N terminal region, called the RHD (Rel Homology Domain). The N terminal part of the RHD contains the DNA-binding domain, whereas the dimerization domain is located in the C-terminal region of the RHD. Close to the C terminal end of the RHD lays the NLS (Nuclear Localization Signal), which is essential for the transport of active NF-κB complexes into the nucleus (Baldwin, 1996a). NF-κB dimers are sequestered in the cytoplasm in un-stimulated cells via non-covalent interactions with a class of inhibitory proteins called I-κB (Baeuerle & Baltimore, 1988b). There are seven IκBs, namely IκB-α, IκB-β, IκB-γ (NEMO), IκB-€, Bcl-3, p100, p105. The IκB have multiple copies of 30 repeating aminoacids called ankyrin repeats, which binds with NF-κB and masks its NLS. Later (Baeuerle & Baltimore, 1988a) reported the inducible activation of NF-κB in response to signals like Phorbol-12 myristate 13 acetate (PMA), which leads to the disruption of cytosolic I-κB. The phosphorylation and degradation via ubiquitination of IκB was later reported (Ghosh & Baltimore, 1990; Kerr et al, 1991) which releases the IκB bound NF-κB. IκB degradation is a tightly regulated process and can be inhibited by proteasome inhibitors (Brown et al, 1995; Chen et al, 1995). The high molecular weight kinases, IKK (IκB kinase), lead to the critical phosphorylation on Ser32 and Ser36 of IκB that initiates its degradation (DiDonato et al, 1997; Mercurio et al, 1997). The Rel/NF-κB proteins can be divided into two groups: Only RelA (p65), RelB and c-Rel contain potent TDs (Trans-activation Domains) within sequences C terminal to the RHD (Ganchi et al, 1992). In contrast, p50 and p52 do not possess TDs, and therefore cannot act as transcriptional activators by themselves (Ganchi et al, 1992).

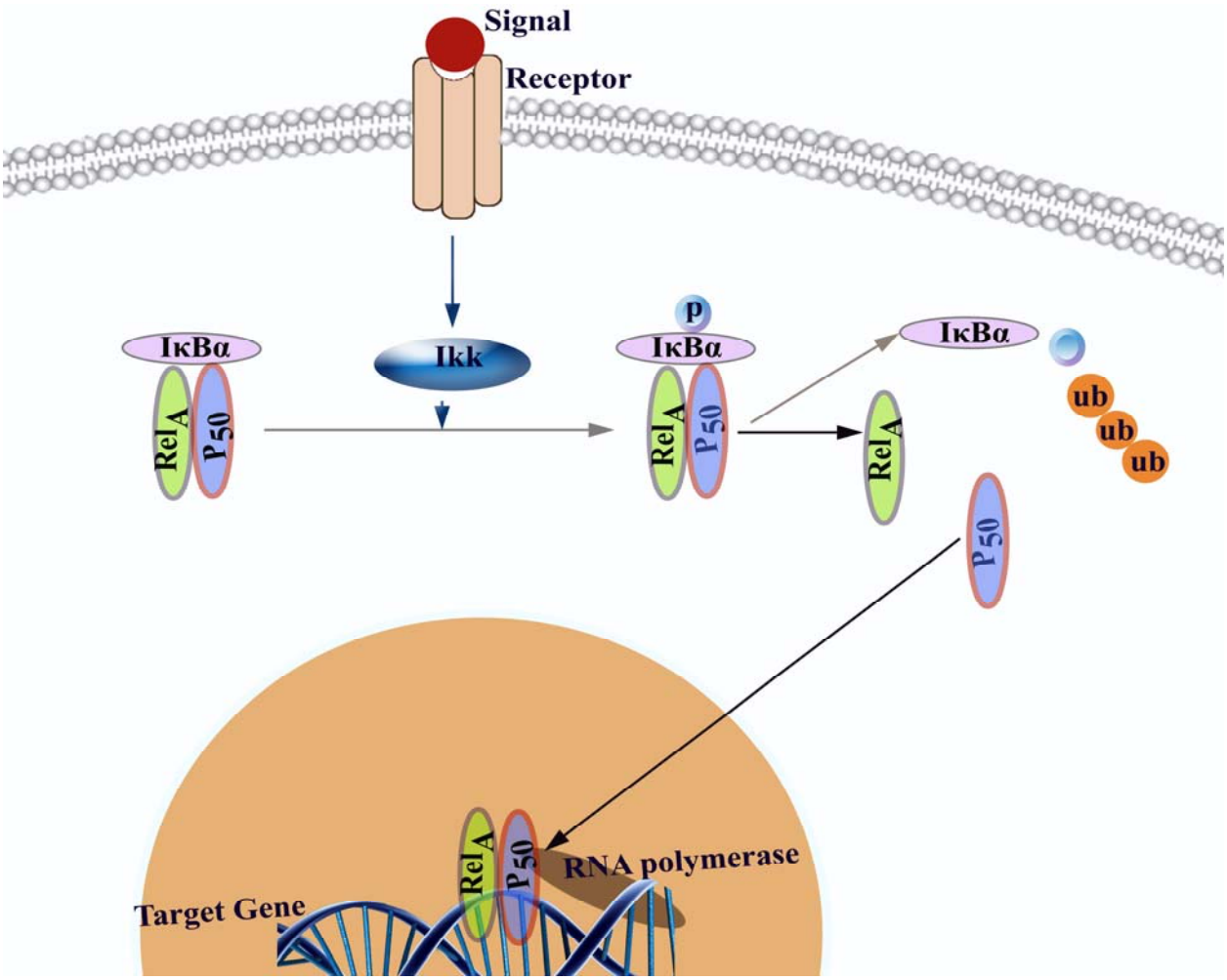


**Figure 1.8**. Mechanism of NF-κB activation. In an inactive stage the components of NF-κB is located in the cytosolic region complexed with the Inhibitor of kappa B (IkB). Upon activation with any extracellular signal, the Inhibitor of kappa B kinases (IKK) is activated and this in-turn phosphorylate the IkB, which results in its ubiquitination. The activated components of NF-κB then translocate to the nucleus and binds to specific DNA sequences called response elements. The DNA/NF-κB complex then recruits other proteins such as co-activators and RNA polymerasewhich results in downstream transcription of the target genes.

NF-κB-1 and NF-κB-2 are produced as p105 and p100 precursors, respectively (Fan & Maniatis, 1991). The NF-κB-1 p105 precursor appears to undergo constitutive processing by the cellular proteasome that removes the C terminal IκB-

like portion to generate p50. NF-κB-2 p100 precursor can be processed to remove the IκB-like C terminus, allowing the active p52 N terminal half to function in transcriptional regulation (Xiao et al, 2004a). Homo- or heterodimers of p50 and p52 were even reported to repress kappaB site-dependent transcription, possibly by competing with other transcriptionally active dimers (e.g. p50/RelA) for DNA binding (Heissmeyer et al, 2001).

NF-κB can be activated by exposure of cells to LPS (Lipopolysaccharides) (Janssens et al, 2002) or inflammatory cytokines such as TNF (Tumour Necrosis Factor) or IL-1 (Interleukin-1), growth factors, lymphokines, oxidant-free radicals, inhaled particles, viral infection or expression of certain viral or bacterial gene products, UV irradiation, B or T-Cell activation, and by other physiological and non physiological stimuli (Dixit & Mak, 2002). The most potent NF-κB activators are the proinflammatory cytokines IL-1 and TNF, which cause rapid phosphorylation of IkBs at two sites within their N terminal regulatory domain (Baldwin, 1996a). TNF, which is the best-studied activator, binds to its receptor and recruits a protein called TRADD (TNF-Associated Receptor Death Domain) (Ermolaeva et al, 2008; Wallach et al, 1999a). TRADD binds to the TRAF2 (TNF Receptor-Associated Factor-2) that recruits NIK (NF-κB -Inducible Kinase) (Hsu et al, 1996; Sakurai et al, 2003). Both IKK1 and IKK2 have canonical sequences that can be phosphorylated by the MAP (Mitogen Activated Protein) kinase NIK/MEKK1 and both kinases can independently phosphorylate IkB-alpha or IkB-beta (Figure 1.8). TRAF2 also interacts with A20, a zinc finger protein whose expression is induced by agents that activate NF-κB. A20 functions to block TRAF2-mediated NF-κB activation (Song et al, 1996). A20 also

inhibits TNF and IL-1 induced activation of NF-κB suggesting that it may act as a general inhibitor of NF-κB activation (Lee et al, 2000).

### 1.2.8. The Akt/PKB signaling pathway

Akt also known as protein kinase B is a serine threonine protein kinase (Bellacosa et al, 1991) that plays a critical role in glucose metabolism, cell proliferation, apoptosis and cell migration (Datta et al, 1999). Akt posses a protein domain called PH domain (plekstrine domain) which have high affinity to phosphoinositides (Franke et al, 1997). Akt is activated by upstream signaling events, as a consequence of the activated second messenger phopholipid kinase phophatidylinositol 3- kinase (PI3K) (Brazil & Hemmings, 2001; Nicholson & Anderson, 2002). Activated receptor tyrosine kinases activate PI3 kinases directly or through tyrosine phosphorylation of scaffolding adaptors like IRIS. The binding of PI3-K generated phospholipids to the PH domain of Akt causes the translocation of Akt from cytoplasm to the plasma membrane (Downward, 1998). This brings Akt in close proximity to membrane bound protein kinase 3- phosphoinositide dependent protein kinase-1 and -2. PI3K phosphorylates $PIP_2$ (phosphatidylinositol 3, 4 bisphosphate) to $PIP_3$ (phosphatidylinositol 3, 4, 5 triphosphate) (Stokoe et al, 1997). These lipids serve as plasma membrane docking sites for proteins that harbor pleckstrin-homology (PH) domains, including Akt and its upstream activator PDK1 (Rong et al, 2001). Once bound to $PIP_3$, Akt can be phosphorylated by its kinases at threonine 308 and serine 473 (Alessi et al, 1997; Sarbassov et al, 2005). Activated Akt can go on to activate or deactivate myriad of substrates. One of the most important functions

signaling downstream directly or indirectly is to regulate the cell survival (Zhou et al, 2000). Akt enhances the survival of cells by blocking the function of pro-apoptotic proteins and processes. Akt negatively regulates the function or expression of several BH3-only proteins, which exert their pro-apoptotic effects by binding to and inactivating pro-survival Bcl-2 family members.

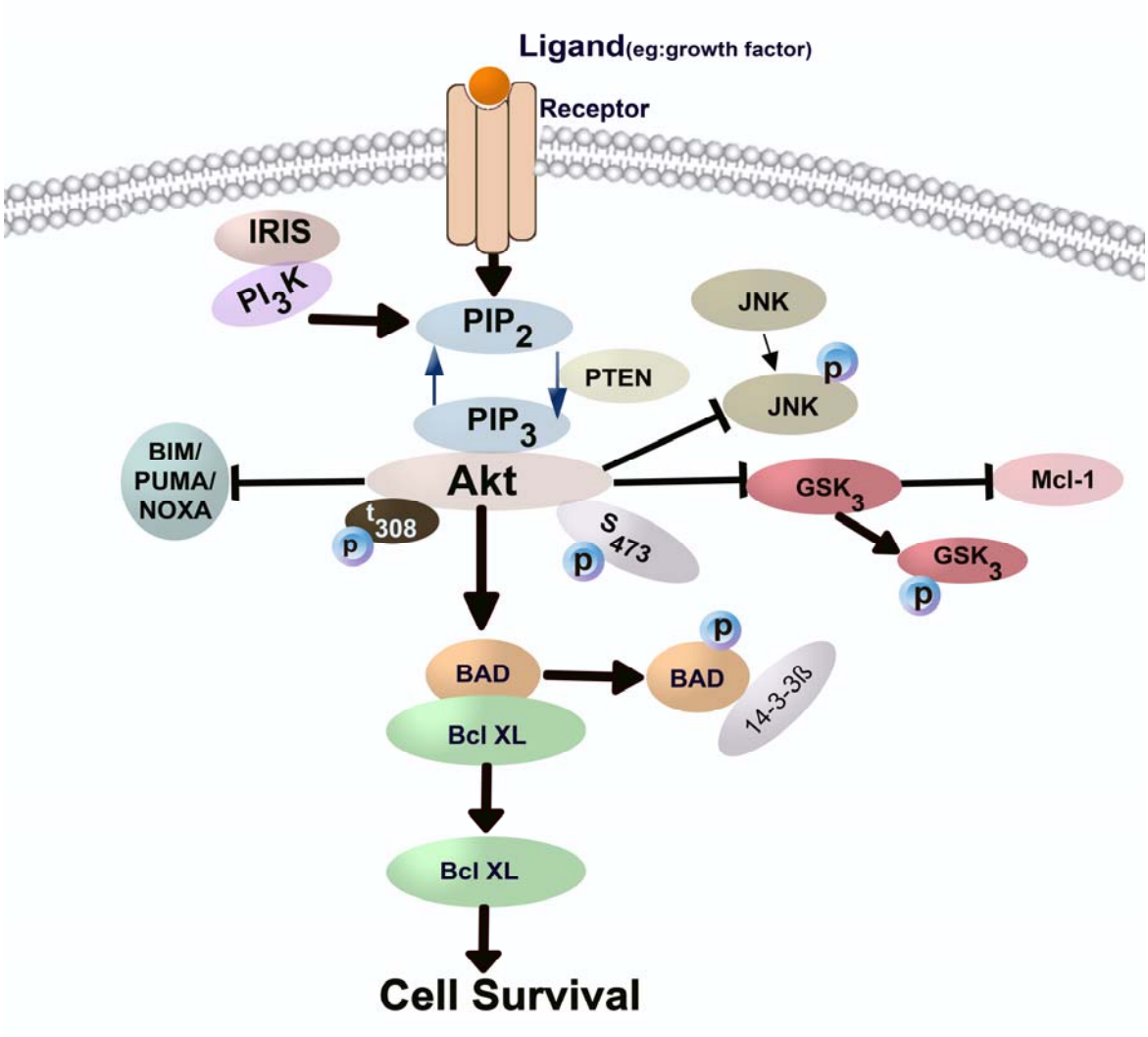


**Figure 1.9**. Overview of the Akt activation and consequences after the binding of diverse array of growth factors and cytokines to the receptor. Binding of p85 subunit of PI3K activates the conversion of $PIP_2$ to $PIP_3$, which recruits the Akt from the cytosol to the plasma membrane where its conformation

change and phosphorylation by PDK1 takes place. This phosphorylates and wide ranges of protein substrates (like Bcl2, BAD, MDM2, GSK3, IKK-ß) thus regulating cell cycle and survival.

For instance, Akt directly phosphorylates and inhibits the BH3-only protein Bad (Datta et al, 1997; Zha et al, 1996). Survival factors stimulate Akt mediated phosphorylation of Bad on S136, and this creates a binding site for 14-3-3 proteins, which triggers release of Bad from its target proteins (Datta et al, 2000). The ability to phosphorylate S136 on Bad is important for the survival effects of Akt on neurons and other cell types (Zhou et al, 2000). Akt also inhibits the expression of BH3-only proteins like Bim through effects on transcription factors, such as FOXO and p53 (Zhu et al, 2008). Akt phosphorylates FOXO1 on T24, S256, and S319, and it phosphorylates FOXO3a and FOXO4 on three equivalent sites (Lee et al, 2011). Akt phosphorylation of FOXO proteins occurs in the nucleus, and, as with Bad, phosphorylated T24 and S256 are bound by 14-3-3 proteins, which displace FOXO transcription factors from target genes and trigger their export from the nucleus (Brownawell et al, 2001). Through this mechanism, Akt blocks FOXO-mediated transcription of target genes that promote apoptosis, cell-cycle arrest, and metabolic processes. Akt also targets the phosphorylation and deactivation of another transcription factor MDM2 that transcriptionally regulates P53 (Mayo & Donner, 2001; Ogawara et al, 2002). BH3-only proteins Puma and Noxa are important transcriptional targets of P53. Another important regulation is the phosphorylation of GSK3 (Fang et al, 2000), which inhibits the pro-survival Bcl-2 family member Mcl-1 (Flusberg et al, 2001). Under certain conditions Akt is also known to activate NF-κB pro-survival pathway also it blocks apoptosis induced by p38/JNK activation (Faissner et al,

2006). The Akt pathway with respect to apoptosis inhibition and cell survival is depicted in Figure 1.9.

### 1.2.9. The extrinsic pathway of apoptosis

The extrinsic pathway begins outside the cell through the activation of specific pro-apoptotic receptors on the cell surface. These extracellular death receptor ligands such as tumor necrosis factor (TNF) (Locksley et al, 2001; Schulze-Osthoff et al, 1998), Fas ligand (FasL)/CD95L, and TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L bind to their respective transmembrane receptors TNFR, Fas/CD95 and DR4/DR5 respectively (Peter & Krammer, 2003; Strasser et al, 2000). Fas, one of the best-characterized death receptors, play crucial roles in a variety of immunological processes. This is reflected in the fact that mutations in the gene encoding Fas underlie most cases of autoimmune lymphoproliferative syndrome (ALPS), an autoimmune disease in children characterized by massive lymphadenopathy, peripheral accumulation of doubly negative T cells, and autoantibody production leading to hepatosplenomegaly, hemolytic anemia, and thrombocytopenia (Straus et al, 1999). In an extensively characterized FasL/FasR and TNF-α/TNFR systems, clustering of the receptors occur together with the binding of the homologues trimeric ligand (Chan et al, 2000). Consequently, cytoplasmic adapter protein gets recruited to the receptor/ligand through the death domain. The binding of TNF-α to the TNFR, with a simultaneous block in protein synthesis, results in the binding of TRADD (TNFR associated death domain) with FADD (Fas associated death domain) and RIP (Hsu et al, 1995). Binding of TNF-α to the TNFR

can lead to activation of NF-κB when protein synthesis is not blocked (Legler et al, 2003). Receptor aggregation in response to the pro-apoptotic Fas ligand binding and TNF initiates the formation of specific intracellular death-induced signaling complexes (DISCs) (Micheau & Tschopp, 2003; Peter & Krammer, 2003), where the adaptor molecule Fas-associated death domain (FADD) serves as platform for recruitment and bringing the pro-caspases to close proximity facilitating autocatalytic activity and activation of caspases-8 and -10 (Figure 1.10). The activation of caspase-8 in turn cleave downstream caspases, such as caspase-3 and caspase-7, to execute cell death; alternatively, caspase-8 can cleave the BH3-only pro-apoptotic protein Bid, which in turn amplifies the cell death signal by causing mitochondrial damage and cell death (Peter & Krammer, 2003). DISC formation is also modulated by several inhibitory mechanisms, including c-FLICE inhibitory protein (c-FLIP), which exerts its effects on the DISC by interacting with FADD to block initiator caspase activation; and decoy receptors, which can block ligand binding or directly abrogate pro-apoptotic receptor stimulation (Ashkenazi, 2008; French & Tschopp, 1999).

It has been shown that activation of the extrinsic pathway through the binding of CD95L/FasL to CD95/Fas can result in 2 apoptotic programs, termed type I and type II. Type I cells are able to overcome the need for mitochondrial amplification of the death signal in CD95-mediated apoptosis by producing sufficient amounts of caspase-8 at the DISC to directly cleave and activate effector caspases and execute cell death (Scaffidi et al, 1998). Because type I cells bypass mitochondrial involvement in CD95-mediated apoptosis, expression of Bcl-2 or Bcl-$_{xl}$ has no inhibitory effect on their apoptotic program. Conversely, type II cells produce minimal

amounts of active caspase-8 at the DISC and require the mitochondrial amplification of the CD95 signal (Scaffidi et al, 1998). The signaling to mitochondria is probably through the pro-apoptotic BH3 domain of the Bcl-2 family member, Bid. The cleavage of Bid by caspase-8 (Li et al, 1998) results in its translocation to the mitochondria where it initiates the release of mitochondrial factors, which in turn augment cell death. Because type II cells rely on the apoptotic function of mitochondria, expression of Bcl-2/Bcl-$_{xl}$ does confer protection from CD95-mediated apoptosis.

An explanation for the differences between type I and type II cells remains unclear, although differential expression of inhibitors of the death receptor signaling cascade, such as c-FLIP or XIAP, has been suggested to play a role (Walter et al, 2008).

### 1.2.10. The intrinsic pathway of apoptosis

As the name suggests the intrinsic pathway is triggered by severe cell stress like DNA damage, a defective cell cycle and detachment from the extracellular matrix, hypoxia and loss of cell survival factors.The intrinsic apoptotic pathway hinges on the balance of activity between pro- and anti-apoptotic members of the Bcl-2 super family of proteins, which act to regulate the permeability of the mitochondrial membrane (Coultas & Strasser, 2003). Members of the Bcl-2 family contain signature domains of homology called Bcl-2 homology (BH) domains as described above. Each of the BH3-only protein acts as sensor to the cellular stress (Bouillet & Strasser, 2002).

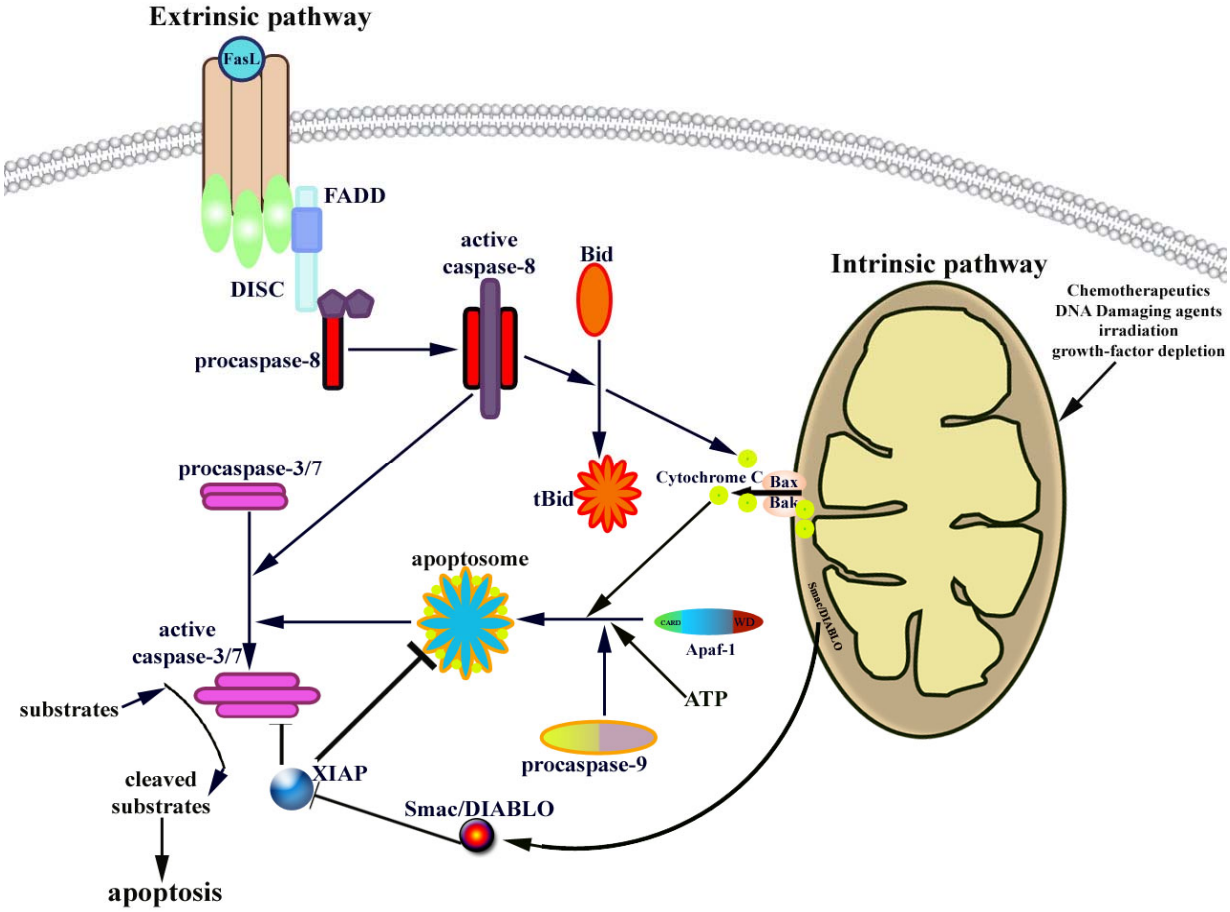


**Figure 1.10.** The intrinsic and extrinsic pathway of apoptosis. The extrinsic pathway is initiated by the death receptor ligation, which induces recruitment of FADD and pro-caspase-8 to death inducing signaling complex. Auto activation of caspase-8 can directly activate caspase-3 in type I cells. In type II cells amplification of the death signal via mitochondria is required which is mediate by Bid. Bax and Bak activation leads to mitochondrial membrane potential loss and apoptogenic factors are released into the cytosol. In the intrinsic pathway death inducing stimuli like DNA damaging agents leads to activation of BH3-only proteins accompanied by the down regulation of Bcl-2 like proteins.

BH3-only proteins activate the multi-BH domain pro-apoptotic proteins Bax and/or Bak, which then allow for permeabilization of the mitochondrial membrane. The anti-apoptotic Bcl-2 proteins Bcl-2 and Bcl-$_{xl}$ act to prevent permeabilization of the outer mitochondrial membrane by inhibiting the action of the pro-apoptotic Bcl-2

proteins Bax and/or Bak (Figure 1.10). Upon membrane permeabilization, cytochrome c and the pro-apoptotic protein SMAC/DIABLO are then able to translocate from the intermembrane space of the mitochondria into the cytosol. As part of the intrinsic apoptotic pathway, the SMAC/DIABLO protein released from the mitochondria promotes apoptosis by directly interacting with IAPs and disrupting their ability to inactivate the caspase enzymes (Srinivasula et al, 2000). Hence, the intrinsic pathway is sometimes referred to as the mitochondrial pathway. Upon release from the mitochondria, cytochrome c forms a complex in the cytoplasm with adenosine triphosphate (ATP), an energy molecule, and the adaptor Apaf-1, forming a large multiprotein structure known as the apoptosome. Assembly of the apoptosome is highly regulated and may be driven by nucleotide exchange factors and/or ATPase-activating proteins. The primary function of the apoptosome seems to be multimerization and allosteric regulation of the catalytic activity of caspase-9. Initiator caspase-9 is recruited into the apoptosome and activated from within the adaptor protein complex, which in turn activates the downstream effector caspases-3, -6, and/or -7 (Thornberry & Lazebnik, 1998). Besides the release of cytochrome c from the intra-membrane space, the intra-membrane content released also contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation and chromatin condensation. It is involved in initiating caspase independent pathway of apoptosis.

### 1.2.11. Perforin/granzyme pathway

In the granzyme B pathway, Perforin and granzyme B are released by cytotoxic T lymphocytes when they recognize antigen bearing cells (Zhao et al, 2006). Granzyme B is exophytically released through the plasma membrane with the aid of

the trans-membrane pore forming protein perforin (Shi et al, 1997). Once inside the cell it can directly activate caspase-3 or specifically cleave Bid and initiate the intrinsic pathway of cell death (Goping et al, 2003). Granzyme B can cleave proteins at aspartate residues and thus activate pro-caspase-10 and can cleave factors like ICAD (Sakahira et al, 1998).

### 1.3. Manipulation of host cell death pathway during microbial infections

Other than maintaining the homeostasis of the immune system, host cell death is a major factor determining clinical progression and disease severity in host pathogen interactions. The clearance of infected host cells is necessary to inhibit microbial replication and dissemination. Several forms of host cell death have been described during infection, as part of the host defense system or as a component of the survival strategy of the pathogen. The type of death the cell undergoes depends on the nature of the pathogen, pathogen load and site of infection. It is thus not surprising that pathogens have evolved an array of toxins and virulence factors to modulate host cell death pathways. Apoptosis, necrosis, and pyroptosis constitute the three major cell death modes for elimination of infected cells. The cellular factors that comprise the cells death machinery and are prone to manipulation by pathogens are summarized in Figure 1.11, reviewed in (Bohme & Rudel, 2009; Lamkanfi & Dixit, 2010).

#### 1.3.1. Regulation of apoptosis during infection

Cells induce intrinsic apoptosis pathway to restrict the growth and reproduction of many pathogens. Recently it was shown that induction of intrinsic

pathway limited the facultative intracellular pathogen *Legionella pneumophila* (Nogueira et al, 2009). Defective or delayed apoptosis can lead to amplified pathogenesis and tissue damage (Marriott et al, 2005). The extrinsic apoptosis pathway equally contributes to the resistance against pathogenesis, as deletion of FasL prevented apoptosis followed by *Pseudomonas aeruginosa* infection and this lead to hyper susceptibility to sepsis-associated lethality (Grassme et al, 2000; Jones et al, 2002). The induction of apoptosis is however not always protective to the host as the pathogens can hijack the apoptotic machinery to eliminate the immune response. Many pathogens like *Staphylococcus aureus* and *Listeria monocytogenes* produce toxins and other virulence factors that activate the host apoptotic machinery (Weinrauch & Zychlinsky, 1999), while other pathogens like *Yersinia enterocolitica* and *Salmonella typhimurium* interfere with the NF-κB and MAP kinase signaling cascades and induces cell death (Collier-Hyams et al, 2002; Monack et al, 1996). A well known example of such significant disease pathogenesis and tissue damage is the rapid depletion of lmyphocytes in patients infected with HIV (Badley et al, 2000). Obligate and facultative intracellular pathogens also benefit for preventing apoptosis to facilitate survival and replication. For instance the obligate intracellular pathogen like *Rickettsia rickettsii* stimulates NF-κB signaling in infected vascular endothelial cells in order to prevent host cell death and to continue replicating unabated (Clifton et al, 1998). *Coxiella burnetii,* protects infected cells from apoptosis during the early invasive stages of infection, presumably by blocking cytochrome c release from mitochondria (Luhrmann & Roy, 2007). Infection with *Neisseria gonorrhoeae* leads to the down-modulation of pro-apoptotic proteins Bim and Bad which accounts for

inhibition of host PCD. Another successful strategy to promote host cell survival is the phosphorylationof the extracellular signal regulated kinase Erk and Akt induced by a multitude of bacteria, including *Neisseria gonorrhoeae* (Howie et al, 2008), *Coxiella burnettii* (Voth and Heinzen 2009), and *Salmonella enterica* serovar *Typhimurium* (Knodler et al, 2005a). Thus, the induction or inhibition of host cell apoptosis is dependent on a delicate balance of pro- and anti-apoptotic strategies during host-pathogen interactions (Figure1.11).

### 1.3.2. Regulation of necrosis during infection

Reports demonstrating the induction or inhibition of programmed necrosis are very limited. A vaccinia virus strain expressing the CrmA homolog B13R/Spi2 was recently demonstrated to induce necrosis in adipocytes and hepatocytes in wild-type mice, while necrosis was markedly attenuated in RIP3 knockout mouse (Cho et al, 2009). It was also reported that in murine cytomegalovirus infection RIP3 induced necrosis is suppressed by the viral inhibitor of RIP activation (Upton et al, 2010). The tools for characterizing and interfering with necrotic cell death are currently being developed like the recently generated RIP1-specific small molecule inhibitors (Degterev et al, 2008). Necrotic cell death was also shown in murine myoblast cells exposed to alpha toxin of the bacterial pathogen *Clostridium septicum*, the causative agent of clostridial myonecrosis (Kennedy et al., 2009). The reports investigating the *in vivo* consequences of inducing necrosis suggest that it may contribute to the capability of host to limit and clear the infection by removing intracellular replication

niches and by enhancing the host's defensive responses through the release of pro-inflammatory cytokines and endogenous danger signals.

### 1.3.3. Regulation of pyroptosis during infection

*Shigella flexneri*, the etiological agent of bacillary dysentery, was the first pathogen reported toinduce pyroptosis in infected macrophages (Hilbi et al, 1998). Subsequent studies demonstrated pyroptotic death of macrophages and dendritic cells infected with *Salmonella typhimurium, Pseudomonas aeruginosa*, and *Legionella pneumophilab* (Monack et al, 2001; van der Velden et al, 2003). All these pathogens induce caspase-1 activation through the Ipaf inflammasome. With the notable exception of *Shigella*, this happens following delivery of bacterial flagellin into the macrophage cytosol. Because *Shigella* lacks flagellin, it remains to be determined how this pathogen induces activation of the Ipaf inflammasome. As with necrosis, the destruction of intracellular replication niches and the release of inflammatory cytokines and endogenous alarmins such as HMGB1 may represent key mechanisms by which pyroptosis may contribute to the anti-infectious responses of the host.

### 1.3.4. Apoptosis and host cell modulation by *Chlamydiales*

Obligate intracellular bacteria, *Chlamydia* confer resistance to the host cell against a variety of apoptotic stimuli, including the action of cytotoxic T cells (Fan et al, 1998b). The bacteria are thus able to maintain a long-term relationship inside the host cell and complete their replication cycle inside the cell, at the end of which numerous elementary bodies are produced and released to infect other cells. *Chlamydia* can sometimes develop long-term chronic infections that could contribute

to diseases such as atherosclerosis. Protection of the host cell against apoptosis would acquire greater significance for the bacteria under such conditions. It is not surprising, therefore, that persistently infected cells have also been shown to be potently resistant to various forms of apoptosis (Dean & Powers, 2001). Apoptosis resistance has been attributed to all major chlamydial species viz. *Chlamydophila pneumoniae, Chlamydia trachomatis, Chlamydophila psittaci, Chlamydophila caviae,* as well as *Chlamydia muridarum* (Coutinho-Silva et al, 2001; Fan et al, 1998b; Rajalingam et al, 2001a; Zhong et al, 2006). Of these, *C. trachomatis* and *C. pneumoniae* have been widely studied for their strong anti-apoptotic effect and relevance to human diseases, reviewed under Sharma and Rudel (2009). The chlamydial infection protects cells against different forms of apoptosis described above (extrinsic, intrinsic and granzyme B), mediated by various stimuli including staurosporine (STS), TNF-α, etoposide, granzyme B/perforin, and UV light (Fan et al, 1998b; Fischer et al, 2001; Rajalingam et al, 2001a) (Figure1.11). *Chlamydia* has evolved several strategies to escape host immune response. *Chlamydia* can down regulate the expression of MHC class I and II molecules, thus avoid the recognition of host cells by CD+ T cells (Zhong et al, 1999; Zhong et al, 2000), they also release some soluble factors that prime the by-stander uninfected cells for less MHC class I expression which would promote more rapid immune evasion upon pathogen entry and help promote unfettered spread of infections (Ibana et al, 2011). Analysis of apoptosis pathway in *Chlamydia* infected cells showed that they resist the cytochrome c release from the mitochondria to the cytosol, caspase-9 activation and processing of caspase-3 was prevented as expected (Fan et al, 1998b). Later it was

also found that the infected cells fail to achieve activation of Bax and Bak, the regulators of mitochondria permeabilisation (Xiao et al, 2004b). *Chlamydia* infection leads to the *Chlamydia* protease like activity factor (CPAF) dependent broad scale degradation of BH3-only proteins (Dong et al, 2005; Paschen et al, 2008), but later Rajalingam et al (2008a) reported that there was no noticeable degradation of these pro-apoptotic proteins. It was recently reported that persistant chlamydial infection does not depend on CPAF for resistance to apoptosis (Rodel et al, 2012). *Chlamydia* infected cells resist host apoptotic machinery at different levels, not only at mitochondria level but also downstream of it (Fischer et al, 2001). *Chlamydia* also interferes with the host protein synthesis, upregulation and stabilization of certain anti-apoptotic proteins like cIAP-1, cIAP-2, XIAP and Mcl-1 (Paland et al, 2006b; Rajalingam et al, 2008a; Rajalingam et al, 2006a). Moreover, the significance of the anti-apoptotic proteins in conferring apoptosis resistance became clear from the RNAi technology reported in Rajalingam et al (2006a). RNAi screen to identify the critical host factors revealed Bcl-2 and Mcl-1 as the targets. Later it was observed that activation of Raf/MEK/ERK pathway is required for the stabilization of Mcl-1 (Rajalingam et al, 2008a). One another host factor that was activated and stabilized Mcl-1 was the transcription factor HIF-1α (Sharma et al, 2011).

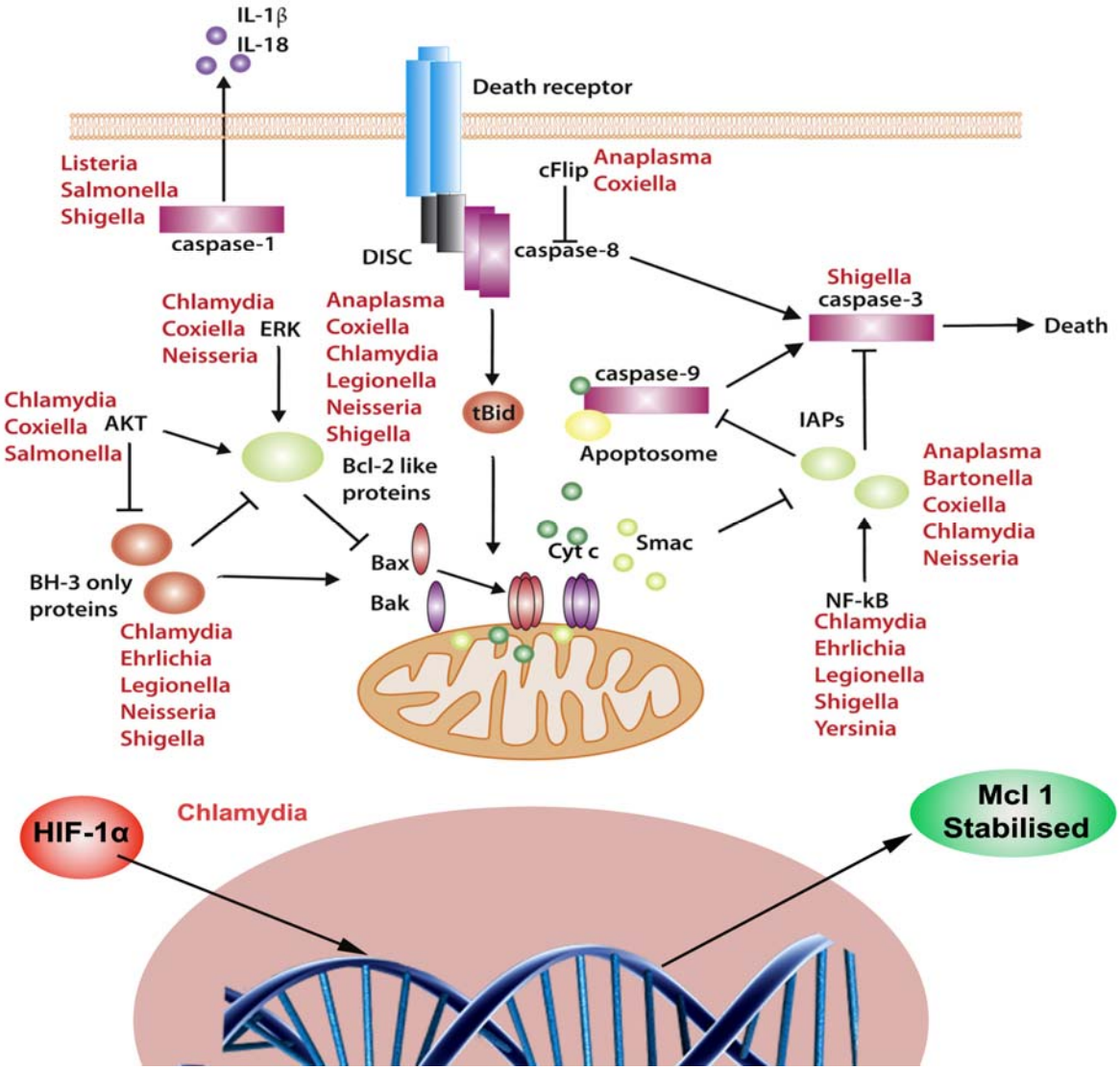


**Figure 1.11**. Modulation of cell death signaling cascades by bacterial pathogens. (Modified from (Bohme & Rudel, 2009)). Apoptosis is activated by intrinsic /extrinsic pathway. Bacterial pathogens (indicated in red) interfere at several points with either the regulators or executioners of the pathway.

## 1.4. Aim of the work

The present study aims at investigating the evolutionary conservation of infection induced cell death inhibition among *Chlamydiales*. The apoptotic response of human cells infected with the *Chlamydia*-like organism *Simkania negevensis* (Sn) was under study. Sn maintains a long-term relationship with the host cells, as they can infect and remain upto 14 days in cell cultures. This bacterium ensures to keep the cell healthy and alive until it completes its life cycle. This aroused the curiosity as how this pathogen regulates the host signaling and remains hidden from the host immune response. Hence, this study was aimed to find if and how Sn infection regulates the signaling pathways in host cells. The different cell death pathways upon induction with various cell death inducers in Sn-infected cells were aimed for investigation. It was interesting to explore how similar was the pathways subverted in Sn-infected cells compared to *Chlamydia*, as they belong to the same family. This work supports the hypothesis of evolutionary conserved signaling pathways to apoptosis resistance as common denominators in the order *Chlamydiales*.

# 2. Materials and methods

## 2.1. Materials

### 2.1.1. Cell lines used in the study

| Cell lines | Features | Origin | Source/Donor |
|---|---|---|---|
| HeLa 229 | Cervical adenocarcinoma | Human | ATCC CCL-2.1 |
| HEp-2 cells | Laryngeal carcinoma | Human | ATCC CCL-23 |
| HT1080 Wild type | Connective tissue (fibrosarcoma) | Human | ATCC CCL-121 |
| HT1080 (IκB mutant) | Connective tissue (fibrosarcoma) with IκB under super repressor | Human | ATCC CCL-121 |

**Table 2.1**. Human tissue culture cell lines used in the study

### 2.1.2. Bacterial strain used in the study

| Bacteria | Strain | Source/Donor |
|---|---|---|
| *Simkania negevensis* | Z | ATCC VR-1471™ |

**Table 2.2.** Bacterial strain used in the study

### 2.1.3. Amoebal strain used in the study

| Amoeba | Strain | Source/Donor |
|---|---|---|
| *Acanthamoeba castellanii* | C3 | ATCC 50739 ™ |

**Table 2.3.** Amoebal strain used in the study

### 2.1.4. Cell culture medium and buffers

| Medium | Ingredients |
|---|---|
| Cell culture media (HeLa 229 cells) | 1x RPMI 1640 + L-Glutamax + 25 mM HEPES (GIBCO,Germany),10 % (v/v) heat inactivated FCS (Biochrom) |
| HT 1080 WT and IκB mutants | 1x RPMI 1640+ L-Glutamax + 25 mM HEPES (GIBCO, Germany),10 % (v/v) heat inactivated FCS (Biochrom) |
| *Simkania* culture media | 1x RPMI 1640+ L-Glutamax + 25 mM HEPES (GIBCO, Germany), 10 % (v/v) heat inactivated FCS (Biochrom), Penicillin (100 U/ml), Streptomycin (100 µg/ml), Vancomycin (50µg/ml), Gentamycin (8 µg/ml), Cycloheximide (1 µg/ml) |
| Freezing media | 1x RPMI 1640 + L-Glutamax + 25 mM HEPES (GIBCO, Germany), 10 % (v/v) heat inactivated FCS(Biochrom), 10 % (v/v) DMSO |
| Transfection media | Cell culture medium + 20% (v/v) Optimem (GIBCO) |
| SPG buffer | 0.22 M sucrose, 10 mM $Na_2HPO_4$, 3.8 $KH_2PO_4$, 5 mM Glutamate |
| PEI stock solution | Dissolve PEI powder to a concentration of 2 mg/ml in water which has been heated to 80°C. Allow solution to cool to room temperature.Adjust pH to 7.0 with 5 M HCl. Filter sterilize. Freeze aliquots at ¯80°C. |

| | |
|---|---|
| Amoebal culture medium<br>(Peptone yeast extract glucose (PYG)) | Peptone (20g), glucose (18g), yeast extract (2g), sodium citrate (1g), $MgSO_4$ $7H_2O$ (980mg), $Na_2HPO_4$ $7H_2O$ (355mg), $KH_2PO_4$ (340mg) per liter of medium. pH-6,7. |
| Encystment medium | 95 mM NaCl, 5 mM KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$, 20 mM Tris–HCl, pH 9.0. |

**Table 2.3.** List of medium and buffers used for cell cultures.

### 2.1.5. Buffer and Solutions

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

### 2.1.5. i. Buffer for Immuno fluorescence

| Buffer | Ingredients |
|---|---|
| PBS (1x) (/L) | 8 g NaCl, 0.2 g KCl, 2.68 g $Na_2HPO_4$ (1xH2O), 0.24 g $KH_2PO_4$,adjust to pH 7.4 |
| Fixing buffer (/L) | 1x PBS + 4% (w/v) PFA, adjust to pH 7.2 |
| Permeabilization buffer | 1x PBS + 0.2% (v/v) TritonX 100 |
| Blocking buffer | 1x PBS + 10% (v/v) FCS |
| Mowiol mounting medium | 2.4 g Mowiol 488, 6 g glycerol, 6 ml $H_2O$, 12 ml 0.2 M Tris/HCl, adjust to pH 8.5 |

**Table 2.4.** Buffers and components used for immunofluorescence.

### 2.1.5.ii. Buffer for Immunoblotting

| Buffer | Ingredients |
|---|---|
| 10 x SDS buffer (/L) | 30.275 g Tris, 144 g glycine, 10 g SDS |
| 10x Semi dry buffer (/L) | 24 g Tris, 113 g glycine, 2 g SDS |
| 1x Semi dry transfer buffer | 1x semi dry buffer + 20% (v/v) methanol |
| 10x TBS (/L) | 60.5 g Tris, 87.6 g NaCl, adjust to pH 7.5 with HCl |
| $TBST_{20}$ | 1 x TBS + 0.5% (v/v) $Tween_{20}$ |
| 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 | 100 mM Tris/HCl [pH 6.8], 20% (v/v) glycerol, 4% (w/v) SDS, 1.5% (v/v) 2-mercaptoethanol, bromophenol blue |
| Lysis buffer for phospho protein | 20 mM HEPES, 400 mM NaCl, 10 mM KCl, 20% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 1x Complete, 1x PhosSTOP |
| 12% SDS lower gel solution | For 10 ml: 3.3 ml H2O, 4 ml 30% (v/v) acrylamide:bisacrylamide 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 H2O, 0.83 ml 30% (v/v) acrylamide: bisacrylamide 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 |

**Table 2.5.** Buffers and components used for immunostaining.

### 2.1.6. Commercial Kits used in the study

| Name | Purpose | Company/Donor |
|---|---|---|
| a.Caspases Glo-8 assay | Caspases-8 activation | Promega |
| b. DeadEnd™ Fluorometric TUNEL Kit | Measure apoptosis | Promega |

**Table 2.6.** List of commercial kits used in the study.

### 2.1.7. Inhibitors, Inducers used in the study

| Name | Purpose | Company / Donor |
|---|---|---|
| a. TNF-α | Apoptosis inducer | BD Pharmingen |
| b. LY 294002 | PI3 kinase inhibitor | Cell Signaling |
| c. CAPE | NF-κB inhibitor | Sigma |
| d. IKK-2 inhibitor IV | NF-κB inhibitor | Calbiochem |

**Table 2.7.** List of inducers and inhibitors used in the study.

### 2.1.8. Fine chemicals

| Compound | Manufacturer |
|---|---|
| a. BMH | Sigma |
| b. Complete protease inhibitor | Roche |
| c. DRAQ5 | Alexis |
| d. ECL immunoblotting substrate | Thermo |
| e. Hoechst 33342 | Sigma |
| f. Syto 82 | Invitrogen |
| g. Phalloidin | Invitrogen |

| | |
|---|---|
| h. Lipofectamine$^{TM}$ 2000 | Invitrogen |
| i. PhoSTOP (phosphatase inhibitor) | Roche |

**Table 2.8.** List of fine chemicals used in the study.

### 2.1.9. Antibodies

| Name | Source | Company | Product # | Dilution | Application |
|---|---|---|---|---|---|
| Akt | rb | Abcam | 93856 | 1:1000 | IB |
| ß-Actin | ms | Sigma | A5441 | 1:3000 | IB |
| Bad | rb | Cell signaling | 9292 | 1:1000 | IB |
| Bax | rb | Abcam | 16910-50 | 1:1000 | IB/IF |
| Bak | rb | Santa Cruz | 526 | 1:1000 | IB |
| Bcl-2 | ms | Santa Cruz | 509 | 1:1000 | IB |
| Bid | rb | Santa Cruz | 11423 | 1:1000 | IB |
| Bim | rb | Santa Cruz | 2819 | 1:1000 | IB |
| Bmf | rb | Cell signaling | 4692 | 1:1000 | IB |
| Caspase-3 | rb | Cell Signaling | 9665 | 1:1000 | IB |
| Cl caspase-3 | rb | Cell Signaling | 9661 | 1:200 | IF |
| Caspase-9 | rb | Cell signaling | 9502 | 1:1000 | IB |
| cIAP-1 | rb | Abcam | 2399 | 1:1000 | IB |
| cIAP-2 | rb | Abcam | 23423 | 1:1000 | IB |
| Chla Hsp 60 | ms | ThermoScientific | MA3-023 | 1:3000 | IB/IF |
| Cytochrome C | rb | Santa Cruz | 7159 | 1:1000 | IB/IF |
| Erk | rb | Cell Signaling | 9108 | 1:1000 | IB |

| | | | | | |
|---|---|---|---|---|---|
| IκB-α | rb | Cell Signaling | 9242 | 1:1000 | IB |
| Mcl-1 | ms | Abcam | 31948 | 1:1000 | IB |
| Mek | rb | Santa Cruz | 252 | 1:1000 | IB |
| NF-κB | rb | Santa Cruz | 372 | 1:1000 | IB/IF |
| PARP | rb | Santa Cruz | 7150 | 1:1000 | IB |
| p Akt | rb | Cell Signaling | 4060 | 1:1000 | IB |
| p Erk | ms | Cell Signaling | 9106 | 1:1000 | IB |
| p Mek | rb | Cell Signaling | 2354 | 1:1000 | IB |
| Puma | rb | Cell Signaling | 4976 | 1:1000 | IB |
| Sn | rb | | | 1:50 | IF |
| Tom40 | rb | Santa Cruz | 11414 | 1:500 | IB |

**Table 2.9.** List ofPrimary antibodies used for Immunoblotting (IB), Immunofluorescence(IF).

| Secondary antibody | Source | Company | Dilution | Application |
|---|---|---|---|---|
| ECL anti-mouse IgG HRP linked | Goat/Donkey | GE healthcare | 1:2500 | IB |
| ECL anti-rabbit IgG HRP linked | Sheep /Donkey | GE healthcare | 1:2500 | IB |
| Anti mouse Cy2 linked | Goat | Dianova | 1:200 | IF |
| Anti rabbit Cy2 linked | Donkey | Dianova | 1:200 | IF |

**Table 2.10**. Secondary Antibody used in the study.

## 2.1.10. 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$^{TM}$ J-25I centrifuge (Beckman Coulter), cold centrifuge 5417R (Eppendorf), Owl Hep semidry electro-blotting system (Thermo), PerfectBlue$^{TM}$ Dual Gel Twin PAGE chambers (Peqlab Biotechnology), DM1500 confocal microscope (Leica), confocal Leica TCS SP5, DMR epifluorescence microscope (Leica), DMIL light microscope (Leica), plate reader infinite M 200 (TECAN), Thermo-mixer comfort (Eppendorf), 2100 Bioanalyzer (Agilent Technologies), G –storm GS1 thermo cycler (GR1), Step One Plus RT PCR System (Applied Biosystems), Nano drop 1000 spectrophotometer (Peqlab Biotechnlogy), ultra low temperature freezer (New Brunswick scientific), liquid Nitrogen tank (CBS 2300 series).

### 2.1.11. Software used

Windows XP, Microsoft Office 2007, EndNote X5, Adobe Photoshop CS4, Corel Draw X4, LAS-AF confocal microscopy software, ImageJ.

## 2.2. Methods

### 2.2.1. Cell culture

All cells were cultured in 75 $cm^2$ cell culture flasks (Greiner) in the corresponding medium as described above (see Table 2.3). The cells were maintained in a humidified incubator (Thermo) at 37 °C with 5 % CO2. The cells were in culture only until ten passages. Trypsin-EDTA was used for splitting the cells upon confluence. The adherent epithelial cells were washed with sterile PBS (GIBCO, Invitrogen) once and detached by incubation with 1 ml Trypsin/EDTA (GIBCO, Invitrogen) per flask. Trypsinization was stopped after 3-5 minutes by re-suspending the cells in FCS containing medium. 10-20 % of cells were transferred into a fresh cellculture plate further cultured in fresh medium.

### 2.2.1a. Amoebal culture

*Acanthamoeba castellanii,* originally isolated as a cell culture contaminant was obtained from American Type Culture Collection (Mannasas, VA, USA). The amoebae were cultured axenically in peptone yeast glucose (PYG) medium at 25$^oC$. Encystment was induced by washing the cells with phosphate buffer saline and incubating in encystment medium for 16h.

### 2.2.2. Cryo stocking of cell lines

For long term storage, and as the cells were maintained for no more than ten passages the same trypsinization procedure was performed to detach cells from cell culture plates, transferred to a 15 ml tube (Greiner) with 5 ml RPMI1640 + 10% FBS. After centrifugation at 800 rpm (Hettich) for 5 min, the cell pellet was resolved in 3 ml

stocking medium with 10% DMSO (cryoprotectant) and aliquoted into 1 ml per cryo tube, gradually cooled to -80°C in isopropanol after which the cryo tubes were transferred to liquid nitrogen tank where the temperature is -186°C. For thawing of cell stocks, frozen cells were rapidly thawed at 37 °C and immediately transferred into pre-warmedmedium for re-culture. Cells are usually seeded in plates one night before performing experiments.

### 2.2.3. Infection with *Simkania negevensis*

The infection procedure was similar to as described above, but the media was 1x RPMI 1640+ L-Glutamax +25mM HEPES (GIBCO, Germany) with 10 % (v/v) heat inactivated FCS (Biochrom) and penicillin (100 U/ml), streptomycin (100 µg/ml), vancomycin (50 µg/ml), gentamycin (8 µg/ml) (to prevent any contamination). The media also contained cycloheximide (1 µg/ml); an effective inhibitor of protein biosynthesis in eukaryotes, at a lower concentration restricts the cell division. Since, Sn needs nearly 5 days to complete the life cycle at a MOI 1 the use of cycloheximide was inevitable.

### 2.2.4. Preparation of bacterialstocks

Owing to the obligate intracellular life style, *Chlamydiales* cannot be cultivated *invitro* on solid or in liquid media. Therefore, infectious EB had to be prepared from infected cell cultures and stored as frozen stocks for later usage. In order to prepare a stock of *Simkania negevensis* Z, HeLa 229 or Hep-2 cells were seeded in a 75 $cm^2$ cell culture flask, infected at MOI 1, and incubated at 35°C and 5% CO2 for 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 EB was transferred to twelve 150 $cm^2$ culture flasks of HeLa 229 or Hep-2 cells for propagation of infection in a dilution of 1:100. After 72 h of incubation, the cells were lysed as above. To remove cell debris, the suspension was centrifuged at 2,000 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 bacterial pellet was washed once with SPG (sucrose phosphate glucose) buffer, centrifuged as above, and resolved in 5 ml SPG buffer (Table 2.3). In order to separate clumps of bacterial EB, the bacterial suspension was passed through a 20 then 18 gauge needle for several times. The 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.

### 2.2.5. Titration of bacterial stocks

To determine the appropriate amount of bacterial suspension to reach a MOI of 1 (one inclusion-forming unit (IFU) per cell) freshly prepared stocks of *Simkania negevensis* were titrated. Therefore HeLa 229 cells were seeded in 24-well plates on glass cover slips, the next day, were infected with different dilutions of the bacterial suspension, as described before. The cells were fixed after 72 hpi, with 4% PFA in PBS for 20 min at RT, washed twice with PBS and taken up for immunostaining.The cells were permeabilised with 0.2% Triton X 100 for 20 min. To prevent unspecific binding of the antibody the cells were incubated in 2% FBS in PBS for 1 h at RT then incubated with a mouse anti-chlamydial heat shock protein 60 (cHsp60) (1:500) or Sn

antibody (1:50) 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) (1:3000) for 30 min at RT, washed twice with PBS, and mounted with Mowiol mounting medium (Table 2.4). Bacterial inclusions and cellular nuclei were counted in an epifluorescence microscope (Leica) with 40x fold magnification to calculate the concentration of the stocks.

The MOI was calculated using the formula:

| IFU/ml= Number of inclusions in a 40X microscopic field X 1916.28 X dilution factor X 1 |
|---|

The factor 1 is used if 1 ml of the media is used and if 500 µl is used the factor 2 is used for calculation MOI.

### 2.2.6. Apoptosis induction

To study if Sn could resist apoptotis, different apoptotic inducers like tumor necrosis factor α (TNF-α) applied together with cycloheximide (chx), required to prevent the survival pathway and staurosporine (STS) was used. After 3 dpi with Sn the media was removed and the cells were either treated with 20 ng/ml of TNF-α/3 µg/ml of chx or 1 µM of STS in 500 µl fresh RPMI1640 + 5% FBS. These cells were incubated at 35°C for 4 h or 6 h respectively for TNF-α/chx and STS.

### 2.2.7. Assays to measure apoptosis

#### 2.2.7.1. Hoechst or DRAQ5 staining

Following the indicated treatment, cells were stained with 20 µM Hoechst 33258 or the far-red fluorescent DNA dye DRAQ5 at a dilution of 1:500 for 30 min at 37°C on glass slides. After fixing with 4% PFA, cells were stained and washed with PBS. Nuclear morphological changes (fragmentation or condensation) were determined under an epifluorescence microscope. At least 300 nuclei per sample were counted.

**2.2.7.2. TUNEL assay**

Fragmentation of chromosome by endonucleases is a hallmark of the later stage of apoptosis that results in a multitude of 3'-hydroxyl termini of DNA ends. This property can be used to identify apoptotic cells by labeling the DNA nicks with fluorescent-tagged deoxyuridine triphosphate nucleotides (F-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3' hydroxyl ends of double- or single-stranded DNA and generates DNA strands with exposed 3'-hydroxyl ends. Addition of the deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, an anti-BrdU antibody using standard immune histochemical techniques can detect BrdU. Non-apoptotic cells do not incorporate the F-dUTP because of absence of exposed 3'-hydroxyl DNA ends. Hence, 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-2 nick endlabeling (TUNEL) method using the Dead End$^{TM}$ Fluorometric TUNEL Kit (Promega). This method allows for detection of apoptotic cells on single cell level (Gavrieli et al, 1992). Therefore, cells were seeded on glass cover slips, infected or mockinfected, and

treated with TNF-α/chx for 4 h. Cells were fixed with 4% PFA for 20 min at 35°C and labeled according to the manufacturer`s instructions. Briefly, cells were permeabilized with 0.2% [v/v] Triton X100 in PBS for 5 min, washed once with PBS, and stained with 1 µg/ml Hoechst 33324 (1:3000) 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 for 60 min at 37°C in a humidified chamber. 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 40x fold magnification.

#### 2.2.7.3. Luminescent caspases-8 activity assay

The autoprocessing of procaspase-8 into its active form is a prerequisite for the apoptotic actions of the initiator caspase (Kang et al, 2008). To determine the activity of caspase-8 in Sn-infected TNF-α/chx 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 (Thornberry et al, 1997). According to the instructions, 15,000 HeLa 229 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 4 h after TNF-α/chx induction. 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 light exposure duration of 1 sec/well.

#### 2.2.7.4. Cytochrome c release

The cells were infected and treated with TNF-α/chx induction as described above. After 4 h of induction the cells were fixed with 4% PFA and taken up for immunostaining with cytochrome c antibody. The absence of cytochrome c in the mitochondria indicated a loss of membrane potential.

#### 2.2.7.5. Active caspase-3 staining

The cells were infected and treated with TNF-α/chx induction as described above. After 4 h of induction the cells were fixed with 4% PFA and taken up for immunostaining with cleaved or active caspases-3 antibody. Uninfected cells induced with TNF-α/chx were taken as the positive control. Active Caspases-3 could be found on the surface of apoptotic cells.

#### 2.2.7.6. Activation of Bax

The cells were infected and treated with TNF-α/chx induction as described above. After 4 h of induction the cells were fixed with 4% PFA and taken up for immunostaining with active Bax antibody. Uninfected cells induced with TNF-α/chx were taken as the positive control. Active Bax could be found accumulated on the mitochondria in apoptotic cells. The cells were analyzed under confocal microscope.

#### 2.2.7.7. Heterodimerisation of Bax and Bak

Upon induction of apoptosis, Bax and Bak change their conformation and homo-oligomerize to permeabilize mitochondria (as described under 1.1.5.1.b). Hence it was necessary to investigate if Sn-infected cells upon apoptosis induction can prevent the oligomerisation of Bax and Bak. HeLa 229 cells were grown in 35 $cm^2$ plates to 60-70 % confluency. Apoptosis was induced as described above or mock

induced in the presence of 3 µg/ml cycloheximide. Supernatant was collected and the remaining cells were scraped with a rubber police and washed in PBS by centrifugation at 1,000 rpm for 3 min at 4°C. For mitochondrial isolation, the pellet was resuspended in Buffer A (20 mM Hepes pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF and 2 mg/ml BSA) and left on ice for 15 min. Cells were homogenized 20 strokes with a homogenizer and pelleted at 800 g for 5 min at 4°C. Mitochondria containing supernatant was collected and centrifuged again at 10,000 g for 10 min at 4°C. Mitochondrial pellet was resuspended in Buffer B (Buffer A without BSA), quantified using a Nanodrop (Peqlab, Germany) and stored at -80°C. Supernatant containing cytoplasmic proteins was kept in cytochrome c release assays. Mitochondria (60 µg) were pelleted (10,000 g, 10 min, 4°C) and washed once with SET-buffer (and resupended in 95 µl SET-buffer. Cross-linking was done with BMH (Pierce, Rockford) at a final concentration of 1 mM for 30 min at RT. For quenching of the cross linker 2 µl DTT (2.5 mM) were added and samples were incubated for 5-10 min on ice. Cross-linked mitochondria were pelleted (14,000 rpm, 5 min, 4°C) and washed once in SET-buffer, resuspended in 30 µl Laemmli-buffer and analyzed by western blotting.

### 2.2.8. Infectivity assays

In order to investigate whether the production of simkanial progeny is altered by NF-κB activation, an infectivity assay was carried out. Therefore, bacterial inclusions were ruptured at the end of the developmental cycle and the particles were transferred to uninfected cells for a second round of infection. The primary infection is

described in section 2.2.3. At the end of the developmental cycle the inclusions were lysed by either incubating at -80°C for 1 h followed by incubation at 35°C and ruptured by pipetting the cells up and down or by glassbeads. Supernatant containing EB was transferred to freshly plated HeLa cells on glass cover slips in different dilutions. Infected cells were incubated for 72 h at 35°C and fixed in 4% PFA. Cells were stained for chlamydial Hsp60 and counterstained with Hoechst 33324 for DNA.

### 2.2.9. Inhibitor treatment

For inhibition of NF-κB activation, cells were treated with 25 μM Caffeic Acid Phenethyl Ester (CAPE) (Sigma) in DMSO in 500 μl of medium. Stock solution of CAPE was made by dissolving CAPE in DMSO to make 25 mM, hence1:1000 of stock (25 μM) was used in each experiment. CAPE is a cell permeable active component of propolis from honeybee hives, a potent and specific inhibitor of NF-κB activation. Control cells were treated with solvent alone. After 5 h of CAPE treatment the infected cells were induced with TNF-α/chx for apoptosis. This set up was also used for Sn for its effect on infection and infectivity.

To inhibit the PI3 kinase, a cell permeable, potent and specific phosphatidylinositol 3- kinase inhibitor LY294002 (Calbiochem) was used. It acts by binding to the ATP binding site of the enzyme. Stock solution of the inhibitor was made in DMSO. LY294002 was used at varying concentration of 0.1,1 and 10 μM and the controls were treated with the solvent alone to investigate the importance of this survival signal in apoptosis resistance.

### 2.2.10. 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 that 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$^{TM}$ 2000 (Invitrogen) or Polyethylenimine (PEI).

### 2.2.10.1. Polyethylenimine (PEI) transfection

Cells (70-80 % confluence) were seeded one day before in 24 well cell culture plates. On the next day, cells were washed with PBS and incubated in 5% FCS medium immediately before transfection. About 1 µg DNA was prepared in 100 µl Optimem transfection medium (GIBCO) for each sample and incubated at RT for 10 min after vortexing. In the meantime, 0.9 mg/ml PEI was prepared in 100 µl optimum (Table 2.3). PEI was reported to have higher efficiency in forming complexes with nucleic acids and was better delivered into the cells, PEI also have reduced toxicity as it reduces free polycations in the transfection media (Godbey et al, 1999). The samples were vortexed and incubated at RT for another 20 min. The mixture was eventually added to the cells for transfection. The cell culture medium was replaced after 4-6 h with normal medium containing 10 % FCS.

### 2.2.10.2. Lipofectamine$^{TM}$ 2000 DNA delivery (Invitrogen)

In each transfection, the DNA (µg) and Lipofectamine$^{TM}$ 2000 (µl) ratio was usually 1:2 to 1:3. One day before transfection, cells were seeded into 6-well plates to gain 70% confluency. 20 pM siRNAs were incubated in 200 µl Optimem transfection medium containing 4 µl Lipofectamine$^{TM}$ 2000 for 25 min at RT before dropwise addition to 800 µl fresh RPMI medium containing 5% FBS. The media was removed

and replaced with 10% FCS media after 4-6 h. After 24 h post transfection, cells were infected with *Simkania* and experiments were performed as described before.

### 2.2.12. Microscopy

#### 2.2.12.1. Leica Fluoresence microscopy

Nuclear fragmentation or condensation was detected by Leica DM RBE (Bensheim, Germany) fluorescence microscopy, following Hoechst staining as described in 2.2.7.1.

#### 2.2.12.2. Confocal microscopy

Confocal microscopes from DM1500 (Leica) was used in this study. Cells growing on a cover slip were washed and fixed, following the indicated treatment. After fixation, cells were permeabilized with PBS containing 0.2 % Triton X 100 for 30 min. Before incubation with the primary antibody listed in Table 2.9 (1 h at RT), cells were blocked with 5 % FCS for 45 min. Cells were washed with PBS after incubation with the primary antibody, and further incubated with the corresponding secondary antibody (fluorescence conjugated) at RT for 1 h. Finally, cells were washed 3 times in PBS and once with distilled water to remove the PBS and mounted on a glass slide with Mowiol. Samples were kept at 4°C until analysis. Cells were inspected under laser scanning confocal microscope (Leica) with 60x fold magnification and an additional 1.5x software magnification. Images were further processed with the software Image J.

#### 2.2.12.3. Electron microscopy (in collaboration with Prof. George Krohne)

The HeLa 229 cells were infected either with Ctr or with Sn for 24 h or 72 h respectively. The infected cells were washed twice with PBS solution and fixed with

gluteraldehyde at 4$^{o}$C for 1 hr. The fixed cells were washed with cocodylate buffer and taken for preparation of the sample.

### 2.2.13. SDS-PAGE and Western blotting

Protein levels or post-translational modifications such as phosphorylation were detected using the sodium-dodecylsulfate polyacrylamide electrophoresis (SDS-PAGE). Gels of different density were prepared according to the table below. The proteins migrate in an electric field according to their size, which is mediated by SDS providing a uniform negative charge to the proteins. Different percentage of gels was used depending on the size of protein to be separated.

| **Solution components for Separating gel** | **6%** | **8%** | **10%** | **15%** | **Solution components for Stacking gel (5%)** |
|---|---|---|---|---|---|
| $H_2O$ | 5.3 | 4.6 | 4.0 | 2.3 | 6.8 |
| 30% acrylamide mix | 2.0 | 2.7 | 3.3 | 5.0 | 1.7 |
| 1.5M Tris (pH 8.8) | 2.5 | 2.5 | 2.5 | 2.5 | 1.0 M Tris (pH6.8)<br>1.25 |
| 10% SDS | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| 10% APS | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| TEMED | 0.008 | 0.006 | 0.004 | 0.004 | 0.01 |

**Table 2.11.** Components of SDS PAGE.

Following the indicated treatment, the cells 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 phosphoproteins, lysis buffer containing phosphatase inhibitors was used (see Table 2.5) pipetting up and down, and transferred to a reaction tube (Eppendorf). Cell debris was removed by centrifugation for 5 min at 13,000g 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°C for 5 min and stored at $^{-}20^{o}$C until used. Denatured protein lysates were loaded onto the gels, besides the pre stained protein ladder (Fermentas, #SM0671). Gels were subjected to 80 volts for about 10-15 min until the proteins move into the separating gel, followed by a complete run at 120 volts for about 45-50 min. Subsequently, proteins were transferred to a methanol activated PVDF membrane (GE Healthcare) in a semidry electro blotting system (Thermo) with a current of 1.0 mA per $cm^2$ membrane for 2 h. Membranes were rinsed in $TBST_{20}$ (See Table 2.4) 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 respective primary antibodies listed in table 2.9, overnight at 4°C. After washing the membrane thrice for 10 min in $TBST_{20}$, antibody-antigen complexes were detected by respective HRP-linked donkey anti- rabbit or sheep anti-mouse secondary antibodies (GE Healthcare) listed in table 2.10. Further the blot was washed thrice 10 min each in $TBST_{20}$ before developing in the x-ray filim (Konika Minolta) or in the developing machine (ChemoCam Imager INTAS). For re-

probing with other antibodies, membranes were re-activated with 100% methanol and incubated in stripping buffer (Table 2.3) for 30 min at RT, after which the membrane was blocked for 1 h and then taken up for primary and secondary antibody for detection (as described above).

### 2.2.14. EMSA (in collaboration with Dr. Bhupesh K Prusty)

HeLa 229 cells were infected with Sn from day 1 to day 5; the infected cells with control were taken up for electro mobility shift assay. The cells were treated with ice cold hypotonic buffer containing (20 mM HEPES, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 mM PMSF and cocktail of protease inhibitors). After 15 min incubation in ice, the lysates were centrifuged at 850g for 15 min at $4^0$C. The supernatant which contain the cytoplasmic extract was removed and the pellet was washed with hypotonic buffer and treated with 2.5 times the pellet size of nuclear extraction buffer (20 mM HEPES, 25% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 mM PMSF and cocktail of protease inhibitors) after 1 h the extraction mixture was centrifuged at 18000g for 15 min at $4^o$C. The supernatant was stored at $^-80^o$ C. The oligo nucleotides described in materials were annealed and labeled with ($\gamma^{32}$ P) ATP by T4 polynucleotide kinase and gel purified in 15% polyacrylamide gel. The binding reaction was performed in a 25 µl reaction mixture containing 50% glycerol, 60 mM HEPES, 20 mM HCl, 300 mM KCl, 5 mM EDTA, 5 mM DTT, 100 µg of BSA/ml, 2.5 µg of (poly dI-dC) and 10 µg of nuclear extract. After 5 min, 10,000 cpm of ($\gamma^{32}$ P) ATP 5' end labeled double stranded oligonucleotide probe was incubated for 25 min at RT.

The DNA protein complex was resolved in 4.5% non-denaturing PAGE dried and exposed to X-ray filims.

### 2.2.15. RNA Isolation

RNA isolation was performed for quantification of specific transcripts by quantitative reverse transcription polymerase chain reaction (qRT-PCR). HeLa cells in 6-well plates were infected with Sn from day 1 to day 5. 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 12,000g 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 7,500g for 5 min. The RNA pellet was air dried, dissolved in 50 µl DEPC $H_2O$, and stored at -80°C until further use.

### 2.2.16. DNA digestion

In order to remove contaminating DNA from isolated RNA, DNA was digested with the TURBO DNAfree™ 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 10,000g for 15 min. The supernatant was transferred to a fresh reaction tube.

### 2.2.17. Copy (c) DNA synthesis

For qRT-PCR, isolated mRNA was reverse transcribed into copy DNA (cDNA) with the Superscript VILO$^{TM}$ cDNA Synthesis Kit (Invitrogen) according to the manufacturer´s instructions. 4 µl 5 x VILO Reaction Mix and 2 µl enzyme Mix was added to 2 µg RNA and DEPC-H2O 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.

### 2.2.18. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To quantify the amount of specific mRNA populations, 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 dd$H_2O$ was added to a total volume of 20 µl. Each sample was analysed in triplicates.

### 2.2.19. Statistical analysis

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

# 3. Results

## 3.1. Establishing *S negevensis* infection in cell cultures

*S. negevensis* strain Z was obtained from ATCC (ATCC VR-1471$^T$). The bacterium was cultivated as explained in the methods (2.2.3) and was titrated as explained in (2.2.4). HeLa 229 cells were plated in 12 well dishes with glass slides and were infected with Sn at a MOI of 1. After 72 hpi the cells were observed under light microscope. The cells were filled with tar like patches mostly in the perinuclear region in the cytoplasm. With increasing time of infection these patches extended and occupied a large proportion of the cytoplasm. At later time, some so called 'inclusions' looked empty, however when observed under phase contrast microscope fine rapidly moving particles could be observed. The cells were attached and still harbored the bacteria until 5 dpi, after which the cells were lysed and the bacterial particles were released into the medium. At a lower MOI the infection could proceed to nearly 8 days until the cells lysed as observed by (Kahane et al, 2002). The cells in later stages of infection looked more flat and expanded in morphology. It was also noticable that they stopped dividing as soon as they were infected and a visible inclusion could be seen. An electron microscopy of the Sn infected cells is shown in Figure 3.1b. All the cells that were tested were infectable with Sn (Figure 3.1a).

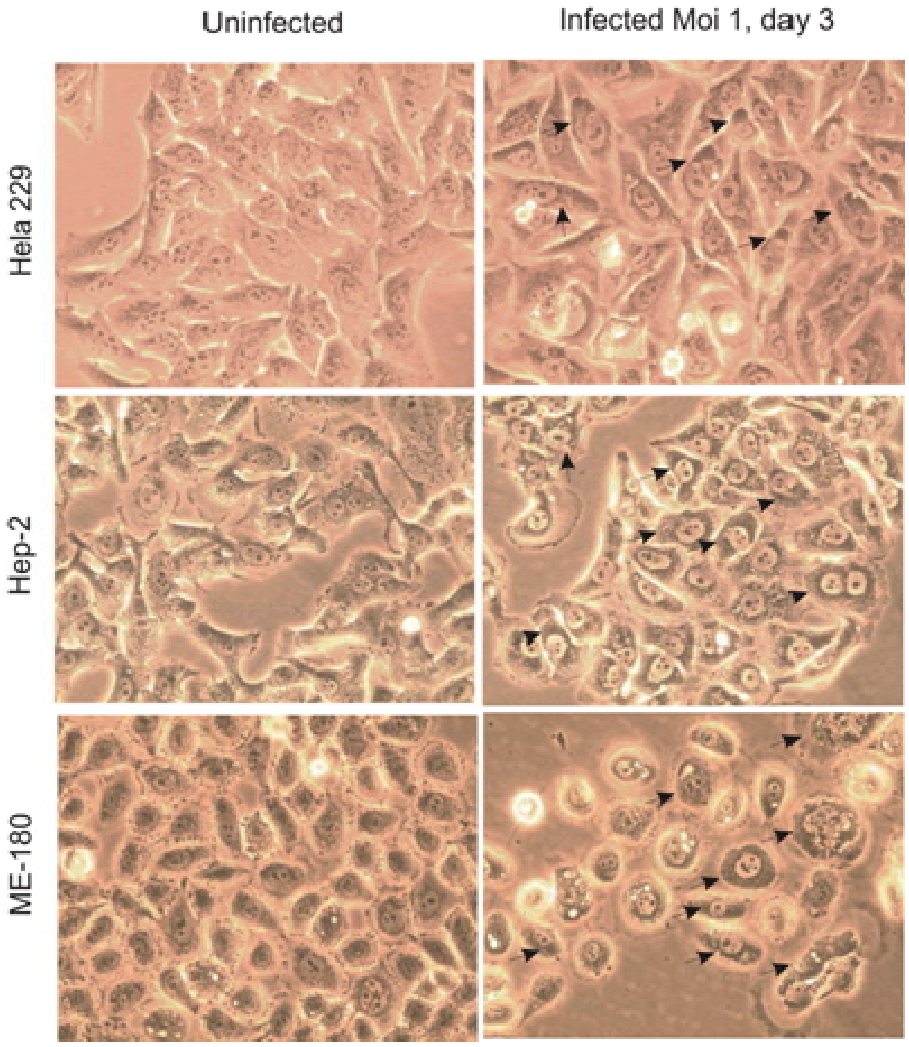


**Figure 3.1a.** Different cell lines like HeLa 229, HEp-2 and ME-180 were tested for Sn infection. Phase contrast images were taken after 3 dpi. The dark tar like patches in the peri-nuclear region of the cells indicates the Sn inclusions (indicated by arrows).

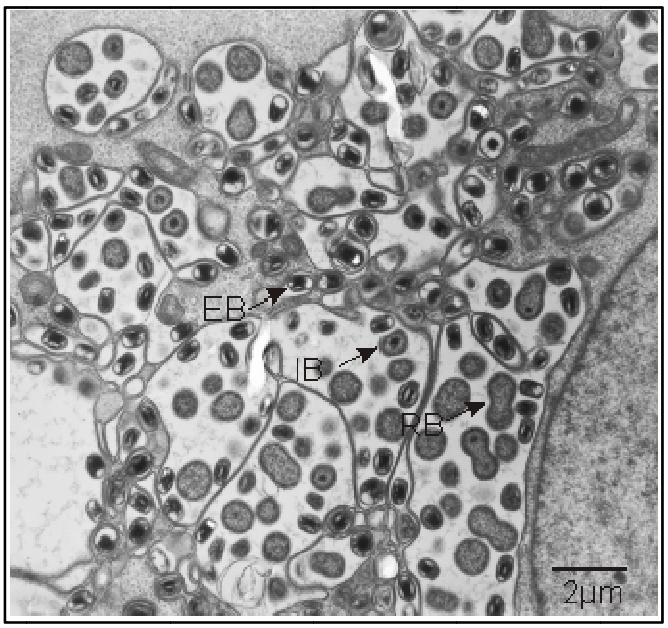

**Figure 3.1b.** Transmission electron micrograph of Sn in membranous inclusion of an infected cervical cancer cell. Black arrowheads indicate rod-shaped mature elementary bodies (EB), dividing spherical reticulate bodies (RB) and intermediate bodies (IB). Bar 2 µm

## 3.2. *S. negevensis* infected cells are resistant to apoptosis

Apoptosis resistance is a hallmark of *Chlamydia* infection but has so far not been investigated for other family level related bacteria like *Simkania negevensis*. To test whether *Simkania* confers apoptosis resistance during infection, apoptosis was induced with human tumor necrosis factor alpha (TNF-α) in the presence of cycloheximide (TNF-α/chx) in infected and non-infected HeLa cells. The cells were fixed and stained with Hoechst to detect chromatin condensation (Figure 3.2a).

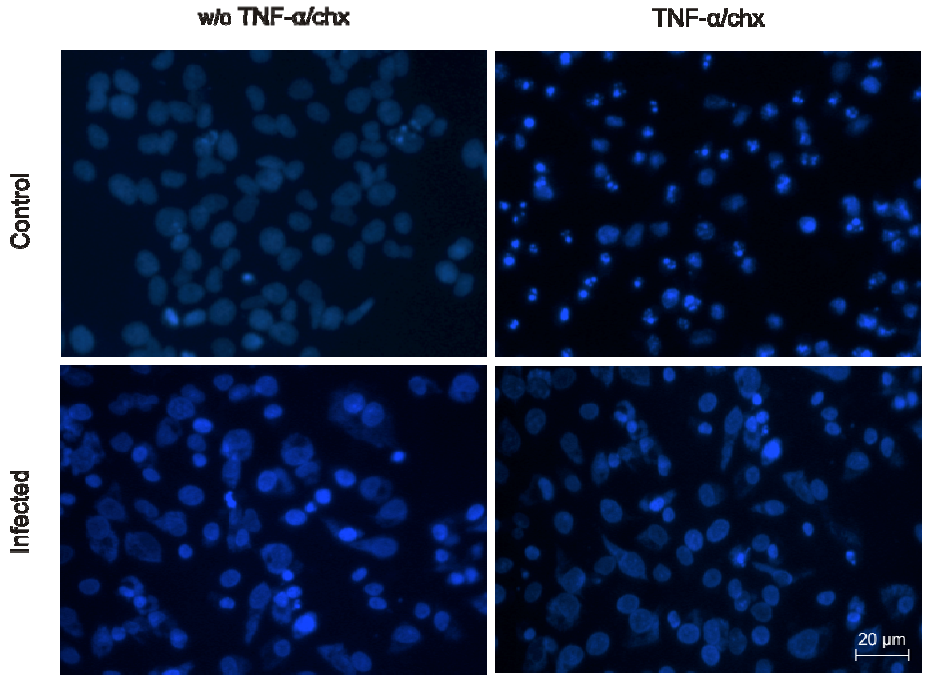


**Figure 3.2a.** HeLa 229 was infected with Sn for 72 h. The control and infected cells were treated with 20 ng/ml of TNF-α and 3 µg/ml of chx in 5% FCS media for 4 h, after which the cells were fixed with 4% PFA and stained with Hoechst or Draq5 and analysed under confocal microscope. The total

number of cells and apoptotic cells were counted and the percentage of apoptotic cells were calculated by dividing the total number of apoptotic cells by the total number of cells. Bar 20 µm. n=3.

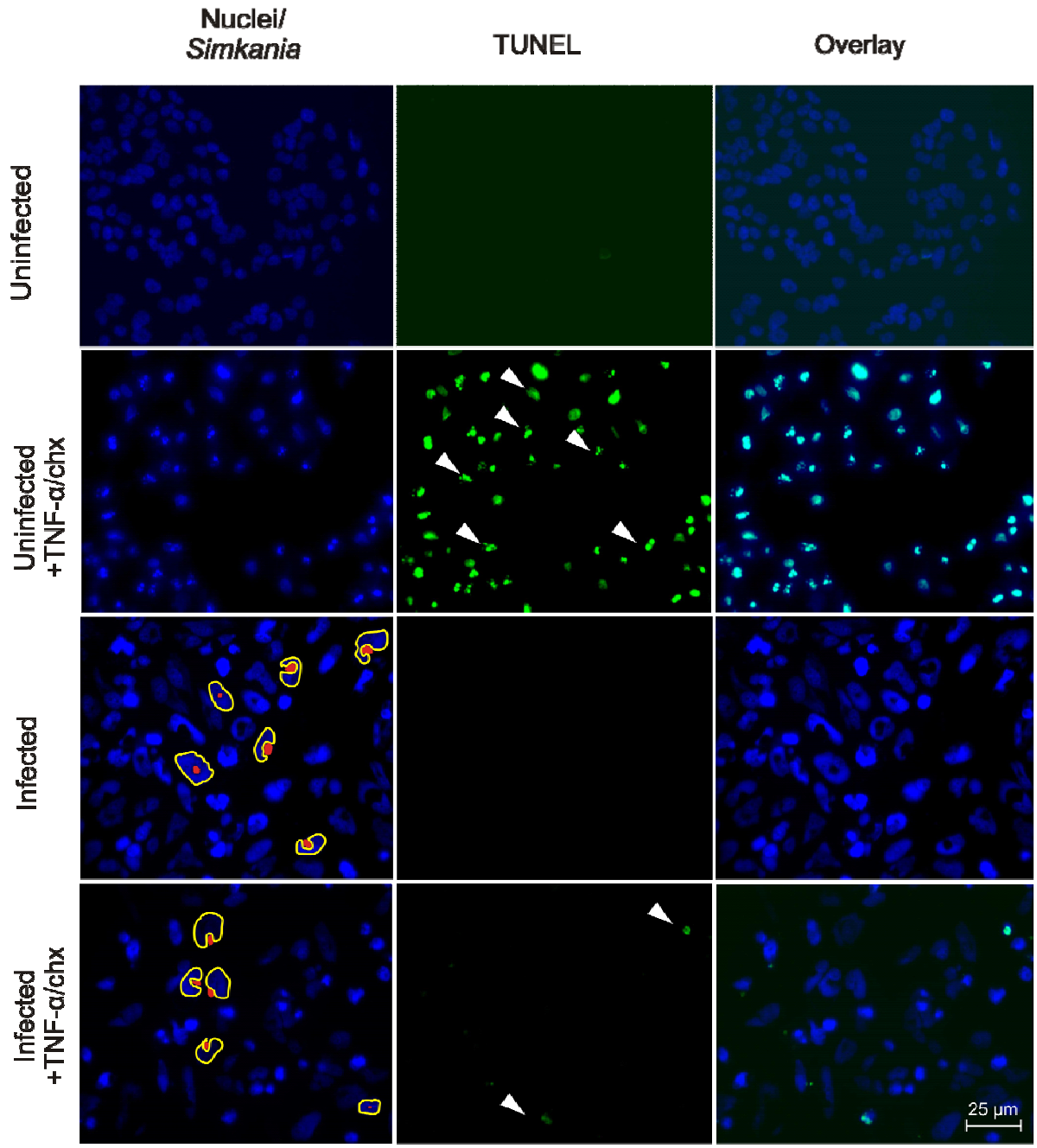


**Figure 3.2b.** *Simkania negevensis* infected HeLa cells are resistant to apoptosis induced by TNF-α. HeLa cells with or without Simkania infection (MOI 1) were treated with 20 ng/ml TNF-α with 3 µg/ml chx or with carrier for 4 h. Samples were stained with Hoechst (blue) and TUNEL(green) and viewed under a epifluorescence microscope. Bar 25 µm. (n=2).

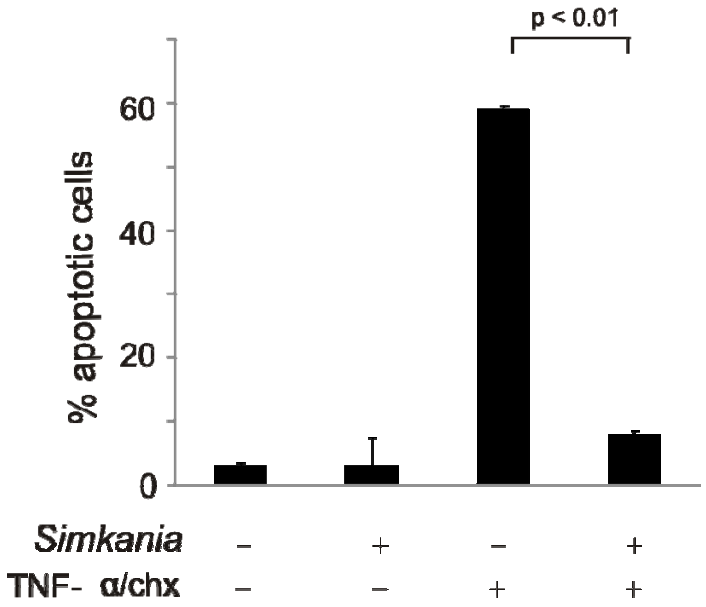


**Figure 3.2c.** Bar diagram displaying the quantitative analysis of the experiment shown in Figure 3.2b. Cells from five random fields were counted under a 40x objective and the percentage of apoptotic cells was calculated. *Simkania* infection significantly decreased the number of apoptotic cells upon TNF-αstimulation to nearly background levels (n = 2).

In a similar experiment apoptotic cells were quantified by terminal transferase mediated dUTP nick end labeling (TUNEL) assay (Figure 3.2b). A nearly complete block of apoptosis induced by TNF-α was observed at day 3 dpi illustrated by a reduction in the number of apoptotic cells from ~60% to ~10% in infected cells (Figure 3.2c). These results suggested an anti-apoptotic activity in cells infected with *Simkania*.

## 3.3. Sn resists apoptosis in a time and MOI dependent manner

To investigate if the resistance to apoptosis in Sn-infected cells depends on the establishment of the inclusion in the cells and the number of EB infecting the cell, time frame infections were carried on. Two conditions were considered under study. In the first case HeLa 229 cell were plated on glass slides and infected with different

MOI of Sn ranging from 0.5 to 20 for 72 h and the cells were induced for apoptosis using TNF-α/chx for 4 h. The cells were fixed and stained as described before (3.2) and the percentage of apoptotic cells was calculated for analysis. Results are shown in Figure 3.3a. In the second case HeLa 229 cells were infected with Sn at a MOI of 1 and apoptosis was induced after different time points i.e., day 1, 3 and 5. Percentage of apoptotic cells were calculated and analysed (Figure 3.3b).

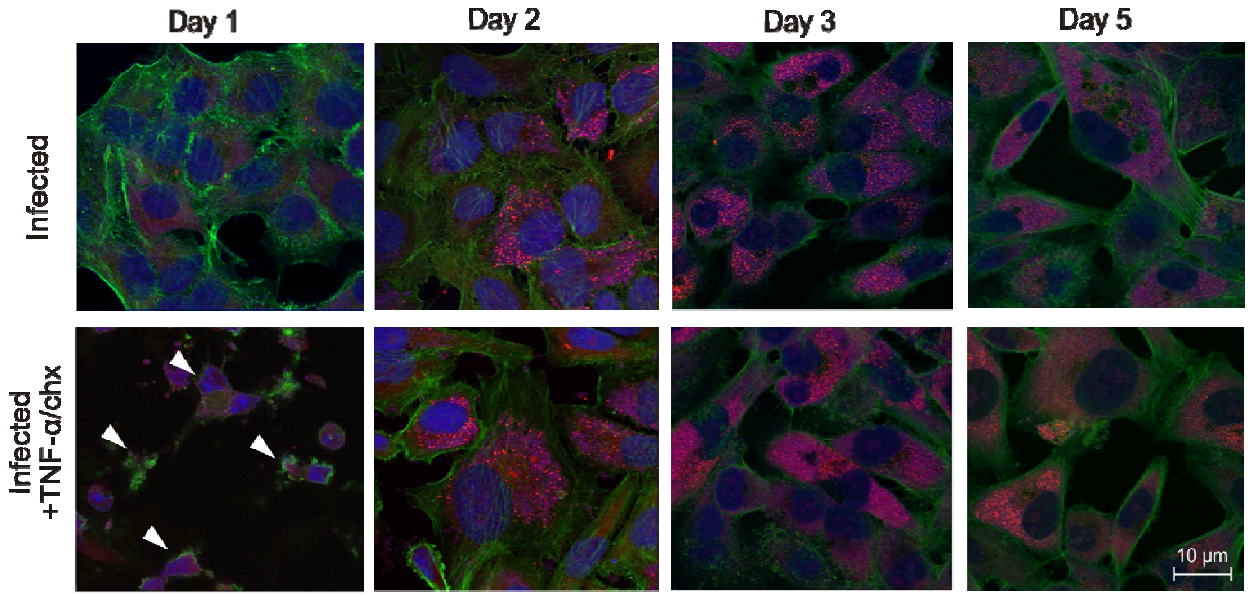


**Figure 3.3a.** Immunofluorescence analysis of the time and dose dependency of *Simkania*-mediated anti-apoptosis. HeLa cells were infected with *Simkania* for 1–5 days, MOI 0.5, 5.0, 10 and 20, induced with 20 ng/ml TNF-α/3 µg/ml chx or with carrier for 4 h and stained with Draq5 (nuclei+inclusions, blue), Syto 82 (accumulates in *Simkania*, red) and Phalloidin (actin, green). Images shown are from an experiment with MOI 0.5; MOI 5, 10 and 20 are not shown. Infection led to apoptosis resistance from day 1 on. Bar-10 µm, n = 3

Time course experiments showed time-dependent resistance for TNF-α/Chx-induced apoptosis at a multiplicity of infection of 0.5 beginning at 1 dpi and was similar to non-treated cells at day 3 with <10% apoptotic cells (Figure 3.3c). Further,

apoptosis resistance was found to be dependent on the MOI, cells infected with higher MOI acquired apoptosis resistance much faster as demonstrated in a quantification of TNF-α/chx-induced apoptosis at day 1 using MOI ranging from 0.5 to 20 (Figure 3.3c).

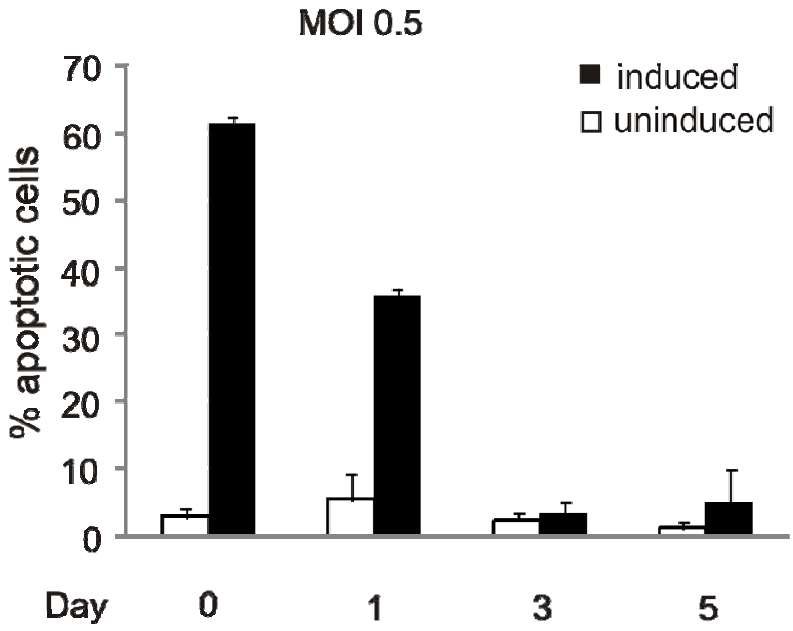


**Figure 3.3b.** Bar diagram displaying the quantitative analysis of the time dependency at MOI 0.5 from the experiment show in Figure 3.3a. Cells from five random fields were counted under a 40X objective and the percentage of apoptotic cells was calculated. Apoptosis resistance increased till day 3; towards the end of the infection cycle apoptosis sensitivity slightly increased until day 5. At higher MOI infected cells became more susceptible to apoptosis towards end cycle. n = 3.

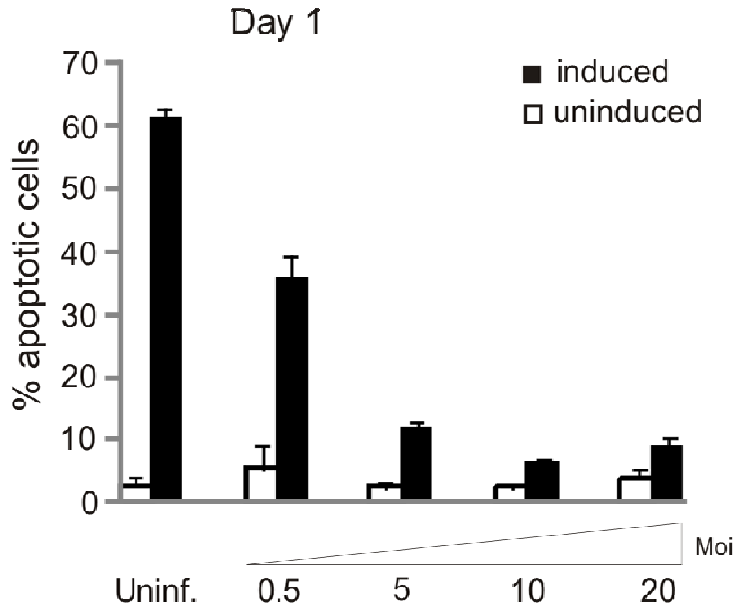


**Figure 3.3c.** Bar diagram displaying the quantitative analysis of the MOI dependency at day 1. Apoptosis resistance was found to be clearly dose dependent, increasing to MOI 10. At MOI 20 increased apoptosis sensitivity was observed. n = 3.

## 3.4. Sn infected cells resist apoptosis induced by Staurosporine

To test whether the intrinsic apoptosis pathway is inhibited, *Simkania*-infected cells were treated with the broad range kinase inhibitor staurosporine (STS). HeLa 229 cells were plated and infected with Sn at a MOI of 1 for 3 days and induced with 1 µM STS for 5 h. After the treatment the cells were fixed and stained as described before. And the percentage of apoptotic cells was calculated. Infection reduced the apoptotic population in STS-treated cells in the same manner as observed in TNF-α/chx-treated cells (Figure 3.4). Taken together, *S.negevensis* inhibits both TNF-α/chx and STS-induced apoptosis in a time and infection dose-dependent manner

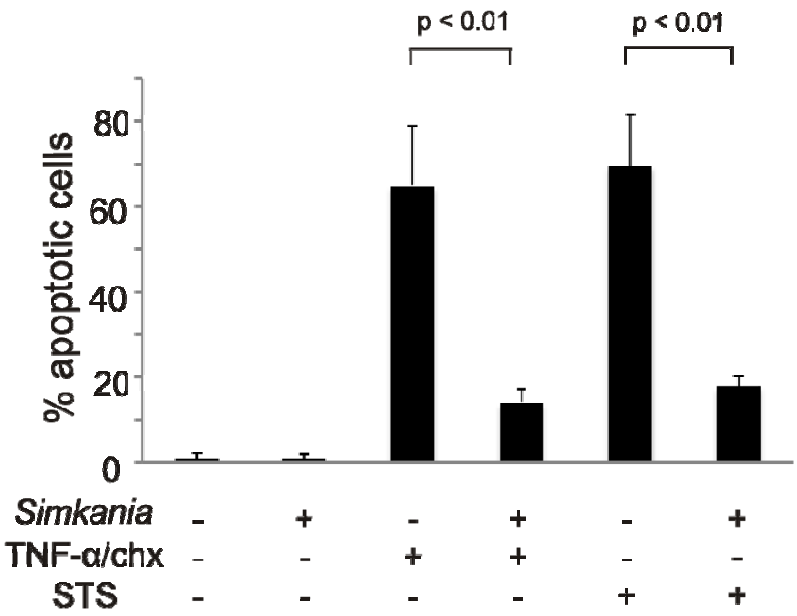


**Figure 3.4.** Bar diagram displaying the quantitative analysis of an infection/apoptosis induction experiment using either TNF-α/chx or STS. HeLa cells with or without *Simkania* infection (MOI 0.5) were treated with 20 ng/ml TNF-α/3 µg/ml chx, STS 1 µg/ml or with carrier for 4 h. Samples were stained with Hoechst 33258 (images not shown), five random fields were counted under a 40x objective and percentage of apoptotic cells was calculated. *Simkania* infection significantly decreased the number of apoptotic cells upon TNF-α/chx or STS stimulation, (n = 2).

## 3.5. TNFR is not shed during Sn infection

*Chlamydia trachomatis* infection differentially regulates the surface expression of TNFR1 and TNFR2 on the cell surface (Paland et al, 2008). TNFR is produced as a membrane anchored protein whose extra cellular domain can be released by proteolysis, a process called protein ectodomain shedding. In the case of TNFR this process is mediated by TNF-α converting enzyme (TACE) (Black et al, 1997). Upon *Chlamydia* infection, the bactericidal action of secreted TNF-α (a major weapon of immune system) is regulated by the activation of TACE and shedding of

TNFR from the cell surface (Paland et al, 2008). This could also explain one of the mechanisms by which *Chlamydia* resist apoptosis induced by TNF-α. As Sn was also found to resist apoptosis induced by TNF-α, curiosity raised the question if TNF-α, binds to TNFR, and signals the survival pathway. Hence HeLa cells were plated and infected with Sn for 72 h and induced with TNF-α in the absence of chx for different time points ranging from 5 min to 60 min. The cell lysate was taken up for western blots (as described in Methods 2.2.13). MEK and ERK were phosphorylated in Sn infected cells as well as the uninfected cells, which indicated that Sn activates survival pathway that may serve to complete its life cycle (Figure 3.5). This also indicated that Sn infected cells have intact TNFR1 on cell surface and they have normal TNFR1 signaling upon TNF-α induction.

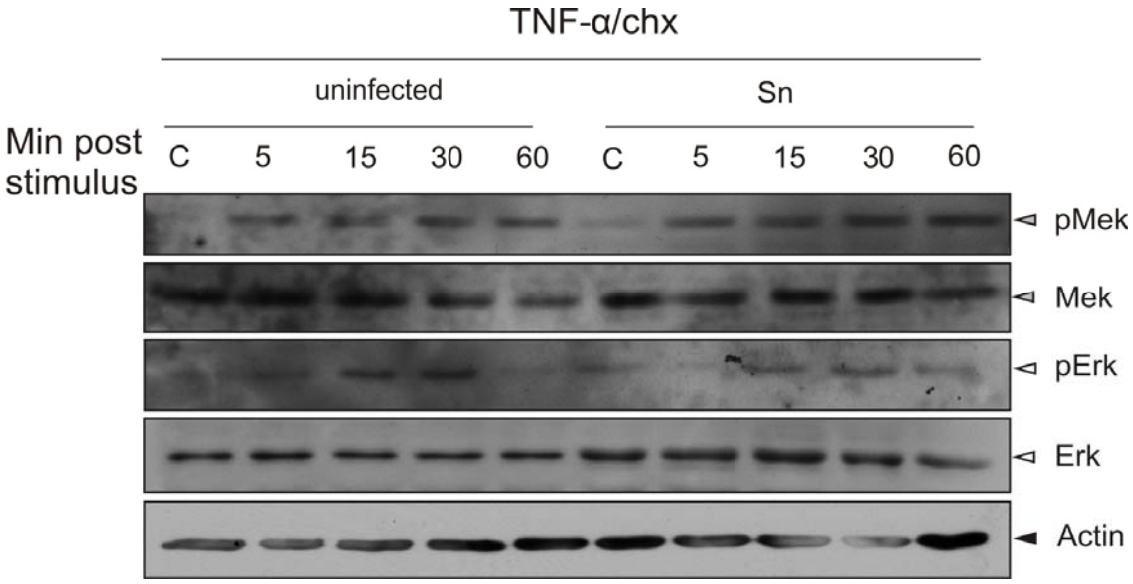


**Figure 3.5.** TNF receptor is activated in *Simkania* infected cells**.** HeLa cells with or without *Simkania* infection (MOI 1) were treated with 20 ng/ml TNF-α without chx for 5, 15, 30 and 60 min to activate the MEK-ERK pathway. The figure shows the phosphorylation of MEK (grey arrowheads) and ERK (white arrowheads), indicating that the TNF receptor is active. Actin is used as the loading control. n = 2.

### 3.6. Caspase maturation isdifferentialy regulated in *Simkania*-infected cells

TNF-α-induced apoptosis depends on a well characterized signaling cascade (Wallach et al, 1999b) and is thus suited to more precisely define the block in apoptotic signaling in *Simkania*-infected cells. To investigate whether *Simkania* blocks the proteolytic cleavage and activation of caspases, infected and TNF-α/chx treated cells were tested for caspase processing and activity. Both caspase-3 cleavage and activity were inhibited by *Simkania* infection (Figure 3.6a and 3.6b). The treated and infected cells were also taken up for immunostaining, and the percentage of apoptosis was counted under the confocal microscope. This revealed the infected cells showed only ~11 % of activated caspase-3 against ~35 % of the uninfected induced cells (Figure 3.6c). Caspase-3 cleavage leads to proteolytic degradation of key proteins such as the nuclear enzyme poly-ADP ribose polymerase (PARP). PARP degradation was inhibited in *Simkania*-infected TNF-α/chx-induced cells (Figure 3.6a), confirming a strongly reduced caspase-3 activity in infected cells.

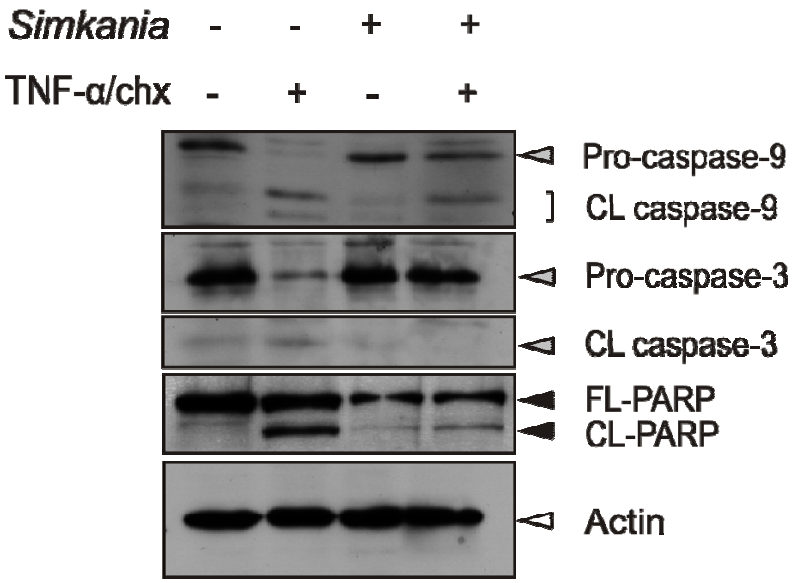

**Figure 3.6a.** HeLa cells with or without Sn infection (MOI 0.5, 3 days) were treated with 20 ng/ml TNF-α/3 μg/ml chx for 4 h before analysis. Immunoblot showing the activation status of caspases-3, -8 and -9 (grey arrowheads) as well as PARP (black arrowheads) cleavage. Caspase-9 and -3 were not found to be cleaved in infected cells compared to uninfected induced cells. PARP is also not cleaved nor activated on induction in Sn-infected cells. Actin (white arrowhead) is used as the loading control. n=2

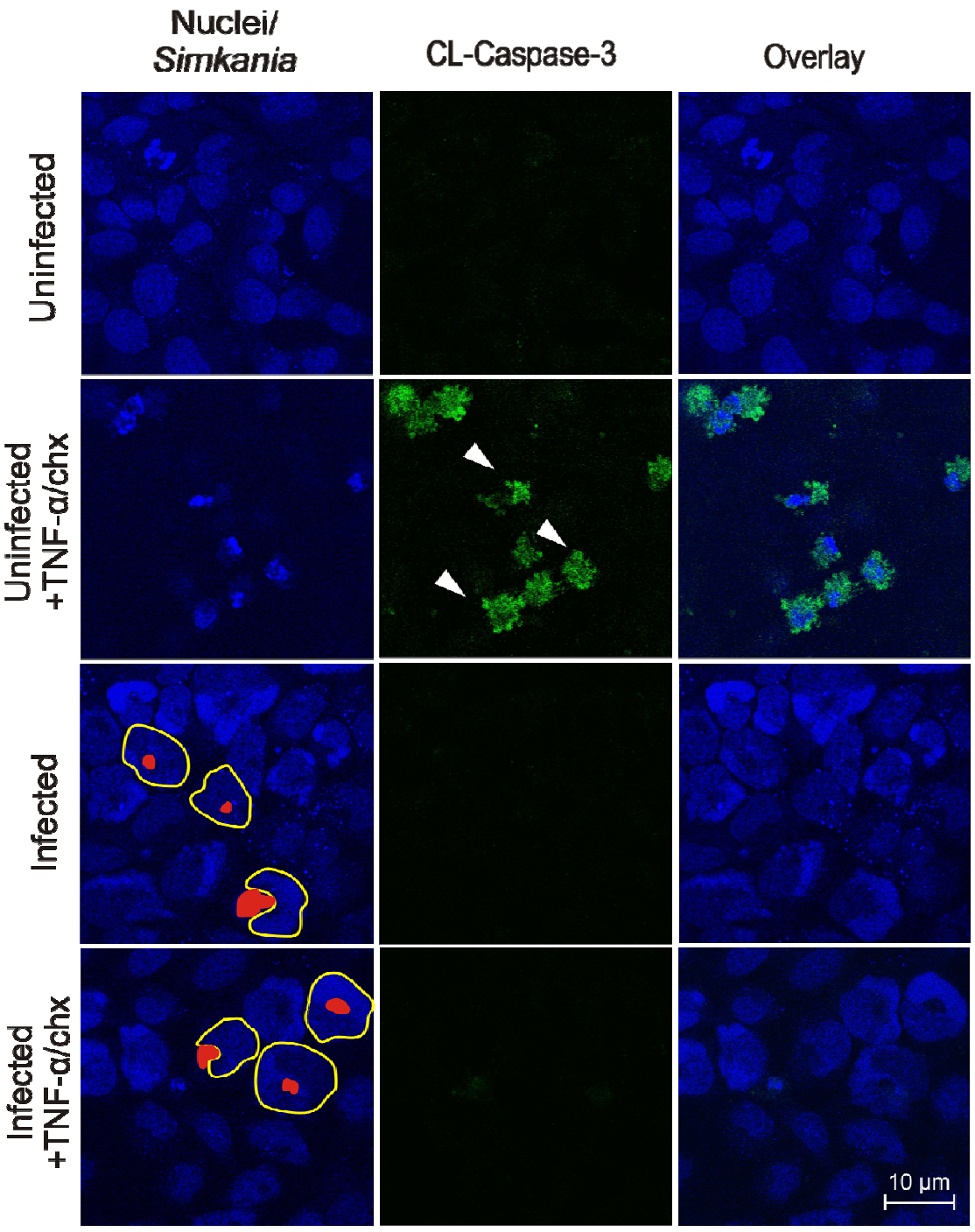

**Figure 3.6b.** Immunofluorescence analysis showing that caspase-3 is not activated in Sn-infected cells on induction with TNF-α/chx. Nuclei and bacteria were stained with Draq5 (blue) and activated caspase-3 was detected with an antibody specific for the cleaved protease (green, CL-Caspase 3). White arrowheads mark active caspase-3. Bacterial inclusions are exemplified by yellow open and nuclei by red filled circles. Bar-10 µm, n = 2.

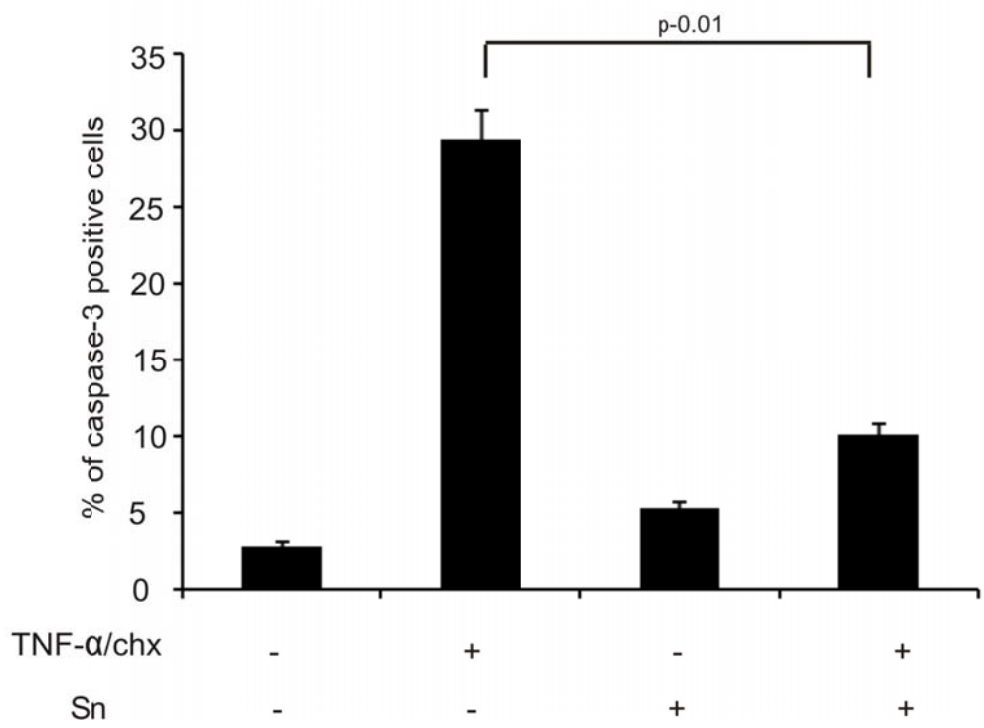


**Figure 3.6c.** Bar diagram displaying the quantitative analysis of the caspase-3 activation at MOI 0.5 from the experiment shown in Figure 3.6b. Cells from five random fields were counted under a 40x objective and the percentage of caspase-3 positive cells was calculated. *Simkania*-infection strongly reduced caspase-3 activation. n = 3.

To investigate if Sn-infection affects caspase-9 and caspase-8, processing or activity of the respective Caspases were tested. Interestingly, while caspase-9 processing was blocked, caspase-8 activity was not affected by infection with *Simkania* (Fig. 3.6a and 3.6d), demonstrating the selective inhibition of caspases in

cells infected with *Simkania*. Rather, Sn infections lead to the activation of caspase 8 even without apoptosis induction.

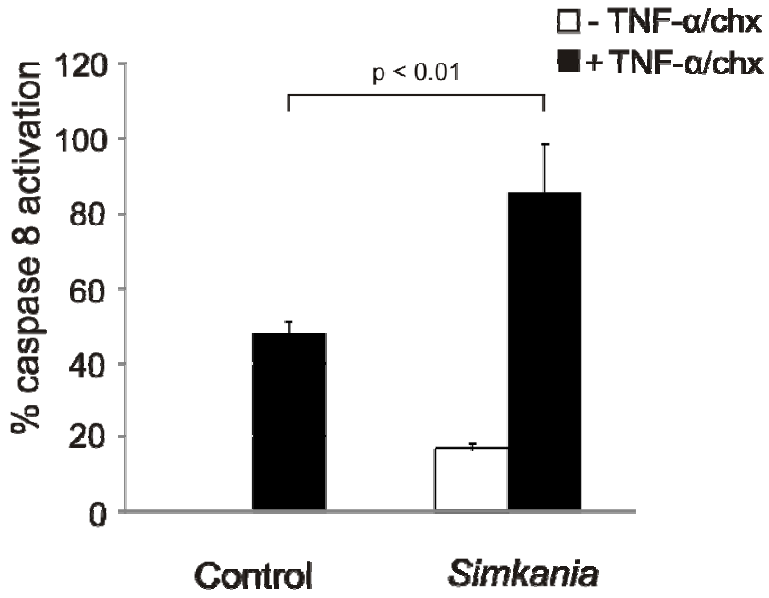


**Figure 3.6d.** Bar diagram showing the result of a caspase-8 activity assay (caspase-8 glo). Activation of caspase-8 is not blocked by Sn infection. n = 3.

## 3.7. *Simkania* infection inhibits apoptosis on the upstream of mitochondria.

Block of caspase-9/-3 in the presence of active caspase-8 is indicative of an apoptosis inhibition at or upstream of the mitochondria (Lakhani et al, 2006). When the cell death pathway is activated, activation and heterodimerisation of various pro-apoptotic proteins lead to the mitochondrial membrane potential loss. Mitochondrial cytochrome *c* is translocated to the cytosol of cells undergoing apoptosis where it participates in caspase-9 activation (Kluck et al, 1997). Hence, it was tested if Sn infection can prevent cytochrome *c* release from the cells treated with TNF-α/chx induction. While TNF-α/chx treatment induced the release of cytochrome *c* from mitochondria of uninfected cells, this was not the case in *Simkania*-infected cells (Fig.

3.7a, 3.7b). Failure to release cytochrome *c* indicated incomplete activation of Bax and/or Bak upon apoptosis induction. Bax, once activated undergoes a conformational change permitting insertion into the mitochondrial membranes. First, it was excluded that either Bax or Bak are differentially transcribed during infection (data not shown). Then whether Bax translocation into mitochondria is affected in Sn-infected cells upon TNF-α/chx -induced apoptosis was tested. Immunostaining confirmed strongly reduced levels of translocated Bax in infected cells upon apoptosis induction (Fig. 3.7d). Once activated Bax and Bak form oligomers in the mitochondrial outer membrane leading to the release of pro-apoptotic factors like cytochrome c. Bax and Bak oligomerisation was done via mitochondria isolation and *in vitro* cross-linking using BMH (as decribed in methods 2.2.7.7). Neither Bax nor Bak were found to form oligomers after TNF-α/chx treatment in *Simkania*-infected cells in comparison to non-infected cells (Fig. 3.7c). Thus, *Simkania* infection inhibits Bax translocation to the mitochondria as well as Bax/Bak oligomerisation.

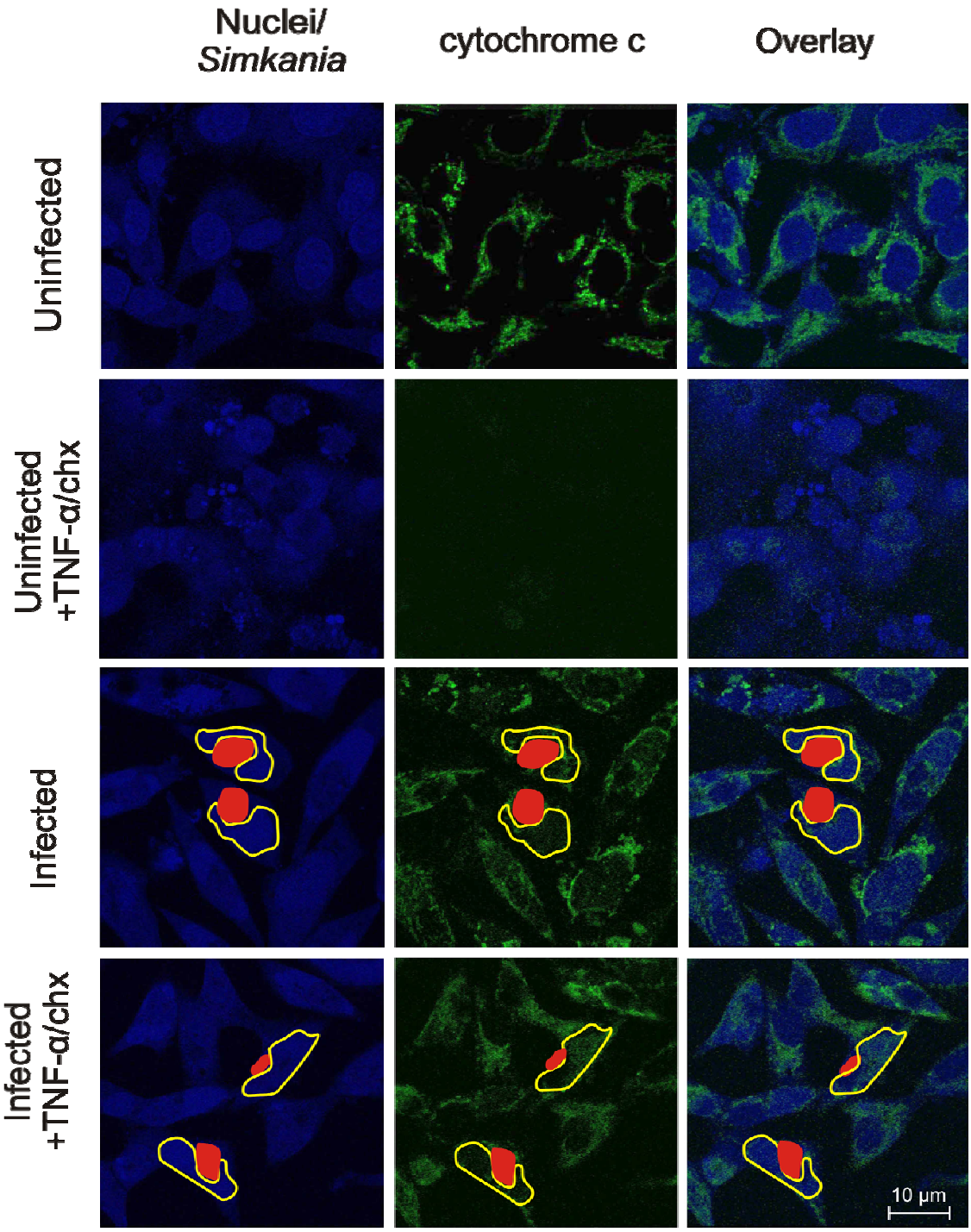


**Figure 3.7a.** Immunofluorescence staining on the cytochrome c distribution after *Simkania* infection on HeLa cells with or without *Simkania* infection (MOI 1, 3 days) were treated with 20 ng/ml TNF-α/3 µg/ml chx or carrier for 4 h before analysis. Cells were stained for cytochrome c (green), *Simkania*/nuclei (blue) and viewed with a confocal microscope. Upon apoptosis induction cytochrome c is released from the mitochondria (uninfected+TNF-α/chx), this was not the case for infected cells (infected+TNF-α/chx). Bar- 10 µm.

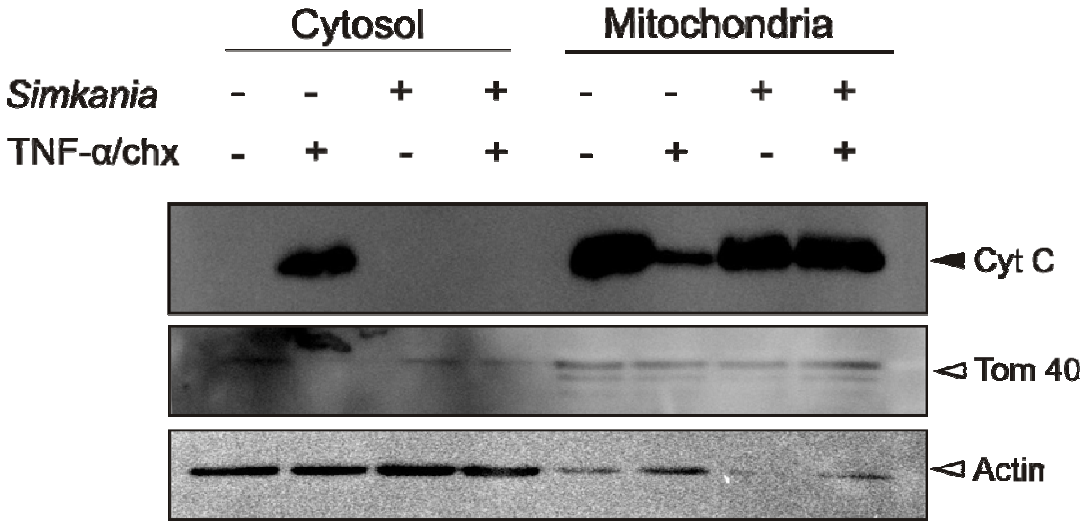


**Figure 3.7b.** Western blot analysis for cytochrome c release. Cytosol and mitochondria were isolated as described inthe experimental procedures section and cytochrome c was detected by Western blot. cytochrome c release was completely blocked by *Simkania* infection. Tom40 was used as a marker for mitochondria and Actin as a marker for cytosol. n=3

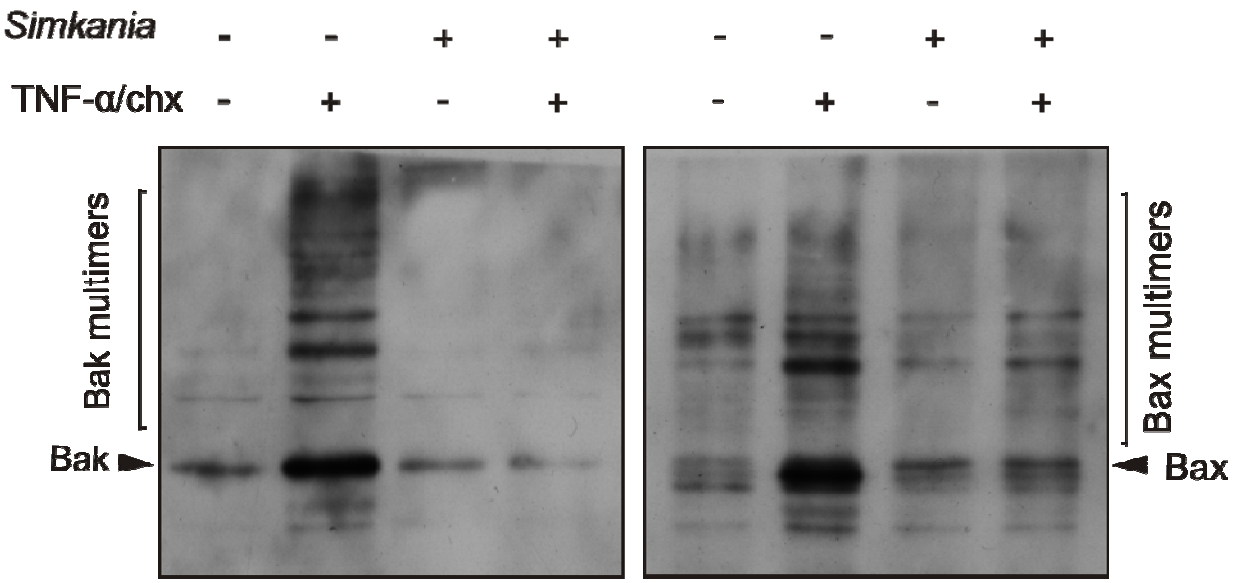


**Figure 3.7c.** Immunoblot analysis of Bax and Bak after mitochondrial isolation and *in vitro* cross linking. HeLa cells with or without *Simkania* infection (MOI 1, 3 days) were treated with 20 ng/ml TNF-α/3 µg/ml chx or carrier for 4 h before mitochondria isolation, *in vitro* crosslinking and analysis. Bax and

Bak (black arrows) are not activated in *Simkania*-infected induced cells as shown by background Bax and Bak oligomerisation (brackets). Equal loading was verified by Bradford assay and Tom 40 western blot. Input Bak, Bax and Actin showed no differences in expression or loading. n = 2.

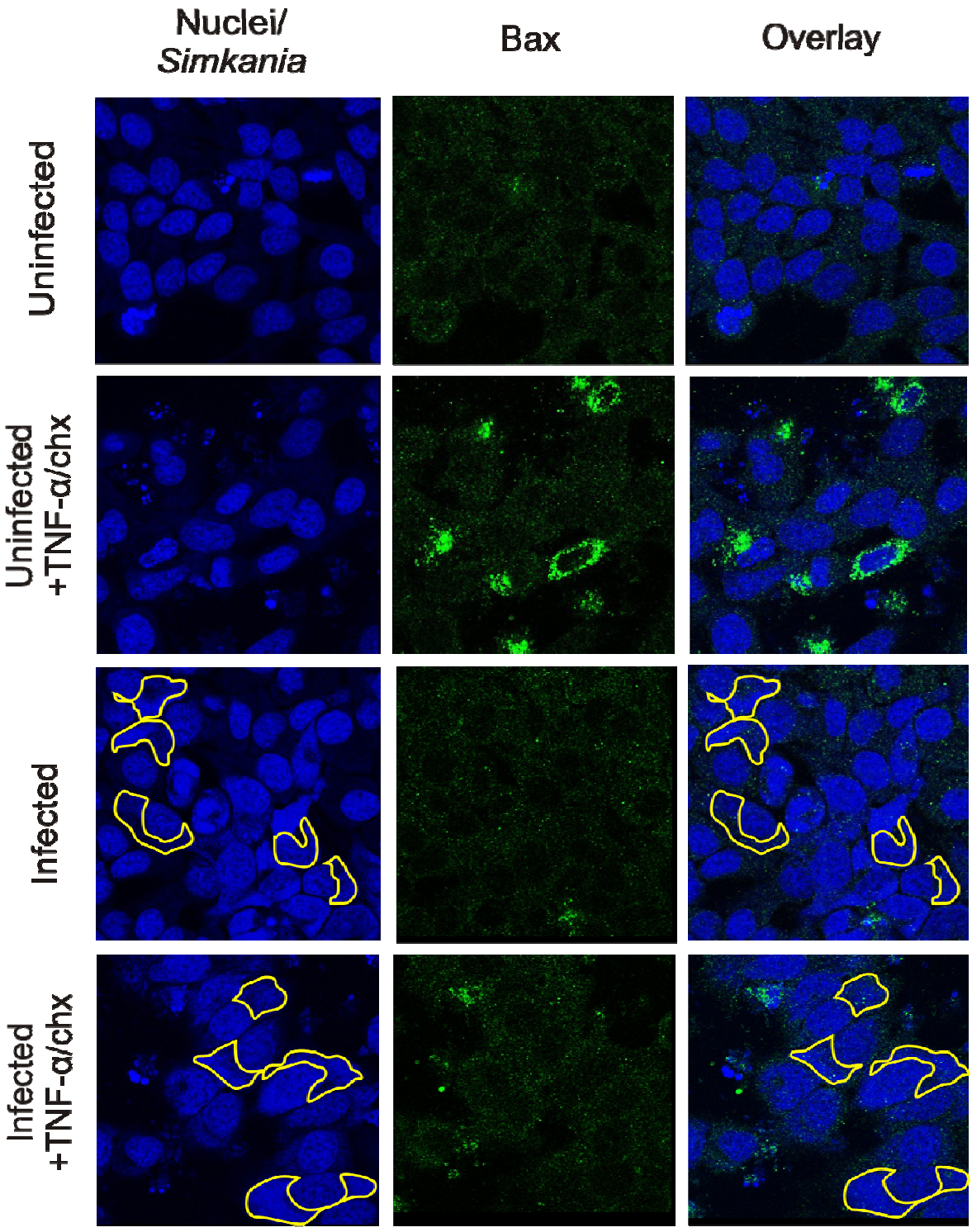

**Figure 3.7d.** Immuno fluorescence staining of active Bax with a conformation sensitive antibody after *Simkania* infection. HeLa cells were treated as described in Figure 3.7a. Images show mitochondrial localization of active Bax (green) in control-induced cells. Bax was not activated in TNF-α/chx treated *Simkania*-infected cells, n=3.

## 3.8. Pro-apoptotic BH3-only proteins are not degraded in *Simkania* infected cell

Inhibition of apoptotic signaling upstream of mitochondria has previously been shown to involve Bcl-2 family members in *Chlamydia*-infected cells (Fischer et al, 2004b). One mechanism involves the degradation of pro-apoptotic BH3-only proteins acting to either directly or indirectly induce Bak and/or Bax oligomerisation and mitochondrial outer membrane permeabilization (Dong et al, 2005; Ying et al, 2005).

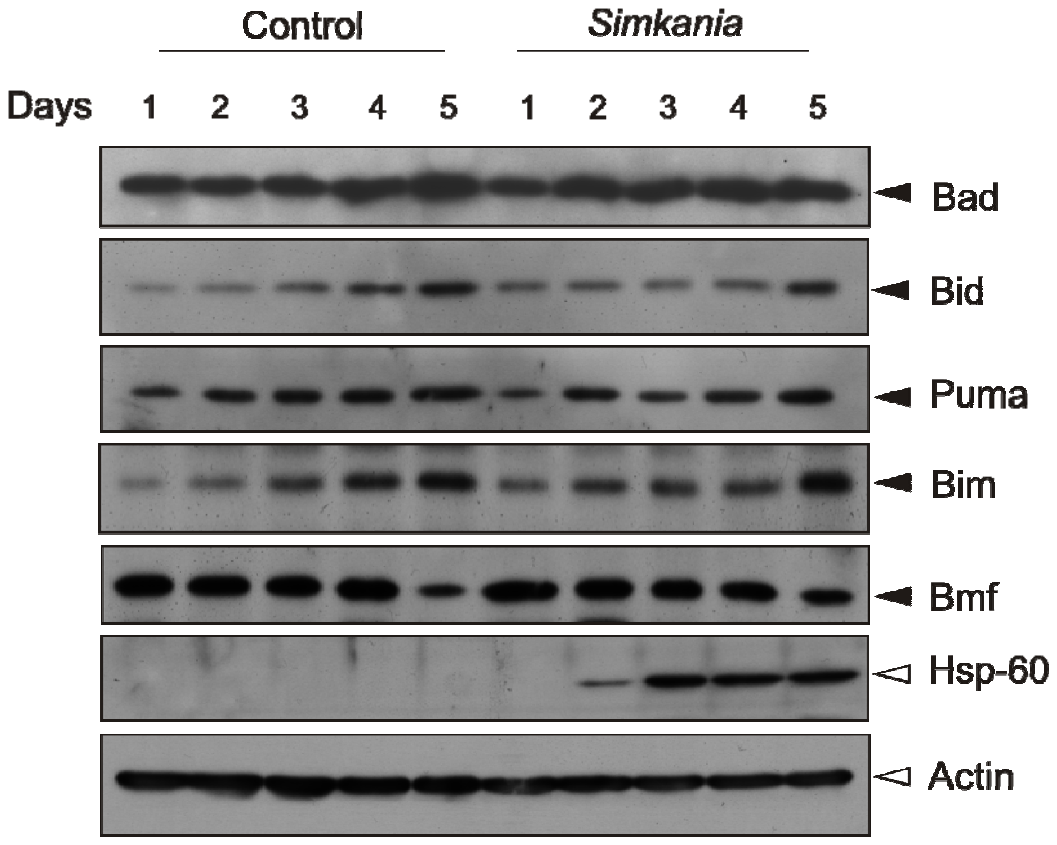

**Figure 3.8.** Immunoblot analysis of the pro-apoptotic BH3-only Bcl-2 family members during S*imkania* infection. HeLa cells were infected with *Simkania* or Mock (control) (MOI 1) in a time course experiment before analysis. The BH3-only proteins (Bad, Bid, Puma, Bim, Bmf, black arrowheads) are not degraded in *Simkania*-infected cells. Hsp 60 and Actin (white arrowheads) were used as loading controls. n = 2

Hence it was tested whether this is similar in *Simkania* infection. HeLa 229 cells were infected with Sn for different time points from day 1 to day 5, the cell lysates were blotted against Bad, Bid, Bim, Puma and Bmf antibody. No degradation of the BH3-only proteins was detectable (Figure 3.8). This proves that the resistance to apoptosis in Sn-infected cells does not depend on the regulation of the pro apoptotic BH3-only proteins.

## 3.9. Anti-apoptotic Bcl-2 family members are not regulated in Sn-infected cells

The regulation of anti-apoptotic Bcl-2 family members Bcl-2 and Mcl-1 were also tested during the time course of Sn infection. Mcl-1 has previously been found to be up regulated and stabilized in *C. trachomatis* infection, Mcl-1 was also critical for apoptosis resistance in Ctr-infected cells (Bohme et al; Rajalingam et al, 2008b; Sharma & Rudel, 2009).

Interestingly, Mcl-1 and Bcl-2 levels remained constant in a time course infection experiment (Fig. 3.9), indicating that regulation of the Bcl-2 family members does not account for the apoptosis resistance in *Simkania* infection.

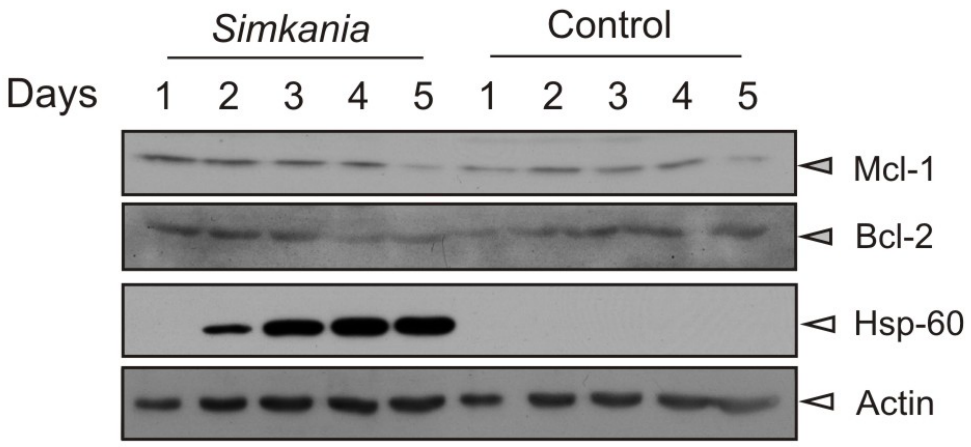


**Figure 3.9**. Immunoblot analysis of the anti-apoptotic/pro-survival signaling during *Simkania* infection. HeLa cells were infected with *Simkania* or Mock (control) in a time course experiment before analysis. Anti-apoptotic Bcl-2 family members like Mcl-1 or Bcl-2 (grey arrowheads) are not regulated. Akt is strongly activated during infection (black arrowheads). Hsp 60 and Actin (white arrowheads) were used as loading controls. n = 2.

## 3.10. NF-κB is activated upon Sn infection

NF-κB comprises a family of highly regulated dimeric transcription factors that play a pivotal role in inflammatory responses and immunological reactions and are required for apoptosis resistance in *C. pneumoniae* infection (Paland *et al.*, 2006). To test the relevance of NF-κB signaling in *Simkania* infection, the degradation of IκB-α after *Simkania* infection was tested. The HeLa cells infected with Sn for 72 h were induced with TNF-α in the absence of chx for time points, ranging from 5 min to 60 min. The cells lysates were blotted and probed against IκB antibody, to find that IκB was degraded in induced cells. Surprisingly the uninduced Sn-infected (72 h) cells also lacked the expression of IκB, which is the clear indication that NF-κB is activated upon Sn infection (Figure 3.10a).

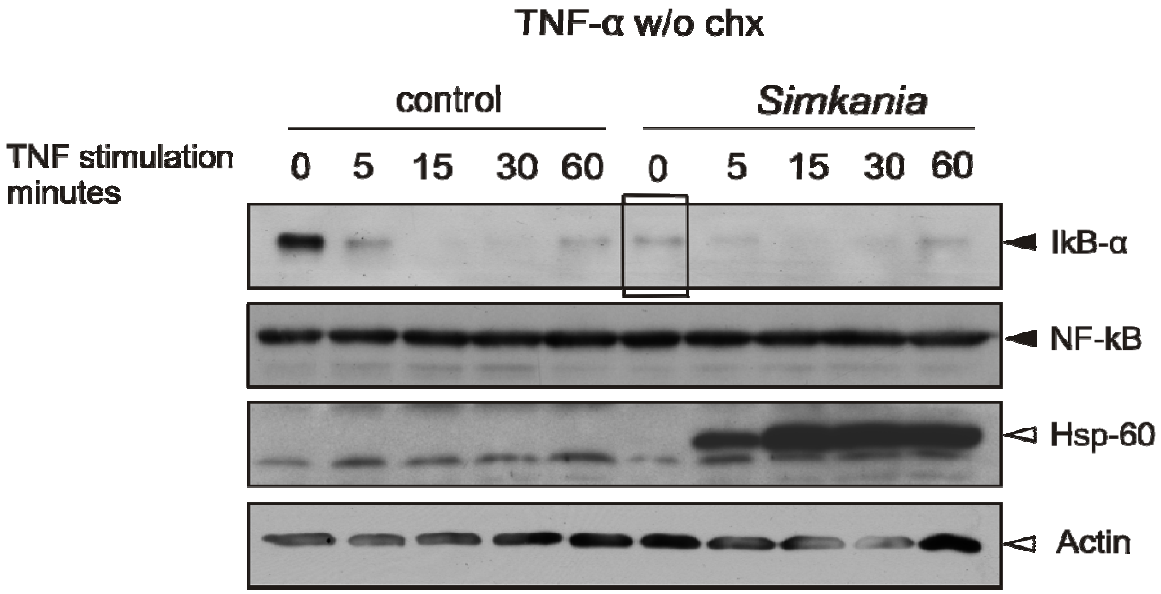


**Figure 3.10a.** HeLa cells with or without *Simkania* infection (MOI 1) were treated with 20 ng/ml TNF-α without chx for 5, 15, 30 and 60 min to activate the MEK-ERK pathway. The regulation of IκB is presented above. The figure shows that even without any TNF-α activation after 3 dpi IκB is ubiquitinated and degraded (black arrowheads) and the total p65 remains unaffected, indicating that the NF-κB is activated upon infection. Actin is used as the loading control, n = 2.

Infected cells were nearly completely depleted of IκB-α similarly as non-infected control cells upon TNF-α stimulation without chx. Hence to further investigate and confirm, Sn-infected cells (72 hpi) were stained for p65 (RelA) an important subunit of NF-κB, which gets transported to the nucleus upon NF-κB activation. Figure 3.10b shows that NF-κB gets clearly activated upon Sn infection.

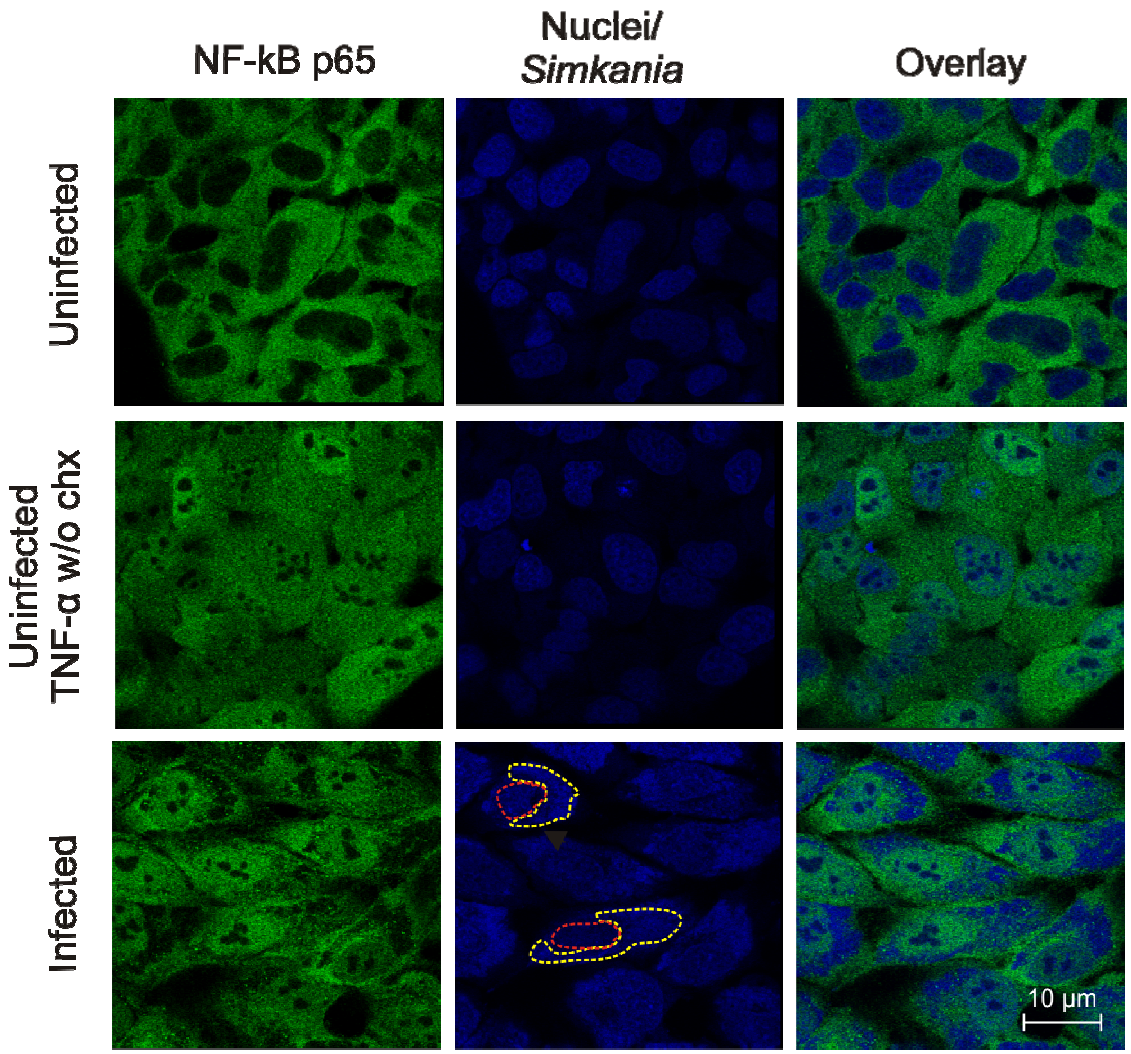


**Figure3.10b.** Immunofluorescence staining of p65 component of NF-κB in uninfected, uninfected TNF-α treated and 3 days Sn infected cells. P65 (green) is detected to be in the nucleus in the TNF-α treated control cells as expected, while the same is also detected in Sn infected cells. P65 is hardly activated in the control cells. The nucleus is labeled with Draq5 (blue), which intercalates in the DNA of the cell as well as the bacteria, n=2.

Consistent with the activation, NF-κB is translocated to the nucleus in infected cells similar as upon TNF-α stimulation. To investigate the binding of NF-κB subunits to the nuclear DNA, Electro Mobility Shift Assay (EMSA) was performed (in collaboration with Dr. Bhupesh Prusty) as described in the Method (2.2.14). The

Figure 3.10c shows the EMSA result, which indicates that NF-κB gets activated, revealing increased binding of NF-κB to an oligonucleotide comprising an NF-κB consensus motif upon 48 hpi with Sn and it is the maximum at 72 hpi at which they resist apoptosis. At the later time point of 4 dpi, the activation clearly goes down.

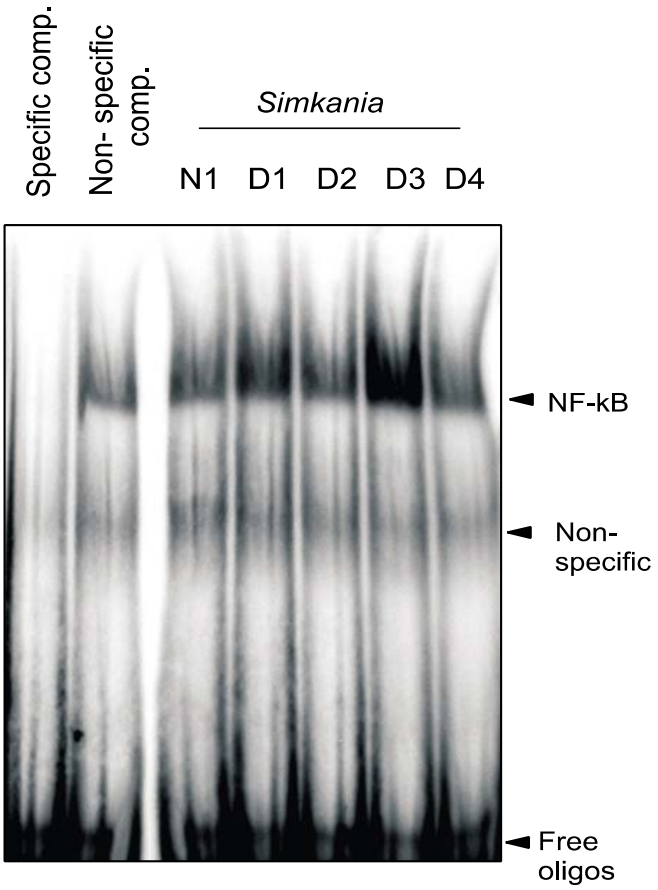


**Figure 3. 10c.** To confirm if NF-κB binds to the nucleus in infected cells, the HeLa 229 cells were infected with Sn from day 1 to day 5 and the cells were taken up for EMSA (in collaboration with Dr. Bhupesh K Prusty). The nuclear extract was made and incubated against radioactive labeled probes. The samples were run on 6 % PAGE and developed. EMSA clearly shows that NF-κB is activated upon infection with Sn, n=2

## 3.11. NF-κB activation is necessary for apoptosis resistance

The next question that rose was if NF-κB activation was required for the apoptosis resistance in Sn-infected cells. HeLa 229 cells were plated and treated with NF-κB specific inhibitor CAPE, a cell permeable active component of propolis from honeybee hives.

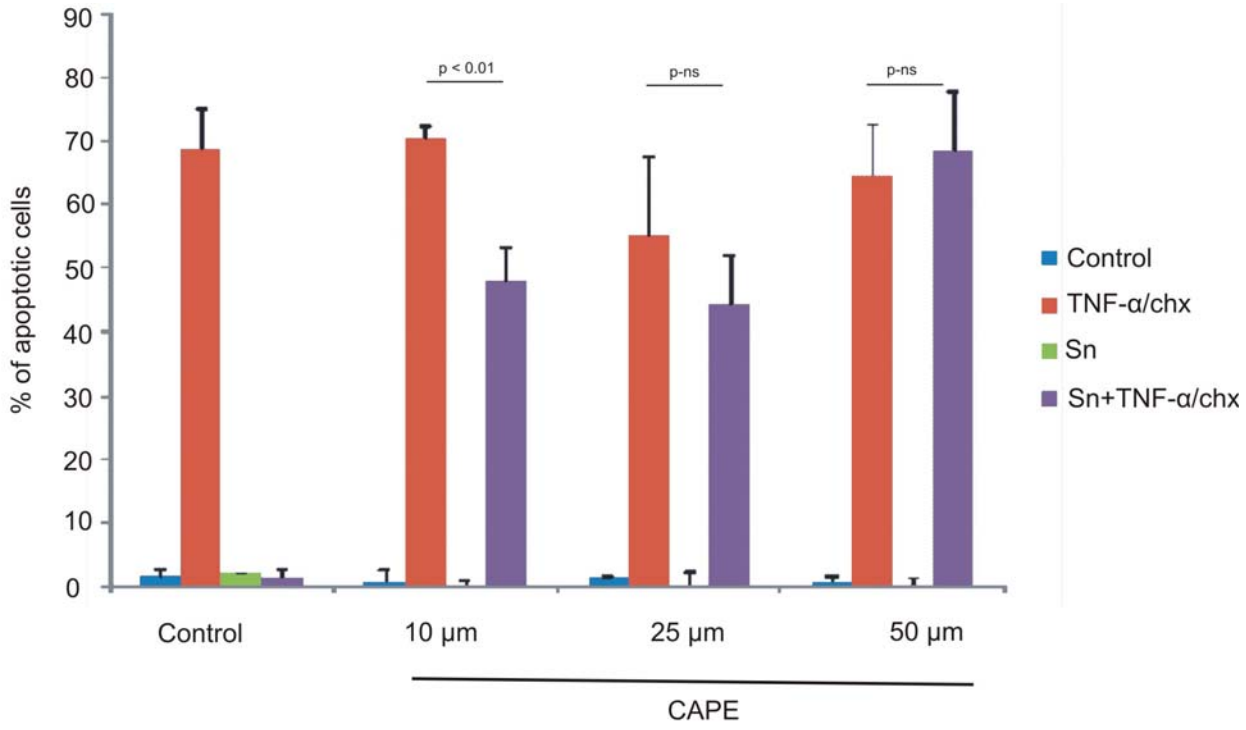


**Figure 3.11a.** Bar diagram showing the percentage of apoptotic cells. Sn infected HeLa 229 cells were treated with CAPE (NF-κB Inhibitor) on 3 dpi for 5h and induced with 20 ng/ml TNF-α/3 mg/ml chx or carrier for 4 h before analysis. The cells were fixed and stained for Hoechst, the apoptotic and healthy cells were counted under 40x magnification, five fields per sample, n = 2, Error bars = SE n = 2.

The HeLa 229 cells were plated and treated with 25 µM of CAPE for 5 h after 3 dpi with Sn infection and were induced for apoptosis using TNF-α and chx as described in Methods. After 4 h of induction the cells were harvested for immunoblotting for PARP cleavage (as shown in figure 3.11b). A similar experiment

was performed for immunofluorescence to access the percentage of apoptosis (Figure 3.11a). CAPE treatment led to decrease in apoptosis resistance and the infected cells were more sensitized to apoptosis than the untreated cells.

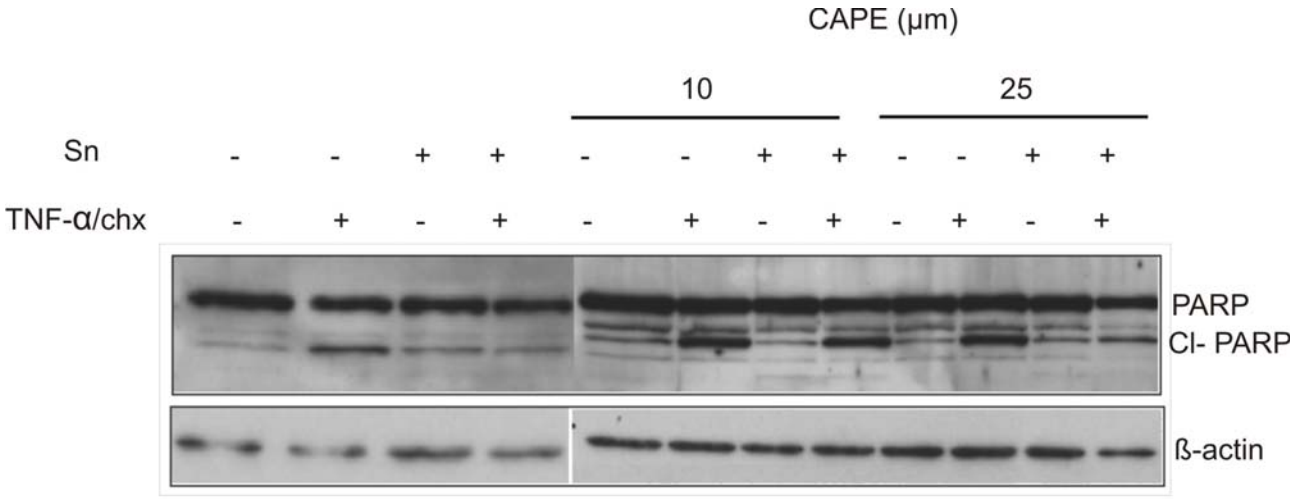


**Figure 3.11b.** Immunoblot analysis of PARP cleavage in *Simkania* infected cells after treatment with NF-κB inhibitor. *Simkania*-infected HeLa 229 cells were treated with CAPE (NF-κB Inhibitor) on 3 dpi for 5 h and induced with 20 ng/ml TNF-α/3 µg/ml chx or carrier for 4 h before analysis. PARP cleavage was a measure of apoptosis sensitization. Treatment with 10 µM CAPE clearly sensitized infected cells to apoptosis. Hsp60 and Actin were used as loading controls.n = 2.

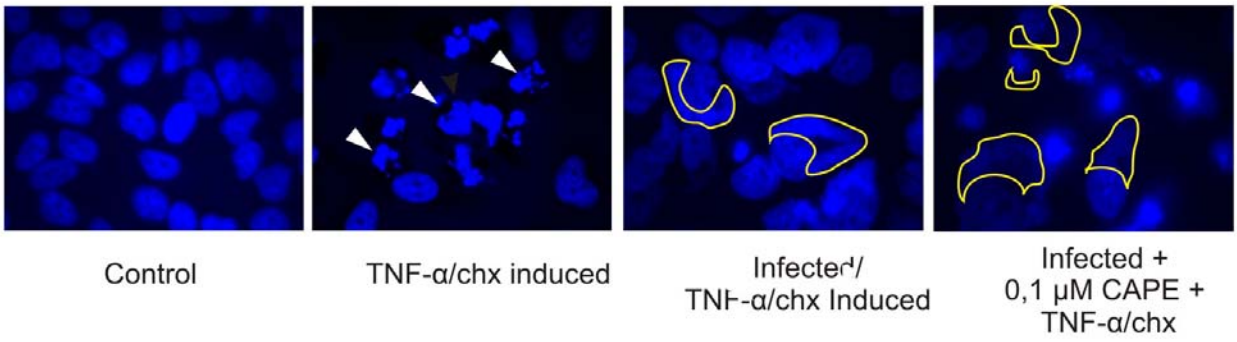


**Figure 3.11c**. Immunofluorescence of samples treated as described in Figure 3.11b. Samples were stained with Hoechst (blue) and viewed under a fluorescence microscope for counting. Hoechst dye stained both HeLa cell nuclei and *Simkania* inclusions. White arrowheads mark example apoptotic cells, n = 2.

## 3.12. NF-κB mutation affects the infectivity of Sn

As NF-κB was strongly activated during infection it was interesting to check whether NF-κB activation is required for the development of bacterial inclusion and infectivity.

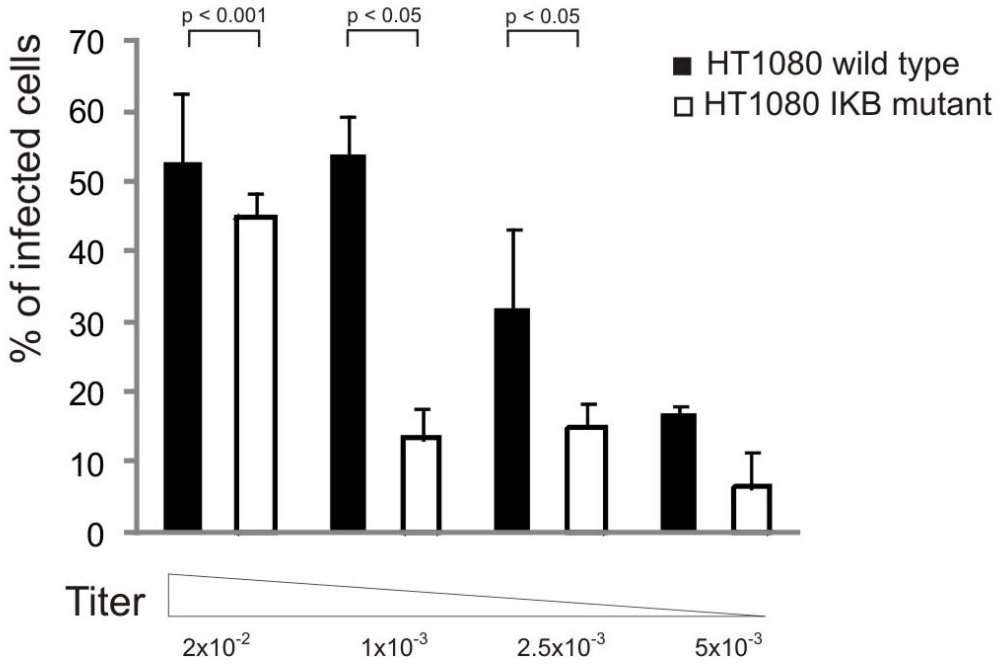


**Figure 3.12a.** Bar diagram showing the *Simkania* infectivity assay. HT1080 wild type (fibrosarcoma cells) and IκB mutant cells were infected with *Simkania* at a MOI 1 for 3 days titrated on fresh HeLa monolayers and stained. *Simkania* positive cells were counted to determine differences in primary infection between the two cell lines (40x magnification, five fields per sample, n=2, Error bars = SE). Bacterial growth was strongly reduced in IκB mutant cells.

Since the developmental cycle of Sn takes 3-5 days, inhibitor treatment was not feasible for this experiment due to toxicity upon long-term treatments. Therefore HT1080 cell line, which expresses a non-degradable IκB derivative, which sequesters NF-κB p65 in the cytosol, was used (Wang *et al.*, 1996).

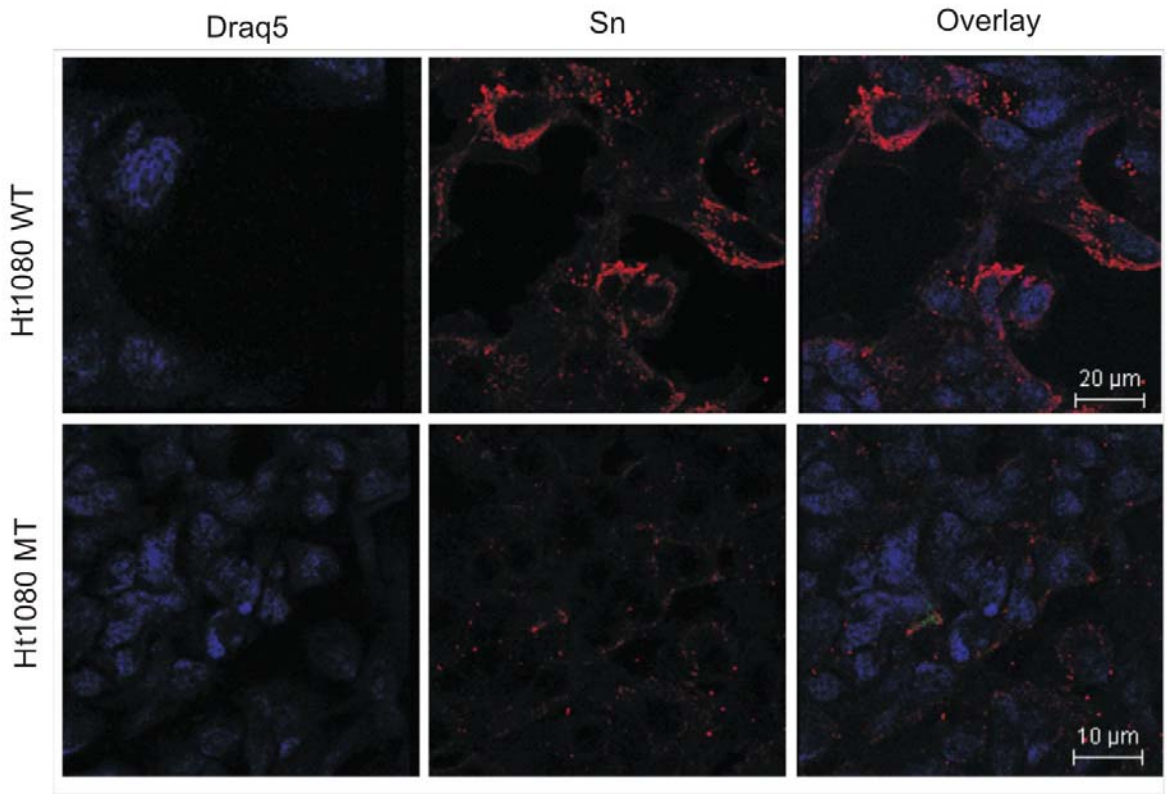


**Figure 3.12b.** Immunofluorescence analysis of HT1080 wt and IκB mutant cells infected with Sn. the cells are stained with Draq5 (blue) and with Sn antibody (red). The HT1080 WT cells are stained more with Sn than the IκB mutant cells showing that the mutants have defect in promoting Sn infection, n=3

The cells were infected with Sn and taken up for infectivity assay. After 72 hpi, the infected cells were lysed using glass beads or freeze thawing at $^{-}80^{o}C$. The lysate was used to infect freshly plated HeLa cells in different dilutions. After second round of infection for 72 h the cells were fixed with 4% PFA and stained with Hoechst and taken up for microscopy. The percentage of infected cells were calculated and used for making the graph as represented in Figure 3.12a. Surprisingly, the expression of non-degradable IκB resulted in decreased infection and infectivity in Sn (Fig. 3.12a), suggesting a role of NF-κB transcribed genes in the replication of Sn.

### 3.13. Akt is activated in *Simkania* infected cell

To further determine the origin of *Simkania*-mediated anti-apoptosis the major survival pathway directly involved in TNFR signaling, PI3 kinase pathway was analysed. It has been reported that Akt/protein kinase B inhibits apoptosis by preventing the release of cytochrome c from mitochondria (Brazil et al, 2002; Kennedy et al, 1999). Since PI3 kinase pathway is a major survival pathway, Sn infected cells after different time points were probed against Akt and pAkt antibody, revealed that Akt was indeed strongly phosphorylated in infected cells (Figure 3.13). The phosphorylation was very prominent after 3 dpi and gradually reduced after day 4. This data very much supports the resistance that this bacterium promotes on apoptosis induction.

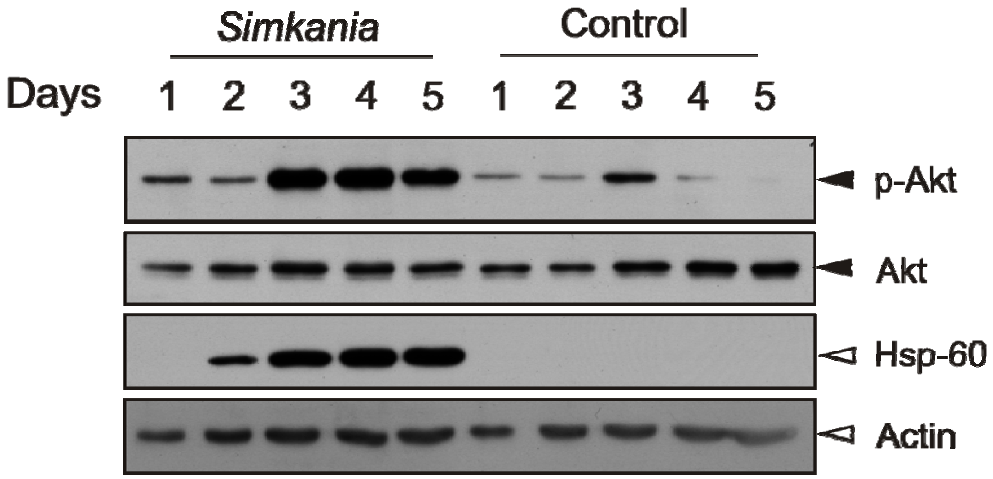


**Figure 3.13.** Immunoblot analysis of the anti-apoptotic/ pro-survival signaling during *Simkania* infection. HeLa cells were infected with *Simkania* or Mock (control) in a time course experiment before analysis. Anti-apoptotic Bcl-2 family members like Mcl-1 or Bcl-2 (grey arrowheads) are not regulated. Akt is strongly activated during infection (black arrowheads). Hsp 60 and Actin (white arrowheads) were used as loading controls, n = 2.

## 3.14. Activation of PI3 kinase pathway is required for apoptosis inhibition in Sninfected cells

As PI3 kinase is a major survival signal, it was important to find if its activation is necessary for the resistance to apoptosis. To test whether activation of Akt is required for apoptosis inhibition, inhibitor experiments were performed. The Akt pathway was investigated by blocking the upstream activator phosphatidylinositol 3-kinase (PI3K) using LY-294002 (Vlahos et al, 1994). The inhibitor is cell permeable and binds directly to the ATP binding site of the enzyme.

Treatment of infected HeLa cells (3 dpi, MOI 1) with LY-294002 (dilution described in 2.2.9) for 2 h before induction of apoptosis resulted in a marked dose-dependent apoptosis sensitization (Figure 3.14a) as judged from PARP cleavage and apoptotic cell count. Different dilutions of the inhibitor were tested to find that the effect is gradual and not an artifact. These results show that the activity of Akt is required in *Simkania*-infected cells to resist apoptosis induction. Different dilutions ranging from 0.1, 1.0 and 10 µM were tested and the percentage of apoptotic cells rose gradually from 14.7 % to 46% and 66% respectively. This data clearly state that PI3 kinase signaling pathway is necessary to block resistance in Sn-infected cells.

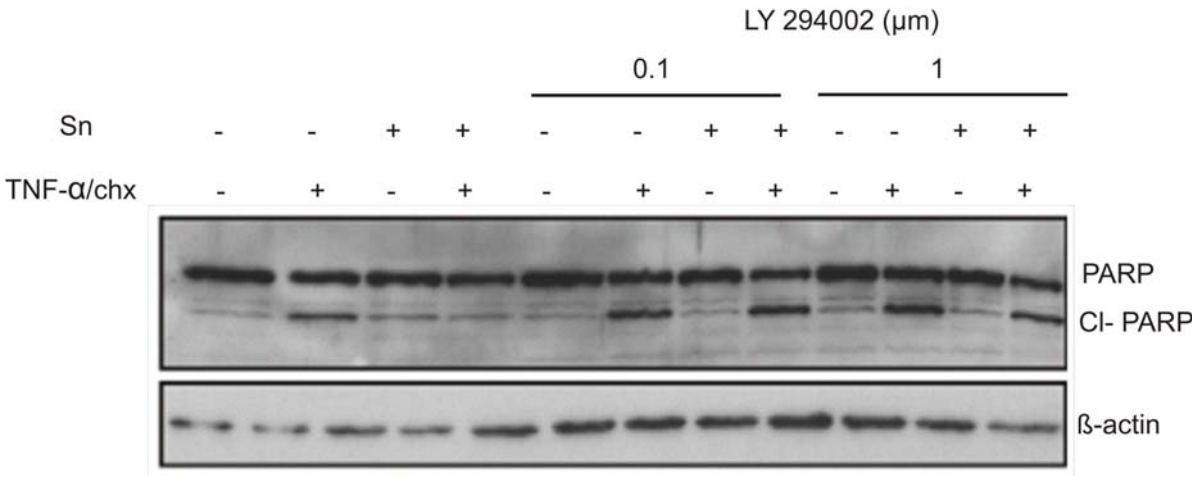


**Figure 3.14a.** Immunoblot analysis of PARP cleavage in *Simkania*-infected cells after treatment with PI3K inhibitor. *Simkania*- infected HeLa 229 cells were treated with Ly294002 (PI3 kinase Inhibitor) on 3 dpi for 2 h and induced with 20 ng/ml TNF-α/3 µg/ml chx or carrier for 4 h before analysis. PARP cleavage was ameasure of apoptosis sensitization. Treatment with 0.1 mM LY29004 clearly sensitized infected cells to apoptosis. Hsp 60 and Actin were used as loading controls, n = 2.

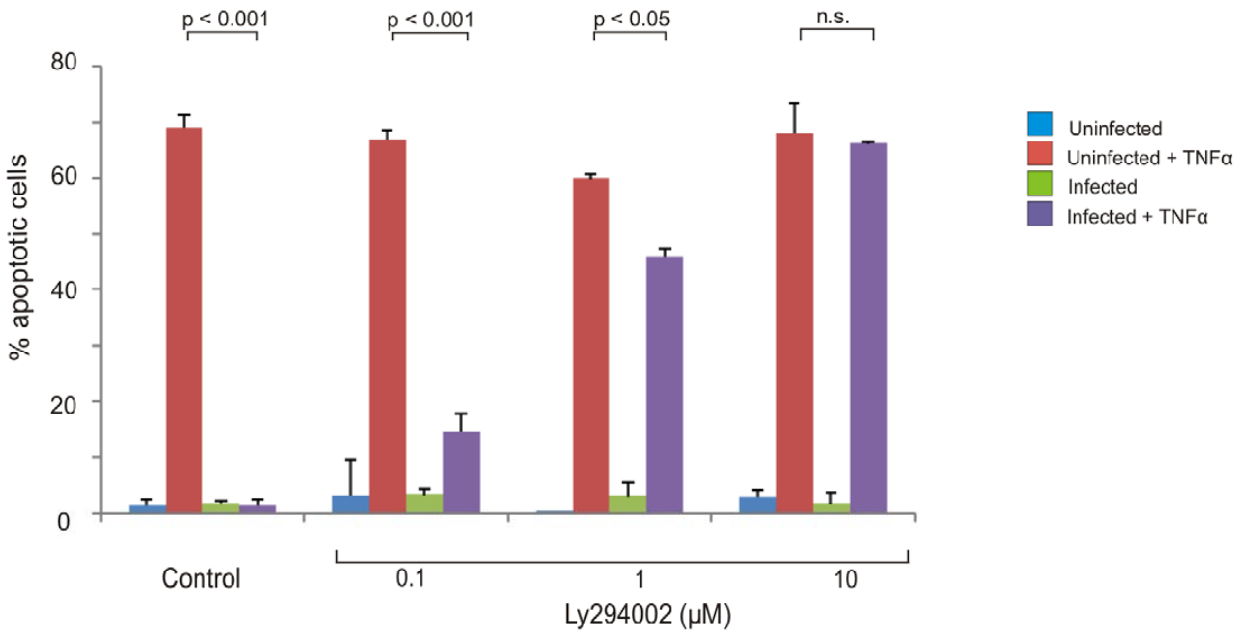


**Figure 3.14b**. Bar diagram showing samples treated similar as in Figure 3.14a but prepared for immunofluorescence analysis. Hoechst stained cells were counted to determine the number of

apoptotic cells (40x magnification, five fields per sample, n = 2, Error bars = SE). 1 mM LY29004 was found to restore apoptosis sensitivity to 70% of control.

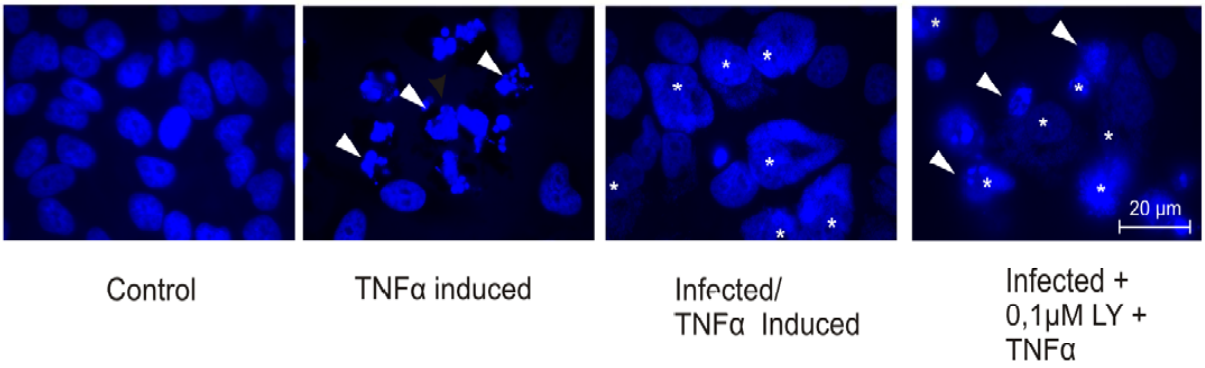


**Figure 3.14c.** Immunofluorescence of samples treated as described in Figure 3.14b. Samples were stained with Hoechst (blue) and viewed under a epifluorescence microscope for counting. Hoechst dye stained both HeLa cell nuclei and*Simkania* inclusions. White arrowheads mark example apoptotic cells. White asticks mark the infected cells. n = 2.

## 3.15. cIAPs are up-regulated and required for apoptosis resistance in Sn-infected cells

It is already reported that Inhibitor of apoptosis proteins (IAPs) plays a vital role in apoptosis inhibition of *C. pneumoniae* and *C. trachomatis*-infected cells (Paland et al, 2006a; Rajalingam et al, 2006b). Hence, the regulation of IAPs in Sn infected cells was under investigation. Interestingly in a time course western blot of the infected sample we found that cIAP-1 and cIAP-2 were up regulated in the course of infection (Figure 3.15a).

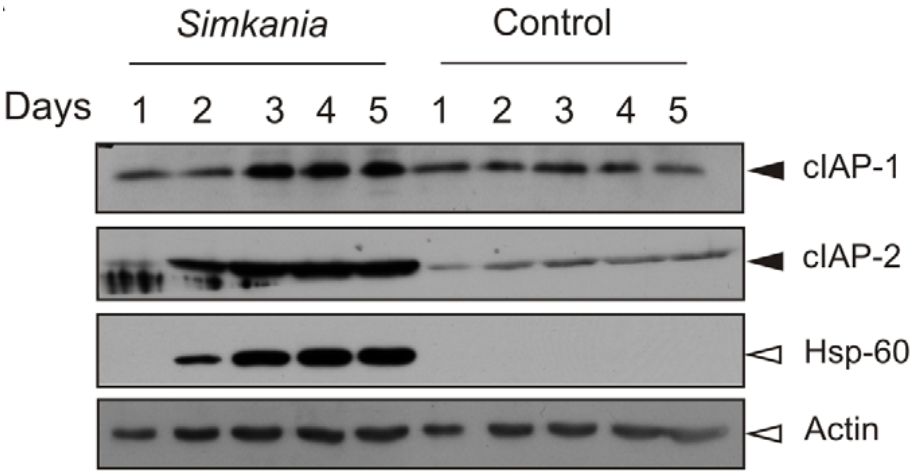


**Figure.3.15a.** Immunoblot analysis of theanti-apoptotic/pro-survival apoptosis regulators during *Simkania* infection. HeLa cells were infected with *Simkania* or Mock (control) (MOI 1) in a time-course experiment before analysis. Anti-apoptotic cIAP-1 and -2 (black arrowheads) are strongly upregulated. Hsp-60 and Actin (white arrowheads) were used as loading controls, n = 2.

The up-regulation or stabilization was well pronounced after 48 hpi. Since the antibody against XIAP did not work, RT PCR was done to analyze the levels of XIAP mRNA over a time frame of infection. HeLa cells were infected with Sn for a time frame of day1 to day 5 and the RNA was isolated as explained above in methods 2.2.15. The RNA was used for real time analysis of expression over time frame. The results show an up regulation of XIAP after 24 hpi. All this data explains how Sn resists apoptosis over a longer time period when compared to other *Chlamydiales.*

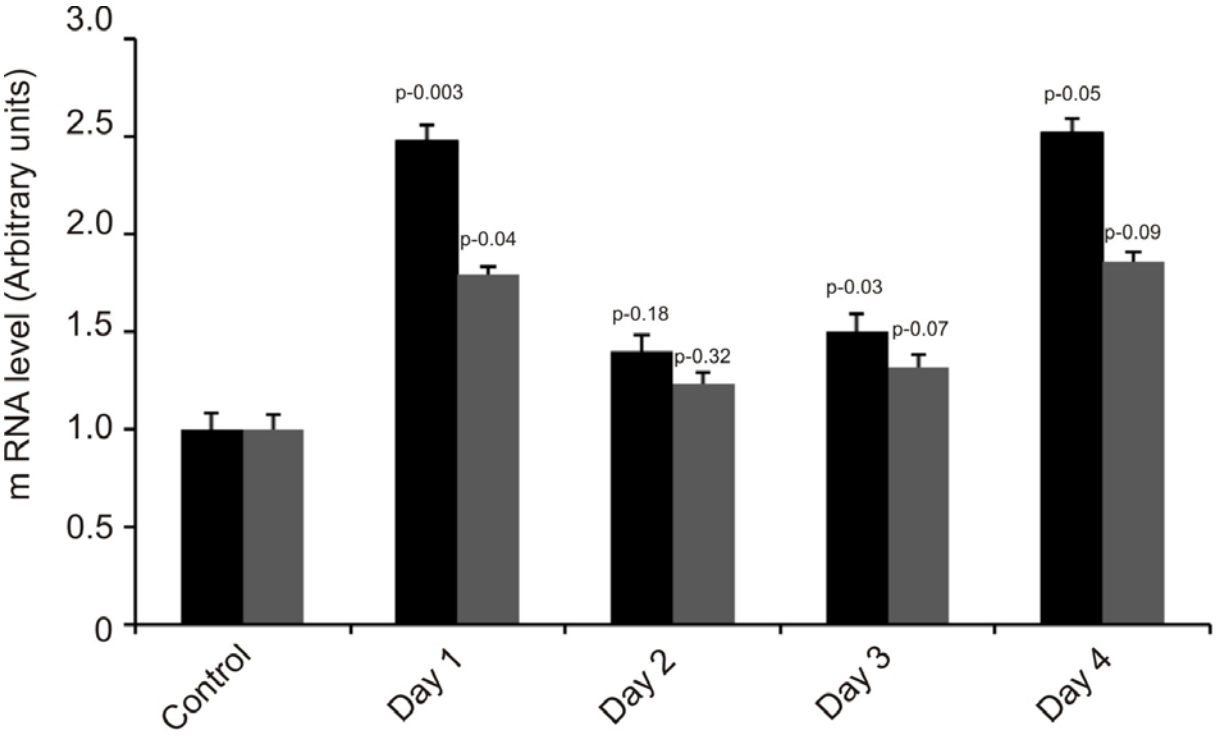


**Figure 3.15b.** HeLa 229 cells were infected with Sn for different days (1-5). The RNA from the cells was isolated as explained in methods. The RNA was converted into cDNA and taken for qRT-PCR to detect the relative amount of the targeted genes. Upon Sn infection the gene expression of cIAP-1 (black) as well as XIAP (grey) was increased. The experiment was repeated three times to calculate the standard deviation.

To prove, if these IAPs are required to maintain the high apoptosis resistance in infected cells. Expression of cIAP-1 and cIAP-2 was silenced in control and infected cells using specific siRNAs and induced with TNF-α/chx for apoptosis.

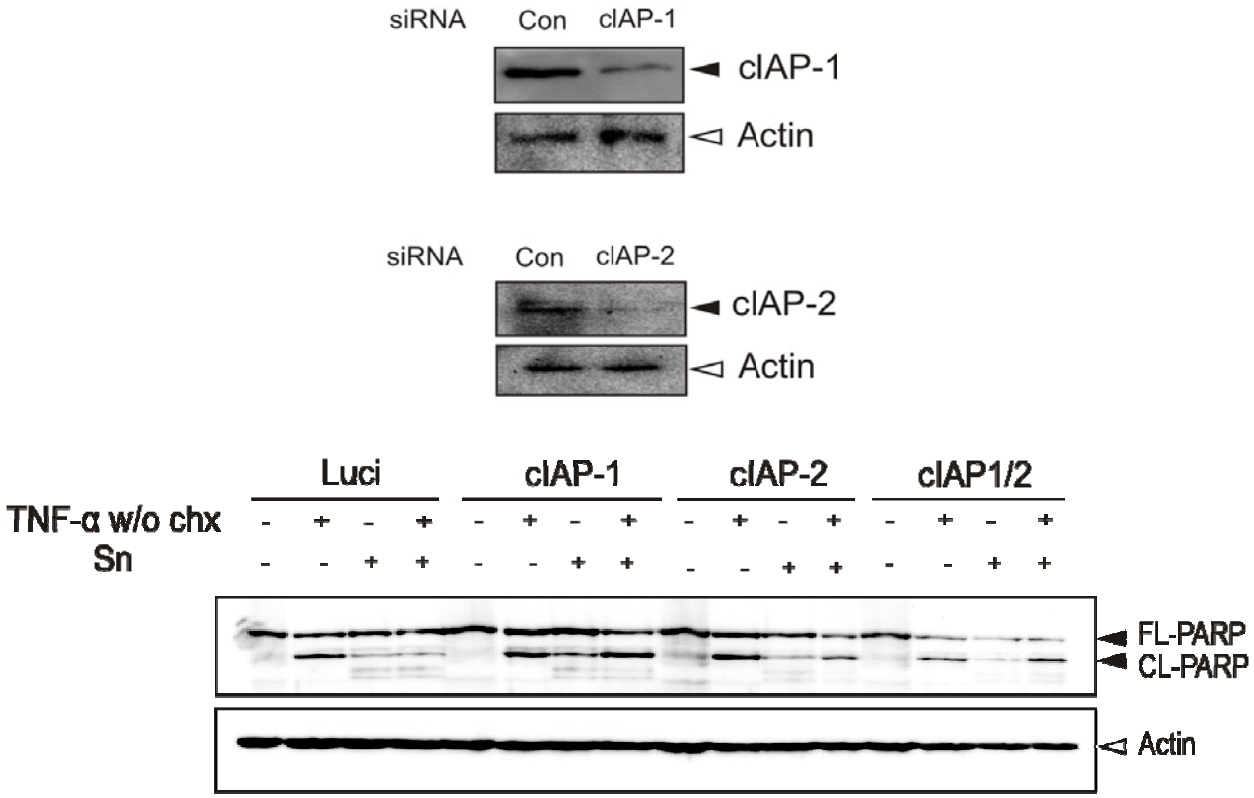


**Figure.3.15c**. Immunoblot confirmation of cIAP-1 and -2 knock down. HeLa cells were transfected with siRNA directed against cIAP-1, -2 or Luciferase (control). cIAP-1 and -2 (black arrowheads) were strongly down regulated at day 3 post transfection. Actin (whitearrowheads) was used as loading control. n = 2. Immunoblot analysis of PARP cleavage in *Simkania* infected cells after cIAP-1 and -2 single or cIAP-1 /-2 double knockdowns. HeLa 229 cells were transfected for 3 days before *Simkania* infection. On day 3 pi cells were induced with 20 ng/ml TNF-α/3 µg/ml chx or carrier for 4 h before analysis. PARP cleavage (black arrowheads) was a measure of apoptosis sensitization. Single knockdown of either cIAP-1 or -2 or both clearly sensitized infected cells to apoptosis. Actin (white arrowhead) was used as loading control. n = 2.

The infected cells were found to be sensitized for apoptosis in the absence of cIAP-1 and cIAP-2 (Figure 3.15c), supporting their role in blocking apoptosis in infected cells. The percentage of apoptotic cells upon TNF-α/chx induction rose from

14 % in untreated infected cells to 48% in cIAP-1 knock down cells, while in the case of cIAP-2 knock down cells, the percentage of apoptotic cells still raised higher to about 54%. This clearly depicts the importance of IAPs in apoptosis resistance in Sn infected cells.

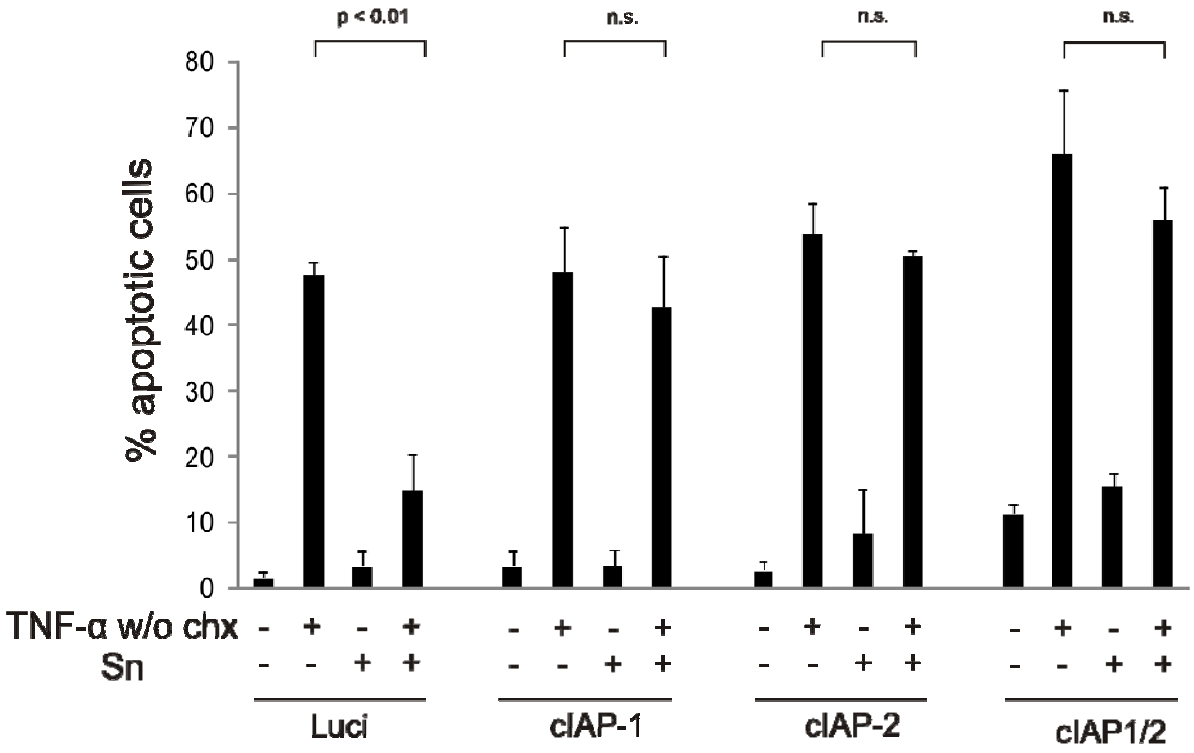


**Figure 3.15d**. Bar diagram showing samples treated similar as in Figure 3.15c but prepared for immunofluorescence analysis. Hoechst stained cells were counted to determine the number of apoptotic cells (40X magnification, five fields per sample, n = 2, Error bars = SE). Single knockdown of either cIAP-1 or -2 or both was found to restore apoptosis sensitivity to 70% of control.

## 3.16. Sn protects *Acanthamoeba castellanii* during starvation

As *A. castellanii* is one of the natural hosts of Sn, it was interesting to investigate the amoeba-Sn interaction. *A castellanii* cells were plated in 12-well plate, after 24 h the amoeba converted from the dormant cyst form to the proliferative

trophozoite form and settled down at the bottom of the plate. The cells were infected with different MOI of Sn ranging from 10 to 100.

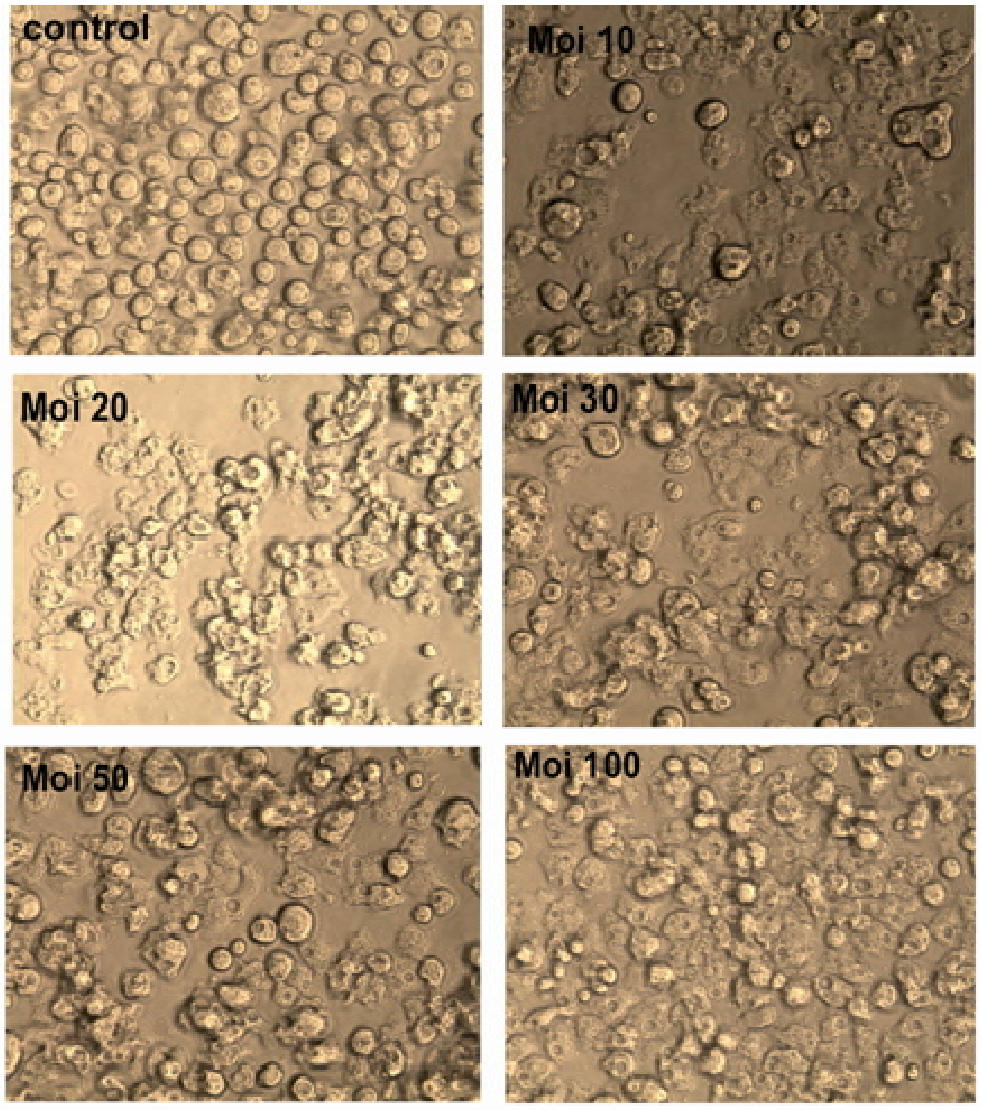


**Figure 3.16.** *Acanthamoeba Castellanii* cells were seeded in 12 well plate and after 24 hpi with different MOI of Sn, the cells were starved for 16h. The uninfected control cells converted into the inactive and harsh environment tolerating csyts form while the Sn infected cells, in a MOI dependent manner continued to survive in the trophozoite form. n=3

After 24 hpi the cells were washed with phosphate buffer saline and incubated in the encystment media for starvation. It was interesting to observe that after 16 h, the starved uninfected cells converted from the trophozoite form to the cyst form

indicating the unfavorable condition for survival. Surprisingly as Figure 3.16 indicates the Sn infected cells could survive in the starving environment and continued in the proliferative and nourishing trophozoite form.The higher the MOI of Sn infection the better was the survival in the trophozoite form, indicating that Sn protects the amoeba to overcome unfavorable environment.

# 4. Discussion

## 4.1. Resistance to apoptosis is highly conserved in the order *Chlamydiales.*

Apoptosis resistance has been a common denominator as an outcome of infection and likely prerequisite for the development of obligate intracellular pathogenic *Chlamydia*. Here, in this study it is demonstrated that like other *Chlamydiales*, the evolutionary related bacterium *Simkania negevensis* is also able to block host cell apoptosis. Sn infection could block the apoptotic pathway at multiple levels by modulation of specific host cell factors. It is evident from the results described above that anti-apoptotic and survival mechanisms are widely conserved in the order *Chlamydiales*. Although the mechanisms involved is not completely revealed, the ability to replicate and maintain long-term relation in the host cell seems to significantly contribute to chlamydial pathogenicity. These intracellular parasites maintain a delicate balance between exploiting and protecting their host by hijacking the signaling pathways in the host cell. Interestingly these pathogens selectively, either up-regulate or stabilize the anti-apoptotic proteins and down-regulate or degrade the pro-apoptotic proteins by means of their proteases. Although *Chlamydiales* are known to resist apoptosis in the productive growth as a path of survival they are also known to inhibit apoptosis during persistent growth or in phagocytes but induce apoptosis in T cells, which indicates that modulating apoptotic pathways have an immunomodulatory role in chlamydial infections (Miyairi & Byrne, 2006). An exception to this rule may be *Parachlamydia*, which appears to survive in human macrophages, but eventually triggers apoptosis (Greub et al, 2003). The inability to induce apoptosis inhibition may be one reason why *Parachlamydia* does

not replicate in most mammalian cell types (Matsuo et al, 2008). Interestingly Gimenez at al (2011) have reported about cross-talk between intra-amoeba pathogens. They could identify potential horizontal gene transfers between *Legionella* and *Chlamydiales*. But, it is still unclear whether *Simkania* and *Parachlamydia* are able to influence death of amoebae. The type 1 metacaspase in amoeba is known to regulate temperature fluctuations. At a lower temperature, metacaspase is over expressed and this induces the encystation of *Acanthamoeba castellanii.* This is associated with a reduced number of bacteria within amoeba (Ohno et al, 2008). Althought, metacaspases are already known for their role in PCD (Suarez et al, 2004) their natural substrate remained unknown until Sundstrom et al (2009), reported that tudor staphylococcal nuclease (TSN) is cleaved by metacaspases. The human TSN is a substrate of caspase-3 during apoptosis; this impairs its ability to activate mRNA splicing and inhibits its ribonuclease activity. Sn infection could maintain the trophozoite form of the amoeba under starvation, which indicates that Sn could regulate some genes in amoeba for its survival and thus they successfully maintained a symbiotic association. Recently, Song et al (2012) reported that Atg-16 mediated autophagy is required for the encystment of *Acanthamoeba castellanii*. From this data one tempts to assume that Sn infection might also interfere with autophagy, since it is an innate immune mechanism employed by the host cell to clear infection. Sn infection might prevent autophagy and thus hinter the encystment of amoeba. This indicate that Sn have derived its ability to resist apoptosis as well as to evade autophagy, by co-evolving in amoeba. Signaling to cell death inhibition initially could have co-evolved as a consequence of the *Simkania*-amoebae interaction and could

have later been exploited during the exposure to mammalian cells. Adaptation to different hosts may, at least in part, explain the differences we observed in anti-apoptotic signaling between cells infected with members of *Chlamydiales* adapted to amoebae (*Simkania*) and mammalian cells (*C. trachomatis*, *C.pneumoniae*).

### 4.2. Sn resists apoptosis in infected cells in a time and MOI-dependent manner

The apoptosis inhibition in *Chlamydiales* depends on its developmental cycle, with the onset early after EB to RB transition and maximum during RB replication (Fan et al, 1998a). The developmental cycle of *Simkania* lasts up to 10 days and therefore significantly longer than that of *C. trachomatis* (48 h) and *C. pneumoniae* (72–96 h) under comparable infection conditions. It was therefore unexpected to see that even at a MOI of 0.5, conditions, where hardly one EB infects one cell; about 50% of the cell were protected from apoptotic stimuli already at 24 hpi and almost all infected cells were completely protected at 3 dpi. A similar efficient protection at such low MOI has neither been observed with *C. trachomatis* (Fan et al, 1998a) nor with *C. pneumonia* (Fischer et al, 2001; Rajalingam et al, 2001b). The infected cell remained resistant for apoptotic stimuli up to 4 dpi indicating that *Simkania* has evolved highly efficient and long-lasting mechanisms to block host cell death, which is evident from the regulation of the survival factors throughout the completion of its life cycle. Similar to *C. trachomatis* infection, cells infected at higher MOI acquired apoptosis resistance earlier (Figure 3.3c.), which has been suggested to be dependent on the rapid entry of the bacteria into the replicative phase of the developmental cycle (Fan et al, 1998a). Interestingly, similar as with *C. trachomatis*, infection with *Simkania* at a higher MOI up to 100 had no visible cytotoxic effect, whereas significant cytotoxicity

already starting at a MOI of 10 has been reported for *C. pneumoniae* infection (Rajalingam et al, 2001b). The infected cells blocks apoptosis even though bacteria were cultured in cycloheximide containing media (an inhibitor of host cell protein biosynthesis). However it was observed that block in upregulation of proteins like IAPs and inhibiting the activation of survival factors like NF-κB sensitized the infected cells to apoptosis. This indicates that host protein production is also required for imparting resistance. Different to what has been reported for *C. trachomatis* infected McCoy cells, Sn did not induce apoptosis in neighboring uninfected cells during any stage of the infection, excluding paracrine apoptosis induction (Schoier et al, 2001). Rather in Sn infection studies, only the non-infected cells tend to apoptosis upon induction, as the bacteria infects the cells asynchronously.

**4.3. Sn infection can resist apoptosis signaling upstream of mitochondria**

Despite differences in the kinetics of the onset, efficiency and duration of apoptosis resistance, all the above data demonstrate a block in apoptotic signaling at the extrinsic and intrinsic levels. The mechanism underlying apoptosis resistance in *Simkania*-infected cells may thus be very similar to those in cells infected with *C. trachomatis* and *C. pneumoniae*, in which apoptotic signaling via mitochondria is prevented (Fischer et al, 2004a; Rajalingam et al, 2008a). Sn may preserve the mitochondrial function and prevent mitochondrial cytochrome c release by three potential mechanisms. It can block the upstream steps that can perturb mitochondrial function.The degradation or down regulation of pro-apoptotic BH3-only proteins is one of the sensible ways. Similar to the report on *C. trachomatis*-infected cells (Rajalingam et al, 2008a), this complete and strong block of apoptotic signaling was

maintained in the infected cell despite of unchanged levels of BH3-only proteins. In contrast, others have found that chlamydial protease-like activating factor (CPAF) was responsible for degradation of BH3-only proteins in cells infected with *C. trachomatis*, (Dong et al, 2005; Fischer et al, 2004b) indicating that depletion of BH3-only proteins plays a role in blocking apoptosis upstream of the mitochondria in their infection setting. The second way is to up-regulate the expression of anti- apoptotic BH3-only proteins. The mitochondrial membrane proteins Bcl-2 or Bcl-2 like proteins are anti-apoptotic and are able to prevent cytochrome c release and caspase-3 activation. But it is also reported that overexpression of Bcl-2 does not always protect from apoptosis induced by Fas cross-linking (Chiu et al, 1995; Scaffidi et al, 1998; Strasser et al, 1995). Overexpression of Bcl-$_{xl}$ protein levels failed to block apoptosis induced by TNF-α (Erhardt & Cooper, 1996). On examining the level of Bcl-2 in Sn infected cells, stabilization or up-regulation could not be observed. Hence it is not likely that host Bcl-2 or Bcl-2 like molecules solely mediates the Sn anti-apoptotic activity. As it was reported that Mcl-1plays a key role in resisting apoptosis in *Chlamydia* infected cells, it was interesting to check the regulation in Sn infection. Mcl-1 as one of the major inhibitors of Bak and Bax activation was neither upregulated nor stabilized in Sn infected cells. Since, Mcl-1 was not regulated upon infection, activation or oligomerisation of neither Bak nor Bax were found in Sn-infected cell treated with TNF-α/chx and cytochrom*e c* was fully retained in the mitochondria of these cells, which explained an inactive caspase-9 after apoptosis induction. Third possibility of protecting the mitochondrial potential loss is the novel anti-apoptotic factors that could be released by the bacteria into the cytoplasm of the

host cells. As Sn anti-apoptotic activity is found to be MOI and time frame dependent, it becomes clear that Sn translation is important for the process. At a higher MOI the bacteria establish in the host much faster, meanwhile at a MOI of 1, by 72 hpi, the cells harbor visible inclusions hence; the maximum percentage of apoptosis inhibition was observed. Sn could harbor some mammalian anti-apoptotic protein homologues, which could prevent apoptosis as reported in the case of Epstein Barr virus. The BHRF1 in EBV are the homologues of mammalian Bcl-2, and it inhibits apoptosis induced by exogenous stimuli, like DNA damaging agents, the calcium ionophore ionomycin and the immunological apoptosis inducing molecules like TNF-α and Fas (Kelly et al, 2006). Another EBV nuclear antigen EBNA2 interferes with apoptosis induced by some stimuli through intrinsic pathway by sequestering Nur77 to the nucleus and preventing its translocation to the cytoplasm where it can induce cytochrome c release from the mitochondria (Lee et al, 2002). This indicates that the microbial homologues of apoptosis suppressors may be more potent than endogenous proteins. Currently it is not known which among the above-mentioned factors might be responsible for the mitochondrial signaling blockage in Sn-infected cells. Unlike in *C. trachomatis* infection, despite the fact that, the anti-apoptotic BH3-only proteins are not regulated upon Sn infection, Sn can protect the cells from membrane permeabilisation and potential loss. Since Sn infection can prevent the caspase activation downstream of mitochondria, it becomes clear that regulation of signals upstream of mitochondria may not be the only mechanism employed by Sn to achieve the strong inhibition to apoptosis.

### 4.4. Caspases are differentially regulated upon Sn infection

Caspases are regulated during pathogenesis in different ways. *Legionella pneumophila* activates Caspase-1 via flagellum and type IV secretions in response of host IPAF prevented phagosome maturation and led to clearance of infection (Amer et al, 2006). Regulation at the level of these cysteine proteases is critical for extrinsic as well as intrinsic apoptosis pathway. Caspases exist as zymogens are activated by cleavage, which permits their protease activity and degradation of substrates leading to cell death. Infection with any pathogen is recognized by the cells leads to the inflammatory responses and release of cytokines, which binds to its receptor and initiates the cascade of caspase activation. The resistance to apoptosis in Ctr was also accounted on the receptor shedding from the infected cells by activated metalloproteinase, TACE. But upon Sn infection as TNF-α induction lead to a strong downstream cascade of survival signals like MEK and ERK it was clear that the resistance to cell death is not contributed by TACE or other means that lead to reduced display of receptors on cell surface. Activated caspases lead to cell death thereby reducing the chance of pathogen to complete its life cycle. The baculovirus is known to inhibit all types of caspases by a baculoviral p35 protein, by cleaving the reactive site loop and forming a protease-inhibitor complex. In addition cowpox virus inhibits the active site of caspases by serine protease inhibitors. One of the most efficiently used methods by pathogens is to up regulate the cellular inhibitor of caspases, the IAPs. Upon Sn infection by one day of infection itself an upregulation of various IAPs could explain the reduced activation of caspases upon apoptosis induction. But in Sn-infected cells the caspases were differentially regulated. Upon Sn

infection, caspase-8 was activated but the executioner caspase -3 and -9 was prevented from activation upon induction with TNF-α. With the activation and recruitment of complex 1 (consisting of TNFR, TRADD, RIP1 TRAF2), the executioner, caspase-3 normally gets activated. This activation was blocked upon Sn infection, which indicates either the formation of complex 1 is affected or that some bacterial factors directly influence the caspase maturation downstream. The data presented here and the previously published work demonstrates that IAPs play a major role in maintaining apoptosis resistance in cultured cells infected with *C. trachomatis* and *C. pneumonia* (Rajalingam et al, 2006a). IAPs are important regulators of apoptosis that bind directly to caspase-3 and -9 with their BIR domain and thus block their activation. During Sn infection IAPs gets upregulated as shown in the figures, until the pathogen lyse the cells. Hence this could be a possible mechanism by which caspase activation is inhibited. But recently some reports have raised doubts over the ability of IAPs in directly inhibiting the catalytic activity of caspases (Eckelman & Salvesen, 2006). But, abrogation of IAPs using siRNA sensitizes the Sn infected cells to apoptosis. Hence analyzing the apoptosis pathway in IAP knockdown scenario could reveal the dependence of caspase activation on IAPs. However, recent data obtained in pulmonary infection experiments with *C. pneumoniae* in knockout mice suggest an important function of cIAP-1 in innate immune signaling in macrophages (Prakash et al, 2009). A role of IAPs in innate immune signaling is consistent with recent reports demonstrating a role of both, cIAP-1 and cIAP-2 in innate immune signaling rather than in directly inhibiting caspase activation (Gyrd-Hansen & Meier, 2010). Infection may directly influence IAP stability

as these proteins possess E3 ligase activity and control their own stability and the stability of other IAPs (Vaux & Silke, 2005b). In this context the previous description of IAP-IAP hetero complexes implemented in IAP stabilization in *C. trachomatis*-infected cells may also be relevant for *Simkania* infection (Rajalingam et al, 2006a).

### 4.5. Caspase-8 is activated upon Sn infection

Unlike other *Chlamdiales*, the caspase-8 was found to be activated even in the Sn infected cells without any apoptosis induction but still prevented cells death. During Sn infection the cells were maintained in RPMI containing 5% FCS and 1µg/ml of cycloheximide. Addition of protein synthesis inhibitor reduces the synthesis of the cellular caspase-8 inhibitor, cFLIP, which leads to the accumulation of complex II to the TNFR and leading to caspase-8 activation. A death ligand association independent, activation of caspase-8 is also reported for the induction of autophagy (Laussmann et al, 2011), efficient ways of the host to eliminate pathogens by fusing with endosomes and lysosomes to finally degrade the sequestered material. However Sn should have strategies to modify this pathway as they survive in the host mysteriously. Meanwhile since autophagy is the major pathway for the turnover of cytoplasmic components like mitochondria, peroxisomes, smooth and rough membranes, proteins, lipids, RNA and lysosomes, Sn could also easily modulate autophagy to gain access to nutrients by autophagosomes into the inclusion.

### 4.6. Akt is activated upon Sn infection

As previously described for *C. trachomatis* (Verbeke et al, 2006) and *C. pneumoniae* (Coombes & Mahony, 2002) infection, *Simkania* infection strongly

activates the PI3K-Akt pathway. Activated Akt promotes cell survival through crosstalk with other signaling cascades like the NF-κB pathway (Romashkova & Makarov, 1999). Akt has in addition a role in cell survival by increasing the uptake of nutrients, positive regulation of metabolic pathways and maintenance of mitochondrial membrane potential (Plas & Thompson, 2005). There is also a direct link between Akt and cell survival by phosphorylating and inhibiting the function of pro-apoptotic Bad (Datta et al, 2000). Any change in the Akt-specific phosphorylation of sites S116 and S136 in Bad in the course of *Simkania* infection was not observed (data not shown), as has previously been described for *C. trachomatis* infection (Verbeke et al, 2006). The observation that PI3K inhibitors sensitize cells infected with *C. trachomatis* (Rajalingam et al, 2008a) or *Simkania* to apoptosis speaks in favor for an evolutionary conserved role of this central survival pathway in controlling host cell survival during development and replication of *Chlamydiales* in their mammalian hosts. Activation of Akt can favor high expression of cIAP-1 (Gagnon et al, 2003), consistent with elevated levels of IAPs and increased phosphorylation of Akt during the course of infection until day 5. But it is still unknown about the mechanism that leads to persistent activation of Akt. An obvious reason could be that Sn secretes anti-apoptotic factors through their type III secretion apparatus as reported with other pathogens (Francois et al, 2000; Galan & Collmer, 1999; Navarro et al, 2005; Yuk et al, 2000). *Salmonella enterica* secretes effector protein SopB to induce sustained activation of Akt (Knodler et al, 2005b). Concurrently host cell stress could also result in Akt activation. Signaling through Akt is known to play (Dimmeler et al, 1998) a key role in protecting cells from variety of toxic stresses (Cross et al, 1995; Dent et al,

2003). Obligate intracellular pathogens like *Chlamydia* is known to increase mitochondria metabolism and oxidative stress (Hatch, 1998; Ojcius et al, 1998). In cells exposed to oxidative stress down regulation of PI3K/Akt pathway results in high level of apoptosis (Taylor et al, 2005) and interestingly the oxidants stimulate Akt survival pathway (Klotz et al, 2000; Zhuang & Kochevar, 2003). Mechanical stress caused by growing inclusion could also induce phosphorylation of Akt in various cell types (Dimmeler et al, 1998; Kippenberger et al, 2005). Thus it is very well possible that Sn secretes effective proteins or the mechanical stress developed due to thedevelopment of inclusion could lead to this efficient survival signal, the necessity of which is evident from the fact that the treatment of Akt inhibitors in Sn infected cells leads to its sensitization.

**4.7. NF-κB is activated upon Sn infection**

The nuclear factor-kB family of transcription factors plays a central role in the host response to infection by microbial pathogens by orchestrating the innate and acquired host immune responses. Diverse signaling pathways that originate from many different cellular receptors and sensors activate the NF-κB proteins. Many successful pathogens have acquired sophisticated mechanism to regulate this pathway by deploying subversive proteins or hijacking the host-signaling molecule.As cellular immune receptors like Toll Like Receptor (TLRs), cytokine receptors and TNFRsignals via NF-κB after recognizing a variety of pathogen associated molecular patterns (PAMPs). Activation of NF-κB following infection is reported in various pathogens. NF-κB activation leads to the secretion of cytokines and chemokines, as a part of host defense response to trigger host innate immune response. On the other

hand the anti-apoptotic effect of NF-κB is thought to be beneficial for intracellular pathogen that depends on host cell survival (Wan & Lenardo, 2010).

In Sn, biphasic activation of NF-κB could be observed, early phase of infection, shortly after adhesion of the bacteria a transient translocation of the p65 component of NF-κB could be observed (data not shown). It is very likely that this first wave of transient activation is via TLR signaling. At a later time point an unknown factor that depends on bacterial replication induces continuous localization of p65 and permanent degradation or ubiquitination of IκB as well as the up-regulation of various anti-apoptotic genes. Hence, as discussed above this time resolved activation provides insight into two opposing effects of NF-κB activation, initially beneficial for the host via activation of innate immune response and later advantageous for the pathogen, likely being casually involved in anti-apoptotic host cell phenotype. This second wave of activation persisted for a longer time of 72h, atypical with normal cells but a hallmark of certain cancers (Courtois & Smahi, 2006). An important feature associated with activation of NF-κB is the anti-apoptotic phenotype which pathogens exploit to ensure the integrity of the infected cells. Sn infection promoted an up-regulation of cIAP1/2 and XIAP during the same time point as the NF-κB activation. This supports how the pathogen benefits, particularly because unlike other intracellular pathogens Sn needs nearly 5 days or more to complete its life cycle.

Various pathogen factors have been reported for the NF-κB regulation, like *sdbA* and *lubX* mutants failed to elicit constitutive NF-κB activation and also exhibited severe growth defects in *L. pneumophila* (Bartfeld et al, 2009). Later the LegK1 protein was recognized as the direct activator of NF-κB signaling. The pathogens

could target the host ubiquitinylation machinery, which plays a central role. Most of the effector proteins are primarily delivered through the type III secretion system as in *Yersinia* spp, or by type IV secretion system as in *Bartonella* spp. The YopJ/P protein of *Yersinia* block the NF-κB pathway by blocking the pro-inflammatory cytokines, recognized later as cysteine proteases they also have a deubiquitylating and desumoylating activity, which prevents the ubiquitination of IkB (Viboud & Bliska, 2005). In the case of *Salmonella* spp. AvrA and SseL inhibits deubiquitylation of IkB. While the intracellular bacterial pathogen *Chlamydia trachomatis* inhibits NF-κB translocation by Rel-A degradation by the *Chlamydia* specific proteases CT441 (Lad et al, 2007), and chlamydial protease-like activating factor (CPAF) (Christian et al, 2010). The chlamydial deubiquitylase and deneddylase (ChlaDub) binds to IκB and prevents its degradation (Le Negrate et al, 2008). While *Chlamydia pneumoniae*, which lacks ChlaDub, uses an inclusion specific protein encoded by the locus CP0236 to sequester NF-κB activator, ACT1/CIKS, which regulate NF-κB (Wolf et al, 2009). In Sn infection as the second wave of activation persist for a longer time it is very likely that an unknown bacterial protein is playing a crucial role. More interestingly, the NF-κB mutant cells showed a reduced infectivity of Sn as shown in the results. This indicates that some critical survival factor depending on NF-κB is necessary for Sn survival or that NF-κB regulates some cell surface receptors required for bacterial uptake.

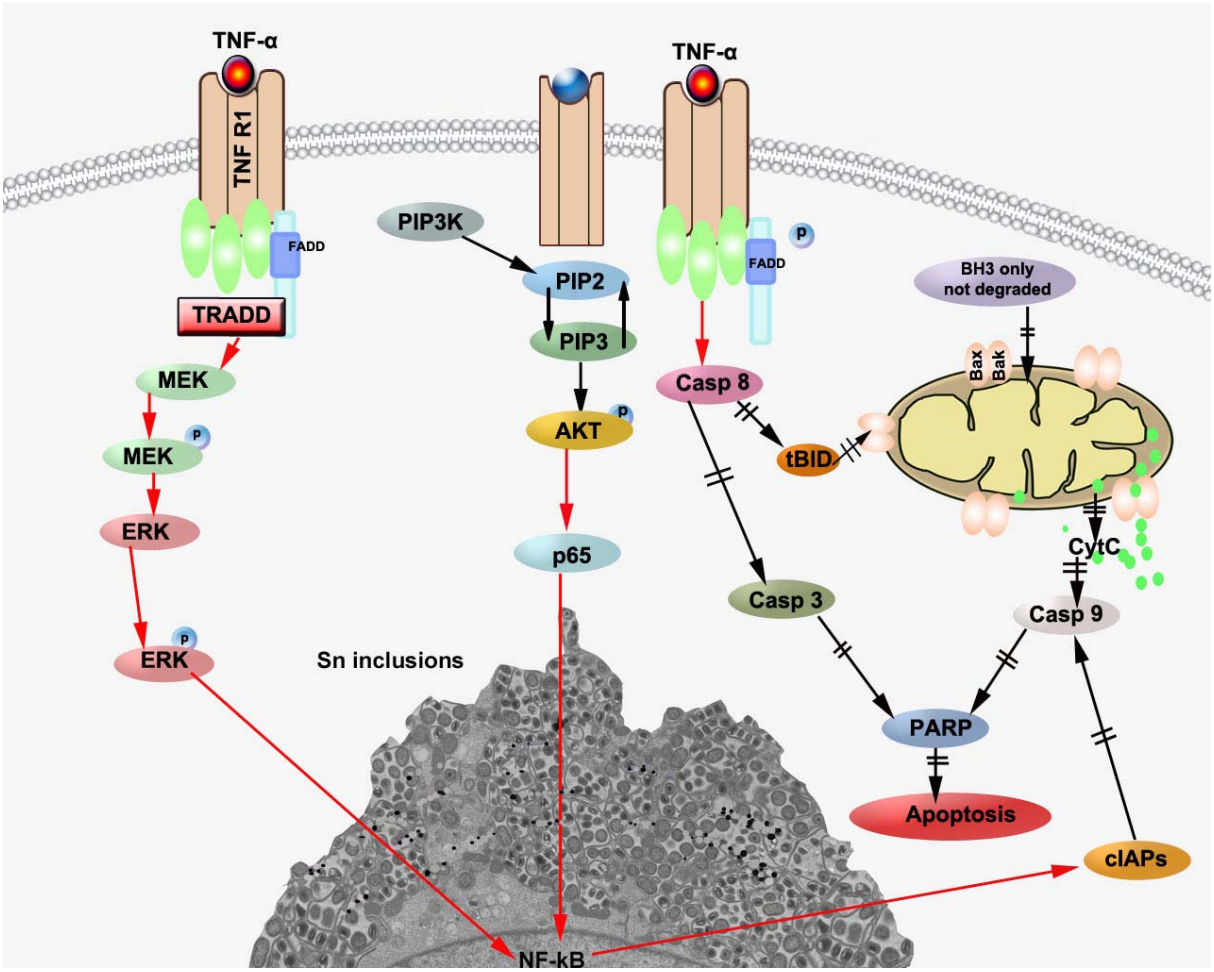


**Figure 4.17**. Overview of anti-apoptotic mechanisms in Sn infected cells: *Simkania* micro-manage the host signaling circuitry to favor the continued survival of the pathogen. Inhibition of apoptosis could be achieved by several different mechanisms. The various survival pathways activated by Sn is shown by red arrows and the pathways that they block to prevent apoptosis is shown by black arrows with cross bars. The black arrows with no bars indicate the possible upstream signals that promote the survival signals in Sn-infected cells. The Sn infection activates survival signal Akt upon infection, which could lead to the activation of NF-κB. This could activate increased transcription of anti-apoptotic genes like IAPs. IAPs prevent the activation of caspases. Although the BH3 only proteins are not degraded upon infection, Sn prevents cytochrome c release from the infected cells which hinters the downstream activation of caspase-9 and effector caspase-3. Sn infection can also activate caspase-8 but do not activate the Bid cleavage and activation thereby protecting the mitochondria.

The figure 3.16 depicts the overall anti-apoptotic and survival signals generated upon Sn infection in cells. The survival signals are persistent throughout the infection.This raises the questions how these long-term signaling events are fueled even several days after the first interaction of the pathogen with the host cell. One could speculate that the bacterial factors play a crucial role in manipulating the host. Like other pathogenic members of the *Chlamydiales, Simkania* has a type III secretion system that may help to transport proteins across the inclusion membrane into the cytosol of the host. Secreted bacterial proteins may take over the pacemaker function in maintaining the host cell in a survival mode to guarantee such a long-term host-pathogen relation.

## 5. Outlook

The present study was an attempt to investigate on the evolutionary conservation of cell death modulation in the *Chlamydiales*. The newly discovered member of *Chlamydiales, Simkania negevensis* was under study. Since this pathogen differ from other *Chlamydiales* in their time span to complete the life cycle, it was obviously interesting to find the survival signals that would play a role. The experiments in this direction revealed that the mechanism of cell death inhibition is highly conserved in the order *Chlamydiales*, but still differ from the other members. Meanwhile, the differential activation of caspases should be given special attention, as the activated caspases in Sn infected cell could reveal more about pathogenesis and authophagy during infection. It is indicated in this work that activates PI3 kinase could lead to persistant NF-κB activation, hence this needs further elucidation.It will be also interesting to investigate the possibility that strong and rather persistent activation of NF-κB could promote development of cancer. One another interesting fact that came across was the reduced infectivity in NF-κB mutant cells, investigation in this direction could reveal the factors that could be necessary for the survival or uptake of the bacteria. Special attention should be given to the yet to be published finding that, Sn resides in the endoplasmic reticulum (results from Dr. Mehlitz) unlike other *Chlamydiales*. This tempts one to think the mechanism that this pathogen would adapt to secrete its effector proteins across multi membranes. It would also be interesting to understand how this pathogen prevents endoplasmic recticulum stress induced cell death. Further studies on amoebae would reveal how this pathogen adapted itself for an obligate intracellular lifestyle and moreover, studies on

*Dictyostelium discoidium*, which could serve as a suitable model organism would reveal more hints on apoptosis, phagocytosis, endocytosis, membrane trafficking, vesicle sorting and pathogenesis.

More than anything the effector proteins that might imitate anti apoptotic host proteins and the one that play a role in maintaining the pathogen as a native component in the cell is yet to be discovered. This study could be further expanded to mouse models, which could reveal how severe could be an acute infection; this could also answer how Sn remained as an emerging pathogen. A closer look into pathogen clearance would be interesting. Since Sn and Cpn are known to occupy the same niche, it would be interesting to see how co-infection works. How the presence and development of one pathogen affects the other? Is there a mechanism of niche protection by these pathogens? Sn transcriptomics and metabolomics could also pave the way to find more interesting candidates that could play role in survival of the pathogen.

## 6. References


Adams JM, Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. *Science* **281:** 1322-1326

Adams JM, Cory S (2002) Apoptosomes: engines for caspase activation. *Curr Opin Cell Biol* **14:** 715-720

Ahmad M, Srinivasula SM, Hegde R, Mukattash R, Fernandes-Alnemri T, Alnemri ES (1998) Identification and characterization of murine caspase-14, a new member of the caspase family. *Cancer Res* **58:** 5201-5205

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* **7:** 261-269

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J (1996) Human ICE/CED-3 protease nomenclature. *Cell* **87:** 171

Amann R, Springer N, Schonhuber W, Ludwig W, Schmid EN, Muller KD, Michel R (1997) Obligate intracellular bacterial parasites of acanthamoebae related to Chlamydia spp. *Appl Environ Microbiol* **63:** 115-121

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59:** 143-169

Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nature medicine* **3:** 917-921

Amer A, Franchi L, Kanneganti TD, Body-Malapel M, Ozoren N, Brady G, Meshinchi S, Jagirdar R, Gewirtz A, Akira S, Nunez G (2006) Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. *The Journal of biological chemistry* **281:** 35217-35223

Annis MG, Soucie EL, Dlugosz PJ, Cruz-Aguado JA, Penn LZ, Leber B, Andrews DW (2005) Bax forms multispanning monomers that oligomerize to permeabilize membranes during apoptosis. *Embo J* **24:** 2096-2103

Antonsson B, Montessuit S, Sanchez B, Martinou JC (2001) Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* **276:** 11615-11623

Ashkenazi A (2008) Directing cancer cells to self-destruct with pro-apoptotic receptor agonists. *Nature reviews Drug discovery* **7:** 1001-1012

Badley AD, Pilon AA, Landay A, Lynch DH (2000) Mechanisms of HIV-associated lymphocyte apoptosis. *Blood* **96:** 2951-2964

Baeuerle PA, Baltimore D (1988a) Activation of DNA-Binding Activity in an Apparently Cytoplasmic Precursor of the Nf-Kappa-B Transcription Factor. *Cell* **53:** 211-217

Baeuerle PA, Baltimore D (1988b) I-Kappa-B - a Specific Inhibitor of the Nf-Kappa-B Transcription Factor. *Science* **242:** 540-546

Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol* **12:** 141-179

Baldwin AS (1996a) The NF-kappa B and I kappa B proteins: New discoveries and insights. *Annu Rev Immunol* **14:** 649-683

Baldwin AS, Jr. (1996b) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annual review of immunology* **14:** 649-683

Bartfeld S, Engels C, Bauer B, Aurass P, Flieger A, Bruggemann H, Meyer TF (2009) Temporal resolution of two-tracked NF-kappaB activation by Legionella pneumophila. *Cellular microbiology* **11:** 1638-1651

Bellacosa A, Testa JR, Staal SP, Tsichlis PN (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254:** 274-277

Best SM (2008) Viral subversion of apoptotic enzymes: escape from death row. *Annual review of microbiology* **62:** 171-192

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385:** 729-733

Bohme L, Albrecht M, Riede O, Rudel T Chlamydia trachomatis-infected host cells resist dsRNA-induced apoptosis. *Cell Microbiol* **12:** 1340-1351

Bohme L, Rudel T (2009) Host cell death machinery as a target for bacterial pathogens. *Microbes Infect* **11:** 1063-1070

Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM, Strasser A (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* **415:** 922-926

Bouillet P, Strasser A (2002) BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *J Cell Sci* **115:** 1567-1574

Brancolini C, Lazarevic D, Rodriguez J, Schneider C (1997) Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. *J Cell Biol* **139:** 759-771

Brazil DP, Hemmings BA (2001) Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem Sci* **26:** 657-664

Brazil DP, Park J, Hemmings BA (2002) PKB binding proteins. Getting in on the Akt. *Cell* **111:** 293-303

Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U (1995) Central of I-Kappa-B-Alpha Proteolysis by Site-Specific, Signal-Induced Phosphorylation. *Science* **267:** 1485-1488

Brown S, Heinisch I, Ross E, Shaw K, Buckley CD, Savill J (2002) Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. *Nature* **418:** 200-203

Brownawell AM, Kops GJ, Macara IG, Burgering BM (2001) Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. *Mol Cell Biol* **21:** 3534-3546

Cartron PF, Gallenne T, Bougras G, Gautier F, Manero F, Vusio P, Meflah K, Vallette FM, Juin P (2004) The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell* **16:** 807-818

Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ (2000) A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288:** 2351-2354

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC (2005a) Differential targeting of prosurvival Bcl-2

proteins by their BH3-only ligands allows complementary apoptotic function. *Molecular cell* **17:** 393-403

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC (2005b) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* **17:** 393-403

Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T (1995) Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev* **9:** 1586-1597

Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Molecular cell* **8:** 705-711

Chinnaiyan AM, Dixit VM (1996) The cell-death machine. *Curr Biol* **6:** 555-562

Chiu VK, Walsh CM, Liu CC, Reed JC, Clark WR (1995) Bcl-2 blocks degranulation but not fas-based cell-mediated cytotoxicity. *J Immunol* **154:** 2023-2032

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* **137:** 1112-1123

Christian J, Vier J, Paschen SA, Hacker G (2010) Cleavage of the NF-kappaB family protein p65/RelA by the chlamydial protease-like activity factor (CPAF) impairs proinflammatory signaling in cells infected with Chlamydiae. *The Journal of biological chemistry* **285:** 41320-41327

Cleary ML, Smith SD, Sklar J (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* **47:** 19-28

Clifton DR, Fields KA, Grieshaber SS, Dooley CA, Fischer ER, Mead DJ, Carabeo RA, Hackstadt T (2004) A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc Natl Acad Sci U S A* **101:** 10166-10171

Clifton DR, Goss RA, Sahni SK, van Antwerp D, Baggs RB, Marder VJ, Silverman DJ, Sporn LA (1998) NF-kappa B-dependent inhibition of apoptosis is essential for host cellsurvival during Rickettsia rickettsii infection. *Proc Natl Acad Sci U S A* **95:** 4646-4651

Collier-Hyams LS, Zeng H, Sun J, Tomlinson AD, Bao ZQ, Chen H, Madara JL, Orth K, Neish AS (2002) Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. *J Immunol* **169:** 2846-2850

Conradt B, Xue D (2005) Programmed cell death. *WormBook : the online review of C elegans biology***:** 1-13

Constable FL (1959) Psittacosis elementary bodies. *Nature* **184(Suppl 7):** 473-474

Cookson BT, Brennan MA (2001) Pro-inflammatory programmed cell death. *Trends in microbiology* **9:** 113-114

Coombes BK, Mahony JB (2002) Identification of MEK- and phosphoinositide 3-kinase-dependent signalling as essential events during Chlamydia pneumoniae invasion of HEp2 cells. *Cellular microbiology* **4:** 447-460

Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2:** 647-656

Coultas L, Strasser A (2003) The role of the Bcl-2 protein family in cancer. *Seminars in cancer biology* **13:** 115-123

Courtois G, Smahi A (2006) NF-kappaB-related genetic diseases. *Cell death and differentiation* **13:** 843-851

Coutinho-Silva R, Perfettini JL, Persechini PM, Dautry-Varsat A, Ojcius DM (2001) Modulation of P2Z/P2X(7) receptor activity in macrophages infected with Chlamydia psittaci. *American journal of physiology Cell physiology* **280:** C81-89

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378:** 785-789

Cuconati A, Mukherjee C, Perez D, White E (2003) DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev* **17:** 2922-2932

Datta SR, Brunet A, Greenberg ME (1999) Cellular survival: a play in three Akts. *Genes Dev* **13:** 2905-2927

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91:** 231-241

Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, Greenberg ME (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Molecular cell* **6:** 41-51

Dean D, Powers VC (2001) Persistent Chlamydia trachomatis infections resist apoptotic stimuli. *Infect Immun* **69:** 2442-2447

Degterev A, Boyce M, Yuan J (2003) A decade of caspases. *Oncogene* **22:** 8543-8567

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nature chemical biology* **4:** 313-321

Degterev A, Yuan J (2008) Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol* **9:** 378-390

Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S (2003) MAPK pathways in radiation responses. *Oncogene* **22:** 5885-5896

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* **388:** 548-554

Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM (1998) Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. *Circulation research* **83:** 334-341

Dixit V, Mak TW (2002) NF-kappaB signaling. Many roads lead to madrid. *Cell* **111:** 615-619

Dong F, Pirbhai M, Xiao Y, Zhong Y, Wu Y, Zhong G (2005) Degradation of the proapoptotic proteins Bik, Puma, and Bim with Bcl-2 domain 3 homology in Chlamydia trachomatis-infected cells. *Infect Immun* **73:** 1861-1864

Downward J (1998) Lipid-regulated kinases: some common themes at last. *Science* **279:** 673-674

Eckelman BP, Salvesen GS (2006) The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *J Biol Chem* **281:** 3254-3260

Eissenberg LG, Wyrick PB, Davis CH, Rumpp JW (1983) Chlamydia psittaci elementary body envelopes: ingestion and inhibition of phagolysosome fusion. *Infect Immun* **40:** 741-751

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494-498

Elwell CA, Ceesay A, Kim JH, Kalman D, Engel JN (2008) RNA interference screen identifies Abl kinase and PDGFR signaling in Chlamydia trachomatis entry. *PLoS pathogens* **4:** e1000021

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391:** 43-50

Erhardt P, Cooper GM (1996) Activation of the CPP32 apoptotic protease by distinct signaling pathways with differential sensitivity to Bcl-xL. *The Journal of biological chemistry* **271:** 17601-17604

Ermolaeva MA, Michallet MC, Papadopoulou N, Utermohlen O, Kranidioti K, Kollias G, Tschopp J, Pasparakis M (2008) Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. *Nat Immunol* **9:** 1037-1046

Everett KD (2000) Chlamydia and Chlamydiales: more than meets the eye. *Vet Microbiol* **75:** 109-126

Everett KD, Bush RM, Andersen AA (1999) Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* **49 Pt 2:** 415-440

Faissner A, Heck N, Dobbertin A, Garwood J (2006) DSD-1-Proteoglycan/Phosphacan and receptor protein tyrosine phosphatase-beta isoforms during development and regeneration of neural tissues. *Advances in experimental medicine and biology* **557:** 25-53

Fan CM, Maniatis T (1991) Generation of p50 subunit of NF-kappa B by processing of p105 through an ATP-dependent pathway. *Nature* **354:** 395-398

Fan T, Lu H, Hu H, Shi L, McClarty GA, Nance DM, Greenberg AH, Zhong G (1998a) Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *The Journal of experimental medicine* **187:** 487-496

Fan T, Lu H, Hu H, Shi L, McClarty GA, Nance DM, Greenberg AH, Zhong G (1998b) Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J Exp Med* **187:** 487-496

Fang X, Yu SX, Lu Y, Bast RC, Jr., Woodgett JR, Mills GB (2000) Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc Natl Acad Sci U S A* **97:** 11960-11965

Fischer SF, Harlander T, Vier J, Hacker G (2004a) Protection against CD95-induced apoptosis by chlamydial infection at a mitochondrial step. *Infection and immunity* **72:** 1107-1115

Fischer SF, Schwarz C, Vier J, Hacker G (2001) Characterization of antiapoptotic activities of Chlamydia pneumoniae in human cells. *Infect Immun* **69:** 7121-7129

Fischer SF, Vier J, Kirschnek S, Klos A, Hess S, Ying S, Hacker G (2004b) Chlamydia inhibit host cell apoptosis by degradation of proapoptotic BH3-only proteins. *The Journal of experimental medicine* **200:** 905-916

Fletcher JI, Huang DC (2006) BH3-only proteins: orchestrating cell death. *Cell Death Differ* **13:** 1268-1271

Flusberg DA, Numaguchi Y, Ingber DE (2001) Cooperative control of Akt phosphorylation, bcl-2 expression, and apoptosis by cytoskeletal microfilaments and microtubules in capillary endothelial cells. *Mol Biol Cell* **12:** 3087-3094

Francois M, Le Cabec V, Dupont MA, Sansonetti PJ, Maridonneau-Parini I (2000) Induction of necrosis in human neutrophils by Shigella flexneri requires type III secretion, IpaB and IpaC invasins, and actin polymerization. *Infection and immunity* **68:** 1289-1296

Franke TF, Kaplan DR, Cantley LC, Toker A (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275:** 665-668

Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, Leonardi A, Tran T, Boyce BF, Siebenlist U (1997) Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* **11:** 3482-3496

French LE, Tschopp J (1999) Inhibition of death receptor signaling by FLICE-inhibitory protein as a mechanism for immune escape of tumors. *J Exp Med* **190:** 891-894

Friedman MG, Dvoskin B, Kahane S (2003) Infections with the chlamydia-like microorganism Simkania negevensis, a possible emerging pathogen. *Microbes Infect* **5:** 1013-1021

Fukuhara S, Rowley JD, Variakojis D, Golomb HM (1979) Chromosome abnormalities in poorly differentiated lymphocytic lymphoma. *Cancer research* **39:** 3119-3128

Gagnon V, St-Germain ME, Parent S, Asselin E (2003) Akt activity in endometrial cancer cells: regulation of cell survival through cIAP-1. *International journal of oncology* **23:** 803-810

Galan JE, Collmer A (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284:** 1322-1328

Ganchi PA, Sun SC, Greene WC, Ballard DW (1992) I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. *Mol Biol Cell* **3:** 1339-1352

Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *The Journal of cell biology* **119:** 493-501

Gaylord WH, Jr. (1954) Intracellular forms of meningopneumonitis virus. *J Exp Med* **100:** 575-580

Ghosh S, Baltimore D (1990) Activation Invitro of Nf-Kappa-B by Phosphorylation of Its Inhibitor I-Kappa-B. *Nature* **344:** 678-682

Gimenez G, Bertelli C, Moliner C, Robert C, Raoult D, Fournier PE, Greub G (2011) Insight into cross-talk between intra-amoebal pathogens. *BMC genomics* **12:** 542

Godbey WT, Wu KK, Hirasaki GJ, Mikos AG (1999) Improved packing of poly(ethylenimine)/DNA complexes increases transfection efficiency. *Gene therapy* **6:** 1380-1388

Goping IS, Barry M, Liston P, Sawchuk T, Constantinescu G, Michalak KM, Shostak I, Roberts DL, Hunter AM, Korneluk R, Bleackley RC (2003) Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. *Immunity* **18:** 355-365

Gordon FB, Quan AL (1965) Occurence of Glycogen in Inclusions of the Psittacosis-Lymphogranuloma Venereum-Trachoma Agents. *J Infect Dis* **115:** 186-196

Graham DM (1965) Growth and neutralization of the trachoma agent in mouse lungs. *Nature* **207:** 1379-1380

Grassme H, Kirschnek S, Riethmueller J, Riehle A, von Kurthy G, Lang F, Weller M, Gulbins E (2000) CD95/CD95 ligand interactions on epithelial cells in host defense to Pseudomonas aeruginosa. *Science* **290:** 527-530

Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* **281:** 1309-1312

Greenberg D, Banerji A, Friedman MG, Chiu CH, Kahane S (2003) High rate of Simkania negevensis among Canadian inuit infants hospitalized with lower respiratory tract infections. *Scandinavian journal of infectious diseases* **35:** 506-508

Greub G (2009) The medical importance of Chlamydiae. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **15:** 2-3

Greub G, La Scola B, Raoult D (2003) Parachlamydia acanthamoeba is endosymbiotic or lytic for Acanthamoeba polyphaga depending on the incubation temperature. *Annals of the New York Academy of Sciences* **990:** 628-634

Gutierrez-Martin CB, Ojcius DM, Hsia R, Hellio R, Bavoil PM, Dautry-Varsat A (1997) Heparin-mediated inhibition of Chlamydia psittaci adherence to HeLa cells. *Microbial pathogenesis* **22:** 47-57

Guttmacher S, Lieberman L, Ward D, Freudenberg N, Radosh A, Des Jarlais D (1997) Condom availability in New York City public high schools: relationships to condom use and sexual behavior. *American journal of public health* **87:** 1427-1433

Gyrd-Hansen M, Meier P (2010) IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. *Nature reviews Cancer* **10:** 561-574

Hackstadt T, Scidmore-Carlson MA, Shaw EI, Fischer ER (1999) The Chlamydia trachomatis IncA protein is required for homotypic vesicle fusion. *Cell Microbiol* **1:** 119-130

Hackstadt T, Scidmore MA, Rockey DD (1995) Lipid metabolism in Chlamydia trachomatis-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. *Proc Natl Acad Sci U S A* **92:** 4877-4881

Hatch GM (1998) Cardiolipin: biosynthesis, remodeling and trafficking in the heart and mammalian cells (Review). *International journal of molecular medicine* **1:** 33-41

Hawkins CJ, Wang SL, Hay BA (2000) Monitoring activity of caspases and their regulators in yeast Saccharomyces cerevisiae. *Methods Enzymol* **322:** 162-174

Heiskanen-Kosma T, Paldanius M, Korppi M (2008) Simkania negevensis may be a true cause of community acquired pneumonia in children. *Scand J Infect Dis* **40:** 127-130

Heissmeyer V, Krappmann D, Hatada EN, Scheidereit C (2001) Shared pathways of IkappaB kinase-induced SCF(betaTrCP)-mediated ubiquitination and degradation for the NF-kappaB precursor p105 and IkappaBalpha. *Molecular and cellular biology* **21:** 1024-1035

Hilbi H, Moss JE, Hersh D, Chen Y, Arondel J, Banerjee S, Flavell RA, Yuan J, Sansonetti PJ, Zychlinsky A (1998) Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. *J Biol Chem* **273:** 32895-32900

Hogan RJ, Mathews SA, Mukhopadhyay S, Summersgill JT, Timms P (2004) Chlamydial persistence: beyond the biphasic paradigm. *Infect Immun* **72:** 1843-1855

Horvitz HR, Shaham S, Hengartner MO (1994) The genetics of programmed cell death in the nematode Caenorhabditis elegans. *Cold Spring Harb Symp Quant Biol* **59:** 377-385

Howie HL, Shiflett SL, So M (2008) Extracellular signal-regulated kinase activation by Neisseria gonorrhoeae downregulates epithelial cell proapoptotic proteins Bad and Bim. *Infect Immun* **76:** 2715-2721

Hsu H, Shu HB, Pan MG, Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84:** 299-308

Hsu H, Xiong J, Goeddel DV (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* **81:** 495-504

Hu Y, Benedict MA, Wu D, Inohara N, Nunez G (1998) Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci U S A* **95:** 4386-4391

Huang DC, Adams JM, Cory S (1998) The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *Embo J* **17:** 1029-1039

Husain S, Kahane S, Friedman MG, Paterson DL, Studer S, McCurry KR, Wolf DG, Zeevi A, Pilewski J, Greenberg D (2007) Simkania negevensis in bronchoalveolar lavage of lung transplant recipients: a possible association with acute rejection. *Transplantation* **83:** 138-143

Ibana JA, Schust DJ, Sugimoto J, Nagamatsu T, Greene SJ, Quayle AJ (2011) Chlamydia trachomatis immune evasion via downregulation of MHC class I surface expression involves direct and indirect mechanisms. *Infect Dis Obstet Gynecol* **2011:** 420905

Janssens S, Burns K, Tschopp J, Beyaert R (2002) Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. *Curr Biol* **12:** 467-471

Jewett TJ, Dooley CA, Mead DJ, Hackstadt T (2008) Chlamydia trachomatis tarp is phosphorylated by src family tyrosine kinases. *Biochem Biophys Res Commun* **371:** 339-344

Jones NL, Day AS, Jennings H, Shannon PT, Galindo-Mata E, Sherman PM (2002) Enhanced disease severity in Helicobacter pylori-infected mice deficient in Fas signaling. *Infect Immun* **70:** 2591-2597

Joseph TD, Bose SK (1991) Further characterization of an outer membrane protein of Chlamydia trachomatis with cytadherence properties. *FEMS microbiology letters* **68:** 167-171

Kahane S, Gonen R, Sayada C, Elion J, Friedman MG (1993) Description and partial characterization of a new Chlamydia-like microorganism. *FEMS microbiology letters* **109:** 329-333

Kahane S, Greenberg D, Friedman MG, Haikin H, Dagan R (1998) High prevalence of "Simkania Z," a novel Chlamydia-like bacterium, in infants with acute bronchiolitis. *J Infect Dis* **177:** 1425-1429

Kahane S, Kimmel N, Friedman MG (2002) The growth cycle of Simkania negevensis. *Microbiology* **148:** 735-742

Kahane S, Metzer E, Friedman MG (1995) Evidence that the novel microorganism 'Z' may belong to a new genus in the family Chlamydiaceae. *FEMS Microbiol Lett* **126:** 203-207

Kang TB, Oh GS, Scandella E, Bolinger B, Ludewig B, Kovalenko A, Wallach D (2008) Mutation of a self-processing site in caspase-8 compromises its apoptotic but not its nonapoptotic functions in bacterial artificial chromosome-transgenic mice. *J Immunol* **181:** 2522-2532

Kelly GL, Milner AE, Baldwin GS, Bell AI, Rickinson AB (2006) Three restricted forms of Epstein-Barr virus latency counteracting apoptosis in c-myc-expressing Burkitt lymphoma cells. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 14935-14940

Kennedy SG, Kandel ES, Cross TK, Hay N (1999) Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. *Molecular and cellular biology* **19:** 5800-5810

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26:** 239-257

Kerr LD, Inoue J, Davis N, Link E, Baeuerle PA, Bose HR, Verma IM (1991) The Rel-Associated Pp40 Protein Prevents DNA-Binding of Rel and Nf-Kappa-B - Relationship with I-Kappa-B-Beta and Regulation by Phosphorylation. *Genes Dev* **5:** 1464-1476

Kippenberger S, Loitsch S, Guschel M, Muller J, Knies Y, Kaufmann R, Bernd A (2005) Mechanical stretch stimulates protein kinase B/Akt phosphorylation in epidermal cells via angiotensin II type 1 receptor and epidermal growth factor receptor. *The Journal of biological chemistry* **280:** 3060-3067

Klotz LO, Schieke SM, Sies H, Holbrook NJ (2000) Peroxynitrite activates the phosphoinositide 3-kinase/Akt pathway in human skin primary fibroblasts. *The Biochemical journal* **352 Pt 1:** 219-225

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275:** 1132-1136

Knodler LA, Finlay BB, Steele-Mortimer O (2005a) The Salmonella effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. *J Biol Chem* **280:** 9058-9064

Knodler LA, Finlay BB, Steele-Mortimer O (2005b) The Salmonella effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. *The Journal of biological chemistry* **280:** 9058-9064

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278:** 294-298

Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. *Physiol Rev* **87:** 99-163

Kumar S, Doumanis J (2000) The fly caspases. *Cell Death Differ* **7:** 1039-1044

Kuo CC, Wang SP, Grayston JT (1973) Effect of polycations, polyanions and neuraminidase on the infectivity of trachoma-inclusin conjunctivitis and lymphogranuloma venereum organisms HeLa cells: sialic acid residues as possible receptors for trachoma-inclusion conjunction. *Infect Immun* **8:** 74-79

Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111:** 331-342

Lad SP, Li J, da Silva Correia J, Pan Q, Gadwal S, Ulevitch RJ, Li E (2007) Cleavage of p65/RelA of the NF-kappaB pathway by Chlamydia. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 2933-2938

Lakhani SA, Masud A, Kuida K, Porter GA, Jr., Booth CJ, Mehal WZ, Inayat I, Flavell RA (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311:** 847-851

Lamkanfi M, Dixit VM (2010) Manipulation of host cell death pathways during microbial infections. *Cell host & microbe* **8:** 44-54

Laussmann MA, Passante E, Dussmann H, Rauen JA, Wurstle ML, Delgado ME, Devocelle M, Prehn JH, Rehm M (2011) Proteasome inhibition can induce an autophagy-dependent apical activation of caspase-8. *Cell death and differentiation* **18:** 1584-1597

Le Negrate G, Krieg A, Faustin B, Loeffler M, Godzik A, Krajewski S, Reed JC (2008) ChlaDub1 of Chlamydia trachomatis suppresses NF-kappaB activation and inhibits IkappaBalpha ubiquitination and degradation. *Cellular microbiology* **10:** 1879-1892

Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, Ma A (2000) Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. *Science* **289:** 2350-2354

Lee JM, Lee KH, Weidner M, Osborne BA, Hayward SD (2002) Epstein-Barr virus EBNA2 blocks Nur77- mediated apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 11878-11883

Lee JW, Chen H, Pullikotil P, Quon MJ (2011) Protein kinase A-alpha directly phosphorylates FoxO1 in vascular endothelial cells to regulate expression of vascular cellular adhesion molecule-1 mRNA. *J Biol Chem* **286:** 6423-6432

Legler DF, Micheau O, Doucey MA, Tschopp J, Bron C (2003) Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. *Immunity* **18:** 655-664

Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2:** 183-192

Li H, Zhu H, Xu CJ, Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94:** 491-501

Lieberman D, Dvoskin B, Lieberman DV, Kahane S, Friedman MG (2002) Serological evidence of acute infection with the Chlamydia-like microorganism Simkania negevensis (Z) in acute exacerbation of chronic obstructive pulmonary disease. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **21:** 307-309

Lieberman D, Kahane S, Friedman MG (1997) Pneumonia with serological evidence of acute infection with the Chlamydia-like microorganism "Z". *Am J Respir Crit Care Med* **156:** 578-582

Lin HS, Moulder JW (1966) Patterns of response to sulfadiazine, D-cycloserine and D-alanine in members of the psittacosis group. *J Infect Dis* **116:** 372-376

Liu X, Zou H, Slaughter C, Wang X (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89:** 175-184

Lockshin RA, Williams CM (1965) Programmed Cell Death--I. Cytology of Degeneration in the Intersegmental Muscles of the Pernyi Silkmoth. *J Insect Physiol* **11:** 123-133

Locksley RM, Killeen N, Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104:** 487-501

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system

neurons. *Proceedings of the National Academy of Sciences of the United States of America* **90:** 7951-7955

Lord SJ, Rajotte RV, Korbutt GS, Bleackley RC (2003) Granzyme B: a natural born killer. *Immunol Rev* **193:** 31-38

Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A* **96:** 11364-11369

Luhrmann A, Roy CR (2007) Coxiella burnetii inhibits activation of host cell apoptosis through a mechanism that involves preventing cytochrome c release from mitochondria. *Infect Immun* **75:** 5282-5289

Marriott HM, Bingle CD, Read RC, Braley KE, Kroemer G, Hellewell PG, Craig RW, Whyte MK, Dockrell DH (2005) Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance. *J Clin Invest* **115:** 359-368

Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, Green DR (1995) Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J Biol Chem* **270:** 6425-6428

Martinou JC, Youle RJ (2006) Which came first, the cytochrome c release or the mitochondrial fission? *Cell Death Differ* **13:** 1291-1295

Matsuo J, Hayashi Y, Nakamura S, Sato M, Mizutani Y, Asaka M, Yamaguchi H (2008) Novel Parachlamydia acanthamoebae quantification method based on coculture with amoebae. *Applied and environmental microbiology* **74:** 6397-6404

Mattson MP, Camandola S (2001) NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Invest* **107:** 247-254

May MJ, Ghosh S (1998) Signal transduction through NF-kappa B. *Immunol Today* **19:** 80-88

Mayo LD, Donner DB (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* **98:** 11598-11603

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A (1997) IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **278:** 860-866

Metzstein MM, Stanfield GM, Horvitz HR (1998) Genetics of programmed cell death in C. elegans: past, present and future. *Trends in genetics : TIG* **14:** 410-416

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* **114:** 181-190

Miller LK (1999) An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends in cell biology* **9:** 323-328

Miyairi I, Byrne GI (2006) Chlamydia and programmed cell death. *Current opinion in microbiology* **9:** 102-108

Monack DM, Detweiler CS, Falkow S (2001) Salmonella pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1. *Cell Microbiol* **3:** 825-837

Monack DM, Raupach B, Hromockyj AE, Falkow S (1996) Salmonella typhimurium invasion induces apoptosis in infected macrophages. *Proc Natl Acad Sci U S A* **93:** 9833-9838

Moulder JW (1991) Interaction of chlamydiae and host cells in vitro. *Microbiol Rev* **55:** 143-190

Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM (1998) An induced proximity model for caspase-8 activation. *J Biol Chem* **273:** 2926-2930

Nascimento-Carvalho CM, Cardoso MR, Paldanius M, Barral A, Araujo-Neto CA, Saukkoriipi A, Vainionpaa R, Leinonen M, Ruuskanen O (2009) Simkania negevensis infection among Brazilian children hospitalized with community-acquired pneumonia. *J Infect* **58:** 250-253

Navarro L, Alto NM, Dixon JE (2005) Functions of the Yersinia effector proteins in inhibiting host immune responses. *Current opinion in microbiology* **8:** 21-27

Nechushtan A, Smith CL, Lamensdorf I, Yoon SH, Youle RJ (2001) Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J Cell Biol* **153:** 1265-1276

Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. *Trends Biochem Sci* **22:** 299-306

Nicholson KM, Anderson NG (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* **14:** 381-395

Nogueira CV, Lindsten T, Jamieson AM, Case CL, Shin S, Thompson CB, Roy CR (2009) Rapid pathogen-induced apoptosis: a mechanism used by dendritic cells to limit intracellular replication of Legionella pneumophila. *PLoS pathogens* **5:** e1000478

Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N, Gotoh Y (2002) Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *J Biol Chem* **277:** 21843-21850

Ohno A, Kato N, Sakamoto R, Kimura S, Yamaguchi K (2008) Temperature-dependent parasitic relationship between Legionella pneumophila and a free-living amoeba (Acanthamoeba castellanii). *Applied and environmental microbiology* **74:** 4585-4588

Ojcius DM, Degani H, Mispelter J, Dautry-Varsat A (1998) Enhancement of ATP levels and glucose metabolism during an infection by Chlamydia. NMR studies of living cells. *The Journal of biological chemistry* **273:** 7052-7058

Paland N, Bohme L, Gurumurthy RK, Maurer A, Szczepek AJ, Rudel T (2008) Reduced display of tumor necrosis factor receptor I at the host cell surface supports infection with Chlamydia trachomatis. *The Journal of biological chemistry* **283:** 6438-6448

Paland N, Rajalingam K, Machuy N, Szczepek A, Wehrl W, Rudel T (2006a) NF-kappaB and inhibitor of apoptosis proteins are required for apoptosis resistance of epithelial cells persistently infected with Chlamydophila pneumoniae. *Cellular Microbiology* **8:** 1643-1655

Paland N, Rajalingam K, Machuy N, Szczepek A, Wehrl W, Rudel T (2006b) NF-kappaB and inhibitor of apoptosis proteins are required for apoptosis resistance of epithelial cells persistently infected with Chlamydophila pneumoniae. *Cell Microbiol* **8:** 1643-1655

Pan G, O'Rourke K, Dixit VM (1998) Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* **273:** 5841-5845

Paschen SA, Christian JG, Vier J, Schmidt F, Walch A, Ojcius DM, Hacker G (2008) Cytopathicity of Chlamydia is largely reproduced by expression of a single chlamydial protease. *J Cell Biol* **182:** 117-127

Peter ME, Krammer PH (2003) The CD95(APO-1/Fas) DISC and beyond. *Cell death and differentiation* **10:** 26-35

Peters J, Wilson DP, Myers G, Timms P, Bavoil PM (2007) Type III secretion a la Chlamydia. *Trends in microbiology* **15:** 241-251

Plas DR, Thompson CB (2005) Akt-dependent transformation: there is more to growth than just surviving. *Oncogene* **24:** 7435-7442

Portera-Cailliau C, Hedreen JC, Price DL, Koliatsos VE (1995) Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **15:** 3775-3787

Prakash H, Becker D, Bohme 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:** e6519

Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, Gotoh T, Akira S, Bouillet P, Strasser A (2007) ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* **129:** 1337-1349

Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DC, Strasser A (2001) Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* **293:** 1829-1832

Rajalingam K, Al-Younes H, Muller A, Meyer TF, Szczepek AJ, Rudel T (2001a) Epithelial cells infected with Chlamydophila pneumoniae (Chlamydia pneumoniae) are resistant to apoptosis. *Infect Immun* **69:** 7880-7888

Rajalingam K, Al-Younes H, Muller A, Meyer TF, Szczepek AJ, Rudel T (2001b) Epithelial cells infected with Chlamydophila pneumoniae (Chlamydia pneumoniae) are resistant to apoptosis. *Infection and immunity* **69:** 7880-7888

Rajalingam K, Sharma M, Lohmann C, Oswald M, Thieck O, Froelich CJ, Rudel T (2008a) Mcl-1 is a key regulator of apoptosis resistance in Chlamydia trachomatis-infected cells. *PloS one* **3:** e3102

Rajalingam K, Sharma M, Lohmann C, Oswald M, Thieck O, Froelich CJ, Rudel T (2008b) Mcl-1 is a key regulator of apoptosis resistance in Chlamydia trachomatis-infected cells. *PLoSONE* **3:** e3102

Rajalingam K, Sharma M, Paland N, Hurwitz R, Thieck O, Oswald M, Machuy N, Rudel T (2006a) IAP-IAP complexes required for apoptosis resistance of C. trachomatis-infected cells. *PLoS Pathog* **2:** e114

Rajalingam K, Sharma M, Paland N, Hurwitz R, Thieck O, Oswald M, Machuy N, Rudel T (2006b) IAP-IAP complexes required for apoptosis resistance of C. trachomatis-infected cells. *PLoSPathog* **2:** e114

Rao L, Perez D, White E (1996) Lamin proteolysis facilitates nuclear events during apoptosis. *J Cell Biol* **135:** 1441-1455

Raulston JE, Paul TR, Knight ST, Wyrick PB (1998) Localization of Chlamydia trachomatis heat shock proteins 60 and 70 during infection of a human endometrial epithelial cell line in vitro. *Infect Immun* **66:** 2323-2329

Rivers TM, Schwentker FF (1932) A Virus Disease of Parrots and Parrakeets Differing from Psittacosis. *The Journal of experimental medicine* **55:** 911-924

Rodel J, Grosse C, Yu H, Wolf K, Otto GP, Liebler-Tenorio E, Forsbach-Birk V, Straube E (2012) Persistent Chlamydia trachomatis Infection of HeLa Cells Mediates Apoptosis Resistance through a Chlamydia Protease-Like Activity Factor-Independent Mechanism and Induces High Mobility Group Box 1 Release. *Infect Immun* **80:** 195-205

Romashkova JA, Makarov SS (1999) NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401:** 86-90

Rong SB, Hu Y, Enyedy I, Powis G, Meuillet EJ, Wu X, Wang R, Wang S, Kozikowski AP (2001) Molecular modeling studies of the Akt PH domain and its interaction with phosphoinositides. *J Med Chem* **44:** 898-908

Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391:** 96-99

Sakurai H, Suzuki S, Kawasaki N, Nakano H, Okazaki T, Chino A, Doi T, Saiki I (2003) Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-kappaB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *J Biol Chem* **278:** 36916-36923

Salvesen GS, Duckett CS (2002a) IAP proteins: blocking the road to death's door. *Nature reviews Molecular cell biology* **3:** 401-410

Salvesen GS, Duckett CS (2002b) IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* **3:** 401-410

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307:** 1098-1101

Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* **275:** 983-986

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. *Embo J* **17:** 1675-1687

Schachter J, Stephens RS, Timms P, Kuo C, Bavoil PM, Birkelund S, Boman J, Caldwell H, Campbell LA, Chernesky M, Christiansen G, Clarke IN, Gaydos C, Grayston JT, Hackstadt T, Hsia R, Kaltenboeck B, Leinonnen M, Ojcius D, McClarty G, Orfila J, Peeling R, Puolakkainen M, Quinn TC, Rank RG, Raulston J, Ridgeway GL, Saikku P, Stamm WE, Taylor-Robinson DT, Wang SP, Wyrick PB (2001) Radical changes to chlamydial taxonomy are not necessary just yet. *Int J Syst Evol Microbiol* **51:** 249; author reply 251-243

Schleifer KH, Stackebrandt E (1983) Molecular systematics of prokaryotes. *Annu Rev Microbiol* **37:** 143-187

Schoier J, Ollinger K, Kvarnstrom M, Soderlund G, Kihlstrom E (2001) Chlamydia trachomatis-induced apoptosis occurs in uninfected McCoy cells late in the developmental cycle and is regulated by the intracellular redox state. *Microbial pathogenesis* **31:** 173-184

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME (1998) Apoptosis signaling by death receptors. *Eur J Biochem* **254:** 439-459

Scidmore MA, Fischer ER, Hackstadt T (1996a) Sphingolipids and glycoproteins are differentially trafficked to the Chlamydia trachomatis inclusion. *J Cell Biol* **134:** 363-374

Scidmore MA, Rockey DD, Fischer ER, Heinzen RA, Hackstadt T (1996b) Vesicular interactions of the Chlamydia trachomatis inclusion are determined by chlamydial early protein synthesis rather than route of entry. *Infect Immun* **64:** 5366-5372

Scott FL, Denault JB, Riedl SJ, Shin H, Renatus M, Salvesen GS (2005) XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. *Embo J* **24:** 645-655

Sen R, Baltimore D (1986) Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* **47:** 921-928

Sharma M, Machuy N, Bohme L, Karunakaran K, Maurer AP, Meyer TF, Rudel T (2011) HIF-1alpha is involved in mediating apoptosis resistance to Chlamydia trachomatis-infected cells. *Cell Microbiol* **13:** 1573-1585

Sharma M, Rudel T (2009) Apoptosis resistance in Chlamydia-infected cells: a fate worse than death? *FEMS immunology and medical microbiology* **55:** 154-161

Shi LF, Mai S, Israels S, Browne K, Trapani JA, Greenberg AH (1997) Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. *Journal of Experimental Medicine* **185:** 855-866

Shi Y (2002) Mechanisms of caspase activation and inhibition during apoptosis. *Molecular cell* **9:** 459-470

Shroff EH, Snyder CM, Budinger GR, Jain M, Chew TL, Khuon S, Perlman H, Chandel NS (2009) BH3 peptides induce mitochondrial fission and cell death independent of BAX/BAK. *PLoS One* **4:** e5646

Song HY, Rothe M, Goeddel DV (1996) The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation. *Proc Natl Acad Sci U S A* **93:** 6721-6725

Song SM, Han BI, Moon EK, Lee YR, Yu HS, Jha BK, Danne DB, Kong HH, Chung DI, Hong Y (2012) Autophagy protein 16-mediated autophagy is required for the encystation of Acanthamoeba castellanii. *Molecular and biochemical parasitology*

Srinivasula SM, Datta P, Fan XJ, Fernandes-Alnemri T, Huang Z, Alnemri ES (2000) Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J Biol Chem* **275:** 36152-36157

Steinhusen U, Weiske J, Badock V, Tauber R, Bommert K, Huber O (2001) Cleavage and shedding of E-cadherin after induction of apoptosis. *J Biol Chem* **276:** 4972-4980

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277:** 567-570

Strasser A, Harris AW, Huang DC, Krammer PH, Cory S (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *The EMBO journal* **14:** 6136-6147

Strasser A, O'Connor L, Dixit VM (2000) Apoptosis signaling. *Annual review of biochemistry* **69:** 217-245

Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W (1999) An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann Intern Med* **130:** 591-601

Su H, Raymond L, Rockey DD, Fischer E, Hackstadt T, Caldwell HD (1996) A recombinant Chlamydia trachomatis major outer membrane protein binds to heparan sulfate receptors on epithelial cells. *Proc Natl Acad Sci U S A* **93:** 11143-11148

Suarez MF, Filonova LH, Smertenko A, Savenkov EI, Clapham DH, von Arnold S, Zhivotovsky B, Bozhkov PV (2004) Metacaspase-dependent programmed cell death is essential for plant embryogenesis. *Current biology : CB* **14:** R339-340

Subtil A, Blocker A, Dautry-Varsat A (2000) Type III secretion system in Chlamydia species: identified members and candidates. *Microbes Infect* **2:** 367-369

Sun C, Cai M, Gunasekera AH, Meadows RP, Wang H, Chen J, Zhang H, Wu W, Xu N, Ng SC, Fesik SW (1999) NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. *Nature* **401:** 818-822

Sundstrom JF, Vaculova A, Smertenko AP, Savenkov EI, Golovko A, Minina E, Tiwari BS, Rodriguez-Nieto S, Zamyatnin AA, Jr., Valineva T, Saarikettu J, Frilander MJ, Suarez MF, Zavialov A, Stahl U, Hussey PJ, Silvennoinen O, Sundberg E, Zhivotovsky B, Bozhkov PV (2009) Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. *Nature cell biology* **11:** 1347-1354

Swanson AF, Kuo CC (1994) Binding of the glycan of the major outer membrane protein of Chlamydia trachomatis to HeLa cells. *Infect Immun* **62:** 24-28

Taylor JM, Ali U, Iannello RC, Hertzog P, Crack PJ (2005) Diminished Akt phosphorylation in neurons lacking glutathione peroxidase-1 (Gpx1) leads to increased susceptibility to oxidative stress-induced cell death. *Journal of neurochemistry* **92:** 283-293

Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. *Science* **281:** 1312-1316

Thornberry NA, Rosen A, Nicholson DW (1997) Control of apoptosis by proteases. *Adv Pharmacol* **41:** 155-177

Ting LM, Hsia RC, Haidaris CG, Bavoil PM (1995) Interaction of outer envelope proteins of Chlamydia psittaci GPIC with the HeLa cell surface. *Infect Immun* **63:** 3600-3608

Tsujimoto Y (1989) Overexpression of the human BCL-2 gene product results in growth enhancement of Epstein-Barr virus-immortalized B cells. *Proc Natl Acad Sci U S A* **86:** 1958-1962

Tsujimoto Y, Cossman J, Jaffe E, Croce CM (1985) Involvement of the bcl-2 gene in human follicular lymphoma. *Science* **228:** 1440-1443

Upton JW, Kaiser WJ, Mocarski ES (2010) Virus inhibition of RIP3-dependent necrosis. *Cell host & microbe* **7:** 302-313

van Delft MF, Huang DC (2006) How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res* **16:** 203-213

van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, Willis SN, Scott CL, Day CL, Cory S, Adams JM, Roberts AW, Huang DC (2006) The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10:** 389-399

van der Velden AW, Velasquez M, Starnbach MN (2003) Salmonella rapidly kill dendritic cells via a caspase-1-dependent mechanism. *J Immunol* **171:** 6742-6749

Vaux DL, Cory S, Adams JM (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335:** 440-442

Vaux DL, Silke J (2005a) IAPs--the ubiquitin connection. *Cell Death Differ* **12:** 1205-1207

Vaux DL, Silke J (2005b) IAPs, RINGs and ubiquitylation. *Nature reviews Molecular cell biology* **6:** 287-297

Verbeke P, Welter-Stahl L, Ying S, Hansen J, Hacker G, Darville T, Ojcius DM (2006) Recruitment of BAD by the Chlamydia trachomatis vacuole correlates with host-cell survival. *PLoS pathogens* **2:** e45

Viboud GI, Bliska JB (2005) Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annual review of microbiology* **59:** 69-89

Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* **269:** 5241-5248

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP (1999a) Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* **17:** 331-367

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP (1999b) Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* **17:** 331-367

Walter D, Schmich K, Vogel S, Pick R, Kaufmann T, Hochmuth FC, Haber A, Neubert K, McNelly S, von Weizsacker F, Merfort I, Maurer U, Strasser A, Borner C (2008) Switch from type II to I Fas/CD95 death signaling on in vitro culturing of primary hepatocytes. *Hepatology* **48:** 1942-1953

Wan F, Lenardo MJ (2010) The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives. *Cell research* **20:** 24-33

Wang LL, Henson E, McClarty G (1994) Characterization of trimethoprim- and sulphisoxazole-resistant Chlamydia trachomatis. *Mol Microbiol* **14:** 271-281

Wang Y, Kahane S, Cutcliffe LT, Skilton RJ, Lambden PR, Clarke IN (2011) Development of a transformation system for Chlamydia trachomatis: restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* **7:** e1002258

Wayne LG, Diaz GA (1987) Intrinsic catalase dot blot immunoassay for identification of Mycobacterium tuberculosis, Mycobacterium avium, and Mycobacterium intracellulare. *J Clin Microbiol* **25:** 1687-1690

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292:** 727-730

Weinrauch Y, Zychlinsky A (1999) The induction of apoptosis by bacterial pathogens. *Annual review of microbiology* **53:** 155-187

Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, Huang DC (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes & development* **19:** 1294-1305

Wolf K, Plano GV, Fields KA (2009) A protein secreted by the respiratory pathogen Chlamydia pneumoniae impairs IL-17 signalling via interaction with human Act1. *Cellular microbiology* **11:** 769-779

Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284:** 555-556

Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* **68:** 251-306

Xiao G, Fong A, Sun SC (2004a) Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation. *J Biol Chem* **279:** 30099-30105

Xiao Y, Zhong Y, Greene W, Dong F, Zhong G (2004b) Chlamydia trachomatis infection inhibits both Bax and Bak activation induced by staurosporine. *Infect Immun* **72:** 5470-5474

Yang T, Kozopas KM, Craig RW (1995) The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol* **128:** 1173-1184

Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* **288:** 874-877

Ying S, Seiffert BM, Hacker G, Fischer SF (2005) Broad degradation of proapoptotic proteins with the conserved Bcl-2 homology domain 3 during infection with Chlamydia trachomatis. *Infect Immun* **73:** 1399-1403

Yuk MH, Harvill ET, Cotter PA, Miller JF (2000) Modulation of host immune responses, induction of apoptosis and inhibition of NF-kappaB activation by the Bordetella type III secretion system. *Mol Microbiol* **35:** 991-1004

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87:** 619-628

Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM (2006) Activated CD4(+)CD25(+) T cells selectively kill B lymphocytes. *Blood* **107:** 3925-3932

Zhong G, Fan T, Liu L (1999) Chlamydia inhibits interferon gamma-inducible major histocompatibility complex class II expression by degradation of upstream stimulatory factor 1. *J Exp Med* **189:** 1931-1938

Zhong G, Liu L, Fan T, Fan P, Ji H (2000) Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon gamma-inducible major histocompatibility complex class I expression in chlamydia-infected cells. *J Exp Med* **191:** 1525-1534

Zhong Y, Weininger M, Pirbhai M, Dong F, Zhong G (2006) Inhibition of staurosporine-induced activation of the proapoptotic multidomain Bcl-2 proteins Bax and Bak by three invasive chlamydial species. *J Infect* **53:** 408-414

Zhou H, Li XM, Meinkoth J, Pittman RN (2000) Akt regulates cell survival and apoptosis at a postmitochondrial level. *J Cell Biol* **151:** 483-494

Zhu S, Evans S, Yan B, Povsic TJ, Tapson V, Goldschmidt-Clermont PJ, Dong C (2008) Transcriptional regulation of Bim by FOXO3a and Akt mediates scleroderma serum-induced apoptosis in endothelial progenitor cells. *Circulation* **118:** 2156-2165

Zhuang S, Kochevar IE (2003) Singlet oxygen-induced activation of Akt/protein kinase B is independent of growth factor receptors. *Photochemistry and photobiology* **78:** 361-371

Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev* **15:** 1481-1486

## 7.1. Curriculum Vitaé

**Biographical data:**

| | | |
|---|---|---|
| Name | : | Karthika Karunakaran. |
| Date of Birth | : | 31 October, 1981. |
| Nationality | : | Indian. |
| Gender | : | Female. |
| Marital Status | : | Married. |
| Permanent address | : | Lerchenweg-14, Wuerzburg D-97074, Germany |
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| E-mail | : | karthikakarunakaran@gmail.com<br>karunakaran@biozentrum.uni-wuerzburg.de |

**Academic Qualification:**

| **Degree/Award** | **Year** | **Institution & Board /University** | **Major Subjects** | **Result** |
|---|---|---|---|---|
| Bachelor of Science (Agriculture) | 2000 - 2004 | GKVK, University of Agricultural Sciences (UAS) , Bangalore, INDIA | Agricultural Sciences | 82% |
| Master of Science (Agricultural Biotechnology) | 2004 - 2006 | Kerala Agricultural University (KAU), Trivandrum, Kerala INDIA | Biotechnology | 92% |
| Graduate Fellowship | May 2007 till date | Graduate School of Life Science, University of Wuerzburg, | Infection Biology, Cell Biology | - |

**Reseach Experience:**

**1) Year : 2004-2007**

Institute : Department of Plant Biotechnology, College of Agriculture, Kerala Agricultural University, Trivandrum, Kerala, INDIA.

Master Thesis : '*Agrobacterium Tumefaciens* mediated genetic transformation in *Dendrobium* variety sonia 17 with 1-aminocyclopropane-1-carboxylic acid (ACC) antisense gene.'

**2) Year : May 2007 - till date.**

Institute : Theoder- Boveri Institute, Department of Microbiology, Biocentre, Am Hubland, Wuerzburg, Germany

Ph.D Thesis : 'Mechanisms of apoptosis regulation in human cells infected with *Simkania negevensis'*
Under Prof. Dr. Thomas Rudel,
Lehrstuhl für Mikobiologie, University of Wuerzburg, Germany

**Awards and fellowships received:**

1) 2000-2004 : All India Scholarship under ICAR (Indian Council of Agricultural Research) For Bachelors in Agriculture (Rank 612)

2) 2004-2006 : All India Scholarship under ICAR (Indian Council of Agricultural Research) For Mastersin Agricultural Biotechnology (Rank 52)

3) 2004-2006 : KAU Award for Masters from the Kerala Agricultural University

4) 2006 : Indira Gandhi Post Graduate Scholarship, Govt. of India.

5) 2007-2010 : The Graduate School of Life Sciences (GSLS) fellowship by The Excellent Initiative of the German Federal and State Government, (Deutsche Forschungsgemeinschaft-DFG)

**Oral presentations and posters accepted:**

**Karunakaran K.** Oral presentation on 'Edible Vaccines' on the theme Sustainable Agriculture as a part of 'Prithvi' 2005 International Global Eco Meet, 21st Feb, 2005, Trivandrum, INDIA.

**Karunakaran K**, Mehlitz A and Rudel T poster on 'The pathogenic mechanisms of an emerging pathogen *Simkania Negevensis'* 2009 meeting on Microbial Pathogenesis and Host Cell Response at Cold Spring Harbor Laboratory, New York, USA.

**Karunakaran K**, Mehlitz A and Rudel T Oral presentation on 'Evolutionary conservation of infection-induced cell death inhibition among *Chlamydiales'*.2010.10th International conference on molecular epidemiology and evolutionary genetics of infectious diseases, Amsterdam, Netherlands.

**Karunakaran K**, Mehlitz A and Rudel T poster presentation on 'Evolutionary conservation of infection-induced cell death inhibition among *Chlamydiales'*.2011 FEMS- Leopoldina- Symposium on Emerging topics in Microbial pathogenesis, würzburg, Germany.

**Conferences and symposia attended:**

1) ICAR National Symposium on Biotechnological Intervention for Improvement of Horticultural Crops: Issues and Strategies, organized by ICAR on10 -12 Jan, 2005, Trissur, INDIA

2) Fellow Retreat of the Graduate School of Life Science, organized by the University of Wuerzburg at Schloss Zeitlitzheim, 27-29 June 2008, Wuerzburg, Germany

3) Third International Symposium organized by the Graduate School of Life Sciences University of Wuerzburg, 8 October 2008, Wuerzburg, Germany

4) Course on European Credit Transfer system Grundstufe 1 from the Zentrum für Sprachen und Mediendidaktik at the University of Wuerzburg, 21 June 2007, Wuerzburg, Germany

5) Workshop on "Presentation Technique" conducted by the Graduate School of Life sciences on 4-5 December 2007, Wuerzburg, Germany

6) Course on "Patent law in Life sciences" conducted by the GSLS, University of Wuerzburg on 30 June 2009, Wuerzburg, Germany

7) A seminar lecture on the "GxP- A Basic Introduction" conducted by Dr. J. Fensterle of Aeterna Zentaris GmbH, Wuerzburg, Germany

8) Leopoldina symposium on Leopoldina- Symposium on Emerging topics in Microbial pathogenesis, würzburg, Germany.

9) Deutcher Chlamydia workshop 2012 at Erfurt, Germany.

## 7.2. List of publications

1. **Karunakaran K,**Mehlitz A, Rudel T.Evolutionary conservation of infection-induced cell death inhibition among *Chlamydiales***.**PLoS One.2011 July; 6(7):e22528.

2. Sharma M, Machuy N, Böhme L**, Karunakaran K**, Mäurer AP, Meyer TF, Rudel T.HIF-1α is involved in mediating apoptosis resistance to *Chlamydia trachomatis*-infected cells.Cell Microbiol. 2011 Oct; 13(10): 1573-85.

3. Mehlitz A, V kozjak-pavlovic, Huber A, **Karunakaran K,**Krohne G, Rudel T. *Simkania negevensis* requires mitofusin-2 for replication in the endoplasmic reticulum. (Manuscript in preparation).

4. Mehlitz A, Banhart S, Thorn H, **Karunakaran K**, Rudel T, Meyer TF. *Chlamydia trachomatis* L2 Tarp regulates immune signaling and survival in epithelial cells via PLCG in a serotype-specific manner. (Manuscript in preparation).