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**Multiple Facets of Neutrophil Apoptosis Modulation by
the Intracellular Pathogens *Anaplasma phagocytophilum*
Chlamydia pneumoniae and *Leishmania major***

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I dedicate my doctoral dissertation work to my late maternal grand father
Sri Atul Krishna Dutta

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Abbreviations

AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
Apaf-1	Apoptosis protease activating factor-1
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BAD	Bcl-2 associated death promoter
Bak	Bcl-2 antagonist killer
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma 2
Bfl-1	A Bcl-2 homolog isolated from human fetal liver
Bid	BH3 interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting mediator of cell death
BH3	Bcl-2 homolog domain-3
Bok	Bcl-2 related ovarian killer
BSA	Bovine serum albumin
CARD	Caspase recruitment domains
Caspases	Cysteine-aspartic acid protease
CREB	Cyclic adenosine monophosphate response element binding
DG	Diacylglycerol
DIABLO	Direct IAP binding protein with low pI
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
DR	Death receptor
DTT	Dithiotheritol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
ERK	Extracellular growth regulated kinase
FACS	Fluorescence activated cell sorter
FADD	FAS associated death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	Formyl-methionine-leucine-phenylalanine
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSK	Glycogen synthase kinase
HGA	Human granulocytic anaplasmosis
HGE	Human granulocytic ehrlichiosis
HRP	Horseradish peroxidase
IFNγ	Interferon gamma
IκB	Inhibitor of kappa B
IL-6	Interleukin-6
IL-8	Interleukin-8
LPS	Lipopolysaccharides
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1

MSK	Mitogen and stress activated kinase
NF-κB	Nuclear factor kappa B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PC	Phosphatidyl choline
PK1	Phosphoinositide dependent kinase 1
PE	Phycoerythrin
PI3K	Phosphatidyl inositol 3 kinase
PKB	Protein kinase B
PKC	Protein kinase C
PMN	Polymorpho nuclear neutrophils
PMSF	Phenylmethanesulfonyl fluoride
PS	Phosphatidyl serine
PSGL-1	platelet selectin glycoprotein ligand 1
PUMA	p53 upregulated modulator or apoptosis
RPMI	Roswell Park Memorial Institute medium
RSK	Ribosomal S6 kinase
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SMAC	Second mitochondria derived activator of caspases
STAT	Signal transducer and activators of transcription protein
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR 1	Tumor necrosis factor receptor 1
TRAIL	TNF related apoptosis inducing ligand
TRAMP	TNF receptor related apoptosis mediating protein
Tris	Tris(hydroxymethyl)aminomethane
XIAP	X-chromosome linked inhibitor of apoptosis protein.

1 Introduction

1.1 Neutrophil granulocytes

The function of the immune system in the body is to provide proper defence mechanisms against invading pathogens. The protection starts with surface barriers which comprised of mechanical, chemical and biological barriers. Close apposition of epithelial cells in the skin provide the mechanical barrier. By the production of various enzymes and anti-bacterial peptides or other substances, the surface barrier restricts propagation of the pathogens. For instance the low pH environment of the stomach maintained by the production of gastric acid limits the multiplication of pathogens. When pathogens are successful in evading the defence of surface barriers they are encountered by innate immune system. The immune system consists of innate and adaptive immune components. Polymorphonuclear neutrophils and macrophages are the cells of the innate immune system.

Haematopoietic cells of the bone marrow are the precursor of neutrophil granulocytes which undergo various morphological stages during maturation and differentiation. In the absence of infection $1-2 \times 10^{11}$ neutrophils are produced per day. However infection leads to a 10 fold increase in the production of neutrophils (Savill et al., 1989). One of the characteristic features of neutrophil granulocytes is spontaneous apoptosis. Alterations of this tight process could cause havoc to the system. PMN are recruited at the site of infection and efficiently eliminate the pathogens with their specialised microbicidal mechanisms. The intracellular killing of the pathogens within the neutrophil occurs either by oxygen dependent mechanisms or by oxygen independent mechanisms. During oxygen independent killing, various bactericidal proteins and peptides are released from the granules which disrupt the integrity of the pathogen membrane. Oxygen dependent killing mechanism is carried out by the generation of toxic reactive oxygen species (ROS) which kill the pathogen by oxidising proteins, nucleic acids and other molecules. Inappropriate phagocytosis leads to the release of toxic materials to the surrounding environment which ultimately results in collateral tissue damage. It has been delineated that human and murine neutrophils express MHC class II antigens which suggests their potential role in antigen presentation (Culshaw et al., 2008; Sandilands et al., 2005). However, further studies are needed to define the role of neutrophils as antigen presenting cells. Neutrophils are short lived cells. They die within 10-24 hours after leaving the bone marrow. However, exposure to pro-inflammatory cytokines at the inflamed site or infection with intracellular pathogens extend the short life span of neutrophils (Aga et al., 2002; Cicco et al., 1990; Kettritz et al., 1998).

1.2 Apoptosis

Apoptosis is the outcome of a genetically controlled death programme of a cell. The term apoptosis was introduced first in 1972 with a description of particular morphological aspects of cell death (Kerr et al., 1972). However, the phenomenon of a cell death programme was first evident in 1890 when William Councilman described the vacuolated acidophilic bodies in liver tissues of yellow fever patients (Thompson, 1995). In recent years, several attempts have been made to redefine the term apoptosis. According to these views apoptosis is a type of cell death which starts with rounding up of the cell, reduction of cellular volume, condensation of chromatin, fragmentation of the nucleus, no or little change in ultra structure of internal organelles and finally the blebbing of the plasma membrane but maintaining the integrity of the membrane (Melino et al., 2005). Previously it was thought that apoptosis is only associated with activation of different cysteine-aspartic acid proteases (caspases) which cleave intracellular proteins resulting into apoptotic morphology. However, recently it has been shown that apoptosis can occur independent of caspase activation (Barbier et al., 2009; Bras et al., 2007). Indeed, 11 different types of death programmes have been reported so far (Melino et al., 2005). According to the new view of apoptosis, caspase activation has been defined as an apoptosis associated caspase activation (Kroemer et al., 2005). In fact both caspase independent and dependent apoptosis programmes generate similar morphologic appearances like phosphatidyl serine flippage at the outer leaflet of the plasma membrane or condensation of the chromatin and fragmentation of the DNA. However, these two apoptotic programmes differ only at the point of enzyme activation. During caspase independent apoptosis, instead of caspases, calpains are activated (Melino et al., 2005).

With the progression of apoptosis, the nuclear envelope disassembles, chromatin condenses and the DNA breaks up into nucleosomal fragments with a length of 180 base pairs. Ca^{2+} and Mg^{2+} dependent endonucleases were suggested for this intranucleosomal cleavage (Wyllie, 1980).

Besides the change of the chromatin and mitochondrial membrane potential, the cell membrane also starts to bleb which generates small apoptotic bodies containing morphologically intact organelles. These bodies are called apoptotic blebs. In the late phase of apoptosis, the blebs are ingested by phagocytes. Asymmetric distribution of phospholipids is observed in plasma membrane where choline containing lipids, phosphatidyl choline (PC) and sphingomyelin are concentrated on outer leaflet whereas aminophospholipids, phosphatidyl serine (PS) and phosphatidyl ethanolamines (PE) are present in abundance on the inner leaflets. Loss of phospholipids asymmetry and appearance of PS in the outer leaflet of the

plasma membrane were found in connection with apoptosis studies (Fadok et al., 1992; Mower et al., 1994; Schlegel et al., 1993). The presence of PS in the outer leaflet of plasma membrane is considered as an early marker for apoptosis (Vermes et al., 1995; Zhang et al., 1997). Flipping of PS molecules from the inner leaflet of the plasma membrane to the outer leaflet occurs due to the decreased function of ATP-dependent amino phospholipid translocase and calcium dependent activity of non specific lipid scrambles (Verhoven et al., 1995).

The programme for apoptosis is genetically regulated. In contrast the necrotic type of death is not genetically controlled but rather accidental. Necrotic death is characterised with swelling of the cell, digestion of chromatin, disruption of the plasma membrane and the membranes of the organelles, which leads to the release of toxic materials in the surrounding milieu. The necrotic death is detrimental to the body since it leads to inflammation and tissue damage. Death programme for apoptosis is a naturally selected process in the course of evolution which is beneficial not only for maintaining the homeostasis but also to restrict pathogen propagation by eliminating infected cells from the body.

1.2.1 Types of apoptosis

During apoptosis, cells undergo various complex biochemical programmes. There are two distinct pathways by which apoptosis can be initiated. These two signalling pathways are called extrinsic or death receptor mediated apoptosis and intrinsic or mitochondrial apoptosis. These two pathways mainly converge in the activation of intracellular caspases which cleaves the intracellular proteins and leads to apoptosis.

1.2.1.1 Extrinsic or death receptor mediated apoptosis

The death receptor mediated apoptosis initiates at the plasma membrane after ligation of pro-apoptotic ligands with specific receptors. For instance, ligation of TRAIL/Apo2L with the death receptors DR4 or DR5 or ligation of CD95/FasL with the FAS/CD95 receptor triggers apoptosis. Death receptors belong to the tumour necrosis factor (TNF) receptor super family which includes FAS (CD95 or Apo-1), TNF-receptor (TNF-R1), death receptor -3 (DR3 or TNF receptor related apoptosis mediating protein (TRAMP) or Apo-3), TNF related apoptosis induction ligand receptor-1 (TRAIL-R1 or DR4), TRAIL-R2 (DR5 or Apo-2), and DR6 (Pitti et al., 1996; Smith et al., 1994). Among these different receptors the best characterized death receptor is FAS or CD95. FAS receptor contains a cytoplasmic death domain (DD) which upon interacting with another adaptor protein, FAS associated death domain (FADD), forms

the death receptor induced signalling complex (DISC) (Boldin et al., 1995; Chinnaiyan et al., 1995). Additionally, FADD contains a death effector domain (DED) which recruits procaspase-8 bound with DED into the DISC. Upon ligation of FAS with the FAS receptor procaspase-8 become proteolytically cleaved and forms the active caspase-8 which then acts on downstream caspases and leads to the apoptosis (French and Tschopp, 2003).

1.2.1.2 Intrinsic pathway of apoptosis

The intrinsic pathway of apoptosis is also called the mitochondrial apoptosis pathway. Loss of mitochondrial membrane potential has been characterised as one of the important sub routine in the cell death programme. Whenever the membrane potential is lost, cytochrome C is released into the cytoplasm from the intermembrane space of mitochondria. This initiates the subsequent down-stream signalling. Reports suggest that mitochondrial permeability transition pore (PTP) is formed at the contact sites between inner and outer membranes (Garrido et al., 2006). In addition to cytochrome C, other proteins like AIF, Smac/DIABLO, endonuclease G and HtrA2/Omi are also released from mitochondria which augment the apoptotic process. Released cytochrome C oligomerizes with apoptotic protease activating factor-1 (Apaf-1) and forms the apoptosome which recruits the seven dimers of caspase-9. Caspase-9 is catalytically processed and subsequently activates caspase-3 which finally leads to apoptosis (Acehan et al., 2002). Upon activation along with cytochrome C both AIF and endonuclease are released from the mitochondria and translocate to the nucleus. This triggers the fragmentation of DNA and condensation of chromatin and ultimately leads to apoptosis (Suzuki et al., 2001).

The central players of the mitochondrial pathway of apoptosis are the Bcl-2 family proteins which were discovered from B cell lymphoma 2. The family comprises both, pro-survival and pro-apoptotic proteins. Mammalian cells contain five distinct types of pro-survival proteins namely Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1 and the function of these proteins antagonize by the pro-apoptotic proteins Bak and Bax. The proapoptotic Bcl-2 member Bax translocates to the mitochondria, oligomerizes, binds to Bak and thereby forms the mitochondrial apoptosis induced channel (MAC). The formation of MAC is facilitated by Bid, and this leads to release of cytochrome C in the cytosol (Dejean et al., 2006).

A member of the anti-apoptotic group of the Bcl-2 family is Mcl-1 which was cloned from myeloid cell line ML-1. This protein has very short half life subjected to proteasomal degradation. Stabilization of Mcl-1 prevents the apoptosis simply by preventing the cytochrome C release by a complex way of regulating the activation of the cascade. This

transient protein is tightly regulated at multiple levels during transcription. Besides the transcriptional control, Mcl-1 has been found to be regulated at translational as well as post translational level. The protein contains multiple sites for phosphorylation. Differential phosphorylation at Thr⁹² as well as Thr¹⁶³ by (ERK1/2) extends the half life while the phosphorylation by GSK3 β at Ser¹⁵⁹ accelerates the turnover rate. Phosphorylation of RSK and Akt inhibits the GSK3 β phosphorylation. Release of cytochrome C is the central step in intrinsic pathway of apoptosis regulated by Bcl-2 protein family. Pro-apoptotic factor Bax and Bak has been reported to be responsible for formation of pore on the mitochondrial membrane but in survival conditions the anti-apoptotic proteins of the Bcl-2 family do not allow to activate Bax as well as Bak. During survival condition, Mcl-1 functions as anti-apoptotic protein by sequestering the pro-apoptotic protein Bak from the mitochondrial membrane and preventing the oligomerization. But upon receiving the apoptotic signal, BH3 only proteins displace the Mcl-1 from Bak which leads to oligomerize Bak on the outer membrane of mitochondria, forms the channel and thereby release the cytochrome C. Mcl-1 also prevents the oligomerization of Bax by heterodimerizing with activator BH3 only proteins which includes tBid, PUMA, and Bim. Whenever the cell receives apoptotic signal, Mcl-1 is displaced from the activator protein groups by the protein NOXA. Activator proteins then allow the Bax to insert into the outer mitochondrial membrane, oligomerize and form pore which finally leads to cytochrome C release (Fig. 1) (Clohessy et al., 2006; Willis et al., 2005).

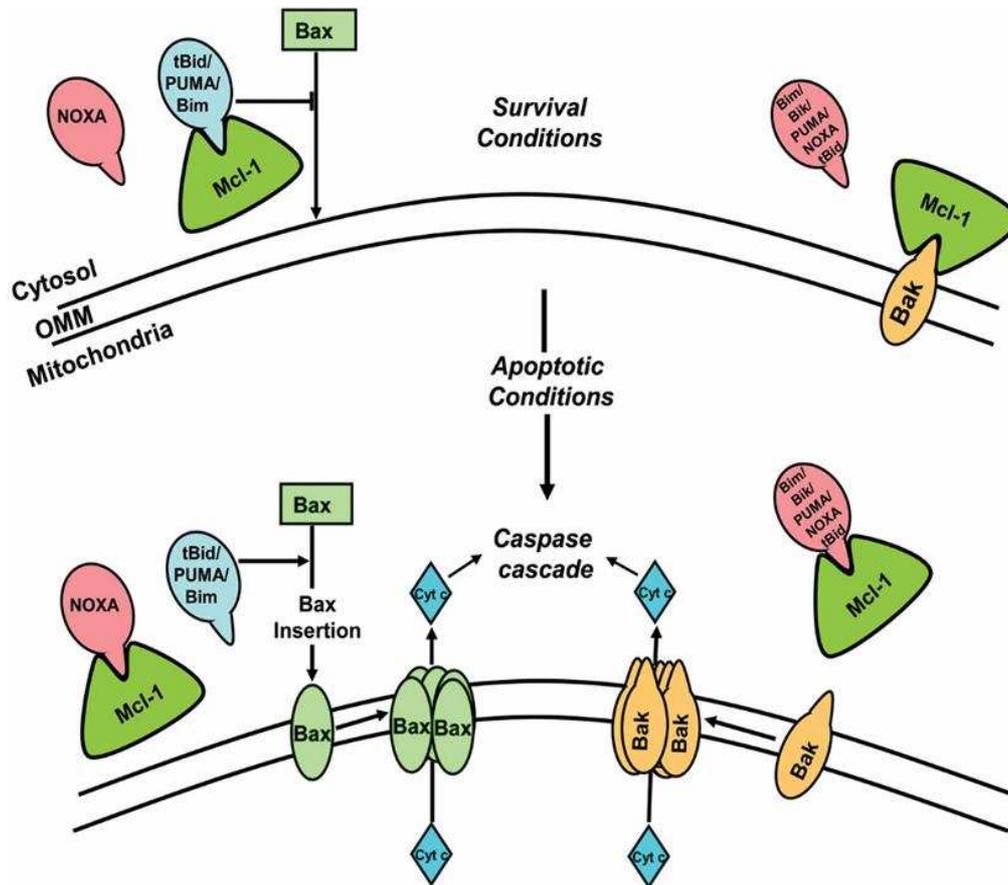


Fig. 1 Mcl-1 regulates the cytochrome C release. Mcl-1 plays two main roles in the cellular apoptosis machinery. Mcl-1 can function as an antiapoptotic factor by remaining bound with Bak on the outer mitochondrial membrane (OMM). However, when apoptotic signals are received, specific BH3-only proteins can displace Mcl-1 from Bak leading to Bak oligomerization and cytochrome c release from mitochondria. Mcl-1 can also display its pro-survival functions by heterodimerizing with activator BH3-only proteins including tBid, PUMA and Bim. In apoptotic conditions, NOXA displaces Mcl-1 from these activator binding partners. Then, Bim, PUMA and tBid can interact with Bax causing its insertion into outer mitochondrial membrane, oligomerization and cytochrome C release (adapted from Akgul C, 2008).

A common link between two pathways of execution of apoptosis is activation of downstream caspases. Signalling via FAS activates the caspase-8 which in turn activates the downstream effector caspases by cleaving caspase-3 (Stennicke et al., 1998). Another molecular link between the death receptor and the mitochondrial pathway is cleavage of cytosolic Bid, a member of BH3 only domain containing subgroup of Bcl-2 family, by caspase-8 (Li et al., 1998; Luo et al., 1998). Whenever cytochrome C is released, the electron transport chain of the mitochondria is disrupted which results into the drop in ATP production. In the presence of cytochrome C in the cytosol, caspase recruitment domain (CARD) of Apaf-1 binds with the CARD domain of caspase-9 which forms the complex apoptosome. The apoptosome associated caspase-9 activates downstream caspases resulting in apoptosis (Li et al., 1997). A variety of growth factors like IL-8, IL-6, GM-CSF and G-CSF delays the neutrophil apoptosis by various signalling mechanisms.

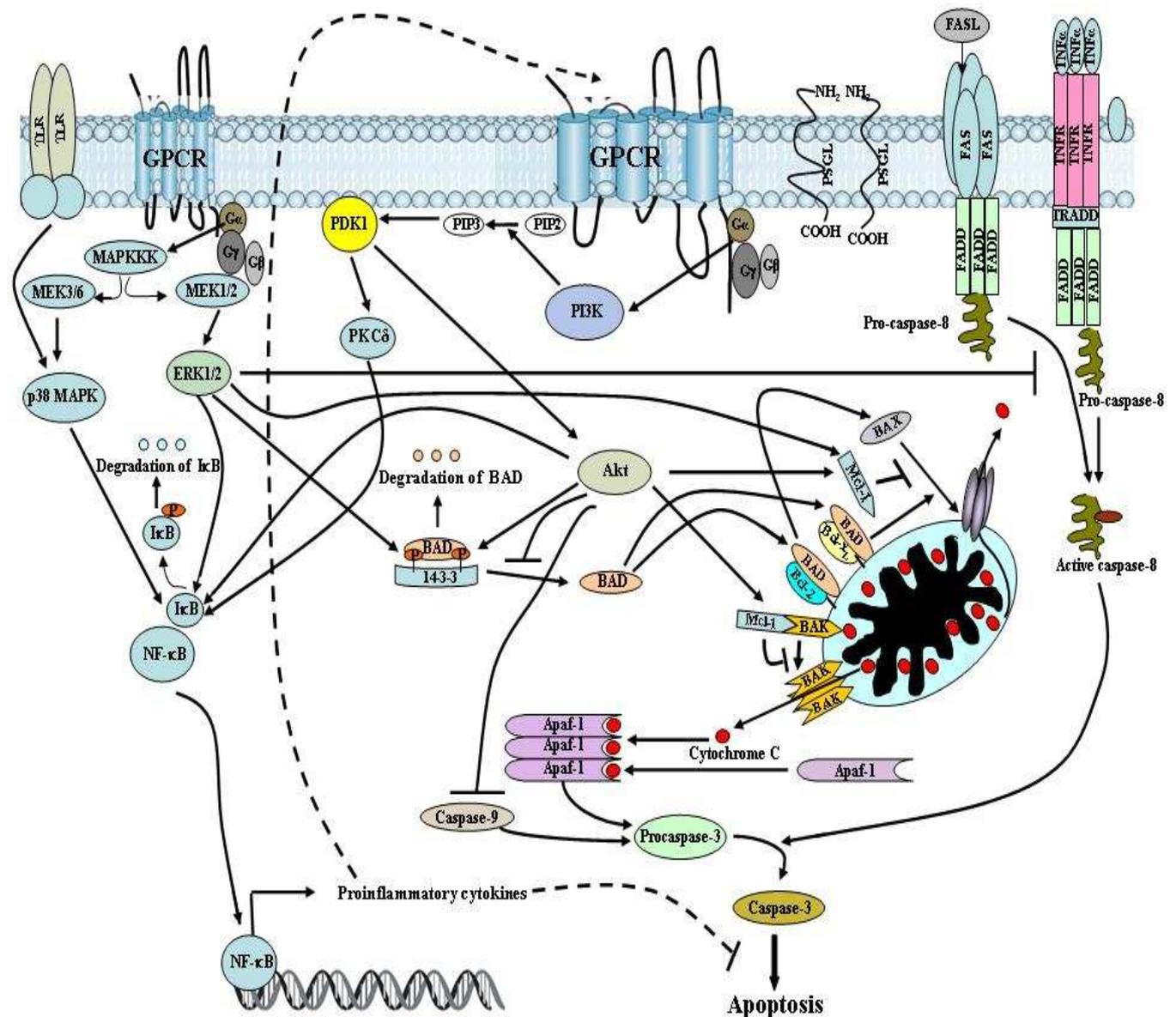


Fig. 2 Partial overview of the two main apoptosis execution pathways and survival signalling pathways. The death receptor pathway is triggered by members of the death receptor super family. Binding of CD95 or TNF α leads to the formation of death inducing signalling complex (DISC). The complex recruited via fas associated death domain (FADD) and multiple procaspase-8 results into caspase-8 activation. The mitochondrial pathway is regulated by Bcl-2 family members. Both pro and anti-apoptotic members of the Bcl-2 family act at the surface of the mitochondria and regulate the release of cytochrome C into the cytosol. Released cytochrome C binds Apaf-1 which then associates with procaspase-9 and forms the apoptosome complex. The apoptosome activates the downstream caspase-3 which finally leads to the apoptosis. Both of the pathways converge at the level of caspase-3. PI3K/Akt and MAPK are the two major survival signals which regulate the apoptotic process by regulating various members of apoptotic cascade. The survival signals also trigger the release of various proinflammatory cytokines via activating NF- κ B which can prevent the apoptosis.

1.3 Survival signalling pathways

Apoptosis is an intricate death programme executed either by intrinsic or extrinsic pathways after receiving the signal for apoptosis thereby maintains the homeostasis. Survival signals, on the other hand, perturb the pathways and inhibit the apoptosis cascade. Signalling via kinases

like phosphatidyl inositol 3 kinase (PI3K), mitogen activated protein kinase (MAPK), inhibit the apoptosis by various mechanisms. Recently, it has been reported that deactivation of Akt is correlated with neutrophil apoptosis (Zhu et al., 2006). This observation shows that signalling via PI3K/Akt is important in delaying the apoptosis. Activation of PI3K/Akt has been shown to inhibit the apoptosis in a variety of cancers cells (Dubrovskaya et al., 2009; Lee et al., 2008; Naughton et al., 2009). Apoptosis was delayed via MAPK pathway in neutrophils exposed to LPS (Sheth et al., 2001). Activation of p38 MAPK has been shown to delay apoptosis in neutrophils whenever infected with an intracellular pathogen (Choi et al., 2005). Another important survival pathway is nuclear factor kappa B (NF- κ B). Activation of NF- κ B controls the transcription of many survival genes of anti-apoptotic members of the Bcl-2 family which in turn regulate the release of the cytochrome C and apoptosis. Various growth factors and cytokines have also been shown to inhibit the apoptosis in different cell types including neutrophils (Cowburn et al., 2002; Dunican et al., 2000; Kettritz et al., 1998; Lindemans et al., 2006). All these factors modulate the apoptotic programme of the cell at various steps regulating the survival pathways showing that survival pathways are upstream of the intrinsic apoptotic cascade and signalling via these pathways indeed dictates the cell's fate. These survival pathways can be divided into two groups namely, the PI3K/Akt pathway and the MAPK pathway. Both of the pathways can regulate the NF- κ B signalling which also regulates the apoptosis.

1.3.1 PI3K/Akt pathway

Signalling via PI3K/Akt is one of the crucial pathways which controls a variety of cellular functions including apoptosis. PI3K has several isoforms. Originally PI3K enzymes were defined on the basis of their ability of phosphorylating different phosphoinositide substrates at 3 position of the inositol ring (Cantley, 2002). Depending on the structural and functional homologies, these enzymes are grouped into three types I, II and III. All these forms have structural homology at their lipid kinase domains at the catalytic subunit but they differ at their regulatory domains. Binding of various stimuli like growth factors and hormones to cell surface receptors activates PI3K which then phosphorylates phosphatidyl inositol bis phosphate (PtdIns(4,5)P₂) and synthesizes phosphatidyl inositol tri phosphate (PtdIns(3,4,5)P₃). PtdIns(3,4,5)P₃ has been shown to inhibit apoptosis in various cell types (Gagnon et al., 2001; Oganessian et al., 2003). Generation of PtdIns(3,4,5)P₃ recruits the protein kinase B (PKB) to the membrane which is considered to be the major target of PI3K. Phosphorylation and activation of PKB depends upon the binding of PtdIns(3,4,5)P₃ to the pH

sensitive domain of the PKB. Both PtdIns(3,4,5)P₃ and PtdIns(4,5)P₂ create a highly complex signalling cascade downstream of PI3K. PI3K regulates proliferation, growth and survival mediated by PKB, the downstream molecule of PI3K (Hawkins et al., 2006).

Protein kinase B (PKB) or Akt is a serine/threonine protein kinase which is considered to be the central regulator of cell growth, survival and proliferation. Three different isoforms have been reported till date (Song et al., 2005). Structural studies revealed that PKB/Akt is comprised of 3 different domains a conserved domain with a specific pH domain, the central kinase domain and the carboxy-terminal regulatory domain. The carboxy domain is responsible for the interaction with other signalling molecules whereas the pH domain interacts with PtdIns(3,4,5)P₃ and becomes activated. Akt was originally cloned as an oncogene in the transforming murine leukemia virus (Staal et al., 1977). The human homologue of akt oncogene, Akt1 and Akt2, were cloned in the primary human gastric adenocarcinoma indicated the importance of akt in the development of human malignancies (Staal, 1987). Activation of Akt depends on the phosphorylation of the kinase. Akt has two phosphorylation sites; serine 473 and threonine 308. Full activation of Akt depends on the phosphorylation of both molecules but phosphorylation of Ser⁴⁷³ depends on phosphorylation of Thr³⁰⁸. Phospho-inositide dependent kinase (PDK1), another serine/threonine dependent kinase, has been shown to regulate the phosphorylation of Akt at Thr³⁰⁸. PDK1 also contains a pH domains similar to Akt. Phosphorylation of PI3K generates PtdIns(3,4,5)P₃ which then binds to the pH domain of PDK1 as well as PKB resulting in the phosphorylation and activation of PKB. Activated PKB, thereby, regulates multiple functions of the cells (Song et al., 2005).

PKB has been shown to regulate the apoptosis either by directly controlling members of the apoptotic cascade for instance BAD, caspase-9 or by controlling the transcription factors responsible for pro-anti apoptotic genes or by regulating the metabolism steps of the cells. In the presence of cytochrome C in the cytosol, Apaf-1 binds the CARD domain of caspase-9 and forms the apoptosome. Upon activation, Akt phosphorylates caspase-9 at serine residue 196 (Ser¹⁹⁶) and attenuates its activity (Cardone et al., 1998). BAD has dual role in the apoptosis. Bound to other members of BH3 only protein family it promotes the activation of Bax and thereby allows Bax and Bak to translocate to the mitochondria leading to the release of cytochrome C which results into apoptosis. However, phosphorylation at serine residue (Ser¹³⁶) by Akt releases from its Bcl-2 bound protein group and remains bound with 14-3-3 adaptor protein in the cytosol and thus prevents apoptosis (Datta et al., 1997).

The calcium independent but PS/DG dependent novel protein kinase C delta (PKC δ) has been shown to regulate apoptosis. PKC is a translocable protein and the translocation is considered as the activation of the protein. In non-activated state PKC δ remains at the cytosol. Upon activation it translocates from the cytosol to the plasma membrane. Recent finding showed activation of PKC δ prevented apoptosis in IFN β treated neutrophils (Wang et al., 2003). Another recent report shows that PKC δ can be activated in TNF α treated neutrophils via PI3K pathway which prevents the neutrophils apoptosis (Kilpatrick et al., 2006).

1.3.2 MAPK pathway

Besides the PI3K/Akt pathway, the MAPK pathway is considered to be the second route in terms of cell survival signalling. Five different families of MAPK have been reported in mammalian cells so far. These are the extracellular signal regulated kinase (ERK1 and ERK2), JUN-N-terminal kinase (JNK1, JNK2, JNK3), the p38 MAPK family (p38 α , p38 β , p38 γ , p38 δ), ERK3/ERK4 and ERK5 (Qi and Elion, 2005). Among the above mentioned members of the MAPK family ERK1/ERK2 and p38 MAPK were found to inhibit the apoptosis (Choi et al., 2005; Wang et al., 2007a; Zhang et al., 2003b). While signalling via JNK induces apoptosis (Kepp et al., 2009), ERK1/2 promotes the cell survival by regulating the Bcl-2 family proteins. BIM, a BH3 only protein, expressed in connection with apoptotic signal like cytokine withdrawal. BIM binds to the pro-survival protein Mcl-1 and thereby it releases the BAX and BAK which then insert into the outer membrane of the mitochondria, oligomerize, form the MAC and ultimately lead to release of cytochrome C. Expression of BIM is dependent on another pro-apoptotic molecule FOXO3A (Fu and Tindall, 2008). ERK1/2 prevents the FOXO3A dependent expression of BIM by phosphorylating FOXO3A which is then destined to proteasomal degradation. BIM is expressed in three splice variants; short (BIM_S), long (BIM_L) and extra long (BIM_{EL}) among which BIM_{EL} is the most abundant. ERK1/2 phosphorylates the BIM_{EL} which leads to dissociation of BIM_{EL} from its pro-survival protein, Mcl-1 and subsequently, targets for degradation.

Another BH3 only protein of Bcl-2 family, BAD, promotes cell death upon binding with Bcl-x_L which alters the membrane potential of mitochondria leads to the release of cytochrome C and finally results in apoptosis. However, upon receiving survival signals BAD becomes phosphorylated and released from Bcl-x_L and subsequently targeted for poly-ubiquitination and proteasomal degradation. BAD has three phosphorylation sites, Ser¹¹², Ser¹³⁶, Ser¹⁵⁵. Akt has been shown to phosphorylate BAD at Ser¹³⁶ residue. The event triggers the release of BAD from the Bcl-x_L and facilitates the binding to 14-3-3 adaptor protein in the cytosol (Datta et

al., 1997). Ribosomal protein S6 kinase (RSK) and mitogen and stress activated kinase 1 (MSK1), the substrates of ERK1/2 and p38 MAPK, phosphorylate BAD at Ser¹¹² thus enhances the sequestration of BAD by 14-3-3 protein. Phosphorylation on its third residue is facilitated by the dual phosphorylation of Ser¹¹² and Ser¹³⁶ and this is catalysed by another kinase, cAMP dependent protein kinase A (PKA) (Bonni et al., 1999). Inhibition of ERK1/2 with MEK inhibitor showed increased in BIM and BAD levels in cancer cells which results into apoptosis of the cells (Wang et al., 2007b).

Mcl-1, an anti-apoptotic member of the Bcl-2 family, plays a crucial role in regulating apoptosis. It prevents the MAC formation on the mitochondrial membrane by preventing the translocation and oligomerization of BAX and BAK and thus inhibits apoptosis. This protein contains a PEST sequence which is present in other proteins which are destined to degradation. Indeed, Mcl-1 has a very short half life, undergoes proteasomal degradation after phosphorylation and subsequent poly-ubiquitination. Mcl-1 has two phosphorylation sites, Ser¹⁵⁹ and Thr¹⁶³. It has been reported that ERK1/2 stabilizes the life span of Mcl-1 by directly phosphorylating it at Thr¹⁶³. However, some reports claim that the phosphorylation at Thr¹⁶³ facilitates the phosphorylation of the second residue Ser¹⁵⁹ which is catalyzed by GSK3 β , but activation of GSK3 β is regulated by the RSK, the direct substrate of ERK1/2, as well as by PKB (Balmanno and Cook, 2009). Therefore, ERK1/2 regulates the intrinsic pathway of apoptosis at various steps. Among the members of the MAPK kinase family, p38 MAPK also plays an important role in regulating apoptosis. It has been shown that p38 MAPK could phosphorylate the pro-apoptotic BAD at Ser¹¹² residue which allows BAD to be sequestered into the cytoplasm by the adaptor protein 14-3-3 and prevents the apoptosis by maintaining membrane potential of mitochondria (She et al., 2002). Caspases are the executors of apoptosis for both intrinsic and extrinsic pathway. Activation of p38 MAPK has been shown to regulate the activity of caspase-8 as well as of caspase-3 (Alvarado-Kristensson et al., 2004). It has also been delineated that p38 MAPK works co-operatively with ERK1/2 and regulates the growth factor induced proliferation of haematopoietic cells (Rausch and Marshall, 1999). Lipopolysaccharide (LPS) treatment has been found to extend the life span of neutrophils and activates the p38 MAPK (Nick et al., 1996). LPS is recognised by the pattern recognition receptor TLR4 and activates NF- κ B in various cell types including neutrophils (Choi et al., 2003; Kim et al., 2006; Lin et al., 2007). The pro-survival function of p38 MAPK is related to the activation of NF- κ B. Activation of p38 MAPK works in association with ERK1/2 to control the phosphorylation and subsequent sequestration of BAD from its Bcl-x_L group.

1.3.3 NF- κ B as a survival signal

Members of the NF- κ B transcription factor family contain specific DNA binding sequences, known as κ B elements, by which they bind to DNA and thereby induce or repress transcription of several genes. In mammalian cells, the NF- κ B family is comprised of 5 members, RelA, RelB, c-Rel, p⁵⁰/p¹⁰⁵ (NF- κ B1) and p⁵²/p¹⁰⁰ (NF- κ B2). In the cytoplasm NF- κ B remains inactivated by the association to the inhibitor of nuclear factor κ B (I κ Bs) (Hayden and Ghosh, 2004). The activation of NF- κ B is carried out in sequential steps by I κ B kinase, the IKK complex, which phosphorylates I κ B, subsequently degrades I κ B by poly ubiquitination. This leads to the release of bound NF- κ B from the complex and facilitates its nuclear translocation where it regulates the transcription of several genes including the genes that controls apoptosis. Activation of NF- κ B has been shown to be regulated by the PI3K/Akt pathway in fibroblast cells (Choi et al., 2004; Cowburn et al., 2004; Romashkova and Makarov, 1999). It has been noticed that the use of specific inhibitors of ERK1/2 as well as of p38 MAPK blocked the activation of NF- κ B suggesting the role of ERK1/2 and p38 MAPK in the regulation of NF- κ B (Kim et al., 2008). The IKK kinase complex, which directly regulates NF- κ B, contains two homologous kinase subunits IKK α and IKK β and the regulatory subunit IKK γ . I κ B proteins are comprised of three functional groups namely I κ B α , I κ B β , I κ B γ . After stimulation by various factors including pro-inflammatory cytokines, they undergo proteasomal degradation by the action of IKK kinase complex. Activation of NF- κ B is associated with the induction of pro-inflammatory cytokines, chemokines, adhesion molecules, and various stress response genes. NF- κ B functions as a important regulator of apoptosis controlling members of the Bcl-2 family (Kim et al., 2008; Riedemann et al., 2004; Stehlik et al., 1998; Takase et al., 2008).

Infection induced inflammation is associated with recruitment of leucocytes which are responsible for destroying the invading pathogens. Most of the infiltrate cells are mostly neutrophils in the acute phase of infection. It has been found that the life span of neutrophils is extended at the sites of inflammation because of the presence of pro-inflammatory cytokines. Release of several pro-inflammatory cytokines has been found to regulate the life span of neutrophils by activation of NF- κ B.

1.3.4 Interleukin-8

The CXC chemokine, interleukin-8 (IL-8), released by a variety of cell types including neutrophils, was first purified from the culture supernatants of lipopolysaccharide stimulated mononuclear cells and described as a neutrophil chemotactic factor (Matsushima et al., 1988;

Yoshimura et al., 1987). Like other chemokines, IL-8 binds to G protein coupled receptors (GPCR). Among all four chemokine subgroups namely CXC, CC, CX₃C and C chemokines, IL-8 is classified in the CXC subgroup. Based upon the systematic nomenclature, IL-8 is also called CXCL8. Mature IL-8 contains 72-77 residues of amino acids. Several cell types including both leukocytes (monocytes, T cells, NK-cells and the neutrophils) and non leukocytes (fibroblasts, epithelial cells) can produce IL-8. In neutrophils preformed IL-8 is rapidly released upon stimulation. The release of IL-8 can be induced by pro-inflammatory cytokines, intracellular infections, bacterial products (LPS) etc.

Induction of IL-8 is associated with NF- κ B activation. Treatment with TNF α induces IL-8 from neutrophils via NF- κ B activation (Cowburn et al., 2004).

IL-8 has been observed to bind to two distinct receptors namely CXCR1 and CXCR2. Upon binding it activates the receptor coupled G α_i proteins which then activate PI3K which in turn generate PIP3 from phospholipase C β and activates Akt. Besides the activation of PI3K/Akt pathway, IL-8 has been shown to activate MAPK as well as ERK1/2 in neutrophils (Knall et al., 1996). IL-8 drives the transmigration of neutrophils into the tissues. In addition it activates various functions of neutrophils like degranulation and mounting oxidative burst. Since neutrophils produce IL-8, IL-8 acts as a feed back loop to attract new neutrophils to the site of infection. IL-8 has been found to delay neutrophils apoptosis (Kettritz et al., 1998).

1.3.5 Interleukin-6

Interleukin-6 (IL-6) is a cytokine which has both anti-inflammatory and pro-inflammatory properties. This cytokine was discovered in 1986 as a B cell stimulating factor (BSF) later on named IL-6. Since IL-6 has a wide range of functions, so initially it was also called with different names according to the research interests of the laboratories. For instance IL-6 has a strong stimulatory effect on growth of murine plasmacytoma and human myeloma. That is why it was referred as hybridoma plasmacytoma growth factor and hepatocyte stimulating factor (Kishimoto, 2006). IL-6 is a pleiotropic cytokine which can regulate the antigen specific immune responses and inflammatory responses as well. IL-6 regulates the proliferation and development of thymic T cells. Synergistically acting with IL-3, it controls the formation of blast T cell colonies and differentiation of macrophages and megakaryocytes. For a long time it was not certain whether IL-6 is released by the neutrophils but several reports clearly show that neutrophils also release IL-6 (Cicco et al., 1990; Lindemans et al., 2006). Glycoprotein 130 (gp130) is the central player which serves as a plasma membrane receptor of IL-6. There are two types of receptors involved in the recognition of IL-6. These

are non-signalling α receptors (IL-6R α , IL-11R α etc) and signal transducing receptors (gp130, oncostatin M receptor (OSMR) and leukemia inhibitory factor (LIF)). Upon binding to signal transducing gp130 receptor IL-6 activates the JAK-STAT pathway. Apart from JAK-STAT signalling it also activates the MAPK pathway. This pro-inflammatory cytokine has been found to prevent apoptosis in the late stage of prostate cancer by stabilizing Mcl-1 (Cavarretta et al., 2008). Exposure to IL-6 also delays spontaneous apoptosis of neutrophils (Kuo et al., 2001; Lindemans et al., 2006).

1.4 Modulation of constitutive apoptosis of neutrophils

Transmigration of leukocytes to the inflammatory site is a characteristic defence mechanism in order to limit the propagation of pathogens. Among the early infiltrating leukocytes neutrophils predominate. Acute inflammatory responses are resolved by progressive decrease of recruiting cells, followed by the removal of apoptotic infiltrate cells by macrophages (Witko-Sarsat et al., 2000). The hallmark of neutrophil biology is its spontaneous apoptosis within 10-24 hours after leaving the bone marrow. Their life span however, can be extended in the inflamed tissues or upon infection with pathogens. Neutrophils have a typical nuclear morphology with multiple lobes joined with interlobular connections. Apoptotic neutrophils represent a variety of morphological characteristics like cell shrinkage, condensation of chromatin and loss of interlobular connections (Cowburn et al., 2002; Moulding et al., 1998; Savill et al., 1993). Rapid randomization of asymmetric distribution of phosphatidyl serine (PS) is a ubiquitous feature of apoptosis in all cell types where PS flips out from the inner leaflet of plasma membrane to the outer leaflet. Apoptotic neutrophils also have PS exposed on their surface (Homburg et al., 1995). Among several molecules used for the recognition of apoptotic cells by the phagocytes, PS holds the strong. Externalisation of PS not only leads to the recognition of apoptotic cells by phagocytes but also provides a convenient method for detecting apoptotic cells even at early phase of apoptosis by using fluorescent labelled annexin-V which specifically binds to PS (Vermes et al., 1995). Apart from the externalisation of PS, downregulation of immunoglobulin super family receptors like CD31, CD50, CD66a, CD66b, CD66c, CD66d, CD66e, CD63, CD87 and other cell surface receptors (e.g. CD15, CD16, CD32, CD35) was observed during neutrophil apoptosis (Dransfield et al., 1994; Homburg et al., 1995). It has been observed that neutrophil apoptosis is delayed at the inflammatory foci. For instance it has been reported that the inflammatory cytokines IL-1, IL-2, IFN γ , G-CSF and GM-CSF prolong the life span of neutrophils (Akgul et al., 2001). IL-8, the prototype of the CXC chemokine family, prevents apoptosis of neutrophil granulocytes

induced by FAS and TNF-receptor mediated pathway (Kettritz et al., 1998; Leuenroth et al., 1998). TNF performs a dual role in regulating neutrophil apoptosis. Treatment with TNF revealed a biphasic effect. At early hours of incubation (4-6 hours) it behaves as a pro-apoptotic while at late hours (24 hours) it inhibits the apoptosis (Dunican et al., 2000). Some recent reports show that TNF α mediated neutrophil survival is mediated by the PI3K/Akt, p38 MAPK, as well as ERK1/2 pathways, which involve the production of IL-8 (Cowburn et al., 2004; Kilpatrick et al., 2006). IL-6 has been found to prolong the neutrophil life span via the PI3K-NF- κ B pathway where the pro-survival Mcl-1 is stabilized and thus prevents the subsequent steps of apoptosis (Lindemans et al., 2006). Several reports show that GM-CSF delays the life span of neutrophils via the PI3K/Akt pathway.

LPS, one of the important components of outer membrane of Gram-negative bacteria, also suppresses the neutrophil constitutive apoptosis partially via the PI3K/Akt and ERK1/2 pathways (Klein et al., 2001). A number of intracellular pathogens delay the spontaneous apoptosis of granulocytes. It is believed that intracellular pathogens adopt this strategy in order to fit better in the intracellular niche. Respiratory syncytial virus inhibits apoptosis by activating PI3K/Akt and NF- κ B pathways. *Anaplasma phagocytophilum*, *Leishmania major*, and *Chlamydia pneumoniae* inhibit the spontaneous apoptosis of neutrophils (Aga et al., 2002; Choi et al., 2005; van Zandbergen et al., 2004). All these reports indicate that perturbation of neutrophil constitutive apoptosis is dependent on various survival signals which are modulated by the interaction of the pathogens or with their host neutrophils.

1.5 Intracellular pathogens infecting neutrophils

Phagocytes protect the body by ingesting and killing the harmful pathogens by their efficient defence mechanisms. However, in the course of evolution a number of pathogens have developed fine strategies by which they manipulate the host defence system and survive even inside phagocytes. The Gram negative bacteria *Anaplasma phagocytophilum* and *Chlamydia pneumoniae* and the protozoan parasite *Leishmania major* are three important intracellular pathogens that survive in neutrophil granulocytes.

1.5.1 *Anaplasma phagocytophilum*

Anaplasma phagocytophilum is the causative agent of human granulocytic anaplasmosis (HGA). The disease was formally called as human granulocytic ehrlichiosis (HGE). The pathogen belongs to the family *Anaplasmataceae* which are members of Gram negative obligate intracellular bacteria. The first report of human infections with an *Anaplasma* species

was evident in 1990 (Dumler et al., 2007). HGA is a tick borne disease. *Anaplasma phagocytophilum* varies in size from 0.2 μm -2 μm . The pathogen completes its life cycle in tick vector and mammalian hosts. Members of the the *Ixodes* genus, *Ixodes scapularis* and *Ixodes pacificus* serve as a tick vector. Infected ticks do not pass the infection to their offsprings and pathogens are transmitted by the bite infected ticks to the mammalian reservoirs thus complete cycles (Nelson et al., 2008). The white footed mouse (*Peromyscus leucopus*) and dusky footed wood rat (*Neotoma fuscipes*) are the mammalian hosts for *A. phagocytophilum* in some parts of the United States. Previously it was thought that human infections are sporadic. However, with the advancement of diagnostic techniques, more and more HGA cases were encountered. The bacterium has established unique tropism for neutrophil granulocytes. It replicates within the parasitophorous vacuole of the host cell and forms a microcolony called morula (Chen et al., 1994). Morulae are round shaped inclusions with 2-7 μm in diameter which can be detected by Romanowsky staining. The pathogen has developed various strategies by which it circumvents and directly inactivates the anti-bacterial mechanisms.

Infection with *A. phagocytophilum* delays neutrophil apoptosis (Choi et al., 2005; Ge and Rikihisa, 2006). However, the molecular mechanism of apoptosis delay is poorly understood. It has been suggested that binding of bacterial proteins to host cell transglutaminase leads to the delay of apoptosis (Yoshiie et al., 2000). Some other reports show that infection with *A. phagocytophilum* alters both of the intrinsic and extrinsic pathways to inhibit apoptosis (Ge and Rikihisa, 2006; Ge et al., 2005). It has also been stated that p38 MAPK plays an important role in delaying apoptosis of neutrophils at the early phase of infection (Choi et al., 2005). In spite of the fact that numerous studies dealt with the *A. phagocytophilum* induced apoptosis delay in neutrophils, little attempts have been made to explore the potential role of survival signals which seem to be important in neutrophil apoptosis (Zhu et al., 2006). A gene expression study shows that the infection with *A. phagocytophilum* induces several anti-apoptotic genes in neutrophils including molecules involved in survival signalling pathways (Lee and Goodman, 2006).

1.5.2 *Chlamydia pneumoniae*

Chlamydia pneumoniae is an obligate intracellular Gram negative bacterium which causes respiratory tract infections like acute and chronic bronchitis, asthma, community acquired pneumonia (CAP). It has also been reported that infection with *C. pneumoniae* can contribute to atherosclerosis and central nervous system disorders (Blasi et al., 2009). *Chlamydia*

pneumoniae was first discovered in 1989 (Persson and Treharne, 1989). The bacterium completes its life cycle in two developmental forms, infective elementary bodies (EBs) and reproductive reticulate bodies (RBs). The bacterium has a unique replication cycle. It multiplies within membrane bound vacuoles of its eukaryotic host cell. Since *C. pneumoniae* is not capable to generate ATP, the bacterium exploits the host cell ATP deposits to fulfil its requirements. Besides ATP, the bacterium also depends on the nucleotide pool of their host cells. *C. pneumoniae* can replicate in various cells like monocytes, macrophages, smooth muscle cells, fibroblast cells and endothelial cells (Krull et al., 2005).

It has been observed that a chlamydial infection prevents the host cell apoptosis. Chlamydial elementary bodies upon contact with endothelial cells significantly increase the phosphorylation and activation of all three groups of MAPK. Activation of ERK1/2 and p38 MAPK is associated with inhibition of apoptosis. Activation of these kinases (p38 MAPK and ERK1/2 only) in turn help in activation and translocation of NF- κ B in *C. pneumoniae* infected cells and leads to the expression of different NF- κ B dependent inflammatory mediators such as IL-8, IL-6, MCP-1 and RANTES (Dechend et al., 1999; Donath et al., 2002; Molestina et al., 2000). Cells infected with other *Chlamydia* species also show resistance to apoptosis induction. Infection with *C. trachomatis* inhibits host cell apoptosis by stabilizing and sequestering the BH3 only proteins Mcl-1 and BAD which prevents release of cytochrome C from mitochondria (Rajalingam et al., 2008; Verbeke et al., 2006). A recent report from our group shows that infection with *C. pneumoniae* multiplies within the neutrophils and extends the life span of granulocytes (van Zandbergen et al., 2004). It has been suggested that this effect is mediated by high amount of IL-8 released from the infected cells at late hours of infection (66 hours). However, the detailed molecular mechanism of *C. pneumoniae* mediated apoptosis inhibition was not studied so far.

1.5.3 *Leishmania*

Leishmaniasis, a parasitic disease distributed in tropical and subtropical areas, caused by protozoan parasite of the genus *Leishmania*. The disease is transmitted to humans by the bite of infected female phlebotomine sandflies, small blood feeding insects which act as a vector in the disease process. Leishmaniasis is not a single disease but a collection of diseases where each type has characteristic clinical manifestations. Depending on the clinical manifestations three different types of leishmaniasis are observed: cutaneous, mucocutaneous and visceral. The cutaneous form of leishmaniasis appears either in localised or diffuse form of skin lesions. The mucocutaneous forms of leishmaniasis cause partial or total destruction of mucous membranes inside the nose, mouth, and throat. The most serious form of

leishmaniasis is the visceral leishmaniasis which is also called kala-azar. Irregular periods of fever, weight loss, anorexia, anemia, hepatomegaly and splenomegaly are the characteristic features. Depending on the zoogeographical distribution *Leishmania* species are divided into two groups: Old World *Leishmania* found in Africa, Asia and Europe. The Old World leishmaniasis group contains five species: *L. major*, *L. tropica*, *L. aethiopica*, *L. donovani* and *L. infantum*. The first three members of this group cause cutaneous leishmaniasis while the remaining two are the causative agent for visceral leishmaniasis. On the other hand *L. mexicana*, *L. amazonensis* and *L. chagasi* belong to the New World *Leishmania* group.

Leishmania are obligate intracellular pathogens present in two developmental forms. The intracellular amastigote form is found in the vertebrate host and the flagellated promastigote form which is predominant in the insect vector. Macrophages are the final host cells for *L. major*. However, after infection neutrophils serve as primary host cells for the parasite (Laufs et al., 2002). Upon infection with *L. major* the life span of neutrophils is extended (Aga et al., 2002) however, the detailed molecular mechanisms of the extension of neutrophils life span is poorly studied.

1.6 Aim of the study

Constitutive apoptosis is a typical feature of neutrophils. However, several reports show that intracellular pathogens modulate this programme and survive inside neutrophils. Although the intracellular pathogens, *A. phagocytophilum*, *C. pneumoniae* and *L. major* have been reported to extend the life span of neutrophils, it is not yet known which survival signals are involved in modulation of neutrophil apoptosis. Therefore, the aim of the present study was to investigate how these pathogens modulate the neutrophil apoptosis from the context of survival signals. Two major survival signalling pathways are known in neutrophils, the PI3K/Akt pathway and the MAPK pathway. In order to investigate the modulation of survival signals by these intracellular pathogens I aimed to study specifically:

Whether these pathogens modulate the PI3K/Akt or the MAPK pathway in order to achieve apoptosis inhibition.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 *Anaplasma phagocytophilum*

The *Anaplasma phagocytophilum* (*Ap*) MRK strain, was kindly supplied by Prof J.S. Dumler (Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA) and was propagated in HL-60 cells in RPMI 1640 medium containing 2 mM L-glutamine and 1 % FCS at 37 °C in a humidified atmosphere containing 5 % CO₂ (Choi et al., 2005). Briefly, *A. phagocytophilum* infected HL-60 cells were thawed from frozen stocks kept in liquid nitrogen, washed with ice cold RPMI 1640 medium containing 2 mM L-glutamine and 10 % FCS by centrifuging at 216 x g for 10 minutes at room temperature. Cell pellet was resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10 % FCS and cells were allowed to grow for 3-6 days. Cells were washed and the subsequent culture was carried out in RPMI 1640 medium containing 2 % FCS supplemented with 2 mM L-glutamine. The culture medium was changed after every 3-4 days. When 30-40 % cells were found infected (determined by observing the presence of morula in the cells) they were used for preparing new frozen stocks.

2.1.2 *Chlamydia pneumoniae*

The *Chlamydia pneumoniae* (*Cp*) bacterial strain CV-6 was isolated from a coronary artery plaque and continuously cultured in HEp-2 cells as described elsewhere (Maass and Harig, 1995; van Zandbergen et al., 2004). Confluent sheets of HEp-2 cells were grown in 24 well plates in Eagle's MEM containing 10 % FCS and non essential amino acids. In order to infect HEp-2 cells with *C. pneumoniae*, bacteria were added to the HEp-2 cell culture, centrifuged at 3000 x g for 45 minutes and incubated at 37 °C in a 5 % CO₂ atmosphere for 72 hours. The infected cells were mechanically disrupted and cell debris was removed by centrifuging at 1000 x g for 10 minutes. *Cp* was concentrated by centrifuging at 24,000 x g for 1 hour and the pellet was resuspended in 0.55 ml PBS. 0.05 ml was taken out from the *C. pneumoniae* suspension and used to inoculate a new monolayer in order to determine the number of inclusion forming unit (IFU) by using a commercial immunofluorescence test. *Chlamydia* suspension with known IFU number of *Chlamydia* in serum free Eagle's MEM supplemented with 1 µg/ml cycloheximide was aliquoted and stored at -80 °C. The aliquots were taken out immediately before the experiments. For all the experiments performed in this study, the ratio between *Chlamydia* IFU and PMN was maintained 5:1.

2.1.3 *Leishmania major*

The *Leishmania major* isolate MOHM/IL/81/FEBNI (Laskay et al., 1995) used in this study was kindly supplied by Dr. F Ebert (Bernhard-Nocht-Institute for Tropical Medicine, Hamburg). This strain was originally isolated from skin biopsy of an Israeli patient. To obtain a continuous pool of infectious parasites, *in vitro* cultures in the stationary growth phase were used to infect BALB/c mice. Amastigotes isolated from spleens of infected mice were cultured *in vitro* in Novy-Nicolle-McNeal biphasic blood agar medium at 27 °C in a humidified chamber containing 5 % CO₂ where they converted into infectious promastigotes.

2.1.4 Laboratory supplies

Cell culture flasks	Greiner bio-one, Frickenhausen
Galss slides superfrost	Menzel, Braunschweig
Gel-loading tips	Eppendorf, Hamburg
Hyperfilm™ ECL	Amersham Biosciences, Freiburg
96-well MaxiSorb™ Microtiter plates,	Nunc, Wiesbaden
Nitrocellulose (NC) membrane:	
Optitran BA-S 83 reinforced NC	Schleicher & Schuell, Dassel
Pipette (5, 10, 25 ml)	Greiner bio-one, Frickenhausen
Pipette filter tips	Nerbe plus, Winsen
Pipette tips	
(1-10 µl, 10-100 µl, 100-1000 µl)	Greiner bio-one, Frickenhausen
Plastic tubes (5 ml Falcon)	BD Biosciences, Heidelberg
Plastic tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht
Reaction tubes (1.5; 2 ml) Biopure	Eppendorf, Hamburg
S-Monovette, 9 ml, lithium-heparin	Sarstedt, Nümbrecht
Tissue culture plates	
(6, 12, 24, 48, 96-well, flat bottom)	Greiner bio-one, Frickenhausen
Transfer pipette 3.5 ml	Sarstedt, Nümbrecht
U-tubes for cytometry	Micronic, Lelystad, The Netherlands
Whatman Paper	Schleicher & Schuell, Dassel

2.1.5 Instruments

Analytical balance BP61S	Sartorius, Göttingen
Balance	Sartorius, Göttingen
Block thermostat	
Unitek™ block thermostat HB 130	Peqlab, Erlangen
Block thermostat TCR 200	Roth, Karlsruhe
Cell counting chambers	Neubauer, Marienfeld
Centrifuges	
Biofuge fresco	Kendro (Heraeus), Langenselbold
Megafuge 2.0R	Kendro (Heraeus), Langenselbold
Multifuge 3 and SR	Kendro (Heraeus), Langenselbold

Microfuge R	Beckmann, Munich
Mikro 12-24	Hettich, Tuttlingen
Centrifuge 5417R	Eppendorf, Hamburg
Cytocentrifuge Cytospin3	Shandon, Frankfurt
CO ₂ - Incubator IG 150	Jouan, Unterhaching
Deep freezer, -20 °C, -70 °C	Liebherr, Ochsenhausen
Densimat	Bio Mérieux, Marcy l'Étoile, France
Electrophoresis chambers	Bio-Rad, Munich
Flow-cytometer FACS-Calibur [®]	Becton Dickinson, Heidelberg
Homogeniser	Glascot, ABCR GmbH, Karlsruhe, Germany
Laminar flow workbench	Biohit, Cologne
Magnetic stirrer: Ikamag, Reo	IKA [®] Labortechnik, Staufen
Microscopes	
Axiovert 25	Carl Zeiss, Jena
AxioStar plus	Carl Zeiss, Jena
AxioCam HRc (Digital Immun- fluorescence-Microscope)	Carl Zeiss, Jena
Multichannel pipette	Eppendorf, Hamburg
pH-meter	Inolab, WTW GmbH, Weilheim
Pipettes	Eppendorf, Hamburg
Power supply P25	Biometra, Göttingen
Ricoh HR-10m camera	Ricoh, Tokyo, Japan
Scanner Umax Astra 6700	Umax Systems GmbH, Willich
Semi-dry protein transfer cell	Bio-Rad, Munich
Shaker Vibrofix VF1 Electronic	Janke & Kunkel IKA [®] Labortechnik, Staufen
Transfer pipette	Brand, Wertheim
Water bath	Köttermann, Uetze (Hänigsen)

2.1.6 Chemicals and laboratory reagents

Acetone	Merck, Darmstadt
Ammonium persulfate	Sigma, Deisenhofen
Annexin V FLUOS	Roche Diagnostics, Mannheim
Aprotinin	Sigma, Deisenhofen
Aqua ad injectabilia	DeltaSelect, Pfullingen
Brain Heart Infusion (BHI)	Becton Dickinson, Heidelberg
Bovine serum albumin (BSA)	Sigma, Deisenhofen
Bromophenol blue dye	Serva, Heidelberg
Calcium chloride	Sigma, Deisenhofen
Chloroform minimum 99 %	Sigma, Deisenhofen
COS blood agar plate	Bio Mérieux, Marcy l'Étoile, France
Crystal violet	Sigma, Deisenhofen
Diaminobenzine (DAB)	Vector laboratories, Burlingame, CA, USA
Diff-Quick [®]	Medion Diagnostics, Duedingen, Switzerland
Dithiothreitol	Sigma, Deisenhofen
EDTA	Sigma, Deisenhofen
EGTA	Sigma, Deisenhofen
FCS	Sigma, Deisenhofen
FCS	Gibco, Karlsruhe
fMLP	Sigma, Deisenhofen
Gentamicin	Sigma, Deisenhofen

Giemsa staining solution, modified	Sigma, Deisenhofen
L-Glutamine	Biochrom, Berlin
Glycerol	Sigma, Deisenhofen
HEPES	Biochrom, Berlin
Histopaque [®] 1119	Sigma, Deisenhofen
IMAGEN [™] Chlamydia	OXOID, Cambridgeshire, UK
IMAGEN [®] Mounting Fluid	Dako, Hamburg
Immersion oil	Carl Zeiss, Jena
Immobilion [™] western	Millipore, MA, USA
Isopropanol	Roth, Karlsruhe
Leupeptin	Calbiochem, Schwalbach
Lipopolysaccharide <i>E. coli</i> 0111:B4	Sigma, Deisenhofen
Lymphocyte separation medium 1077	PAA, Pasching, Austria
Magnesium chloride	Sigma, Deisenhofen
2-Mercaptoethanol	Sigma, Deisenhofen
Methanol	J.T. Baker, Deventer, The Netherlands
MOPS	Roth, Karlsruhe
Paraformaldehyde	Sigma, Deisenhofen
PBS (1 X) sterile solution	Pharmacy of University of Lübeck, Lübeck
PBS (10 X) sterile solution	Gibco, Karlsruhe
PBS, Instamed, Dulbecco w/o Mg, Ca	Biochrom, Berlin
Penicillin/streptomycin	Biochrom, Berlin
Pepstatin-A	Sigma, Deisenhofen
Percoll [®]	Pharmacia, Uppsala, Sweden
PMSF	Sigma, Deisenhofen
Prestained protein molecular weight marker	NEB, Frankfurt am Main
Defibrinated rabbit blood	Elocin-lab GmbH, Mülheim
RPMI 1640 medium	Sigma, Deisenhofen
SDS	Roth, Karlsruhe
Sodium azide	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium orthovanadate	Sigma, Deisenhofen
Sulfuric acid	Merck, Darmstadt
TEMED	Roth, Karlsruhe
Trichloroacetic acid (TCA)	Sigma, Deisenhofen
Tris-(hydroxymethyl)-aminomethane	Roth, Karlsruhe
Triton X-100	Merck, Darmstadt
Trypan blue solution 0.4 %	Sigma, Deisenhofen
Tween -20	Sigma, Deisenhofen
Vectastain [®] ABC kit	Vector laboratories, Burlingame, CA, USA

2.1.7 Primary antibodies and dilutions used

Rabbit anti-human β -actin (1:1000)	NEB, Frankfurt am Main
Rabbit anti-human phospho-Akt (1:2000)	NEB, Frankfurt am Main

Rabbit anti-human phospho-Erk1/2 (1:1000)	NEB, Frankfurt am Main
Mouse anti-human phospho IκB (1:1000)	NEB, Frankfurt am Main
Rabbit anti-human phospho p38 MAPK (1:1000)	NEB, Frankfurt am Main
Mouse anti-human FAS (1 μg/100μl)	Dako, Hamburg
Rabbit anti-human Mcl-1 (1:1000)	NEB, Frankfurt am Main
Rabbit anti-human phospho PDK1 (1:1000)	NEB, Frankfurt am Main
Rabbit anti-human phospho PI3K (1:1000)	NEB, Frankfurt am Main
Rabbit anti-human PKCδ (1:1000)	Santacruz, Heidelberg

2.1.8 Secondary antibodies and dilutions used

Goat anti-rabbit IgG-HRP conjugate (1:5000)	Santacruz, Heidelberg
Rabbit anti-mouse IgG-HRP conjugated (1:4000)	NEB, Frankfurt am Main
Rabbit anti-mouse IgG-FITC.conjugated (1:200)	Dako, Hamburg

2.1.9 Inhibitors

BAY 11-7082 (IκB inhibitor)	Calbiochem, San Diego, CA, USA
LY294002 (PI3K inhibitor)	NEB, Frankfurt am Main
Rottlerin (PKCδ inhibitor)	Calbiochem, San Diego, CA, USA
SB203580 (p38 MAPK inhibitor)	Calbiochem, San Diego, CA, USA
U0126 (MEK1/2 inhibitor)	NEB, Frankfurt am Main
MG-132 (IκB inhibitor)	Calbiochem, San Diego, CA, USA

2.1.10 Ready to use kits

Proteome profiler™ array	R&D systems, Wiesbaden
Human IL-6 DuoSet®	R&D systems, Wiesbaden
Hu IL-8 Cytoset	Biosource, CA, USA

2.1.11 Cytokines

rh GM-CSF	R&D systems, Wiesbaden
rh TNFα	R&D systems, Wiesbaden

2.1.12 Softwares

Statistical analysis software

GraphpadPrism®, Version 4.01	San Diego, CA, USA
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Instrument softwareCellQuestpro[®] software

Becton Dickinson, Heidelberg

2.1.13 Solutions, Buffers and media**Annexin buffer**

10 mM HEPES/NaOH, pH 7.4

140 mM NaCl

5 mM CaCl₂**Blocking solution (Western blot)**

TBS + 0.1 % Tween 20 + 5 % BSA

Novy-Nicolle-Mcneal blood agar medium

50 ml defibrinated rabbit blood

50 ml PBS

2 ml penicillin / streptomycin

200 ml brain heart infusion (BHI) medium

(10.4 g agar in 200 ml distilled water)

Wash buffer for immunohistochemistry

PBS (1X) + 0.3 % Tween-20

Lysis buffer for the protein kinase C translocation experiment

20 mM Tris pH 8.0

2 mM MgCl₂

2 mM EDTA pH 8.0

10 mM EGTA pH 8.0

10 µg/ml leupeptin

10 µg/ml aprotinin

6 µg/ml pepstatin

2 mM DTT

10 mM PMSF

Protein sample buffer (4X)

(0.16 M Tris-HCl, 30 % glycerol, 4 % SDS, 0.7125 M β-mercaptoethanol, pH 6.8)

For 10 ml	Final conc	Final Vol.	Stock conc.
Tris HCl, pH 6.8	0.16 M	1.6 ml	1.00 M
Glycerol	30 %	3.0 ml	100 %
SDS	4 % v/v	2.0 ml	20 %
β-Mercaptoethanol	0.7125 M	0.5 ml	14.25 M
Deionized water		2.9 ml	
Bromophenol blue			a little crumb

Tris buffer solution (10X) for 1 litre (TBS)

Tris base	24.22 g
NaCl	80.00 g

Adjust the pH 7.6 with HCl and make up the volume to 1 litre.

5X SDS-PAGE running buffer, pH 8.4 (0.5 litre)

Tris base	7.50 g
Glycine	36.00 g
SDS	2.50 g

Deionized water added to adjust the 0.5 litre

Solutions for SDS polyacrylamide gel

	Separating gel 10 %	Stacking gel 3 %
Acrylamide stock (30 %)		1.665 ml
		0.750 ml
Deionized H ₂ O	2.000 ml	3.000 ml
3 M Tris HCl, pH 8.4	1.250 ml	
0.5 M Tris HCl, pH 6.8		1.250 ml
10 % SDS	0.050 ml	0.050 ml
10 % APS	0.045 ml	0.045 ml
TEMED	0.005 ml	0.005 ml

Stripping buffer

62.5 mM Tris HCl, pH 6.7
2 % SDS
100 mM β -Mercaptoethanol
Distilled H₂O

2.2 Methods

2.2.1 Isolation of neutrophil granulocytes from human blood

Peripheral blood was collected from healthy donors in lithium heparin S-Monovette tubes. 3 ml of lymphocyte separation medium 1077 were layered on 5 ml of Histopaque[®] 1119 in a 15 ml centrifuge tube and 4 ml of blood were layered on that column. The column was set for centrifugation for 25 minutes at 800 x g. The first interphase layer from the top of the column, rich with lymphocytes and monocytes was discarded and subsequent granulocyte rich interphase layers were collected in 50 ml centrifuge tube and washed with PBS for 10 minutes at 250 x g. The pellet was resuspended in RPMI 1640 medium containing 10 % FCS and layered on a discontinuous Percoll[®] gradient consisting of fractions with densities 1,105 g/ml (85 %), 1,100 g/ml (80 %), 1,093 g/ml (75 %), 1,087 g/ml (70 %), 1,081 g/ml (65 %) from the bottom to the top, respectively. The gradient column was centrifuged at 800 x g for 25 minutes. After centrifugation the interphase between the 80 % and 85 % Percoll layers was collected, washed with PBS at 250 x g for 10 minutes and resuspended in RPMI 1640 medium. The viability and purity of the cells were checked by trypan blue exclusion and Diff-Quick[®] staining of cytocentrifuged slides, respectively. Viability and purity was mostly over 99 %. All the above steps were carried out at room temperature.

2.2.2 Diff-Quick[®] staining for cells

1×10^5 cells were taken in 100 μ l medium and cytocentrifuged at 400 x g for 5 minutes using cytocentrifuge Cytospin 3. Slides were air dried, fixed in fixative solution provided in Diff-Quick[®] staining set and subsequently stained according to the manufacturer's protocol.

2.2.3 Preparation of cell free *Anaplasma phagocytophilum* and co-incubation with neutrophils

The infection rate of *A. phagocytophilum* infected HL-60 cells was determined by counting the cells containing morula in Romanowsky stained (Diff-Quick[®]) cytospin preparates. When ≥ 70 % of the cells were found infected they were used for the preparation of cell free *A. phagocytophilum*. Briefly, infected HL-60 cells were centrifuged at a speed of 250 x g for 10 minutes, resuspended in 2 ml of PBS and passed through a 25 gauge needle for 10-14 times. Cellular debris was removed by centrifuging at 750 x g for 10 minutes. The supernatant was collected and centrifuged at 2500 x g for 15 minutes. Pellets obtained in this way were immediately resuspended in PMN cell suspension and incubated for various time points. The

ratio between infected HL-60 cells and PMN was always maintained 1:1 throughout the study.

2.2.4 Co-incubation of neutrophils with *Leishmania major*

7-11 days old stationary phase cultures of *Leishmania major* promastigotes were used for the study, collected from *in vitro* cultures maintained in biphasic NNN blood agar medium. Briefly, *L. major* promastigotes were taken out from each well and washed with complete medium for 10 minutes at 2800 x g. After centrifugation the supernatant was discarded and the pellet was resuspended in complete medium. Promastigotes with active flagellar movement were counted and added to the neutrophil culture in neutrophil to parasite ratio of 1:5.

2.2.5 Co-incubation of neutrophils with *Chlamydia pneumoniae*

For co-incubation experiments with *Chlamydia pneumoniae*, frozen stocks of infected Hep2 cell culture with known IFUs (1×10^8 /ml) were used. Briefly, infected Hep2 cell stocks were thawed and vortexed with glass beads to rupture the cells and added directly to neutrophils at a neutrophil to Cp IFU ratio of 1:5.

2.2.6 Immunohistochemical staining for *A. phagocytophilum*

In order to determine the presence of *A. phagocytophilum* in infected neutrophils, immunohistochemical staining was carried out using a mouse anti *Anaplasma phagocytophilum* antibody kindly provided by Prof J. S. Dumler (Department of Pathology, John Hopkins University School of Medicine, Baltimore, Maryland, USA). Briefly, neutrophils (5×10^6 /ml) were incubated either with *A. phagocytophilum* or left untreated for 18 hours. For immunohistochemical staining 1×10^5 neutrophils from each culture were cytocentrifuged and fixed with methanol for 5 minutes. Subsequently, the cytospin preparates were hydrated by keeping them for 2 minutes in gradually decreasing alcohol solutions (100 %, 95 %, and 70 %). Samples were then incubated for 15 minutes with 3 % hydrogen peroxide (H_2O_2) and washed with distilled water subsequently blocked for 20 minutes in serum and then washed twice with PBS containing Tween-20. A mouse anti *Anaplasma phagocytophilum* antibody was then added in PBS containing 1 % BSA containing PBS and incubated for 1 hour. After 3 times washing with PBS-Tween 20 the samples were incubated with biotinylated rabbit anti mouse IgG for 30 minutes. Slides were then washed 3 times in PBS-Tween-20 buffer and incubated for another 30 minutes with Vectastain[®] ABC kit prepared according to the manufacturer's recommendation. Subsequently a DAB solution was prepared in 0.05 M Tris HCl according to the manufacturer's recommendation and slides

were incubated in DAB solution for 8 minutes in dark. Slides were washed twice with distilled water followed by 30-40 seconds incubation with haematoxylin solution and washed under tap water for 5 minutes. Preparates were dehydrated in gradually increasing percentage of alcohol solutions (70 %, 95 %, and 100 %). Slides were kept for 2 minutes in xylol and mounted.

2.2.7 Immunohistochemical staining for *C. pneumoniae*

For immunohistochemical staining 1×10^5 cells were cytocentrifuged and fixed with methanol. Cells were incubated for 15 minutes at 37 °C with IMAGEN™ Chlamydia test reagent which contains FITC conjugated chlamydial LPS specific mouse monoclonal antibody mixed with Evans blue. Excess reagent was washed with PBS. The slides were subsequently mounted with the mounting fluid provided in the staining kit and viewed under fluorescence microscope.

2.2.8 Cell lysis for immunoblots

Whole cell lysates were prepared following published protocol (Wang et al., 2003). Briefly, after incubation, 3×10^6 cells were spun at 216 x g for 10 minutes, pellets were resuspended in 500 µl of 10 % TCA solution and kept for 10 minutes on ice and subsequently centrifuged for 5 minutes at 14000 x g at 4 °C. The pellets were washed for 2 times with 500 µl 100 % acetone at 14000 x g for 5 minutes at 4 °C, resuspended in 1 fold (1X) sample buffer and boiled for 5-7 minutes at 100 °C.

2.2.9 SDS-polyacrylamide gel electrophoresis and western blotting

Lysates from equal number of cells were added in each lane of the gel and electrophoresis was performed with 75 V constant voltage while running in the stacking gel and with 125 V constant voltage in separating gel. For performing western blot, transblot semidry transfer cell was used where protein was transferred on to a nitrocellulose membrane at 2.6 mA/cm² constant current for 1 hour and 40 minutes. The membranes were blocked in TBS containing 0.1 % Tween-20 and 5 % BSA for one hour on a gentle rocking platform and subsequently incubated with the appropriate primary antibody for 10-12 hours at 4 °C with gentle rocking. After extensive washing with TBS containing 0.1 % Tween-20 for 45 minutes, membranes were incubated for 60 minutes with HRP conjugated polyclonal anti rabbit IgG or anti mouse IgG depending on the primary antibody used. Membranes were washed again and bands were detected using the ECL western blotting detection system (Immobilion™ western). Equal loading was confirmed by stripping the membranes and re-probing with either antibody for

total protein which detects both phosphorylated and non-phosphorylated form of the given protein and/or with an antibody to β -actin.

2.2.10 Separation of cytosolic and membrane fractions

Since activated PKC translocates from the cytosol into the plasma membranes, cytosolic and membrane fractions were isolated by differential centrifugation as described elsewhere (Wang et al., 2003). Briefly, 20×10^6 neutrophils were infected with *Anaplasma* or left untreated. After incubation, pellets were obtained by spinning down at $256 \times g$ for 10 minutes and then washed with ice cold PBS for another 3 minutes at $216 \times g$. The pellets were resuspended in lysis buffer (lysis buffer for protein kinase C translocation experiment) and incubated for 20 minutes on ice. The samples were homogenised by 50 strokes in tight fitting homogenizer. Post nuclear fractions were separated by centrifuging at $500 \times g$ for 10 minutes at 4°C . Supernatants were collected and spun at $100000 \times g$ for 45 minutes at 4°C . After centrifugation, supernatants were used as the cytosolic fractions and the pellets were used as the membrane fractions. Equal loading was confirmed by stripping the membrane and reprobing for β -actin. β -actin bands were observed in the cytosolic fractions which was completely absent in the membrane fractions (data not shown).

2.2.11 Annexin-V-binding assay

Annexin-V exhibits calcium dependent binding to phosphatidylserine expressed on the outer leaflet of the cell membrane of apoptotic cells. Apoptosis was determined by Annexin V FLUOS binding according to the manufacturer's recommendation. Briefly cells were stained with Annexin V FLUOS for 30 minutes in the dark on ice in the binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl_2), washed and resuspended in binding buffer. Propidium iodide (PI) was added at a concentration of $1 \mu\text{g/ml}$ in order to assess the membrane integrity and analysed using a flow cytometer (FACS Calibur; BD Biosciences).

2.2.12 Determination of cytokines in the culture supernatants

In order to determine the cytokine profile in the culture supernatants of neutrophil granulocytes co-incubated either with *A. phagocytophilum* or with *C. pneumoniae*, supernatants were collected after 18 hours and cytokine array was performed according to the manufacturer's protocol (Proteome ProfilerTM, R&D systems, Wiesbaden). The kit provides nitrocellulose membranes spotted in duplicate with selected capture antibodies. The number of antibodies spotted is listed in the following table (Table.1). A transparency overlay template is also provided (Fig. 3). This transparency overlay helps to identify which spot

stands for which cytokine on the photographic film after performing the experiments according to the manufacturer's protocol. To measure the concentrations of IL-6 and IL-8, ELISA was performed according to the manufacturer's instruction.

Coordinate	Target/Control	Alternative Nomenclature
A1, A2	Positive Control	
A3, A4	C5a	Complement Component 5a
A5, A6	CD40 Ligand	CD154
A7, A8	G-CSF	CSF β , CSF-3
A9, A10	GM-CSF	CSF α , CSF-2
A11, A12	GRO α	CXC11
A13, A14	I-309	CCL1
A15, A16	sICAM-1	CD54
A17, A18	IFN- γ	Type II IFN
A19, A20	Positive Control	
B3, B4	IL-1 α	IL-1F1
B5, B6	IL-1 β	IL-1F2
B7, B8	IL-1ra	IL-1F3
B9, B10	IL-2	
B11, B12	IL-4	
B13, B14	IL-5	
B15, B16	IL-6	
B17, B18	IL-8	CXCL8
C3, C4	IL-10	
C5, C6	IL-12p70	
C7, C8	IL-13	
C9, C10	IL-16	LCF
C11, C12	IL-17	
C13, C14	IL-17E	
C15, C16	IL-23	
C17, C18	IL-27	
D3, D4	IL-32 α	
D5, D6	IP10	CXCL10
D7, D8	I-TAC	CXCL11
D9, D10	MCP-1	CCL2
D11, D12	MIF	GIF, DER6
D13, D14	MIP-1 α	CCL3
D15, D16	MIP-1 β	CCL4
D17, D18	SerpinE1	PAI-1
E1, E2	Positive Control	
E3, E4	RANTES	CCL5
E5, E6	SDF-1	CXCL12
E7, E8	TNF-1 α	TNFSF1A
E9, E10	sTREM-1	
E19, E20	Negative Control	

Table.1 Cytokine array coordinates. List of antibodies spotted on the nitrocellulose membrane strip provided in the cytokine array kit (R&D systems, Wiesbaden).

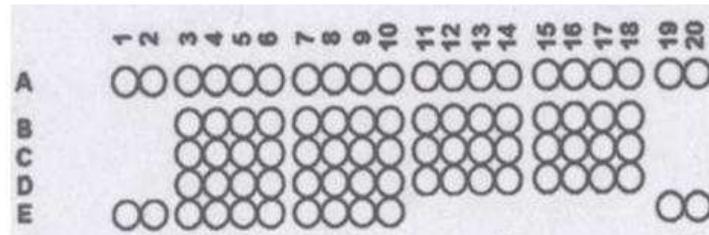


Fig. 3 Transparency overlay template. Human cytokine array transparency overlay template provided by the manufacturer where the each two successive numbers and alphabet co-ordinate represent a single cytokine

2.2.13 Flow cytometric analysis of FAS

Surface expression of FAS was detected by flow cytometric analysis using human anti FAS (CD95) monoclonal antibody conjugated with PE. Neutrophils were incubated with or without *C. pneumoniae* for 18 hours at 37 °C, 5 % CO₂ atmosphere. For staining, 1 x 10⁵ cells were taken in 96 well V bottom plate, washed with FACS buffer at 4 °C and stained either with anti human CD95 monoclonal antibody conjugated with PE or with isotype control antibody for 30 minutes on ice. After incubation cells were washed two times with FACS buffer and fixed with 1 % paraformaldehyde in PBS. Cells were analysed by FACS Calibur[®] using CellQuestpro[®] software.

2.2.14 Statistical analysis

Data from a minimum of 3 independent experiments are presented as mean ± SEM. Statistical evaluation of differences was determined with the Student's t test or two-way ANOVA and Bonferroni post test. Results were considered statistically significant when p<0.05. * or + is used when Student t test was performed (+ is used in certain experimental condition find in the respective figure legend) and # is used and when two-way ANOVA and Bonferroni post test were carried out. *p<0.05, **p<0.005, ***p<0.0005, +p<0.05, ++p<0.005, +++p<0.0005 and #p<0.05

3 RESULTS

3.1 *Anaplasma phagocytophilum* infects neutrophil granulocytes and delays their constitutive apoptosis

A characteristic feature of neutrophil granulocytes is their constitutive apoptosis. The typical morphology of the multilobed nucleus with interlobular connections is lost in cells that undergo apoptosis. These morphological changes can be utilized to differentiate between apoptotic and non apoptotic neutrophils by visual examination. Since previous studies showed that *A. phagocytophilum* can modulate neutrophil apoptosis (Choi et al., 2005; Ge and Rikihisa, 2006), I investigated the ability of the bacterium to delay neutrophil apoptosis. The apoptotic rate was determined after 18 hours incubation of neutrophils with or without *A. phagocytophilum*. It was observed that whereas the majority (60-80 %) of cells incubated in medium alone were apoptotic, a significant reduction of apoptotic rate was noticed in neutrophils after co-incubation with *A. phagocytophilum* (Fig. 4C, D, E). In order to investigate whether the observed anti apoptotic effect was due to the infection of neutrophils with the bacteria, *A. phagocytophilum* was visualised inside the neutrophils by using immunocytochemical staining of *A. phagocytophilum*. The staining revealed the presence of intracellular bacteria in neutrophils after co-incubation with *A. phagocytophilum* (Fig. 4A, B).

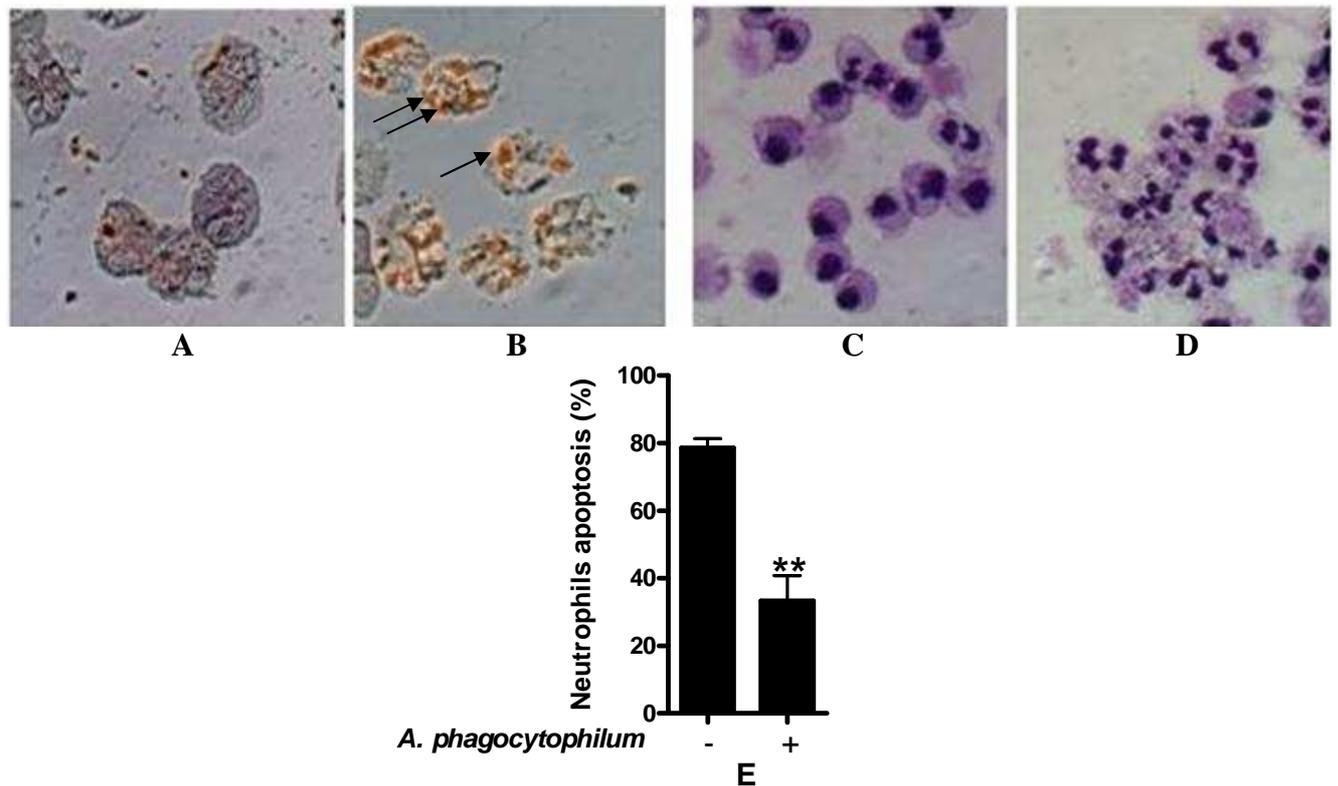


Fig. 4 Co-incubation of neutrophils with cell free *A. phagocytophilum* infects neutrophils and delays their spontaneous apoptosis. Neutrophils were incubated with or without *A. phagocytophilum* for 18 hours. Cells were harvested, cytospin prepares were prepared, and stained either by Diff-Quick[®] or used for immunocytochemical staining of *A. phagocytophilum* as detailed in the materials and methods section. Representative pictures of immunocytochemical staining of non-infected (A) and infected cells (B) (arrow mark showing the infection as revealed by the immunocytochemical staining) and Diff-Quick[®] staining (non infected cells (C) and infected cells (D)) are shown. Percentage of apoptotic cells (E) was shown here obtained from microscopic counting of minimum 200 cells from 3 independent experiments. Values are given in mean \pm SEM and ** $p < 0.005$.

3.1.1 Infection with *A. phagocytophilum* upregulates the phosphorylation of PI3K in neutrophils

Spontaneous apoptosis is a unique feature of neutrophil granulocytes (Webb et al., 2000). Neutrophil apoptosis is, however, delayed in the presence of pro-inflammatory modulators at the site of infection (Lee et al., 1993; Wei et al., 1996) or upon challenge with intracellular pathogens (Aga et al., 2002; van Zandbergen et al., 2004). Phosphatidyl inositol 3 kinase (PI3K) is a critical survival signalling molecule comprised of regulatory (p85) and catalytic subunits (p110). Phosphorylation of the regulatory subunit is crucial for the activation of PI3K. Upon activation PI3K phosphorylates various phosphoinositides in the plasma membrane which initiates a complex signalling cascade by activating the downstream serine threonine protein kinase B (PKB) and thus leads to cell survival. To investigate whether activation of PI3K plays a role in the *A. phagocytophilum*-induced apoptosis delay in

neutrophils, the phosphorylation status of PI3K was determined by performing western blot analysis at different time points after infection. The antibody used in these experiments was directed against the phosphorylated form of the regulatory subunit of human PI3K. Enhanced phosphorylation of PI3K was observed 3 h and 18 h after *A. phagocytophilum* infection as compared to neutrophils incubated in medium alone (Fig. 5).

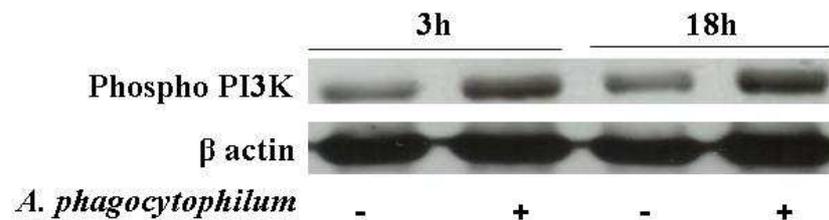


Fig. 5 **Enhanced phosphorylation of PI3K in neutrophils infected with *A. phagocytophilum*.** Neutrophils were cultured with or without *A. phagocytophilum* for the indicated time points. Whole cell lysates were prepared following the protocols described in the methods section, separated in 10 % denaturing SDS PAGE gel and immunoblotted with a rabbit anti-human PI3K antibody which detects the p85 regulatory subunit only when phosphorylated. Equal loading was confirmed by stripping and reprobing the blot with rabbit anti human beta actin antibody. Representative blots from three independent experiments are shown.

3.1.2 Infection with *A. phagocytophilum* upregulates the phosphorylation of protein kinase B (PKB) in neutrophils

Protein kinase B (PKB) which is also known as Akt, is a serine threonine kinase, downstream of PI3K and considered to be the central player in cell survival and proliferation. Upon phosphorylation Akt controls a large number of downstream effector molecules such as BAD, caspases, and SAPK to exert an anti-apoptotic effect (Song et al., 2005). Akt has two phosphorylation sites, Thr³⁰⁸ and Ser⁴⁷³. Full activation of Akt requires phosphorylation at Ser⁴⁷³ which, however, depends on the phosphorylation of Thr³⁰⁸. To assess whether infection with *A. phagocytophilum* in neutrophils affects the phosphorylation of Akt, western blot analysis was carried out. The results show significant upregulation of Akt phosphorylation at 30 minutes, 1 hour and 1.5 hours in *A. phagocytophilum* infected neutrophils as compared to neutrophils incubated only in medium. However, no phosphorylation was detected in *A. phagocytophilum* infected neutrophils after 3 hours (Fig. 6).

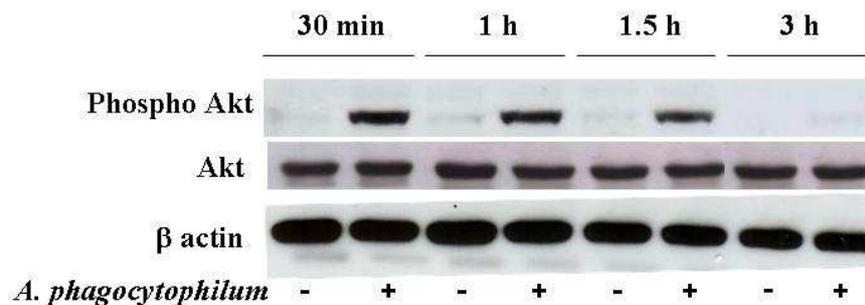


Fig. 6 Infection of neutrophils with *A. phagocytophilum* leads to the phosphorylation of Akt. Neutrophils were cultured with or without *A. phagocytophilum* for various time points. Whole cell lysates were separated in 10 % SDS PAGE, electroblotted and probed with rabbit anti human Akt antibody which detects Akt phosphorylated at Ser⁴⁷³ site. Equal loading was shown by stripping and reprobing the blot with rabbit anti human beta actin. Equal loading was further confirmed by running equal amounts of the lysates in separate 10 % SDS PAGE, blotted and probed with rabbit anti human Akt which detects both phosphorylated and non phosphorylated Akt. The blot shown here is representative of five independent experiments.

3.1.3 Infection with *A. phagocytophilum* maintains the phosphorylation of phosphoinositide dependent protein kinase 1 (PDK1) in neutrophils

It has been well documented that full activation of Akt depends on the phosphorylation on its both residues Thr³⁰⁸ and Ser⁴⁷³ (Alessi et al., 1996). Phosphorylation at Thr³⁰⁸ site is catalyzed by PDK1 which is downstream of PI3K in the PI3K-Akt mediated survival pathway. Phosphorylation of Akt depends on the phosphorylation of an intermediate kinase, PDK1 (Wick et al., 2000). Transient early phosphorylation of Akt was observed 30–90 min after *A. phagocytophilum* infection (Fig. 6). However, interestingly enhanced phosphorylation of PI3K, which is upstream of Akt, was observed as late as 18 hours after *A. phagocytophilum* infection. Since PDK1 is downstream of PI3K and upstream of Akt and enhanced phosphorylation of PI3K was observed for 18 h, I investigated the activation state of PDK1 in *A. phagocytophilum* infected neutrophils. Phosphorylation status of PDK1 in *A. phagocytophilum* infected neutrophils was determined by western blot analysis of whole cell lysates. After 1 hour of incubation phosphorylated PDK1 was observed both in infected and non-infected neutrophils. However, phosphorylated PDK1 could not be detected in non infected neutrophils after 22 hours, whereas phosphorylation status of PDK1 was maintained until 22 hours in cells co-cultured with *A. phagocytophilum* (Fig. 7).

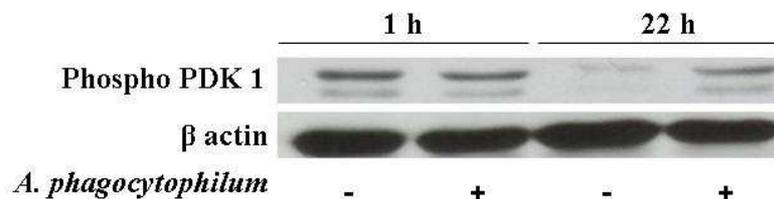


Fig. 7 Phosphorylation of PDK1 is maintained for up to 22 hours in neutrophils infected with *A. phagocytophilum*. Neutrophils were cultured with or without *A. phagocytophilum* for 1 hour or 22 hours respectively. Phosphorylation of PDK1 was determined by western blot analysis. Proteins from whole cell lysate was separated in 10 % denaturing SDS PAGE, electroblotted and probed with a rabbit polyclonal anti-human PDK1 antibody which recognizes phosphorylated PDK1. Equal loading was shown by reprobing the blot with rabbit anti-human beta actin antibody. The blot shown is representative for four experiments.

3.1.4 Infection with *A. phagocytophilum* activates protein kinase C δ (PKC δ) in neutrophils

Protein kinase C (PKC), a member of the lipid dependent serine threonine kinase family, is a crucial player in the regulation of a wide range of cellular activities notably differentiation, survival and apoptosis (Deacon et al., 1997). In its native state PKC δ is localised in the cytosol. Upon activation PKC δ translocates to the plasma membrane. Activation of PKC δ leads to extended life span of neutrophils by inducing the nuclear factor κ B (NF- κ B) (Kilpatrick et al., 2002; Wang et al., 2003). In order to investigate the role of PKC δ in the *A. phagocytophilum*-induced apoptosis delay, activation of PKC δ was assessed in infected cells by determining translocation of PKC from the cytosol to the cell membrane fraction. Cytosolic and cell membrane fractions were isolated from infected and noninfected neutrophils by using differential centrifugation. The phorbol ester PMA was used as a positive control in the experiment. Western blot analysis shows enhanced presence of PKC δ in the membrane fraction of neutrophils infected with *A. phagocytophilum* as compared to the neutrophils cultured in medium alone (Fig. 8). It indicates that infection with *A. phagocytophilum* activates PKC δ in neutrophils.

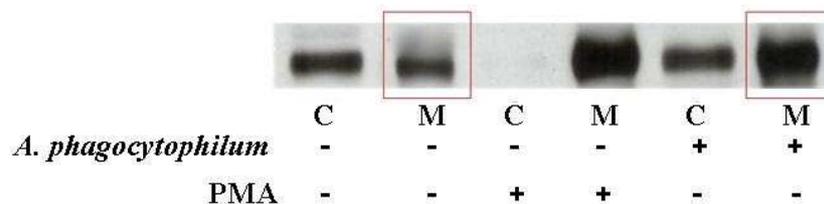


Fig. 8 Infection of neutrophils with *A. phagocytophilum* activates the PKC δ . Neutrophils were incubated with or without *A. phagocytophilum* for 3 hours. Cells were lysed in PKC translocation buffer following the protocol described in methods and materials section. Membrane and cytosolic fractions of the cells were prepared by ultracentrifugation and analysed by western blotting after separating the proteins with a 4-12 % gradient SDS-PAGE. Blots were probed with polyclonal rabbit anti-human PKC δ antibody. Incubation with 10 nM phorbol myristate acetate (PMA) for 5 minutes was used as the positive control for PKC δ activation. Bands marked in red boxes show the membrane translocation of PKC δ in infected and non infected neutrophils. A representative blot from three independent experiments is shown here.

3.1.5 Infection with *A. phagocytophilum* induces the phosphorylation of extracellular growth regulated kinases (ERK) in neutrophils

Extracellular growth regulated kinase (ERK) is a member of the mitogen activated protein kinase family (MAPKs). ERK is a serine threonine kinase, activated by a variety of stimuli. Mammalian cells contain five different types of ERK among which ERK1 and ERK2 regulate cellular differentiation and proliferation (Qi and Elion, 2005). ERK promotes cell survival by regulating several members of the Bcl-2 family. Similarly to PI3K/Akt, ERK1/2 regulates cell death regulator proteins such as BIM, BAD and Mcl-1 in order to prevent apoptosis. Some of the recent studies suggest that, in addition to its role to regulate Bcl-2 family members, ERK1/2 also inhibits the activation of both caspase-8 and caspase-9 (Perianayagam et al., 2004; Strasser et al., 2000). Western blot experiments were carried out to examine whether the ERK pathway is involved in the modulation of apoptosis in *A. phagocytophilum* infected neutrophils. Enhanced phosphorylation was observed in *A. phagocytophilum* infected neutrophils after 1 h and 3 h of infection. No phosphorylation was detected in neutrophils incubated in medium alone (Fig. 9)

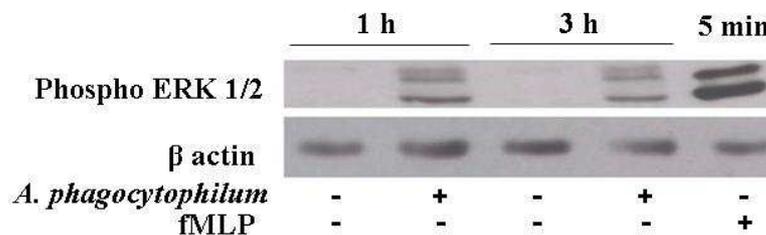


Fig. 9 Infection with *A. phagocytophilum* induces the phosphorylation of ERK1/2 in neutrophils. Neutrophils were incubated with or without *A. phagocytophilum* for the indicated time points and phosphorylation of ERK1/2 was assessed by western blot analysis of whole cell lysates prepared following the protocol detailed in the materials and methods section. The membranes were probed with rabbit anti human ERK1/2 antibody which detects ERK1/2 when phosphorylated. Equal loading was confirmed by stripping the blot and reprobing with rabbit anti human beta actin antibody. A representative blot of 3 independent experiments is shown. Incubation with 1 μ M fMLP for 5 minutes was used in the experiment as a positive control for ERK1/2 phosphorylation.

3.1.6 Infection with *A. phagocytophilum* activates NF- κ B in neutrophils via the PI3K/Akt pathway

Nuclear factor- κ B (NF- κ B) is a transcription factor which tightly regulates the expression of numerous genes involved in host immune responses, inflammatory reactions, apoptosis, proliferation and differentiation (Aggarwal, 2000; Barkett and Gilmore, 1999). In its non-activated state NF- κ B is localised in the cytoplasm of the cell in close association with inhibitory kappa B (I κ B) (Henkel et al., 1993). During NF- κ B activation, the inhibitory subunit I κ B gets phosphorylated for polyubiquitination and subsequent degradation and thus allows the free NF- κ B to translocate to the nucleus. The NF- κ B pathway has been shown to be involved in the delay of neutrophil apoptosis during infection with respiratory syncytial virus (Lindemans et al., 2006) and following exposure to the pro-inflammatory cytokines GM-CSF and TNF- α (Cowburn et al., 2004). In order to decipher whether NF- κ B is activated in *A. phagocytophilum* infected neutrophils, I κ B phosphorylation was assessed by western blotting. The results revealed enhanced phosphorylation of I κ B in *A. phagocytophilum* infected neutrophils as compared to the cells incubated in medium alone (Fig. 10). This reflects the activation of NF- κ B in infected cells. Since activation of PI3K-Akt pathway was observed in *A. phagocytophilum* infected neutrophils therefore, I wanted to investigate whether PI3K plays any role in regulating NF- κ B activation in *A. phagocytophilum* infected neutrophils. Neutrophils were pre-incubated with 25 μ M of the PI3K inhibitor LY294002 for 30 minutes and subsequently infected with *A. phagocytophilum*. Activation of NF- κ B was determined by western blot analysis of phosphorylated I κ B. Activation of NF- κ B was completely abolished by treatment with the PI3K inhibitor LY294002 (Fig. 10)

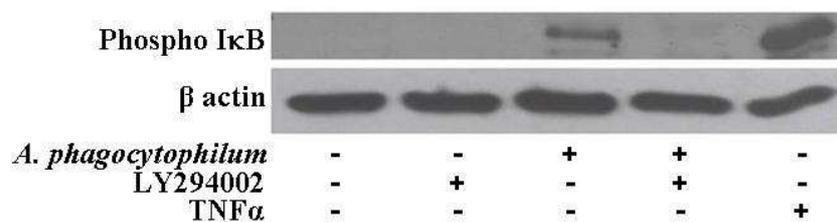


Fig. 10 Infection with *A. phagocytophilum* results in phosphorylation of I κ B in neutrophils Neutrophils were either pretreated with the PI3K inhibitor LY294002 for 30 minutes or left untreated and then incubated with or without *A. phagocytophilum* for 18 hours. Whole cell lysates were prepared, separated in 10 % denaturing SDS PAGE, electroblotted and probed with mouse anti human I κ B antibody which detects I κ B only when phosphorylated. Equal loading was confirmed by reprobing the blot with rabbit anti human beta actin antibody. The blot shown here is representative for three independent experiments. TNF α (100 ng/ml) was used as a positive control for I κ B phosphorylation.

3.1.7 Inhibition of PI3K and PKC δ reverses the *A. phagocytophilum* induced apoptosis delay in neutrophils

Activation of PI3K and PKC δ was observed in *A. phagocytophilum* infected neutrophils. Experiments with specific inhibitors were carried out to investigate whether these kinases are involved in the delay of apoptosis. The infection inhibited neutrophils apoptosis significantly as compared to control cells (Fig. 11A, B). Pre-incubation for 30 minutes with the PI3K specific inhibitor LY294002 or with the PKC δ specific inhibitor rottlerin reversed the pathogen-mediated apoptosis delay (Fig. 11A, B). The inhibitors alone had no effect on the rate of constitutive neutrophil apoptosis. (Fig. 11A, B).

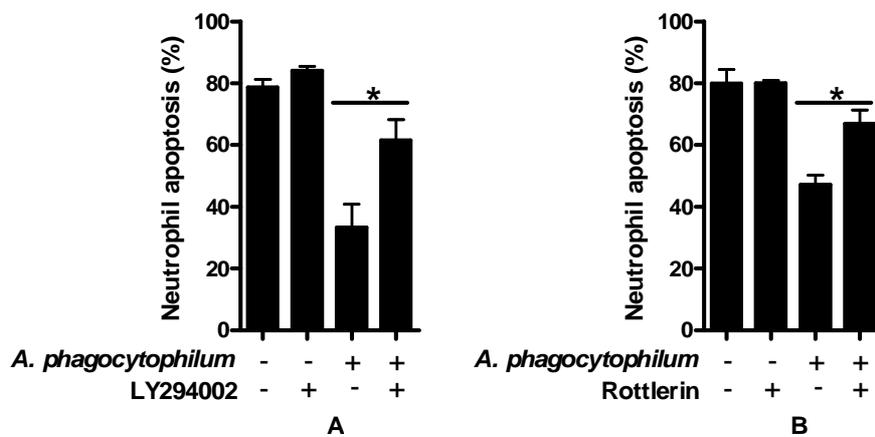


Fig. 11 Treatment with the PI3K inhibitor LY294002 and with the PKC δ inhibitor rottlerin reverses the antiapoptotic effect of *A. phagocytophilum* in neutrophils. Neutrophils (5×10^6 /ml) were incubated with or without *A. phagocytophilum* in the presence or absence of the PI3K inhibitor LY294002 (25 μ M) (A) or the PKC δ inhibitor rottlerin (10 μ M) (B) at 37 $^{\circ}$ C for 18 hours. Data represented here is the percentage of apoptotic cells determined by microscopic evaluation of minimum 200 cells from 4 independent experiments. Values given here are mean \pm SEM * p < 0.05

3.1.8 Infection with *A. phagocytophilum* stabilizes Mcl-1 by a PI3K/Akt dependent manner

Myeloid cell leukaemia-1 (Mcl-1) is a member of the anti-apoptotic subgroup of the Bcl-2 protein family. Mcl-1 has a very short half life of 1-6 hours in granulocytes being subjected to proteasomal degradation (Edwards et al., 2004). Exposure of neutrophils to proinflammatory cytokines like GM-CSF or to various pathogenic microorganisms extended the life span of neutrophils by stabilizing the expression of Mcl-1 via a PI3K dependent manner (Derouet et al., 2004; Lindemans et al., 2006). Infection with *A. phagocytophilum* was shown to stabilize the expression of Mcl-1 in neutrophils (Choi et al., 2005). However, it has not been investigated which pathway is involved in the stabilization. To investigate if stabilization of Mcl-1 in *A. phagocytophilum* infected neutrophils occurs via the PI3K/Akt pathway, cells were pre-incubated with 25 μ M of the PI3K inhibitor LY294002 for 30 minutes and the expression of Mcl-1 was assessed by western blotting. The results show that the infection-mediated stabilization of Mcl-1 was abrogated after treatment with the PI3K inhibitor. Therefore, it can be concluded that infection with *A. phagocytophilum* stabilizes Mcl-1 in neutrophils via the PI3K/Akt pathway (Fig. 12).

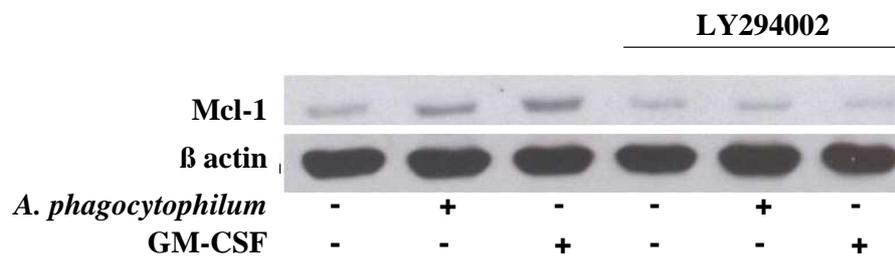


Fig. 12 Pretreatment with the PI3K inhibitor LY294002 abrogates the *A. phagocytophilum* induced stabilization of Mcl-1 in neutrophils. Neutrophils were cultivated with or without 25 μ M of the PI3K inhibitor LY294002 for 30 minutes and then incubated with or without *A. phagocytophilum* at 37 $^{\circ}$ C for 6 hours. Whole cell lysates were prepared and separated in 10 % denaturing SDS PAGE, electroblotted and probed with rabbit anti human Mcl-1 antibody. Equal loading was confirmed by reprobing the blot with rabbit anti human beta actin antibody. Representative picture from three independent repetitive experiments is shown. GM-CSF (5 ng/ml) was used as a positive control for Mcl-1 stabilization.

3.1.9 Infection with *A. phagocytophilum* induces the release of the pro-inflammatory cytokine IL-8 from neutrophils.

Upon stimulation with pro-inflammatory agents such as GM-CSF or after infection with various pathogens, neutrophils release a variety of pro-inflammatory cytokines. These cytokines can extend the life span of neutrophils in an autocrine manner (Dunican et al., 2000; van Zandbergen et al., 2004). Experiments were carried out to characterize the cytokine profile of *A. phagocytophilum* infected neutrophils. Neutrophils were co-incubated with or without *A. phagocytophilum* for 18 hours at 37 °C. A cytokine array, which can detect 36 cytokines simultaneously, was used to determine the cytokine profile in culture supernatants of infected neutrophils. Among others, the results show the presence of high level of IL-8 in the supernatant of infected neutrophils (Fig. 13A). Measurement of IL-8 by using ELISA confirmed the finding of the array experiment. The results show high levels (~80 ng/ml) of IL-8 in the culture supernatant of *A. phagocytophilum* infected neutrophils (Fig. 13B). This observation is in accordance with an earlier observation where it has been shown that the infection with *A. phagocytophilum* induces IL-8 (Choi et al., 2005).

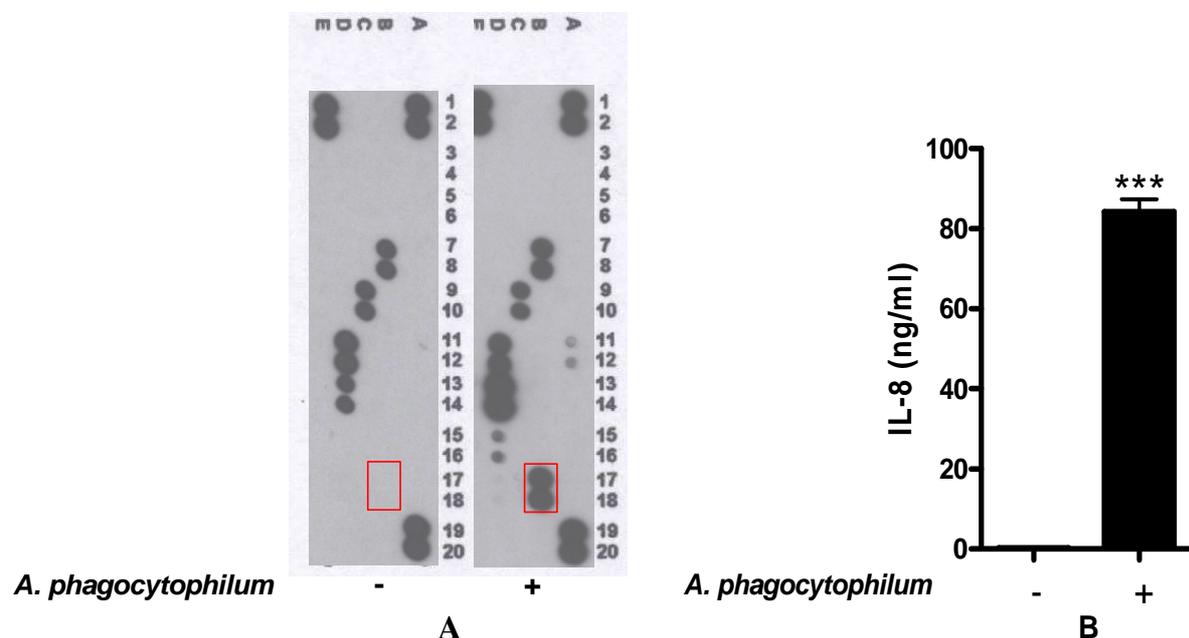


Fig. 13 Release of IL-8 from neutrophils after infection with *A. phagocytophilum*. Neutrophils were incubated with or without cell free *A. phagocytophilum* for 18 hrs. After incubation supernatants were collected and cytokine array was performed according to the manufacturer's instructions (A). B17 and B18 (red frames) represent IL-8. The IL-8 content in the supernatants was measured by ELISA from 3 independent experiments (B).

3.1.10 *A. phagocytophilum* induced IL-8 production depends on the activation of NF- κ B, PI3K/Akt, PKC δ , and ERK1/2 pathways

Interleukin-8 (IL-8) is a prototype chemotactic cytokine which attracts neutrophils. Infection with *A. phagocytophilum* induces high amount of IL-8 release. It has been documented that TNF-treated neutrophils release IL-8 in a NF- κ B dependent manner. A number of kinases have been reported to regulate IL-8 release via the NF- κ B dependent pathway (Jo et al., 2004; Kim et al., 2005; Profita et al., 2008). PI3K/Akt, ERK1/2, and PKC δ have been shown to regulate the activation of NF- κ B in neutrophils (Kilpatrick et al., 2006; Lindemans et al., 2006; Ozes et al., 1999; Wang et al., 2003). In order to investigate whether the *A. phagocytophilum* induced IL-8 production is regulated via the NF- κ B activation, an inhibitor approach was taken. Using the NF- κ B inhibitor BAY 11-7082, significant inhibition of IL-8 release was observed in *A. phagocytophilum* infected neutrophils (Fig. 14). In addition, LY294002, U0126 and rottlerin, inhibitors for PI3K, MEK1/2 and PKC δ , respectively, also inhibited significantly the IL-8 release from *A. phagocytophilum* infected neutrophils (Fig. 14).

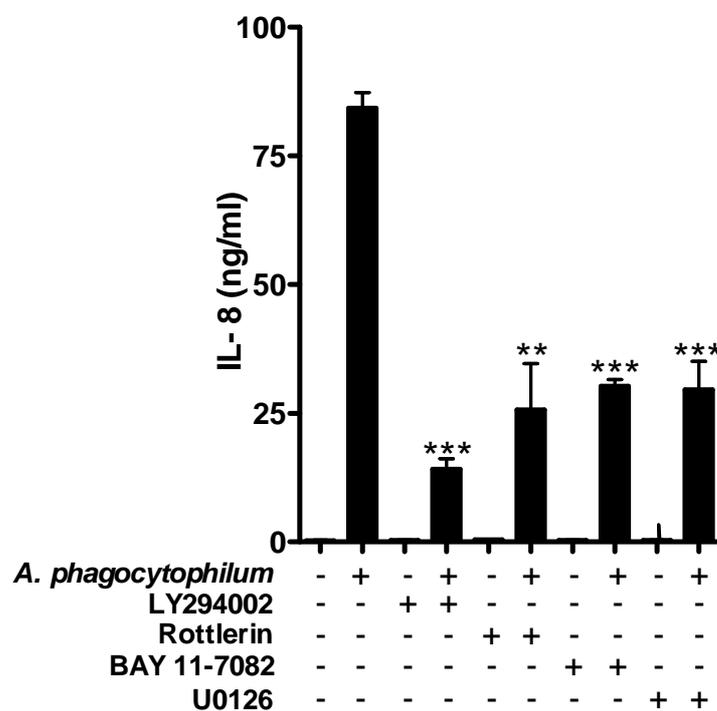


Fig. 14 IL-8 production depends on the activation of PI3K/Akt, ERK1/2, PKC δ and NF- κ B pathways in *A. phagocytophilum* infected neutrophils. Neutrophils (5×10^6 /ml) were preincubated with pharmacological inhibitors of PI3K (LY294002, 25 μ M), MEK1/2 (U0126, 10 μ M), PKC δ (rottlerin, 10 μ M), NF- κ B (BAY 11-7082, 0.5 μ M) for 30 minutes. Cells were then incubated with cell free *A. phagocytophilum* for another 18 hours. Supernatants were then collected and IL-8 was measured by ELISA. Values given here are mean \pm SEM from three independent experiments, ** $p < 0.005$, *** $p < 0.0005$, as compared to IL-8 release of *A. phagocytophilum* infected neutrophils.

3.1.11 Supernatants of *A. phagocytophilum* infected neutrophils delay the spontaneous apoptosis of neutrophils

At inflammatory foci neutrophil granulocytes come in contact with a wide range of pro-inflammatory cytokines like GM-CSF and IL-8. Both GM-CSF and IL-8 have been reported to extend the life span of neutrophils (Kettritz et al., 1998; Klein et al., 2000). Since IL-8 has been shown to delay the apoptosis (Kettritz et al., 1998) and a high amount of IL-8 was detected in the culture supernatants of *A. phagocytophilum* infected neutrophil granulocytes (Fig), experiments were performed to test whether culture supernatants have any effect on neutrophil apoptosis. Supernatants from *A. phagocytophilum* infected neutrophil granulocytes were collected after 18 hours. Freshly isolated granulocytes were incubated in the presence of these supernatants for 12 hours. Apoptosis was measured by using annexin-V and PI staining. The results show a significant inhibition of apoptosis in the presence of supernatants as compared to the cells incubated in medium only (Fig. 15A). Although IL-8 has been reported to delay neutrophils apoptosis, I investigated whether the concentration of IL-8 which is present in the culture supernatant was sufficient to delay the apoptosis programme of granulocytes. 100 ng/ml of recombinant IL-8 led to significant apoptosis inhibition of neutrophils (Fig. 15B).

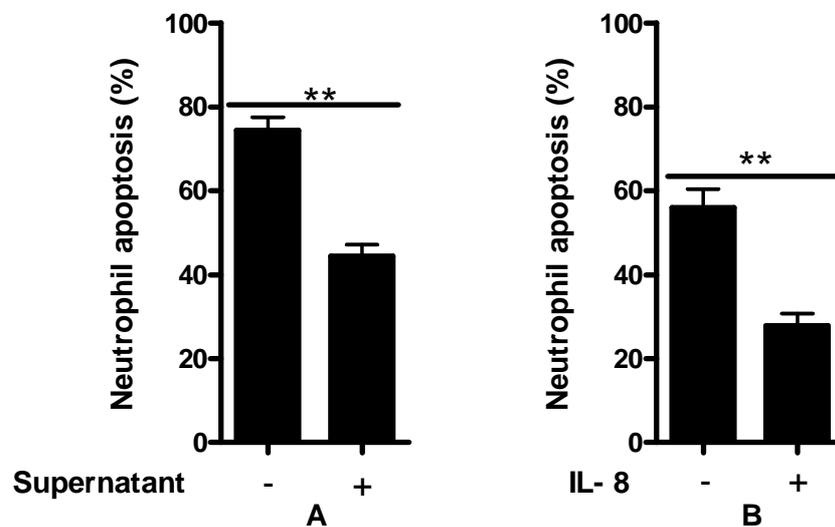


Fig. 15 Inhibition of neutrophil apoptosis by culture supernatant of *A. phagocytophilum* infected neutrophils and by recombinant IL-8. Neutrophils were incubated with *A. phagocytophilum* for 18 hours; supernatant was collected, centrifuged at 20,000 x g for 10 minutes twice and used to incubate freshly isolated neutrophils for 12 hours at 37 °C (A). Neutrophils were incubated with or without recombinant IL-8 (100 ng/ml) for 12 hours (B) at 37 °C. Rate of apoptosis was determined by annexin V and PI staining as detailed in the material and methods section. Data represented here is the percentage of apoptotic cells (annexin positive but PI negative cells were considered only) from 3 independent experiments. Values given here are mean \pm SEM, **p<0.005

3.2 Co-incubation with *Chlamydia pneumoniae* infects neutrophil granulocytes and delays their constitutive apoptosis

In previous studies from our group it was shown that the obligate intracellular Gram negative bacterial pathogen *Chlamydia pneumoniae* infects granulocytes and delays their spontaneous apoptosis (van Zandbergen et al., 2004). However, the mechanism of *C. pneumoniae* induced apoptosis delay of neutrophils remained unclear. I intended to study the molecular mechanism how this pathogen delays the spontaneous apoptosis. Neutrophils were incubated with or without *Chlamydia pneumoniae* for 18 hours and apoptosis rate were determined morphologically. The results show a clear inhibition of apoptosis in neutrophils co-incubated with *C. pneumoniae* as compared to neutrophils incubated in medium alone (Fig. 16E). In order to check whether *Chlamydia* infects neutrophils, an immunofluorescence staining was carried out using a Chlamydia staining kit. Results of the immunofluorescence staining have revealed brightly stained *C. pneumoniae* in neutrophils. This proves that incubation of *C. pneumoniae* infects neutrophils. Representative pictures of immunofluorescence (Fig 16A, B) and Diff-Quick[®] stained (Fig 16C, D) non infected (Fig 16A, C) and infected (Fig 16B, D) neutrophils are shown.

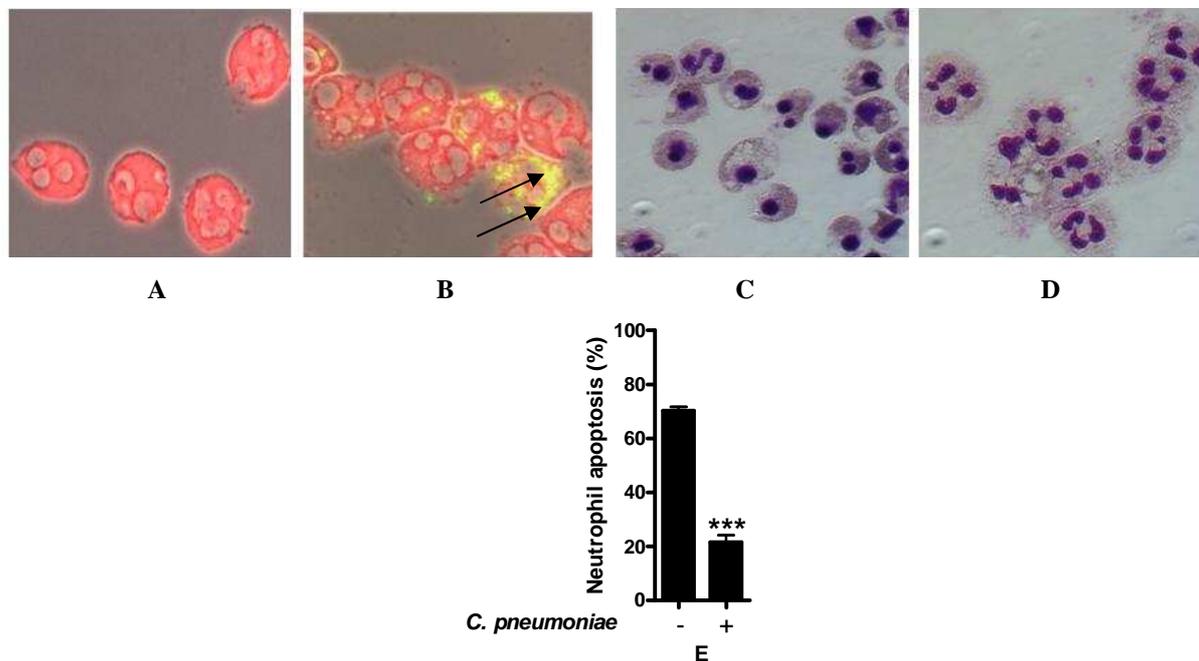


Fig. 16 *C pneumoniae* infects neutrophils and delays their spontaneous apoptosis. Neutrophils were incubated with or without *C. pneumoniae* for 18 hours. Cells were harvested, cytospin preparates were stained either by Diff-Quick[®] (C, D) or used for immunocytochemical staining for *C. pneumoniae* (A, B) as detailed in the materials and methods section. Representative pictures of both immunocytochemical and Diff-Quick[®] stained non-infected (A, C) and infected (B, D) cells are shown. Immunofluorescence staining revealed brightly stained *C. pneumoniae* pointed out by arrow in the infected cells (B). Percentage of apoptotic cells shown here (E) obtained from microscopic counting of a minimum of 200 cells in 4 independent experiments. Values are given in mean \pm SEM. *** $p < 0.0005$

3.2.1 Infection of neutrophils with *Chlamydia pneumoniae* upregulates the phosphorylation of Akt

The serine threonine kinase Akt is considered to be the cell signalling hub because of its versatile functions. Among other functions it plays a critical role in controlling cell survival (Manning and Cantley, 2007). Akt can extend the life span of cells by directly regulating various downstream effector proteins. For instance, Akt prevents the pro-apoptotic function of BAD (member of Bcl-2 family) by phosphorylating at the ser¹³⁶ residue. This event enhances the release of the BAD protein from its original Bcl-X_L bound mitochondrial membrane association to the cytoplasm where it binds the adapter protein 14-3-3 (Datta et al., 1997). Akt has also been shown to control mitochondrial apoptosis by preventing cytochrome C release (Kennedy et al., 1999; Whitlock et al., 2000) and by processing caspase-9 (Jeong et al., 2008). Since the life span of neutrophils was extended after infection with *Chlamydia pneumoniae* I aimed to investigate whether the infection activates Akt signalling in neutrophils to achieve this delaying effect. To address this question I performed western blotting from whole cell lysates of neutrophils incubated with or without *C. pneumoniae* and checked the phosphorylation status of Akt. The results show a marked enhancement of phosphorylation of Akt in neutrophils incubated with *C. pneumoniae* (Fig. 17).

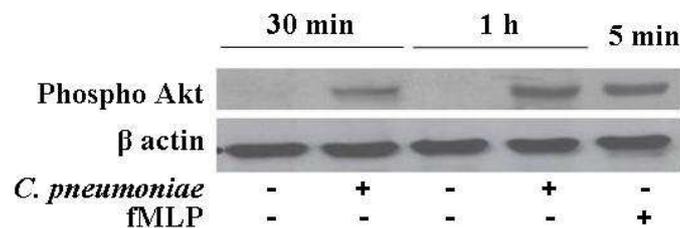


Fig. 17 Infection with *C. pneumoniae* upregulates the phosphorylation of Akt in neutrophils. Neutrophils were cultured with or without *C. pneumoniae* for the indicated time points and western blot analysis was performed from whole cell lysates. Proteins were separated in 10% SDS PAGE, electroblotted and probed with rabbit anti human Akt antibody which detects Akt only when phosphorylated at Ser⁴⁷³ site. Equal loading was shown by stripping and reprobing the blot with rabbit anti human beta actin antibody. The blot shown here is representative of four independent experiments. Incubation with 1 μM fMLP for 5 minutes was used in the experiment as a positive control.

3.2.2 Infection of neutrophils with *Chlamydia pneumoniae* upregulates the phosphorylation of ERK1/2

Mammalian cells contain five distinct families of mitogen activated protein kinases (MAPK), namely extracellular signal regulated kinses (ERK1 and ERK2), p38 MAPK (p38α, p38β, p38γ and p38δ), JUN N-terminal kinases (JNK1, JNK2 and JNK3), ERK3/4 and ERK5 (Qi and Elion, 2005). ERK1 and ERK2 have been found to regulate various cellular functions including survival (Jeong et al., 2008; Subramanian and Shaha, 2007). ERK can regulate apoptosis at different levels upstream or downstream of mitochondria as delineated in

previous studies (Gardai et al., 2004; Perianayagam et al., 2004). Activation of ERK1/2 has been reported to regulate neutrophil apoptosis in different pathological conditions or after exposure to mediators such as GM-CSF (Derouet et al., 2004; Iwase et al., 2006). As infection with *Chlamydia* delays the spontaneous apoptosis in neutrophils, I aimed to investigate whether activation of ERK1/2 is involved in the delaying effect of apoptosis on neutrophils. Western blot analysis of whole cell lysates was carried out to assess the phosphorylation status of ERK1/2. The results show a strong upregulation of ERK1/2 phosphorylation in *C. pneumoniae* infected neutrophils compared to the cells incubated in medium alone (Fig. 18).

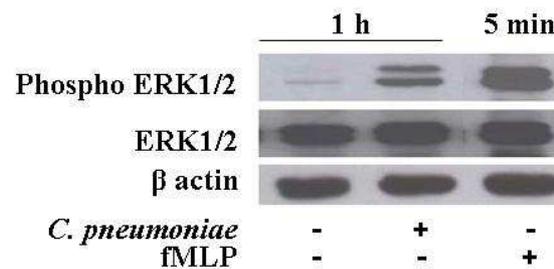


Fig. 18 Infection with *C. pneumoniae* enhances the phosphorylation of ERK1/2 in neutrophils. Neutrophils were incubated with or without *C. pneumoniae* for 1 hour and phosphorylation of ERK1/2 was assessed by western blot analysis of whole cell lysates probed with a rabbit anti human ERK1/2 antibody which detects ERK1/2 only when phosphorylated. Equal loading was confirmed by stripping the blot and reprobing with a rabbit anti human antibody which detects both the phosphorylated and non-phosphorylated ERK1/2 first and then with an anti β -actin antibody. A representative blot of 3 independent experiments is shown. Incubation with 1 μ M fMLP for 5 minutes was used in the experiments as a positive control.

3.2.3 Infection of neutrophils with *C. pneumoniae* enhances the phosphorylation of p38 MAPK

p38 MAPK, a member of the MAPK family, exerts both anti-apoptotic (Alvarado-Kristensson et al., 2004; Choi et al., 2005) and pro-apoptotic effects in different cell types including neutrophils (Alvarado-Kristensson et al., 2002; Aoshiba et al., 1999). Cell fate depends upon the tight balance between the levels of ERK1/2 and p38 MAPK (Suh, 2002). Since ERK1/2 has been observed to be upregulated in *C. pneumoniae* infected neutrophils I addressed the question whether p38 MAPK is activated after infection. Activation of p38 MAPK was investigated by assessing the phosphorylation of p38 MAPK by western blotting. The results show enhanced phosphorylation of p38 MAPK in infected cells after 30 minutes and 1 hour of infection as compared to cells incubated in medium alone.

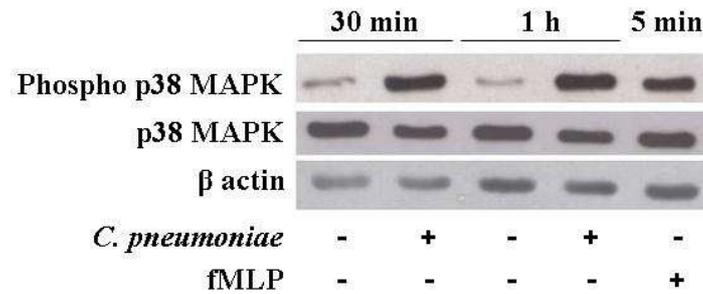


Fig. 19 Infection with *C. pneumoniae* enhances the phosphorylation of p38 MAPK in neutrophils. Neutrophils were incubated with or without *C. pneumoniae* for the indicated time points and phosphorylation of p38 MAPK was assessed by western blot analysis of whole cell lysates probed with a rabbit anti human p38 MAPK antibody which detects p38 MAPK only when phosphorylated. Equal loading was confirmed by stripping the blot and reprobing with a rabbit anti human p38 MAPK antibody which detects both phosphorylated and non-phosphorylated p38 MAPK and then with an anti human β -actin antibody. A representative blot of 3 independent experiments is shown here. Incubation with 1 μ M fMLP for 5 minutes was used in the experiments as a positive control for p38 MAPK phosphorylation.

3.2.3 Inhibition of PI3K/Akt and ERK1/2 but not p38 MAPK and PKC δ signalling reverses the *Chlamydia* induced apoptosis delay in neutrophils

Signalling via the PI3K/Akt axis is considered to be the major survival signal in several cell types including neutrophils. It has been shown that activation of PI3k/Akt as well as ERK1/2 results in extended life span of neutrophils. Although activation of PI3K/Akt and ERK1/2 pathway was observed in *C. pneumoniae* infected neutrophils (Fig. 20) it has not been clarified whether these pathways indeed have any effect on delaying of apoptosis in *Chlamydia* infected neutrophils. In order to decipher whether these pathways are important in exerting *Chlamydia* induced delaying of neutrophil apoptosis, an inhibitor approach was taken. Cells were pre-incubated with LY294002, U0126, SB 203580 and rottlerin, the inhibitors of PI3K, MEK1/2, p38 MAPK, PKC δ , respectively, for 30 minutes or left untreated and subsequently incubated with or without *C. pneumoniae* for 12 hours at 37 °C. Apoptosis rate was assessed by annexin-V and PI staining. Treatment with the PI3K inhibitor LY294002 (Fig. 20A) and with the MEK1/2 (upstream kinase of ERK1/2) inhibitor U0126 (Fig. 20B) reversed the *C. pneumoniae* induced apoptosis delay in neutrophils. However, the delaying effect was not altered when the cells were pretreated with the p38 MAPK inhibitor SB203580 (Fig. 20C) or with the PKC δ inhibitor rottlerin (Fig. 20D). The inhibitors alone had no effect on the rate constitutive apoptosis of neutrophil granulocytes (Fig. 20 A-D). These results suggest that infection with *C. pneumoniae* exerts the apoptosis delay in neutrophils by modulating PI3K/Akt and ERK1/2 pathways.

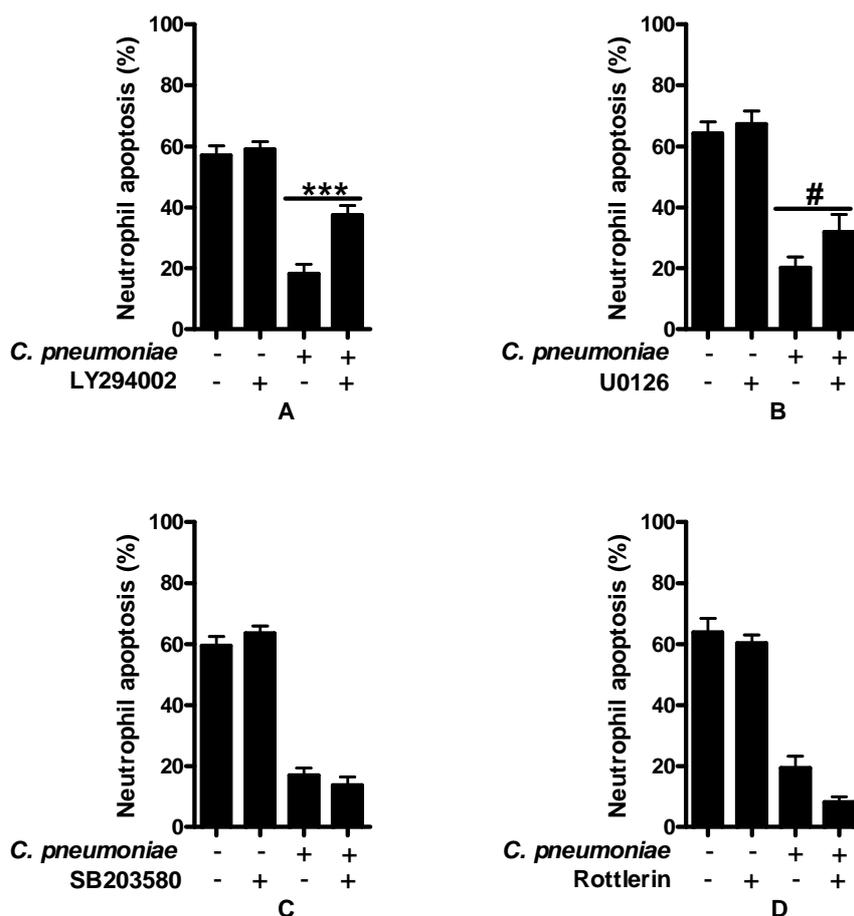


Fig. 20 Inhibitors of PI3K and MEK 1/2 but not of p38 MAPK and PKC δ reverse the *C. pneumoniae* induced anti-apoptotic effect in neutrophils. Neutrophils ($5 \times 10^6/\text{ml}$) were incubated with or without *C. pneumoniae* in the presence or absence of the PI3K inhibitor LY294002 (25 μM) (A) or the MEK1/2 inhibitor U0126 (10 μM) (B) or the p38 MAPK inhibitor SB203580 (10 μM) (C) or the PKC δ inhibitor rottlerin (10 μM) (D) at 37 $^\circ\text{C}$ in a 5% CO_2 atmosphere for 12 hours. Cells were harvested and annexin-V and PI staining were carried out to assess neutrophil apoptosis. Data presented here is the percentage of apoptotic cells (annexin positive but PI negative cells) from 8 independent experiments. Values given here are mean \pm SEM, *** $p < 0.0005$, # $p < 0.05$

3.2.4 Infection with *C. pneumoniae* stabilizes Mcl-1 expression via the PI3K/Akt pathway

Mcl-1, an antiapoptotic protein of the Bcl-2 protein family with a very short half life, undergoes rapid proteasomal degradation. It has been revealed that Mcl-1 contains characteristic protein sequences (PEST sequence) found among various unstable proteins which is considered as a probable reason for its short life span (Akgul, 2008). Mcl-1 prevents cytochrome C release from the mitochondria by directly interacting with pro-apoptotic members of the Bcl-2 family (Akgul, 2008). Rapid degradation of Mcl-1 has been observed in neutrophils. However exposure to various pro-inflammatory mediators and infections with certain intracellular pathogens extend the neutrophil life span by stabilizing Mcl-1 (Bouchard

et al., 2004; Choi et al., 2005; Leuenroth et al., 2000). Moreover, Mcl-1 has been reported to be a critical factor for prevention of apoptosis in *Chlamydia trachomatis* infected Hela cells (Rajalingam et al., 2008). All this background information intrigued me to investigate whether stabilization of Mcl-1 occurs in *C. pneumoniae* infected neutrophils and whether the PI3K/Akt pathway plays any role in the stabilization of Mcl-1. The question was addressed by western blot analysis to detect Mcl-1 in whole cell lysates of neutrophils incubated with or without *C. pneumoniae*. To investigate the role of PI3K/Akt, the PI3K inhibitor LY294002 was used in the experiments. Western blotting revealed that Mcl-1 is stabilized in infected neutrophils (Fig. 21). However, no Mcl-1 stabilization was observed after treatment of 25 μ M of PI3K inhibitor LY294002 (Fig 21). Therefore, it can be concluded that infection with *C. pneumoniae* stabilizes the Mcl-1 in neutrophils via the PI3K/Akt pathway.

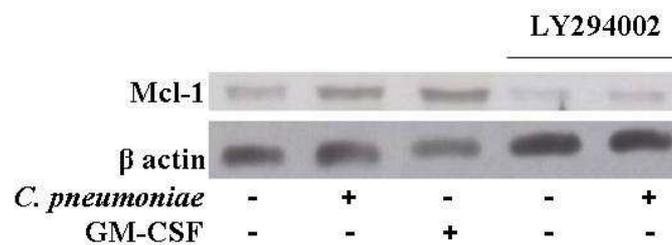


Fig. 21 Infection with *C. pneumoniae* results in Mcl-1 stabilization in neutrophils in a PI3K-dependent manner. Neutrophils were incubated for 6 hours with or without *C. pneumoniae*, either pretreated with the PI3K inhibitor LY294002 for 30 minutes or left untreated and whole cell lysates were prepared, electroblotted and probed with a rabbit anti human Mcl-1 antibody. Equal loading was confirmed by stripping and reprobing the blot with a rabbit anti human beta actin antibody. A representative blot from three independent experiments is shown here.

3.2.5 Infection with *C. pneumoniae* activates NF- κ B in neutrophils via the PI3K/Akt pathway

Nuclear factor kappa B (NF- κ B) has been shown to regulate neutrophil apoptosis. For instance, under hypoxic conditions or at high temperature the life span of neutrophils have been shown to be extended via NF- κ B (Kettritz et al., 2006; Walmsley et al., 2005). The NF- κ B pathway was shown to play a role in the apoptosis delay after exposure of neutrophils to inflammatory mediators like TNF α , IFN β or after infection with intracellular pathogens (Cowburn et al., 2004; Lindemans et al., 2006; Wang et al., 2003). On the other hand it has been reported that inhibition of NF- κ B accelerates neutrophil apoptosis (Choi et al., 2003). Moreover, activation of NF- κ B has been found to be responsible for apoptosis resistance in *Chlamydia pneumoniae* infected epithelial cells (Paland et al., 2006). Based on this background information, I aimed to investigate NF- κ B activation in *C pneumoniae* infected neutrophils. Since the PI3K/Akt pathway was found activated (Fig. 17) and PI3K/Akt is known to activate NF- κ B (Lindemans et al., 2006), I also wanted to investigate whether the

PI3K/Akt pathway is required for the regulation of NF- κ B activation in *C. pneumoniae* infected neutrophils. I addressed both questions by performing western blot experiment for phosphorylated I κ B using whole cell lysates of neutrophils incubated with or without *C. pneumoniae* with or without the PI3K inhibitor LY294002. The results show a marked upregulation of I κ B phosphorylation in *C. pneumoniae* infected neutrophils. This effect was abrogated after treatment with the PI3K inhibitor (Fig. 22).

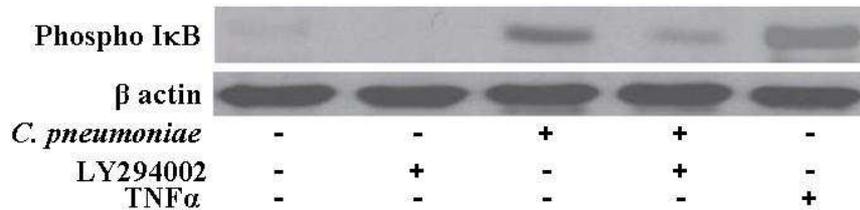


Fig. 22 Pretreatment with PI3K inhibitor LY294002 abrogates the *C. pneumoniae* mediated activation of NF- κ B in neutrophils. Neutrophils were incubated with or without *C. pneumoniae*, either pretreated with the PI3K inhibitor LY294002 for 30 minutes or left untreated and whole cell lysates were prepared. Proteins were separated in 10% SDS PAGE, electroblotted and probed with a mouse anti human I κ B antibody which detects I κ B only when phosphorylated. Equal loading was confirmed by stripping and reprobing the blot with rabbit anti human beta actin antibody. A representative blot from three independent experiments is shown. TNF was used as positive control for NF- κ B activation.

3.2.6 The *C. pneumoniae* induced apoptosis delay of neutrophils is NF- κ B dependent

Previous studies clearly indicate that activation of NF- κ B in neutrophils exert an anti apoptotic effect (Cowburn et al., 2004; Lindemans et al., 2006). I have also found that NF- κ B is activated in neutrophils after infection with *C. pneumoniae* (Fig. 22). However, it is not known whether activation of NF- κ B is essential for *C. pneumoniae* induced neutrophil survival. In order to delineate this, BAY 11-7082, a pharmacological inhibitor of I κ B phosphorylation, was used. Briefly, neutrophils were treated with 0.5 μ M of BAY 11-7082 for 30 minutes or left untreated and subsequently incubated with or without *C. pneumoniae* for 12 hours. The apoptosis rate of neutrophils was determined by using annexin-V and PI staining. It was observed that treatment with I κ B inhibitor partially but significantly reversed the *C. pneumoniae* induced apoptosis delay (Fig. 23). Therefore, the result suggests that activation of NF- κ B plays a role in apoptosis delay in *C. pneumoniae* infected neutrophils.

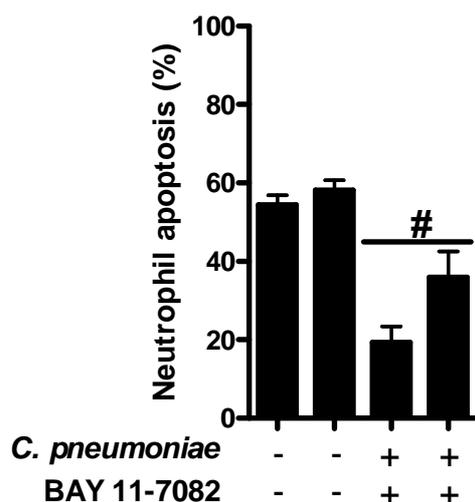


Fig. 23 Inhibition of I κ B phosphorylation partially reverses *C. pneumoniae* induced delaying of apoptosis in neutrophils. Neutrophils (5×10^6 /ml) were pretreated with 0.5 μ M of the I κ B inhibitor BAY 11-7082 for 30 minutes or left untreated. Subsequently the cells were incubated with or without *C. pneumoniae* for 12 hours at 37 °C. Cells were then harvested and the rate of apoptotic cells was determined by annexin-V and PI staining. Data presented here is the percentage of apoptotic cells (annexin positive but PI negative cells were considered only) from 3 independent experiments. Values given here are mean \pm SEM, # $p < 0.05$.

3.2.7 Infection with *C. pneumoniae* induces IL-6 and IL-8 release from neutrophil granulocytes

Neutrophils release a variety of pro-inflammatory cytokines upon exposure to stimuli such as GM-CSF, G-CSF or upon infection with several intracellular pathogens. Infection with respiratory syncytial virus induces IL-6 release from neutrophils (Arnold et al., 1994; Lindemans et al., 2006). Stimulation with GM-CSF has been shown to induce IL-6 release from neutrophils (Cicco et al., 1990). Recently IL-6 was shown to inhibit the apoptosis of neutrophils and other cell types (Asensi et al., 2004; Kuo et al., 2001; Lindemans et al., 2006; Wei et al., 2001).

Another chemokine, IL-8, is also secreted by neutrophils. Upon stimulation with pro-inflammatory cytokines, and bacterial products (LPS) or after infection with intracellular pathogens IL-8 is released from an intracellular pool. Recently it has been shown that infection with *C. pneumoniae* also induces IL-8 release from neutrophil granulocytes (van Zandbergen et al., 2004). It has been well documented that exposure to pro-inflammatory cytokines like IL-8, GM-CSF or G-CSF delays the apoptosis of neutrophils (Kettritz et al., 1998; Klein et al., 2000; Maianski et al., 2004). All this background information intrigued me to investigate the cytokine profile of *C. pneumoniae* infected neutrophils early (6-18 hours) after infection.

In order to analyse the cytokine profile of neutrophil supernatants, a cytokine array assay was carried out which can detect 36 different cytokine simultaneously. The assay revealed the presence of high amount of IL-6 in the supernatants of *C. pneumoniae* infected neutrophils as compared to the non infected neutrophils (Fig. 24A). The array results showed also the presence of IL-8 in the supernatants. Quantitative determination of IL-6 and IL-8 by ELISA confirmed the results of the array experiments showing the presence of high amounts of both cytokines in the supernatants of *C. pneumoniae* infected neutrophils (Fig. 24B, 24C).

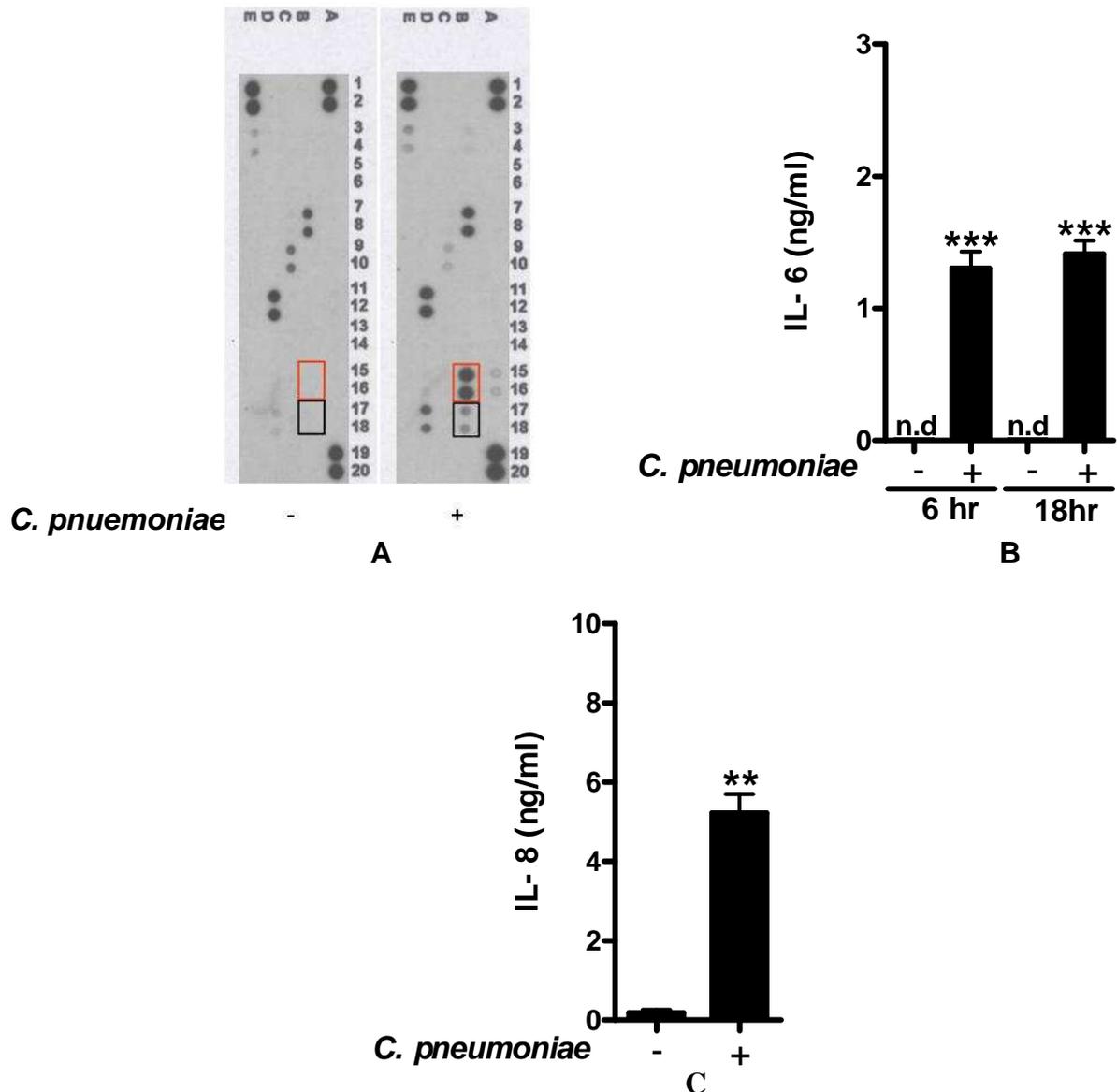


Fig.24 Release of IL-6 and IL-8 from neutrophils after infection with *C. pneumoniae*. Neutrophils were incubated with or without *C. pneumoniae* at 37 °C for the indicated time points and supernatants were collected. Cytokine array was performed according to the manufacturer's instructions using the supernatants (A). B15 and B16 (red rectangles) represent IL-6 and B17 and B18 (black rectangles) represent IL-8. The IL-6 and IL-8 contents in the supernatants were measured by ELISA (B, C respectively). (n.d: not detectable). Values given here are mean \pm SEM from three independent experiments ** $p < 0.005$, *** $p < 0.0005$.

3.2.8 The *C. pneumoniae* induced IL-8 production depends on the activation of the NF- κ B, PI3K/Akt and ERK1/2 pathways

Previously it was reported that TNF α treatment induces IL-8 release from neutrophils in a NF- κ B dependent manner (Cowburn et al., 2004). PI3K/Akt and ERK1/2 were shown to regulate NF- κ B activation in neutrophils (Kilpatrick et al., 2006; Ozes et al., 1999; Wang et al., 2003). Experiments with pharmacological inhibitors were carried out to elucidate which signalling pathways are involved in the *C. pneumoniae*-induced IL-8 release by neutrophils. Using the NF- κ B inhibitors BAY 11-7082 and MG-132, a significant reduction in IL-8 production was observed. Similarly, inhibition of PI3K and ERK1/2 with LY294002 and U0126, respectively, also significantly reduced the *C. pneumoniae*-induced IL-8 release (Fig. 25)

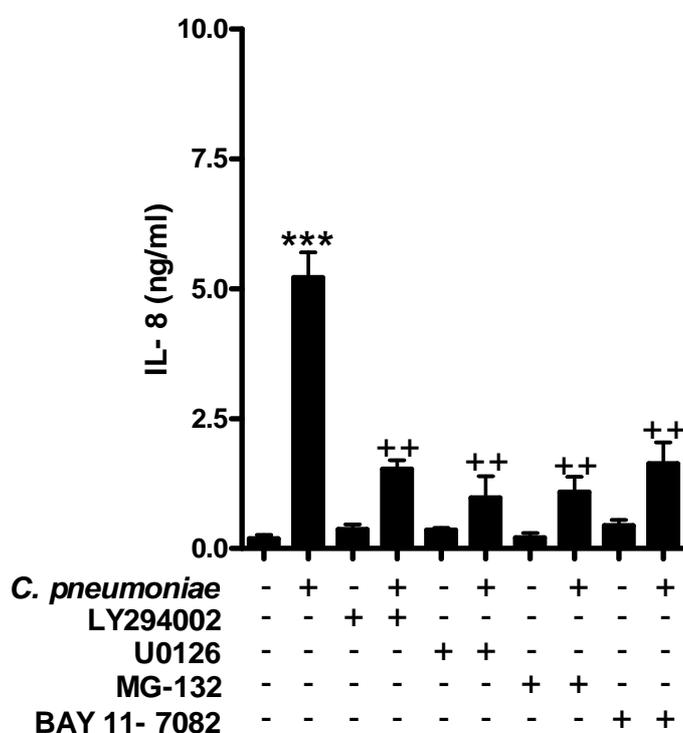


Fig. 24 IL-8 production depends on the activation of PI3K/Akt, ERK1/2 and NF- κ B pathways in *C. pneumoniae* infected neutrophils. Neutrophils (5×10^6 /ml) were preincubated with or without pharmacological inhibitors of PI3K (LY294002, 25 μ M), MEK1/2 (U0126, 10 μ M), NF- κ B (BAY 11-7082, 0.5 μ M and MG-132, 10 μ M) for 30 minutes. Cells were then incubated with *C. pneumoniae* for 18 hours. Supernatants were then collected and IL-8 was measured by ELISA. Values given here are mean \pm SEM from three independent experiments. *** $p < 0.0005$ as compared to the neutrophil incubated in the medium alone. ++ $p < 0.005$ as compared to IL-8 release of *C. pneumoniae* infected neutrophils

3.2.9 Incubation of neutrophils with culture supernatant of *Chlamydia pneumoniae* infected neutrophils delays the apoptosis

Exposure to pro-inflammatory cytokines results in the delay of constitutive apoptosis of neutrophil granulocytes. Infection of neutrophils with *C. pneumoniae* was reported to release

high amounts (~50-100 ng/ml) of IL-8 at a later time point (66 hours) of infection (van Zandbergen et al., 2004). It has been also demonstrated that supernatants of *Chlamydia* infected neutrophils collected 66 hours after infection could inhibit the neutrophil spontaneous apoptosis and this inhibition was mediated by IL-8 present in the supernatants (van Zandbergen et al., 2004). However, it has not been investigated whether supernatants of *C. pneumoniae* infected neutrophils early (6-18 hours) after infection has the potential to inhibit neutrophil apoptosis. Since high amounts of IL-6 was found in early 6 hours and IL-6 have been reported to inhibit neutrophils apoptosis (Lindemans et al., 2006) therefore it is important to know whether supernatants of *C. pneumoniae* infected neutrophils early after 6-18 hours does also play any role in inhibiting the neutrophil apoptosis. Experiments were carried out by incubating neutrophil granulocytes with supernatants of *C. pneumoniae* infected neutrophil collected 6 and 18 hours after infection. The apoptosis rate was assessed by using annexin-V and PI staining. Neutrophils incubated in the culture supernatant showed significantly reduced apoptosis compared to the cells incubated in medium only (Fig. 26).

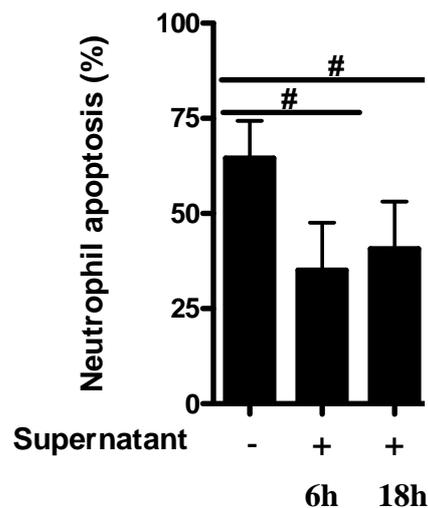


Fig.26 Culture supernatants of *C. pneumoniae* infected neutrophils delay neutrophil apoptosis. Neutrophils were incubated with *C. pneumoniae* for 6 and 18 hours, supernatant was collected, centrifuged at 20000 x g for 10 minutes twice and used to incubate freshly isolated neutrophils for 12 hours at 37 °C. The rate of apoptosis was determined by annexin-V and PI staining. Data presented here is the percentage of apoptotic cells (annexin-V positive but PI negative cells were considered only) from 3 independent experiments. Values given here are mean \pm SEM, #p<0.05 (Two way ANOVA with Bonferoni post tests).

3.2.10 Infection with *C. pneumoniae* leads to reduced surface expression of FAS (CD95) in neutrophils

A characteristic feature of neutrophils is their constitutive apoptosis. The complex process of apoptosis can be initiated via death receptor signaling where ligation of death receptors is considered to be the crucial step. This is called the extrinsic pathway of apoptosis. On the

other hand apoptosis could be triggered by perturbation of intracellular homeostasis which is called the intrinsic pathway of apoptosis. Both pathways converge at the executional caspase-3 signalling and finally lead to apoptosis (Ferri and Kroemer, 2001). Recently it has been shown that infection with *C. pneumoniae* delays the spontaneous apoptosis of neutrophils (van Zandbergen et al., 2004). Since neutrophils express enhanced levels of FAS on their cell surface during apoptosis and FAS expression is an important step in their spontaneous apoptosis (Daigle and Simon, 2001; Ge and Rikihisa, 2006; Scheel-Toellner et al., 2004), I investigated whether infection with *C. pneumoniae* affects FAS expression on neutrophils. FAS expression was analysed by flow cytometric analysis using an anti FAS antibody conjugated with RPE. The results show a significantly lower expression of FAS in *C. pneumoniae* infected neutrophils compared to the control where cells were incubated in medium alone (Fig. 27). Therefore, results show that apart from modulating intrinsic pathway of apoptosis, infection with *C. pneumoniae* also extends the life span of host cell neutrophil modulating extrinsic pathway of apoptosis.

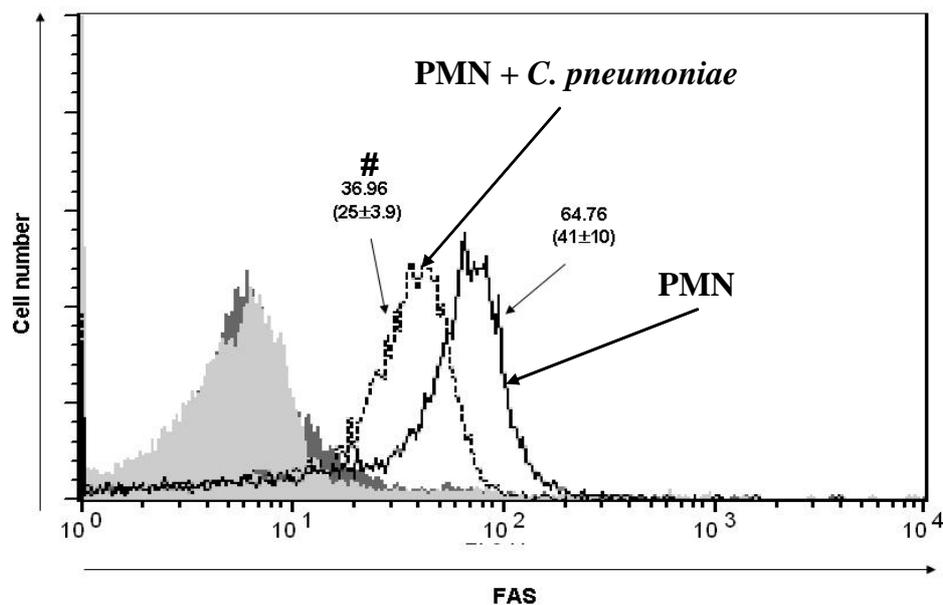


Fig. 27 Infection with *C. pneumoniae* decreases surface expression of FAS on neutrophils Neutrophils were incubated with or without *C. pneumoniae* for 16 hours and expression of FAS was assessed by staining the cells with RPE conjugated anti CD95 antibody. Staining with isotype control antibody for both uninfected and infected neutrophils are given by light and dark histograms respectively. The histograms and mean fluorescence intensities are from one representative experiment. In the brackets mean \pm SEM of the mean fluorescence intensities of three experiments are given. # $p < 0.05$

3.3 *Leishmania. major* promastigotes infect neutrophil granulocytes and delay their spontaneous apoptosis

As described above, infection on neutrophils with the two bacterial pathogens *A. phagocytophilum* and *C. pneumoniae* leads to apoptosis delay and, as a consequence, to extended life span of these otherwise short-living cells. In addition, it has been shown by our group that the protozoan parasite *L. major* can infect neutrophils which also leads to apoptosis delay (Aga et al., 2002; Laufs et al., 2002). However, the molecular mechanism of this apoptosis delay is still unclear. I aimed to study which molecular signalling mechanisms are modulated in neutrophils by *L. major* in order to prevent apoptosis. Neutrophils were infected with *L. major* promastigotes for 18 hours and the rate of apoptosis was assessed. The results showed a marked inhibition of neutrophil apoptosis after infection with *L. major* promastigotes (Fig. 28A, B, and D). Cytopsin prepares of neutrophils revealed morphological intact intracellular parasites in neutrophils (Fig. 28C). These results confirm former findings that *L. major* infects neutrophils and delays their apoptosis.

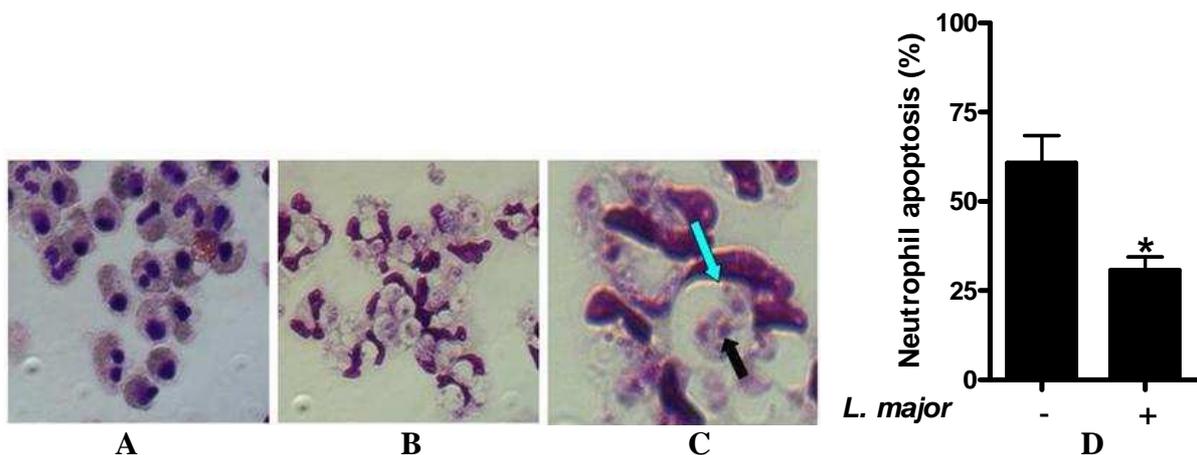


Fig. 28 *L. major* promastigotes infect neutrophil granulocytes and delay their apoptosis. Neutrophils were incubated with or without *L. major* promastigotes for 18 hours at 37 °C. Cells were then stained with Diff-Quick®. Representative pictures of non-infected (A) and infected cells (B, C) are shown. Black and blue arrows show nuclear and kinetoplast DNA of the parasite, respectively. The rate of apoptotic cells (D) was determined by microscopic counting of minimum 200 cells from 3 independent experiments. Values are given as mean \pm SEM, * $p < 0.05$

3.3.1 Infection with *L. major* does not lead to phosphorylation of PI3K and Akt in neutrophils

Activation of PI3K/Akt is a pivotal pathway and a major cell survival signal as Akt phosphorylates a variety of substrates which directly interact with the apoptosis cascade. Activation of Akt depends on the phosphorylation of PI3K and upon activation it inhibits the apoptosis by modulating various targets such as BAD, FKHR and caspase-9 (Brunet et al., 1999; Datta et al., 1997; Perianayagam et al., 2004). Akt has been reported to inhibit the

cytochrome C release which is one of the crucial steps in the intrinsic pathway of apoptosis (Kennedy et al., 1999). Since infection with *L. major* delays neutrophils apoptosis, I aimed to investigate whether *L. major* infection activates PI3K/Akt in neutrophils by applying western blot analysis. No enhancement of phosphorylation of either PI3K (Fig. 29A) or Akt (Fig. 29B) was observed in *L. major* infected neutrophils.

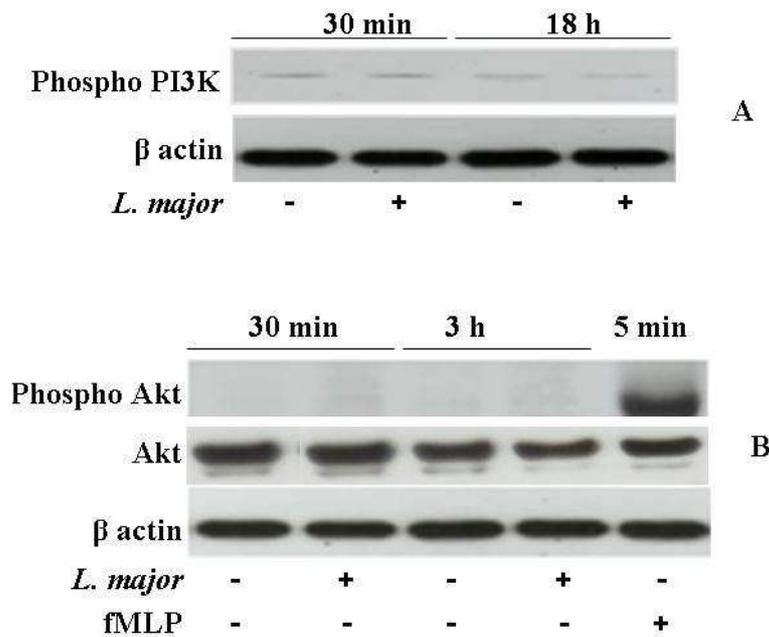


Fig. 29 Infection with *L. major* does not lead to the phosphorylation of PI3K and Akt in neutrophils. Neutrophils were incubated with or without *L. major* for the indicated time points and the phosphorylation of PI3K and Akt was assessed by performing western blot analysis with a rabbit anti human PI3K antibody which detects the p85 regulatory subunit of PI3K only when phosphorylated (A) or with a rabbit anti human Akt antibody which detects Akt only when phosphorylated at Ser⁴⁷³ site (B). Equal loading was confirmed by stripping and reprobing the blots with a rabbit anti human beta actin antibody. A representative blot of 3 independent experiments is shown. For the Akt blot equal loading was further confirmed by probing with a rabbit anti human Akt which detects both phosphorylated and non phosphorylated Akt. Incubation with 1 μM fMLP for 5 minutes was used in the experiment as a positive control for the phosphorylation of Akt

3.3.2 Infection with *L. major* upregulates the phosphorylation p38 MAPK and ERK1/2 in neutrophils

p38 MAPK and ERK1/2 have been reported to act as anti-apoptotic signals in neutrophils. Exposure to various mediators or bacterial constituents such as GM-CSF or LPS activates these kinases in neutrophils (Coxon et al., 2003; Guo et al., 2006; McLeish et al., 1998). Activation of p38 MAPK has been observed in *A. phagocytophilum* infected neutrophils

where the pathogen delays the apoptosis via this pathway (Choi et al., 2005). Activation of ERK1/2 has been shown to regulate neutrophil apoptosis by regulating phosphorylation of BAD (Perianayagam et al., 2004). Since infection with *L. major* extends the life span of neutrophils, I investigated whether the infection activates the members of MAPKs to employ its anti-apoptotic effect. The question was addressed by performing western blot analysis of whole cell lysates of neutrophils incubated with or without *L. major* for 30 minutes. The results show a rapid and strong enhancement of phosphorylation of both p38 MAPK (Fig. 30A), and ERK1/2 after infection (Fig 30B).

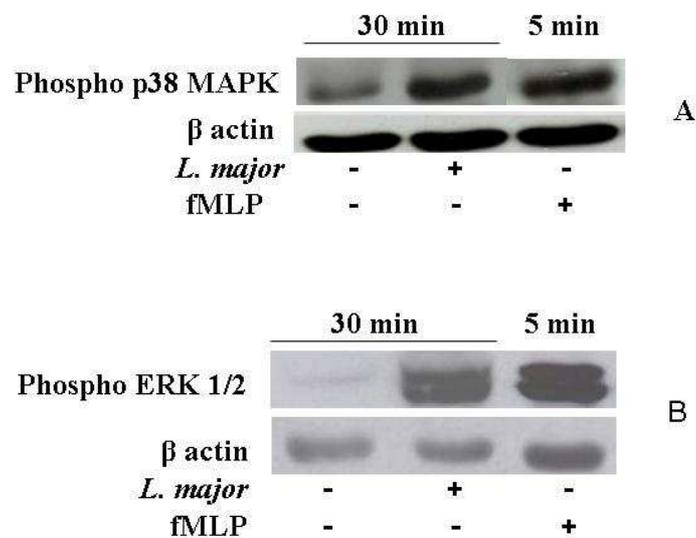


Fig. 30 Infection with *L. major* enhances the phosphorylation of p38 MAPK and ERK1/2 in neutrophils. Neutrophils were incubated with or without *L. major* for 30 minutes and phosphorylation of p38 MAPK and ERK1/2 was assessed by performing western blot analysis probed either with a rabbit anti human p38 MAPK antibody or with a rabbit anti human ERK1/2 antibody which detects p38 MAPK (A) and ERK1/2 (B) respectively only when phosphorylated. Equal loading was confirmed by stripping and reprobing the blot with a rabbit anti human beta actin antibody. A representative blot of 3 independent experiments is shown.

3.3.3 Inhibition of ERK1/2 reverses the *L. major* induced apoptosis delay in neutrophils

Upregulation of ERK1/2 was observed in *L. major* infected neutrophils (Fig. 30B). ERK1/2 has been shown to regulate apoptosis by several pathways (Gardai et al., 2004; Perianayagam et al., 2004). Activation of ERK1/2 has also been reported to delay neutrophil spontaneous apoptosis which suggests the ERK1/2 plays crucial role in regulating neutrophil apoptosis (Derouet et al., 2004). In order to decipher whether activation of ERK1/2 observed in *L. major* infected neutrophils plays any role in apoptosis delay, an inhibitor approach was taken. Cells were pre-incubated with the MEK1/2 (upstream kinase of ERK1/2) specific inhibitor U0126 for 30 minutes and subsequently incubated with *L. major* for 18 hours. Rate of apoptosis was

assessed by flow cytometry after staining with annexin V and PI. Treatment with the MEK1/2 inhibitor reversed the *L. major*-induced apoptosis delay in neutrophils. (Fig. 31)

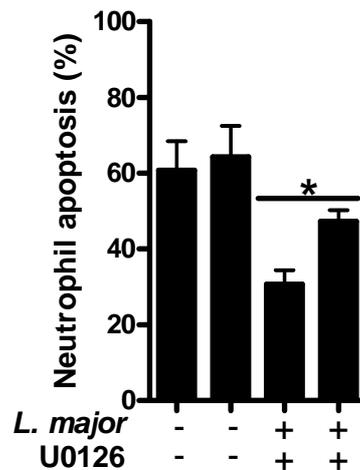


Fig. 31 Treatment with the MEK1/2 inhibitor U0126 prevents *L. major*-mediated apoptosis inhibition in neutrophils. Neutrophils ($5 \times 10^6/\text{ml}$) were incubated with or without *L. major* in the presence or absence of the MEK 1/2 inhibitor U0126 ($10 \mu\text{M}$). Cells were harvested after 18 hours and the rate of apoptosis was assessed by annexin V and PI staining from 3 independent experiments. Values given here are mean \pm SEM. * $p < 0.05$

3.3.4 Inhibition of p38 MAPK does not reverse the *L. major* induced apoptosis delay in neutrophils

As described above (Fig.30A), infection with *L. major* leads to activation of p38 MAPK in neutrophils. However, p38 MAPK activation does not appear to play a role in the *L. major* mediated apoptosis delay, since the infection-mediated apoptosis delay was not reversed when the cells were pretreated with the p38 MAPK inhibitor SB203580 (Fig. 32).

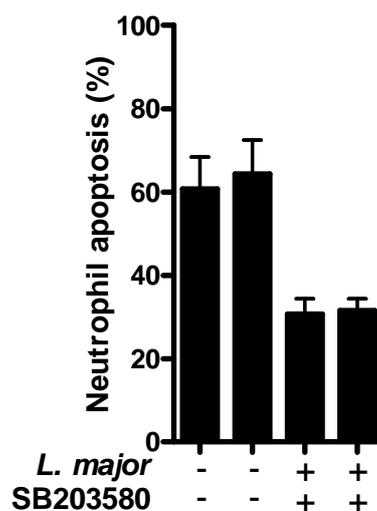


Fig. 32 Treatment with the p38 MAPK inhibitor SB203580 does not prevent the *L. major*-mediated apoptosis inhibition in neutrophils. Neutrophils ($5 \times 10^6/\text{ml}$) were incubated with or without *L. major* in the presence or absence of the p38 MAPK inhibitor SB203580 ($10 \mu\text{M}$). Cells were harvested after 18 hours and the rate of apoptosis was assessed by annexin V and PI staining from 3 independent experiments. Values given here are mean \pm SEM.

4 Discussion.

Spontaneous apoptosis is the characteristic feature of neutrophil granulocytes. Infection with *Anaplasma phagocytophilum*, *Chlamydia pneumoniae* and *Leishmania major* modulates this spontaneous apoptosis and extends the life span of neutrophils (Aga et al., 2002; Choi et al., 2005; Ge and Rikihisa, 2006; van Zandbergen et al., 2004). Although it has been shown that all these intracellular pathogens modulate the spontaneous apoptosis of granulocytes the molecular mechanism of this modulation is still unclear.

Here in this study I addressed the question how all these pathogens modulate the apoptotic process of granulocytes. From the result it is clear that both *A. phagocytophilum* and *C. pneumoniae* activate the PI3K/Akt and the ERK1/2 pathway in order to achieve apoptosis inhibition. However, infection with *L. major* does not activate the PI3K/Akt pathway instead activates ERK1/2 and p38 MAPK. Common feature of all these three pathogens is that they modulate the death receptor mediated extrinsic pathway since all of them decrease the FAS expression as is revealed from my results and some former observations of our group.

Neutrophils are the most abundant cell type among circulating leukocytes in the body which constitute the first line of defence against the invading pathogens. They are recruited to the infected tissue in high number where their function is to engulf, kill and finally digest the pathogens. The striking feature of this cell type is that they have a very short half life and die within 10-24 hours after leaving the bone marrow. This feature of spontaneous apoptosis has been selected in course of evolution which prevents the release of toxic materials from neutrophils into the surrounding tissue thereby minimize the chance of unwanted inflammation. However, a number of pathogens have evolved elegant strategies to survive within the intracellular milieu of neutrophil granulocytes. Inhibition of apoptosis of infected cells appears to be a common mechanism of intracellular pathogens to secure the longevity of their host cell. Intracellular pathogens such as *Trypanosoma cruzi*, *Toxoplasma gondii*, *Coxiella burnetii* inhibit their host cell apoptosis (Nakajima-Shimada et al., 2000; Voth et al., 2007; Vutova et al., 2007).

Two major pathways have been reported so far by which apoptosis could be initiated namely intrinsic or mitochondrial apoptosis and extrinsic or death receptor mediated apoptosis. During intrinsic pathway of apoptosis integrity of the mitochondrial membrane is lost which results into release of cytochrome C into the cytosol. In subsequent steps the released cytochrome C oligomerizes with Apaf-1 and forms the apoptosome complex. The apoptosome complex recruits seven dimers of active caspase-9 which subsequently activates caspase-3 and finally leads to apoptosis (Acehan et al., 2002; Budihardjo et al., 1999; Yuan

and Yankner, 2000). This intrinsic process of apoptosis is tightly regulated by proteins of the Bcl-2 protein family. This protein family consist of both pro-apoptotic (BAX, BID, etc.) and anti-apoptotic (Mcl-1, A1, etc.) members. On the other hand extrinsic apoptosis is initiated at the cell surface with the ligation of FAS–FAS ligand or activation of death receptors which leads to the processing of pro-caspase-8 which then directly activates the downstream executioner caspase-3 and results into apoptosis. Both of the pathways converge at the point of caspase-3. Several survival signals have been shown to perturb the apoptotic pathways by various mechanisms. Signalling via PI3K/Akt or ERK1/2 has been shown to delay apoptosis by stabilizing the anti-apoptotic factor like Mcl-1 which in turn inhibits the release of cytochrome C (Derouet et al., 2004; Rajalingam et al., 2008).

Infection with *A. phagocytophilum* and *C. pneumoniae* was reported to activate the PI3K/Akt and ERK1/2 in neutrophils and inhibition of the PI3K/Akt and the ERK1/2 pathways attenuated the anti-apoptotic effect exerted by these two pathogens. Moreover it was also found that both of the pathogens stabilized the Mcl-1 level via the PI3K/Akt pathway. Therefore it seems that both PI3K/Akt and ERK1/2 pathways are responsible for modulation of intrinsic pathway of apoptosis in *A. phagocytophilum* and *C. pneumoniae* infected neutrophils. Results revealed the activation of p38 MAPK in *C. pneumoniae* infected neutrophils. However, using the p38 MAPK inhibitor SB203580 no increase in apoptosis rate was observed in *C. pneumoniae* infected neutrophil.

Neutrophil granulocytes are the first line of defence against invading pathogens. Constitutive apoptosis is an inherent character of this cell type which is an important cellular event for maintaining the neutrophil number in infection and inflammation. Modulation of this process by intracellular pathogens could lead to the inflammation and tissue damage. Therefore it is important to know in details about the molecular mechanisms how these pathogens modulate this process. Here I tried to address the probable mechanisms by which intracellular pathogens *A. phagocytophilum*, *C. pneumoniae* and *L. major* delay their host cell neutrophil apoptosis.

4.1 Modulation of neutrophils apoptosis by the intracellular pathogen *A. phagocytophilum*

Apoptosis is the final outcome of cell death programme which is an essential strategy for the control of dynamic balance in living systems. It is known till date that there are two major pathways by which apoptosis could be initiated namely 1) intrinsic or mitochondrial and 2) extrinsic or death receptor mediated. Both pathways involve sequential activation of Caspases in two distinct but converging pathways. However, both pathways are tightly regulated by a

variety of survival signals. PI3K/Akt and MAPK are two survival signalling pathways known so far which interact with the apoptotic cascade (Fig. 2). Spontaneous apoptosis is typical feature of neutrophil granulocytes. However, a number of intracellular pathogens have been found to infect and survive within neutrophils and delay their apoptosis (Aga et al., 2002; Choi et al., 2005; van Zandbergen et al., 2004). In this study I have observed that infection with *A. phagocytophilum* delays the spontaneous apoptosis of neutrophil which is in concordance with earlier observations (Choi et al., 2005; Ge and Rikihisa, 2006). Earlier studies concerning the *Anaplasma* infection and neutrophil apoptosis were focused mainly towards unravelling the apoptotic cascade (Ge and Rikihisa, 2006; Ge et al., 2005). It has been shown that infection with *A. phagocytophilum* stabilizes the Mcl-1 in neutrophils (Choi et al., 2005). Only few studies addressed the survival signals involved in the apoptotic inhibition. However, a previous gene expression study shows enhanced expression of PI3K, Akt, PKC δ and NF- κ B in *A. phagocytophilum* infected neutrophils (Lee and Goodman, 2006). Nothing has been studied so far whether infections with *A. phagocytophilum* engage the PI3K/Akt and MAPK signalling pathway in neutrophils. In this study I showed that infection with *A. phagocytophilum* activates the expression of PI3K in neutrophils. Enhanced phosphorylation of PI3K was observed early hours of infection with *Anaplasma* and maintained till 18 hours. This suggests that the PI3K/Akt pathway is involved in delaying of apoptosis in *A. phagocytophilum* infected neutrophils. Upon activation, PI3K phosphorylates phosphatidyl inositol (PtdIns) substrates which produce the second messengers PtdIns(3,4,5)P₃ from PtdIns(3,4)P₂. These messengers recruit and activate downstream kinases such as Akt and PDK1.

PDK1 is present downstream of PI3K which is crucial in PI3K/Akt mediated signalling pathway. Phosphorylation of PDK1 was observed early after infection and was maintained for 22 hours. Akt is present downstream of PI3K and PDK1, activated upon phosphorylation by PDK1. Transient phosphorylation of Akt was noticed in *A. phagocytophilum* infected neutrophil as early as 30 minutes after infection. Enhanced phosphorylation of both PI3K and Akt was observed which suggest that PI3K/Akt pathway is involved in exerting apoptosis delay in *A. phagocytophilum* infected neutrophils. Blocking with the PI3K specific inhibitor LY294002 reversed the *A. phagocytophilum* induced apoptosis delay which further indicates that PI3K/Akt plays a crucial role in apoptosis delay in *Anaplasma* infected neutrophils. Activation of Akt has been observed in other intracellular infection such as *Salmonella* infected epithelial cells prevents apoptosis activating Akt (Knodler et al., 2005).

Akt is considered to be the central regulator which functions in cell proliferation, growth, and survival as well. Akt prevents apoptosis by interacting with various molecules of the apoptosis cascade such as Mcl-1. Mcl-1 is one of the important anti-apoptotic members of the Bcl-2 protein family which prevents the release of cytochrome C. Stabilization of Mcl-1 is essential to prevent cytochrome C release. It has been shown that infection with *A. phagocytophilum* stabilizes the Mcl-1 in neutrophils (Choi et al., 2005). Here I showed for the first time that stabilization of Mcl-1 in *A. phagocytophilum* infected neutrophils occurs via the PI3K/Akt pathway. Activation of PI3K/Akt pathway seems to be essential for preventing the apoptosis in *A. phagocytophilum* infected neutrophils.

Akt exerts its anti-apoptotic effect by targeting a variety of molecules in the apoptotic cascade. In addition, Akt has been shown to regulate activation of NF- κ B by upregulating the phosphorylation of I κ B complex (Mansell et al., 2001; Ozes et al., 1999; Romashkova and Makarov, 1999). I have shown that infection with *A. phagocytophilum* activates NF- κ B in neutrophils and inhibition of PI3K with LY294002 partially prevented this activation. These results indicate that activation of NF- κ B in *A. phagocytophilum* infected neutrophils is PI3K/Akt dependent. Activation of NF- κ B has been shown to induce expression of various pro-survival genes in other infection models (Binnicker et al., 2004; Yanai et al., 2003; Zhang et al., 2004).

Apart from stabilizing Mcl-1, Akt regulates the phosphorylation of BAD at the serine 136 residue by disrupting the heterodimeric association of BAD with Mcl-1 and thus prevents cytochrome C release (Rane and Klein, 2009). Since it has been found that both PI3K and Akt are activated in *A. phagocytophilum* infected neutrophils, it is plausible to hypothesize that activated Akt phosphorylates BAD and prevents the apoptosis in *A. phagocytophilum* infected neutrophils. Although the experiments to investigate whether activation of Akt indeed phosphorylates BAD were not carried out, further experiments could provide the answer. This is the first report where I show that infection with *A. phagocytophilum* activates the PI3K/Akt pathway in neutrophils which is beneficial for the pathogen for their own survival.

Apart from Akt, other substrate of PDK1 is PKC δ . PKC δ also plays a crucial role in neutrophil apoptosis. Activation of PKC δ is characterised by the translocation of PKC δ . In this study I have observed significant translocation of PKC δ from the cytosol to the plasma membrane in *A. phagocytophilum* infected neutrophils. Moreover blocking with the PKC δ specific inhibitor rottlerin infection induced apoptosis delay in *A. phagocytophilum* infected neutrophil was reversed. This strongly suggests that infection with *A. phagocytophilum* inhibits the apoptosis activating PKC δ via the PI3K-PDK1 pathway. Previous studies show

that treatment with type I IFN or TNF α delays the spontaneous apoptosis of neutrophils by activating PKC δ (Kilpatrick et al., 2006; Wang et al., 2003) which further supports my findings that PKC δ could play a major role in the apoptosis delay in *A. phagocytophilum* infected neutrophils.

It might create confusion to the readers that both Akt and PKC δ are substrates of PDK1 and both get activated in *A. phagocytophilum* infected neutrophils. However, it has to be noted that only a transient activation of Akt was observed. The phosphorylation of Akt observed after 30 minutes was completely diminished after 3 hours. In contrary, both of the upstream molecules of Akt; PI3K and PDK1, were still activated after 18 and 22 hours respectively. Activation of PKC δ was also observed at 3 hours time point.

Therefore results clearly suggest that infection with *A. phagocytophilum* activates PI3K in neutrophil which in turn activates the downstream molecule PDK1. PDK1 initially activates Akt which exert apoptosis inhibition of *A. phagocytophilum* infected neutrophils early after infection but at later time points inhibition of apoptosis is mediated at least partially via PKC δ .

Transient activation of Akt was observed in *A. phagocytophilum* infected neutrophils. However, since Akt is considered as a typical signalling hub which regulates a number of targets in the apoptotic cascade to achieve apoptosis inhibition therefore it is plausible to believe that the transient phosphorylation of Akt could be sufficient to exert its anti-apoptotic effect.

As described previously, in addition to PI3K/Akt, MAPK pathway is another survival signalling pathway. ERK1/2 and p38 MAPK are two important members of MAPK family. ERK1/2 prevents apoptosis by regulating Bcl-2 family proteins. Enhanced phosphorylation of ERK1/2 was observed early (30 min) after infection with *A. phagocytophilum* in neutrophils. However, using the MEK1/2 inhibitor, U0126, only a marginal increase of apoptosis was observed (data not shown). Another member of the MAPK family, p38 MAPK, also inhibits the apoptosis. Indeed p38 MAPK mediated apoptosis inhibition was shown in *A. phagocytophilum* infected neutrophils (Choi et al., 2005). However, using p38 MAPK specific inhibitor, SB203580; no increase in the apoptosis rate has been observed (data not shown). Therefore, MAPK pathway apparently does not seem to be responsible for exerting the apoptosis inhibition in *A. phagocytophilum* infected neutrophils.

Upon stimulation, neutrophil produce wide variety of cytokines including copious amount of IL-8 (Scapini et al., 2000). This chemokine attracts neutrophils to the site of infection. In the

present study significant amount of IL-8 (~80 ng/ml) was measured in supernants of *A. phagocytophilum* infected neutrophils after 18 hours. Co-incubation of neutrophils with 80 ng/ml of IL-8 inhibited the constitutive apoptosis (Fig. 15B). Moreover incubation with supernatants of *A. phagocytophilum* infected neutrophils markedly inhibited the spontaneous apoptosis of neutrophil granulocytes. Therefore, it seems that the delaying effect of apoptosis could be mediated at least partially via IL-8 produced by *A. phagocytophilum* infected neutrophils. Several reports also show that IL-8 delays spontaneous apoptosis of neutrophil granulocytes (Kettritz et al., 1998; van Zandbergen et al., 2004). Results are consistent with other finding where it has also been shown that IL-8 is released during infection with *A. phagocytophilum* infected neutrophils (Choi et al., 2005).

However, IL-8 depletion experiments will be necessary to prove the role of IL-8 in *A. phagocytophilum* infected neutrophils. In several infection models activation of NF- κ B has been observed (Binnicker et al., 2004; Yanai et al., 2003; Zhang et al., 2004). It has been shown that generation of inflammatory cytokines by neutrophils such as IL-8 is regulated by NF- κ B (Cloutier et al., 2007). A significant amount of IL-8 release was observed in *A. phagocytophilum* infected neutrophils. Moreover, infection resulted into activation of NF- κ B in neutrophils (Fig. 10). In the present study investigation was carried out to determine whether infection induced IL-8 generation is mediated by NF- κ B. By using inhibitor of NF- κ B pathway a significant inhibition of IL-8 production in *A. phagocytophilum* infected neutrophils was observed. This finding suggests that infection induced IL-8 production indeed mediated via NF- κ B.

Additionally, inhibitor of PI3K, PKC δ and inhibitor of ERK pathway also showed marked inhibition of IL-8 production from infected neutrophils. This suggests that all these kinases regulate the IL-8 release probably by regulating activation of NF- κ B. This is the first report showing clearly that activation of NF- κ B in *A. phagocytophilum* infected neutrophils is mediated via the activation of PI3K, PKC δ and ERK1/2.

The Gram negative bacterium *A. phagocytophilum* established exclusive tropism for neutrophil granulocytes. Neutrophils are short living cells and undergo constitutive apoptosis. However, this obligate pathogen modulates apoptotic signalling of the cell targeting both the intrinsic and the extrinsic pathways and also augments the release of the pro-inflammatory cytokine IL-8. Previously demonstrated that this pathogen prevents the apoptosis of neutrophils activating p38 MAPK pathway (Choi et al., 2005) but here in this study I clearly show that pathogen activates PI3K/Akt, and PKC δ pathways which stabilize Mcl-1, activate NF- κ B and thus regulate the release of IL-8. However, using p38 MAPK inhibitor,

SB203580, no increase in apoptosis rate was observed in infected neutrophils. The reason of discrepancy with former observation (Choi et al., 2005) is unclear. However, the functionality of the inhibitor for blocking p38 MAPK was tested by performing western blot in LPS (positive inducer of p38 MAPK) stimulated neutrophils (data not shown). Therefore from this result it can be concluded that p38 MAPK is not responsible for inhibition of apoptosis here in *A. phagocytophilum* infected neutrophils.

It is not yet clarified which molecules of the bacterium are responsible for recognition and binding to granulocytes. However, it has been reported that Msp2 or p44 protein is predominant on the bacterial surface which acts as a adhesion molecule for entering into the host cell granulocytes upon interacting with fucosylated platelet selectin glycoprotein ligand 1 (PSGL-1) present on the cell membrane (Park et al., 2003). PSGL-1 was shown to be responsible for inducing high amount of IL-8 in HGE infected neutrophils (Akkoyunlu et al., 2001). It is possible that PSGL-1 plays a profound role in activating downstream signalling via the PI3K/Akt pathway. A report about interactions between PSGL-1 and Akt activation in neutrophils (Pluskota et al., 2008b) supports the possibility that interactions of p44 protein and PSGL-1 induce PI3K/Akt activation in *A. phagocytophilum* infected neutrophils. In the present study it has been observed that infection induced IL-8 release was suppressed significantly when the pharmacological inhibitors of PI3K/Akt, PKC δ , MEK1/2 and NF- κ B were used. This suggests that IL-8 release is dependent on the activation of these pathways. It has also been postulated that enhanced production of IL-8 might be another strategy of efficient dissemination of bacterium into the newly arrived neutrophils since IL-8 also increases the phagocytic potential of neutrophils (Carlyon and Fikrig, 2003)

Taken together it can be concluded that infection with *A. phagocytophilum* activates the PI3K/Akt pathway possibly by interacting of p44 surface protein with PSGL-1 which stabilizes Mcl-1. Furthermore, the infection activates NF- κ B via PI3K/Akt which results in the release of IL-8. IL-8 has autocrine and / or paracrine effects in prolonging the lifespan of neutrophil granulocytes. In addition the infection also induces PKC δ and ERK1/2 which possibly delays the spontaneous apoptosis of *A. phagocytophilum* infected neutrophils. Recently ERK1/2 phosphorylation has been reported to inhibit the activation of caspase-8 and caspase-10 in neutrophils (Zhang et al., 2003a). Previously it was shown that *A. phagocytophilum* inhibits the caspase-8 activation in neutrophils (Ge and Rikihisa, 2006). Therefore indirectly it can be concluded that ERK1/2 regulates the caspase-8 activation in *A. phagocytophilum* infected neutrophils and thus delays the apoptosis.

4.2 Modulation of neutrophils apoptosis by the intracellular pathogen *C. pneumoniae*

Neutrophil granulocytes play a critical role by ingesting and killing invading pathogens. Neutrophils are short living cells with characteristic features of constitutive apoptosis. This feature of constitutive apoptosis is beneficial for limiting the propagation of intracellular pathogens. However, a number of intracellular pathogens prolong the lifespan of neutrophil by modulating the molecular mechanisms of death programme (Aga et al., 2002; Ge and Rikihisa, 2006). Intrinsic or extrinsic are the two major pathways known so far by which apoptosis could be initiated but both of the pathways converge at the point of caspase-3. However, the onset of initiation of either of these apoptotic pathways is regulated by two major survival signalling pathways namely PI3K/Akt and MAPK.

Co-incubation with *C. pneumoniae* delayed the spontaneous of apoptosis of granulocytes which supported the former report from our group that *C. pneumoniae* infects and delays the spontaneous apoptosis of granulocytes (van Zandbergen et al., 2004). However, it has not been investigated so far whether infection with *C. pneumoniae* engages the PI3K/Akt and MAPK signalling pathways in neutrophils in order to achieve the apoptosis delay.

Infection with *C. pneumoniae* enhanced the phosphorylation of Akt in neutrophils. Moreover, the use of the PI3K inhibitor LY294002 reversed the *C. pneumoniae* induced apoptosis delay. Since Akt is present downstream of PI3K, the above results clearly indicate that PI3K/Akt pathway is activated in *C. pneumoniae* infected neutrophils. Deactivation of Akt has been reported as an important factor in neutrophils spontaneous apoptosis (Zhu et al., 2006). Several intracellular pathogens have been shown to activate PI3K/Akt pathway and thus prevent the apoptosis in epithelial cells and J774 murine macrophages (Knodler et al., 2005; Mansell et al., 2001; Yilmaz et al., 2004).

It has been delineated that activation of Akt promotes cell survival by blocking the function of pro-apoptotic proteins and augmenting the pro-survival proteins of the Bcl-2 family. Mcl-1 is the member of Bcl-2 family which play an important role in regulation of apoptosis by preventing the release of cytochrome C. Due to rapid proteasomal degradation the half life of Mcl-1 is very short. In the present work it has been found that infection with *C. pneumoniae* stabilized the Mcl-1 expression in neutrophils. Furthermore, inhibition with the PI3K specific inhibitor LY294002 attenuated the Mcl-1 expression observed in *C. pneumoniae* infected neutrophils. These results suggest that activation of PI3K/Akt is required to delay the apoptosis in *C. pneumoniae* infected neutrophils. It has been evident from various previous

reports that activation of PI3K/Akt rescues and stabilizes the Mcl-1 (Derouet et al., 2004; Lindemans et al., 2006).

Apart from Mcl-1, Akt regulates a number of molecules such as the pro-apoptotic BAD. BAD, the BH3 only protein of Bcl-2 family, are directly phosphorylated by Akt at serine 136 residue which allows the BAD protein to remain bound with other adaptor protein 14-3-3 in the cytosol thereby enhances the lifespan as evident in BALB/c 3T3 and 293 cells (Datta et al., 1997). Activation of Akt was observed in *C. pneumoniae* infected neutrophils; therefore, it is possible that Akt phosphorylates BAD in *C. pneumoniae* infected neutrophils to achieve the apoptosis inhibition. However, further experiments should be carried out in order to investigate whether activation of Akt phosphorylates the BAD in *C. pneumoniae* infected neutrophils.

Here I show clearly that infection with *C. pneumoniae* activates the PI3K/Akt pathway which stabilizes the anti-apoptotic protein Mcl-1 in neutrophils which in turn prevents apoptosis.

Akt also regulates the activation of NF- κ B by regulating the phosphorylation of I κ B (Mansell et al., 2001; Ozes et al., 1999). Activation of NF- κ B results in the inhibition of the constitutive apoptosis of neutrophil granulocytes regulating the expression of various survival genes and release of pro-inflammatory cytokines. In the present study, activation of NF- κ B was observed in *C. pneumoniae* infected neutrophils. The activation was abrogated with the treatment of the PI3K inhibitor LY294002 suggesting that activation of NF- κ B is mediated via the PI3K/Akt pathway. Furthermore, inhibition studies with I κ B specific inhibitor BAY 11-7082 partially reversed the infection induced apoptosis delay in neutrophils. This data also supports the view that activation of NF- κ B is responsible at least partially for the apoptosis inhibition in *C. pneumoniae* infected neutrophils.

NF- κ B has been found to be activated via the PI3K/Akt pathway (Lindemans et al., 2006) which further supports my findings. Recently it has been shown that persistent infection with *Chlamydia pneumoniae* in epithelial cells require activation of NF- κ B (Paland et al., 2006).

PKC δ is downstream of PI3K and activated by PDK1, the intermediate kinase in the PI3K-PKC δ pathway. PKC δ plays an important role in the regulation of apoptosis as revealed from previous reports (Pongracz et al., 1999; Wang et al., 2003). Since in the present study transient activation of Akt was observed, I expected that activation of PKC δ might take place. An inhibitor approach was used to study the involvement of PKC δ in regulation of apoptosis in *C. pneumoniae* infected neutrophils. However, Since the PKC δ inhibitor rottlerin did not prevent apoptosis in *C. pneumoniae* infected cells the delaying of apoptosis in *Chlamydia* infected neutrophils is apparently independent of PKC δ activation. However, in order to

confirm the role of PKC δ further translocation experiments should be carried out. All my findings indicate that infection with *C. pneumoniae* activate PI3K/Akt survival pathway. It leads to the stabilization of Mcl-1 and to activation of NF- κ B in neutrophils.

Besides the PI3K/Akt pathway, MAPK pathway is another major survival signalling pathway. Results from this study revealed the activation of ERK1/2 as well as p38 MAPK in *C. pneumoniae* infected neutrophils indicating the involvement MAPK in infection induced apoptosis delay. However, using a p38 MAPK specific inhibitor I could not observe any prevention of apoptosis delay in *Chlamydia* infected cells. However, pre-treatment of neutrophils with the inhibitor of MEK1/2 reversed the pathogen induced apoptosis delay suggesting the role of ERK1/2 in *C. pneumoniae* induced apoptosis delay. ERK1/2 has been shown to prevent apoptosis by phosphorylating BAD at serine 136 position. Phosphorylated BAD prevents the mitochondrial leakage of cytochrome C and subsequently the activation of caspase cascade (Perianayagam et al., 2004). Activation of ERK1/2 also modulates the apoptosis by preventing the activation of caspase-8 (Pluskota et al., 2008a; Zhang et al., 2003a). The phosphorylation of BAD as well as activation caspase-8 was not studied in the present study. However, pronounced activation of ERK1/2 has been observed which suggests that ERK1/2 could probably phosphorylate BAD and prevents the activation of caspase-8. Further experiments could clarify whether activated ERK1/2 phosphorylates the BAD and prevents the activation of caspase-8 in *C. pneumoniae* infected neutrophils.

Western blot analysis of p38 MAPK revealed an increased phosphorylation of the kinase in *C. pneumoniae* infected neutrophils. However, p38 MAPK activation possibly does not contribute to survival effect on infected neutrophils since treatment a p38 MAPK inhibitor did not reverse apoptosis delay in infected neutrophils. Although activation of p38 MAPK was also observed in IL-18 treated neutrophils however, treatment with SB203580 did not reverse the apoptosis delay (Hirata et al., 2008). Therefore it seems that *C. pneumoniae* induced apoptosis delay in neutrophils does not depend on p38 MAPK activation. It is known that p38 MAPK has multiple targets such as MSK1 and MSK2 which regulates the phosphorylation of several factors such as CREB, ATF1 including the phosphorylation of NF- κ B isoform p65 (Roux and Blenis, 2004). Although both MSK1 and MSK2 are downstream targets of p38 MAPK however, these target molecules are also regulated by ERK1/2. Therefore, further experiments are necessary to delineate the anti-apoptotic role of p38 MAPK on *C. pneumoniae* infected neutrophils.

LPS has been shown to activate p38 MAPK in neutrophils (Choi et al., 2005). Since *C. pneumoniae* contains LPS therefore the initial activation of p38 MAPK in *C. pneumoniae* infected neutrophils might be the result of interaction with chlamydial LPS.

A previous report showed that p38 MAPK can inhibit the activation caspase-8 and therefore prevent apoptosis of the neutrophils (Alvarado-Kristensson et al., 2004). As I have not studied caspase-8 activation in the present study therefore I have no proof that activated p38 MAPK plays any role in regulation of caspase-8 in *C. pneumoniae* infected neutrophils.

Activation of NF- κ B has been observed in *C. pneumoniae* infected neutrophils. Since activation of NF- κ B is considered as the hall mark of TLR signalling, therefore I expected that TLR would be involved in exerting apoptosis delay in *C. pneumoniae* infected neutrophils. Moreover it was also shown that neutrophil apoptosis can be delayed by TLR agonists stabilizing Mcl-1 via the PI3K/Akt signalling pathway (Francois et al., 2005). However, using blocking anti TLR 4 and anti TLR 2 antibodies or by using TLR 4 antagonist, I did not observe any effect on the *C. pneumoniae* induced apoptosis delay (data not shown). This suggests that *C. pneumoniae* mediated activation of NF- κ B is independent of TLR 2 and TLR 4.

Activated neutrophils released variety of pro-inflammatory cytokines and the life span of neutrophils were extended *in vitro* in presence of cytokines such as GM-CSF, G-CSF, IL-8, and IL-1 β . In the present study, upon infection with *C. pneumoniae* neutrophils release high amount of IL-6. In a previous study it has been shown that infection with *Chlamydia* induces the IL-6 release (Rizzo et al., 2008). Infection with respiratory syncytial virus also induced high amounts of IL-6 release from neutrophils (Lindemans et al., 2006). IL-6 has been shown to delay neutrophils in several instances suggesting the autocrine or / paracrine effect of IL-6 on neutrophils (Asensi et al., 2004; Fanning et al., 1999; Lindemans et al., 2006; Matsuda et al., 2001; Raffaghello et al., 2008). However, in this present study using recombinant IL-6, I did not observe any inhibition of neutrophil apoptosis (data not shown). In a previous report it has also been shown that recombinant IL-6 alone does not delay neutrophil apoptosis (Parsonage et al., 2008). Although the reason of discrepancy is unknown, I think that a contaminating population of PBMC could play some role in extending the life span of neutrophils. This is assumed because purity of neutrophils was reported ~97% in reports showing that IL-6 prevents neutrophils apoptosis, but purity of neutrophils in my study was over 99.9%. From my results it can be suggested that IL-6 may not delay the apoptosis in *C. pneumoniae* infected neutrophils. However, further experiments should be carried out in order to clarify whether IL-6 can delay neutrophils apoptosis. One thing I want to mention that I

should be careful while discussing the IL-6 data. Because *Chlamydia pneumoniae* infected HEp2 cells also release high amount of IL-6 (Baltch et al., 2003) and in my experiments I have used *C. pneumoniae* infected HEp2 cells lysate. As I have not measured the IL-6 concentration in infected HEp2 cell lysates therefore I can not rule out the possibility that measured IL-6 was from infected HEp2 culture. It is likely to expect release of IL-6 from *Chlamydia pneumoniae* infected neutrophils since infection with *Chlamydia* induces significantly high amount of IL-6 release in other cell types such as HEp2 cells, fibroblasts (Baltch et al., 2003; Rizzo et al., 2008) and neutrophils have been shown to release IL-6 in other infection (Lindemans et al., 2006). Further experiments would be carried out to reveal the fact whether measured IL-6 were released indeed from neutrophils.

In addition to IL-6, infection with *C. pneumoniae* released significantly high amount of IL-8. This is in line with the former report from our group that infection with *C. pneumoniae* release high amount of IL-8 (van Zandbergen et al., 2004). Further experiments with NF- κ B inhibitor, BAY 11-7082 and MG-132 revealed significant inhibition of IL-8 production from *C. pneumoniae* infected neutrophils which clearly suggests that *C. pneumoniae* induced IL-8 release from neutrophils is mediated via NF- κ B activation. Experiments with the inhibitors of PI3K and MEK1/2 also showed marked inhibition of IL-8 production from infected neutrophils. This suggests that *C. pneumoniae* induced IL-8 production is dependent on activation of NF- κ B which is regulated by PI3K/Akt and ERK1/2 pathway.

The extrinsic pathway of apoptosis initiates at the cell surface after interaction of ligands with its appropriate receptors. Neutrophils are found to express constitutively high levels of FAS which upon ligation with FAS ligand activates caspase-8 subsequently caspase-3 and finally leads to apoptosis. Reports show during neutrophils spontaneous apoptosis FAS mediated activation of extrinsic pathway could take place independent of FAS ligation (Scheel-Toellner et al., 2004). My results revealed reduced expression of FAS on *C. pneumoniae* infected neutrophils indicate that infection affects also the extrinsic pathway of apoptosis.

The results suggest that infection of neutrophils with *C. pneumoniae* activates the PI3K/Akt pathway which in turn stabilizes the anti-apoptotic protein Mcl-1 and lead to the activation of NF- κ B. Additionally, infection induces also the ERK1/2 pathway which is partially responsible for delaying of apoptosis. Infection activates NF- κ B via PI3K/Akt and probably via ERK1/2 pathway results in the release of IL-8 which functions possibly in autocrine and / or paracrine manner and extends the life span of neutrophils. High amounts of IL-6 were also observed in culture supernatants of *C. pneumoniae* however, IL-6 does not seem to contribute any role in delaying of apoptosis in infected neutrophils.

4.3 Modulation of neutrophil apoptosis by the intracellular pathogen *L. major*

Apoptosis is a result of a complex death programme which could be initiated by two different executioner pathways such as the intrinsic or mitochondrial and the extrinsic or death receptor mediated pathways. These executioner pathways are regulated by various survival signals. Activation of these signals decides cell fate. There are two major survival signalling pathways: PI3K/Akt and MAPK.

Neutrophils are the short lived cells die within 10-24 hours after leaving the bone marrow. It was reported for the first time from our group that *Leishmania major*, causative agent for cutaneous leishmaniasis, infects neutrophils and inhibits their spontaneous apoptosis (Aga et al., 2002). It has been suggested that the parasite uses neutrophil granulocytes as 'Trojan horses' for silent entry into their final host macrophages (Laskay et al., 2003). Although it has been observed that infection with *L. major* modulates both the extrinsic as well as the intrinsic pathways of neutrophils apoptosis (unpublished data from our laboratory), it is still unclear which survival signals are involved in the *L. major* mediated apoptosis delay. Activation of PI3K/Akt has been observed in connection with apoptosis inhibition by number of intracellular pathogens such as *Salmonella*. Infection with *Salmonella* inhibits the camptothecin induced apoptosis induction in epithelial cells activating the PI3K/Akt pathway (Faherty and Maurelli, 2008). It has been observed that infection with the obligate intracellular Gram negative bacteria *A. phagocytophilum* and *C. pneumoniae* also activate the PI3K/Akt pathway by which the pathogens achieve the apoptosis inhibition (Fig. 5, 6 and 17). Treatment with various modulators like type I IFN, GM-CSF, TNF α inhibits apoptosis by activating the PI3K/Akt pathway in neutrophils (Cowburn et al., 2002; Cowburn et al., 2004; Wang et al., 2003). Although PI3K/Akt pathway is an important survival signal which regulates apoptosis, however, infection with *L. major* does not activate either PI3K or Akt in neutrophils (Fig. 29). Results hint that *L. donovani* induced apoptosis inhibition in neutrophils does not dependent on PI3K/Akt activation. However, these data need to be confirmed.

MAPK activation is another important survival signal which regulates initiation of apoptosis targeting various proteins of apoptotic cascade. In the present study activation of ERK1/2 has been observed in *L. major* infected neutrophils (Fig. 30B). Moreover, using the MEK1/2 inhibitor, U0126, *L. major* induced apoptosis inhibition was partially reversed (Fig. 31) which implies that activation of ERK1/2 is a crucial steps in apoptosis inhibition in *L. major* infected neutrophils.

ERK1/2 enhances the phosphorylation and subsequent degradation of the anti-apoptotic molecule, Mcl-1, thereby prevents the release of cytochrome C. In addition, ERK1/2 also regulates initiation of mitochondrial cascade phosphorylating and inactivating BAD.

Apart from regulating the intrinsic pathway of apoptosis ERK1/2 interferes with TNF α and FAS mediated apoptosis induction inhibiting caspase-8 as well as caspase-10 activation (Tran et al., 2001; Zhang et al., 2003b).

p38 MAPK plays an important role in several cellular functions including apoptosis. In my study, significant upregulation of p38 MAPK was observed in *L. major* infected neutrophils which indicate that p38 MAPK is involved in *L. major* induced apoptosis inhibition. However, using a p38 MAPK specific inhibitor the *L. major* induced apoptosis delay could not be reversed in infected neutrophils. It is known that p38 MAPK activity is critical in variety of cellular responses such inflammatory responses. p38 MAPK has been shown to phosphorylate number of cellular targets including MSK1 and MSK2 which in turn regulates the transcriptional activities. Both MSK1 and MSK2 have been reported to mediate NF- κ B dependent transcription through the phosphorylation of NF- κ B isoform p65 (Vermeulen et al., 2003). Although MSK1/2 is the down stream molecule of p38 MAPK, however, the activation of MSK1/2 depends on both ERK1/2 and p38 MAPK. Therefore it could be assumed that blocking of p38 MAPK was not sufficient enough to block MSK1/2 mediated NF- κ B activation. Simultaneous blocking with U0126 and SB203580, selective blocker for ERK1/2 and p38 MAPK could give the answer whether p38 MAPK is responsible in exerting apoptosis delay in *L. major* infected neutrophils. Activation of p38 MAPK directly phosphorylates and inactivates the function of caspase-8 and caspase-3, thereby prevents the apoptosis of neutrophil granulocytes (Alvarado-Kristensson et al., 2004). Previously it was shown that infection with *L. major* prevents the FAS induced apoptosis in neutrophils (unpublished data of our group) which means that the infection also interferes with the extrinsic pathway of apoptosis in neutrophils (unpublished data of our group). Since activation of p38 MAPK was observed in *L. major* infected neutrophils and knowing that p38 MAPK can interfere with the FAS mediated apoptosis therefore it could be assumed that activation of p38 MAPK inactivate caspase-8 as well as caspase-3 thereby prevents the FAS induced apoptosis. However, this subject has not been addressed experimentally so far.

From this study it became evident that although all three pathogens delay neutrophil apoptosis, there are striking differences regarding which survival signalling pathways are targeted by the pathogens. Infection with *A. phagocytophilum* and *C. pneumoniae* activates the PI3K/Akt pathway whereas infection with the parasite *L. major* does not activate the PI3K/Akt pathway. Moreover, it has also been observed that infection with the two Gram negative bacteria exert the anti-apoptotic effect by modulating similar signalling cascades. Therefore, it is quite plausible that there are some common mechanisms how Gram negative bacteria interact with their host cells leading to the activation of the PI3K/Akt pathway. Activation of the PI3K/Akt pathway indicates the involvement of G-protein coupled receptor (GPCR) signalling. Therefore it is possible that both of the Gram negative bacteria interact with GPCR on the cell surface. Moreover, infection with both *A. phagocytophilum* and *C. pneumoniae* induced the release of high amounts of IL-8 by neutrophils. There are number of receptors on the surface of the cell which are capable of binding IL-8. However, the most extensively studied receptors are CXCR1 and CXCR2 which are indeed G protein coupled serpentine receptors. IL-8 works in an autocrine / paracrine manner and extends the life span of neutrophil. In a previous report it has also been postulated that high amounts IL-8 responsible for recruiting more neutrophils and rapid dissemination of pathogens in fresh neutrophils during *A. phagocytophilum* infection (Akkoyunlu et al., 2001). Previously, high amounts of IL-8 production has been observed in *C. pneumoniae* infection (van Zandbergen et al., 2004). In this study I have clearly showed how these infections regulate the IL-8 production. Therefore, in my view, infection with *A. phagocytophilum* and *C. pneumoniae* initially interacts with GPCR on the cell surface leads to activation of the PI3K/Akt pathway which induce IL-8 release by activating NF- κ B. Interestingly, despite the similarities in intracellular signalling between *A. phagocytophilum* and *C. pneumoniae* infected neutrophils some clear differences have been observed particularly in the pathogens induced cytokine release by PMN. Infection with *C. pneumoniae* induces relatively low concentration of IL-8 release by neutrophils as compared to *A. phagocytophilum* infected neutrophils at early hours of infection. However, previously it has been shown that *C. pneumoniae* induced IL-8 release by neutrophils increases at late hours of infection (42-90 hours). Therefore, the possible explanation for high amounts of IL-8 release by *A. phagocytophilum* infected neutrophils is unique tropism of the pathogens for neutrophil granulocytes where high concentrations of IL-8 provides proinflammatory environment which not only extends the life span of neutrophils but also recruits large number of neutrophils and helps in rapid dissemination of the pathogens. In addition to IL-8 it has been observed that infection with *C. pneumoniae* induces

the release of significantly high amounts of IL-6 by neutrophils at early hours of infection. Induction of IL-6 appears to be a characteristic feature in chlamydial infection as it has been observed during chlamydial infections in several other cell types such as human gingival fibroblasts (Rizzo et al., 2008). However, *C. pneumoniae* infection induced IL-8 release by neutrophil is also regulated via the PI3K/Akt–NF- κ B pathway. Therefore, basic signalling patterns are quite similar for both of these Gram negative pathogens. The initial difference in cytokine production is probably related with adaptation of these pathogens to their respective niches. The final host of *C. pneumoniae* is not neutrophil but rather lung epithelial cells and alveolar macrophages. Therefore, apart from the anti-apoptotic function of IL-6 on neutrophils, the high amounts of IL-6 release observed during *C. pneumoniae* infection might play role in the shift from acute to chronic and persistent infection recruiting the monocytes which could replace the resident alveolar macrophage to monocyte derived macrophage pool in acute respiratory infections. This supports one recent finding that recruited monocytes could replace the resident alveolar macrophages in response to LPS induced lung inflammation (Maus et al., 2006). On the other hand IL-6 has been reported to function in the shift from acute to chronic inflammation changing from neutrophilic infiltrate to monocytic infiltrate (Gabay, 2006). In a recent report it has been clearly depicted that *Chlamydia pneumoniae* uses neutrophils as a hiding place and then silently enters into the macrophages (Rupp et al., 2009).

L. major differs from *A. phagocytophilum* and *C. pneumoniae* regarding the modulation of survival signalling cascades. This intracellular parasite does not activate the PI3K/Akt pathway. Moreover, it has also been observed in previous studies of our group that supernatants of *L. major* infected neutrophil does not prevent neutrophil apoptosis. Although infection with *L. major* leads to IL-8 release by neutrophils, however, IL-8 apparently does not function here at an autocrine / paracrine manner. Activation of the ERK1/2 pathway has been observed which is also via activation of GPCR. However, further stimulation of GPCR in autocrine manner by IL-8 is possibly absent in *L. major* infection.

5 Summary

Neutrophil granulocytes are short living cells. They undergo apoptosis within 10-24 hours after leaving the bone marrow. However, infection with intracellular pathogens *Anaplasma phagocytophilum*, *Chlamydia pneumoniae* and *Leishmania major* inhibits the spontaneous apoptosis of neutrophils. This study addresses the molecular mechanisms by which these pathogens extend the life span of the host cells particularly in the context of survival signals.

The results revealed that infection with Gram negative bacteria *Anaplasma phagocytophilum* and *Chlamydia pneumoniae* share some common mechanisms regarding the anti-apoptotic effect. This includes the activation of the PI3K/Akt pathway which stabilizes the anti-apoptotic protein Mcl-1. Furthermore, PI3K/Akt mediated activation of NF- κ B was observed which resulted in the release of high amounts of IL-8 from both *A. phagocytophilum* and *C. pneumoniae* infected neutrophils. IL-8 has been found to inhibit neutrophil apoptosis suggesting that probably there is an autocrine/paracrine role of IL-8 in delaying neutrophil apoptosis in both *A. phagocytophilum* and *C. pneumoniae* infected cells. Additionally, activation of ERK1/2 was observed which was found to regulate the activation of NF- κ B which in turn regulates the release of IL-8.

Apart from the above mentioned similarities infections with these two pathogens differ in modulation of some other signals. Activation of PKC δ was observed in *A. phagocytophilum* infected neutrophils. Use of the pharmacological inhibitor of PKC δ abrogated the infection induced anti-apoptotic effect in *A. phagocytophilum* infected but not in *C. pneumoniae* infected neutrophils. This implies that the *A. phagocytophilum* induced apoptosis delay depends on PKC δ activation while PKC δ does not seem to be involved in *C. pneumoniae* induced delay of neutrophil apoptosis. Inhibition of PKC δ with specific inhibitor revealed marked reduction in IL-8 production in *A. phagocytophilum* infected neutrophils. This finding further supports the involvement of PKC δ in infection induced apoptosis delay. In addition to IL-8, infection with *C. pneumoniae* leads to release significantly high amounts of IL-6 which probably have some role in infection induced apoptosis delay. However, further experiments are required to prove this hypothesis.

In contrast to *A. phagocytophilum* and *C. pneumoniae*, infection with *L. major* did not activate either PI3K or Akt which implies that the PI3K/Akt pathway is not involved in *L. major* induced apoptosis delay in neutrophils. Instead, activation of both ERK1/2 and p38 MAPK

were important. Using pharmacological inhibitors, the *L. major* induced delay of neutrophil apoptosis was found to be dependent on ERK1/2 but not p38 MAPK activation.

This is a comparative study among three pathogens which delineates how these pathogens modulate the survival signals in neutrophil granulocytes. Both of the bacterial pathogens, *A. phagocytophilum* and *C. pneumoniae*, modulate common mechanisms to induce apoptosis delay via the PI3K/Akt as well as the ERK1/2 pathways. Infection with the protozoan parasite *L. major*, however, modulates only the ERK1/2 pathway. High amounts of IL-8 in the supernatants of *A. phagocytophilum* and *C. pneumoniae* infected neutrophils were found to be responsible for pathogens induced apoptosis inhibition. However, *L. major* infection induced apoptosis inhibition is independent of IL-8 released in the supernatant.

The survival signals are the key regulators of apoptotic cascade. Therefore this approach of study is critical to better understand the molecular mechanisms by which these pathogens modulate the apoptosis.

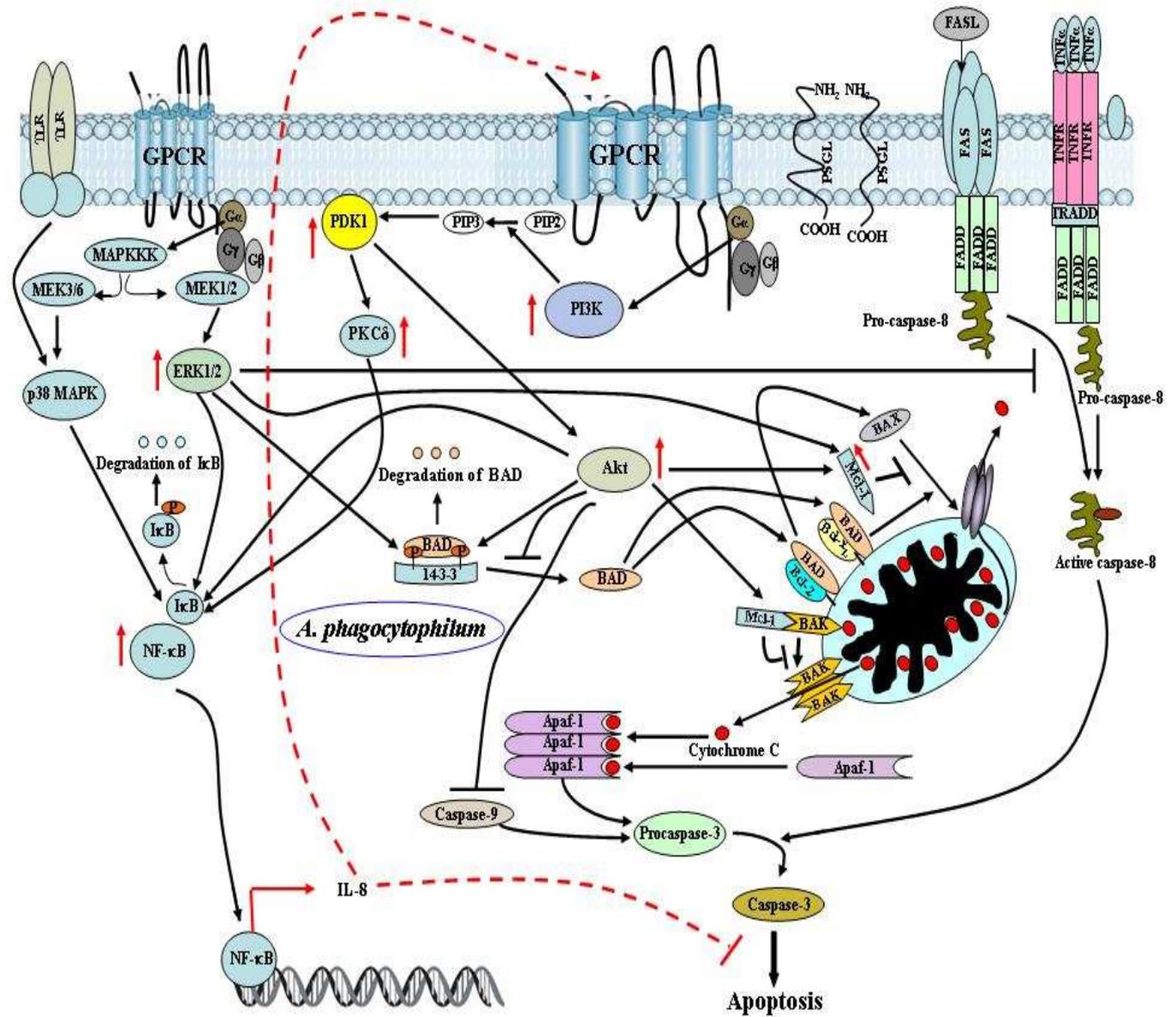


Fig. 31 Summary of survival signalling events in PMN modulated by the infection with *A. phagocytophilum*

Pathway keys

- Activation of survival kinases —————>
- Possible operative mechanism - - - - ->

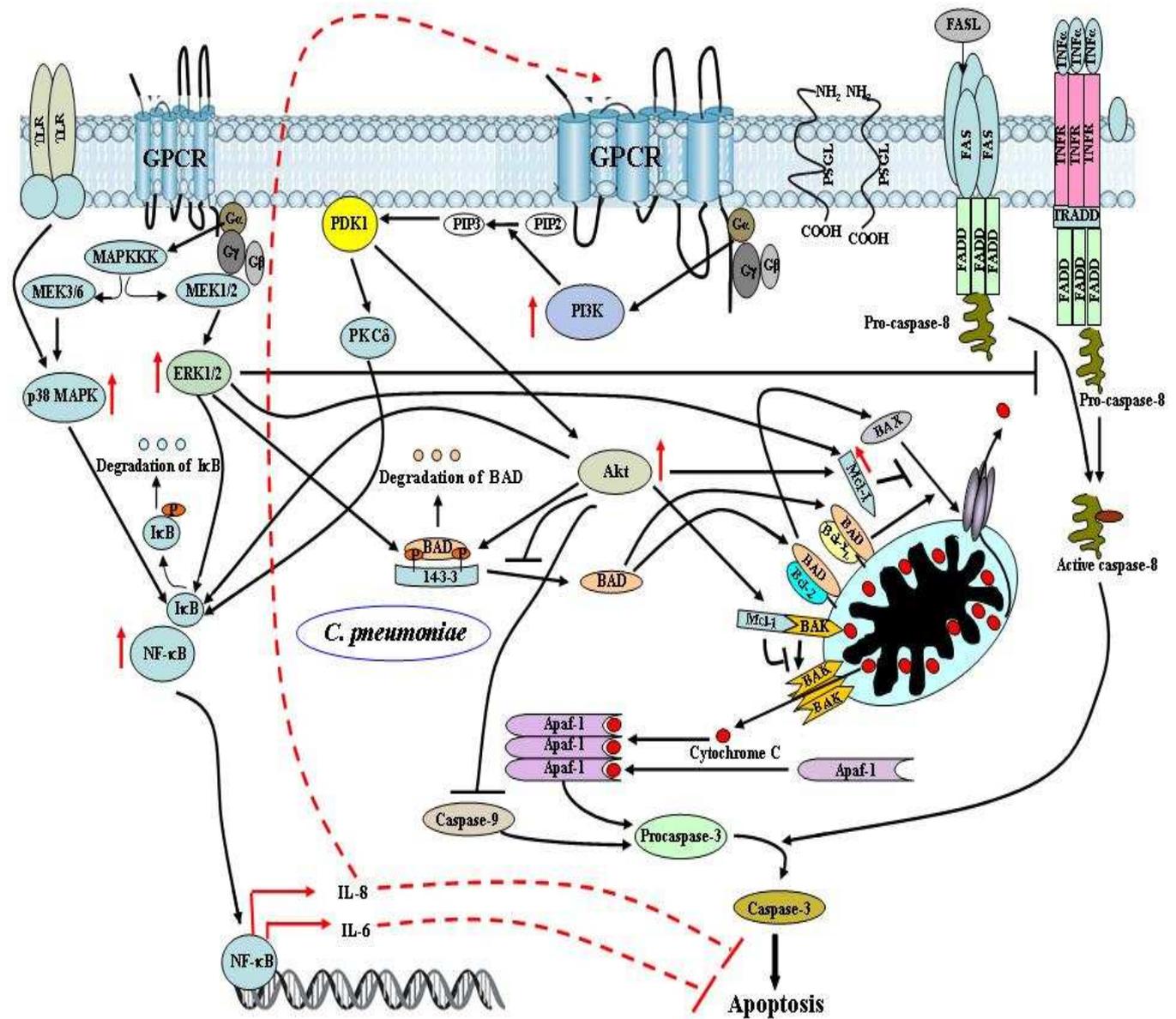


Fig. 32 Summary of survival signalling events in PMN modulated by the infection with *C. pneumoniae*

Pathway keys

- Activation of survival kinases →
- Possible operative mechanism - - - - ->

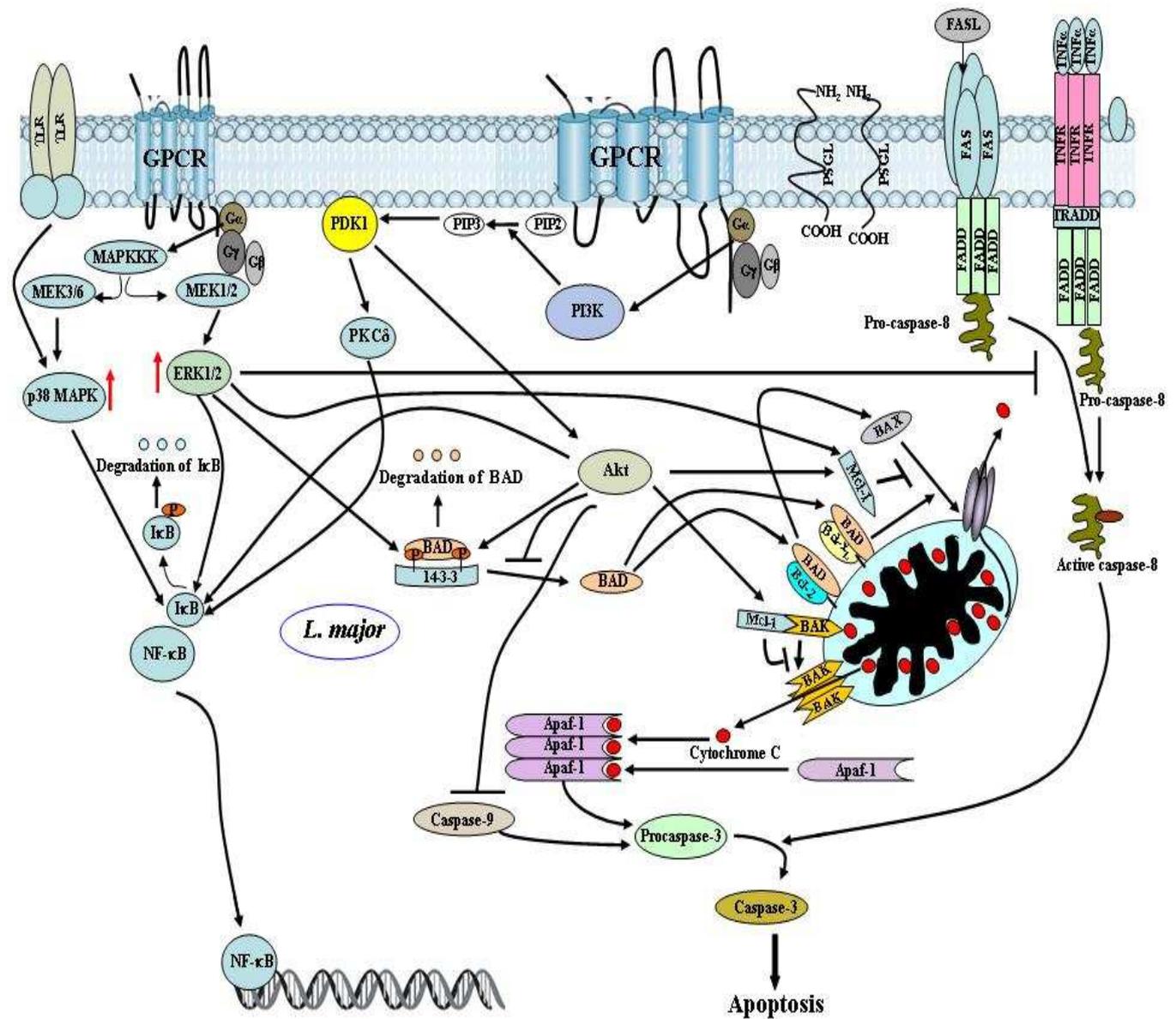


Fig. 33 Summary of survival signalling events in PMN modulated by the infection with *L. major*

Pathway key

Activation of survival kinases →

6 References

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7 List of publications and presentations

PAPERS

- **Impairment of Interferon- γ Signalling in Human Neutrophils Infected with *Anaplasma phagocytophilum***

Uta Bussmeyer^{1*}, **Arup Sarkar**^{1*}, Kirsten Broszat¹, Ger van Zandbergen¹, Christian Bogdan², Behnen-Haerer, Martina, J. Stephen Dumler³, Friederike D. von Loewenich⁴, Werner Solbach¹ and Tamás Laskay.¹ (***Equal first authorship**) (**Submitted**).

- **Phagocytosis of apoptotic cells inhibits pro-inflammatory functions of neutrophil granulocytes**

Lars Esmann*, Christian Idel*, **Arup Sarkar**, Ger van zandbergen, Antje Klinger, Martina Behnen, Werner Solbach (* Equal first authorship) (**Submitted, in revision**).

- ***Anaplasma phagocytophilum* inhibits apoptosis of neutrophil granulocytes through a phosphatidyl-3 kinase and NF- κ B dependent mechanism**

Arup Sarkar, Sonja Möller, Martina Behnen-Haerer, Lars Helberg, Keqing Wang, Janet M Lord, Werner Solbach and Tamás Laskay (**Manuscript under preparation**).

- **Regulation of impaired protein kinase C signaling by chemokines in murine macrophages during visceral leishmaniasis.**

Dey R, **Sarkar A**, Majumder N, Bhattacharyya Majumdar S, Roychoudhury K, Bhattacharyya S, Roy S, Majumdar S. **Infect Immun.** 2005 Dec;73(12):8334-44.

- **Development of a semi-automated colorimetric assay for screening anti-leishmanial agents.**

Ganguly S, Bandyopadhyay S, **Sarkar A**, Chatterjee M. **J Microbiol Methods.** 2006 Jul;66(1):79-86

TALKS / PRESENTATIONS

- **Pathway of inhibition of respiratory burst and its restoration upon IFN γ treatment on *A. phagocytophilum* infected neutrophils, 12th Symposium “Infection and Immune defense” (2008) Rothenfels, Germany (Oral presentation).**

- **Modulation of neutrophils apoptosis by the intracellular pathogens *Anaplasma phagocytophilum* and *Leishmania major*, Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGFI), September 3-6, 2008, Vienna, Austria (Poster presentation).**

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9 Curriculum vitae

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Education

05/06-09/09 **Doctoral** student in the group of Prof Tamás Laskay,
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University of North Bengal
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Research experience

04/02-03/04 **Junior research fellow (JRF) in Council of Scientific and Industrial Research (CSIR), India** project entitled “Chemokine mediated signal transduction in visceral leishmaniasis and possible immunotherapeutic role to control the disease *in vivo*”, Dept of Microbiology Bose Institute, Kolkata, India

03/05-04/06 **Senior research fellow (SRF) in Life Science Research Board (LSRB) Defence Research and Development Organisation (DRDO), India** project entitled “Evaluation of indigenous medicinal plants having immunomodulatory activity in Indian Leishmaniasis”, Dept of Pharmacology, Institute of Post Graduate Medical Education & Research (IPGME&R), Kolkata, India