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Microbe-induced apoptosis in phagocytic cells and its role in innate immunity

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Cover: microscopy image of human neutrophils, collected during the thesis work.

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Abstract

Apoptosis, or programmed cell death, is a controlled process by which aged or damaged cells are eliminated in multicellular organisms. Neutrophils, short-lived phagocytes of the innate immune system, are highly equipped effectors that can sense, locate, ingest and kill bacterial pathogens. Inflammatory mediators and the presence of bacterial products at the foci of infection regulate the function and life span of these cells. Modulation of neutrophil apoptosis and the subsequent clearance by scavenger cells, such as macrophages, is part of a balanced inflammatory process leading to resolution of inflammation. Many pathogens are capable of modulating host cell apoptosis, and thereby influence the progression of disease. Hence, this thesis was aiming at elucidating mechanisms involved in pathogen- and host-modulated apoptosis and its contribution to the inflammatory process.

We found that different routes of bacterial entry, i.e. through invasion or by receptor-mediated phagocytosis, triggered different signaling pathways within phagocytes. Invasion of virulent *Salmonella* caused apoptosis, a process requiring activation of the Rho GTPases Rac1 and Cdc42. On the other hand, phagocytosis of the non-invasive *Salmonella* inhibited apoptosis despite similar intracellular survival as the invasive bacteria. Protection against phagocytosis-induced apoptosis was regulated by tyrosine- and PI3-kinase-dependent activation of AKT (also called PKB for protein kinase B). Furthermore, inhibiting the intraphagosomal production of reactive oxygen species (ROS) in neutrophils during phagocytosis of *E. coli* decreased apoptosis below spontaneous apoptosis, further indicating that both pro- and anti-apoptotic pathways are triggered by receptor-mediated phagocytosis.

Type 1 fimbria-expressing *E. coli* adhering to neutrophils resisted ingestion, and induced a ROS-dependent apoptosis by a cooperative effect of the FimH adhesin and LPS. To explore how compartmentalization of ROS during neutrophil activation was involved in modulating apoptosis, we evaluated the stability of lysosomes. In contrast to phagocytosis of *E. coli*, the adhesive strain induced intracellular nonphagosomal ROS production which triggered early permeabilization and release of lysosomal enzymes to the cytosol. Cathepsin B and/or L were responsible for targeting of the pro-apoptotic Bcl-2 protein Bid,

thereby inducing mitochondrial damage, and apoptosis. These data propose a novel pathway for ROS-induced apoptosis in human neutrophils, where the location of the ROS rather than production *per se* is important.

Moreover, we found that pathogen-induced apoptotic neutrophils, in contrast to uninfected apoptotic neutrophils, activated blood-monocyte derived macrophages to increase their Fc γ RI surface expression and to produce large quantities of the pro-inflammatory cytokine TNF- α . This demonstrates that during the early phase of infection, pathogen-induced neutrophil apoptosis will help local macrophages to gain control over the microbes. Furthermore, we suggest that heat shock protein 60 and 70 represent a stress signal that enables macrophages to distinguish between, and react differently to, uninfected and inflammatory apoptotic neutrophils.

Populärvetenskaplig sammanfattning

Neutrofiler, en sorts vita blodkroppar i det medfödda immunförsvaret, utgör det tidigaste och viktigaste cellsvaret vid en bakterieinfektion. Dessa kortlivade försvarsceller är utrustade med system för att lokalisera, äta upp (fagocytera) och avdöda inkräktande bakterier. Detta sker med hjälp av olika nedbrytande enzymer, anti-bakteriella peptider och förmåga att bilda fria syreradikaler. Livslängden och funktionen hos dessa fagocyter påverkas av bakterieprodukter och andra inflammatoriska ämnen som bildas vid en infektion. Apoptos är en fysiologisk form av celledöd som behövs för normal utveckling och balans i kroppen. Neutrofiler som har utfört sin uppgift går i apoptos och elimineras av vävnadsmakrofager. Vid en bakterieinfektion balanseras således inflammationen av apoptos och medföljande celleliminering, vilket slutligen leder till minskad inflammation. Flera slags bakterier kan dessvärre modulera apoptosförloppet och därmed förvärra sjukdomsbilden. Målsättningen för min avhandling har därför varit att förstå hur olika bakterier påverkar apoptos, samt vilka effekter det har på inflammations-processen.

Min forskning visar att olika upptagsmekanismer för bakterier, som invasion eller receptor-reglerat upptag (fagocytos), aktiverar specifika signalvägar i fagocyter. Salmonella-bakterien använder sig av ett nålkomplex med vilket den injicerar bakterieproteiner, som hjälper den att invadera cellen och inducera apoptos. Däremot hämmas apoptosen vid fagocytos av icke-invasiva Salmonella. Jag visar att detta sker genom aktivering av vissa överlevnadsfaktorer, i det här fallet ett fosforylerande enzym (proteinkinase B).

Jag har i mitt avhandlingsarbete även studerat interaktionen mellan fimbrierade *E. coli*-bakterier och humana neutrofiler. Fimbrier är ytstrukturer som möjliggör stark inbindning av bakterierna till slemhinnan på urinvägar, vilket orsakar urinvägsinfektioner. De fimbriebärande bakterierna fäster till neutrofilerna utan att fagocyteras. Detta leder till bildning av fria syreradikaler med apoptos som följd. I min strävan att förstå hur syreradikalproduktion reglerar apoptos, undersöktes stabiliteten hos lysosomerna. Lysosomer är cellstrukturer som i andra celltyper har visat sig vara känsliga för syreradikalstress. När fimbrierade *E. coli*-bakterier inducerar syreradikalproduktion sker en skada på lysosomerna med läckage av apoptosinducerande

enzymmer, som visar på en ny signalväg för syreradikalinducerad apoptos i dessa försvarsceller.

Vid en inflammation spelar också makrofager en viktig roll. Tidigare forskning har visat att makrofagerna producerar anti-inflammatoriska substanser såsom TGF- β vid upptag av apoptotiska celler. Härvid minskas rekryteringen och aktiveringen av de inflammatoriska cellerna och vävnadsreparationen stimuleras. Dessa studier gäller normalt åldrade neutrofiler. Våra försök visar att makrofager som tar upp bakterie-inducerade apoptotiska neutrofiler aktiveras till att producera proinflammatoriska ämnen (TNF- α), vilket är ett signum för inflammatoriska makrofager. Jag tror att detta är ett sätt för makrofagerna att få kontroll över infektionen, framförallt under dess tidiga förlopp.

Ökad apoptos ses vid AIDS, neurodegenerativa sjukdomar och stroke, medan en minskad eller hämmad apoptos förekommer vid cancer och autoimmuna sjukdomar. Mer kunskap kring hur apoptos regleras kan leda till nya behandlingsmetoder av olika infektiösa och inflammatoriska sjukdomar.

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List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I M. Forsberg, **R. Blomgran**, M. Lerm, SM. Sebti, A. Hamilton, O. Stendahl, and L. Zheng. Differential effects of invasion by and phagocytosis of *Salmonella typhimurium* on apoptosis in human macrophages: potential role of Rho-GTPases and Akt. **J. Leukoc. Biol.** **74(4):620-629; 2003.**

- II **R. Blomgran**, L. Zheng, O. Stendahl. Uropathogenic Escherichia coli triggers oxygen-dependent apoptosis in human neutrophils through the cooperative effect of type 1 fimbriae and lipopolysaccharide. **Infect. Immun.** **72(8): 4570-8; 2004.**

- III **R. Blomgran**, L. Zheng, O. Stendahl. Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. Status: under revision for **J. Leukoc. Biol.**

- IV L. Zheng, M. He, M. Long, **R. Blomgran**, and O. Stendahl. Pathogen-Induced Apoptotic Neutrophils Express Heat Shock Proteins and Elicit Activation of Human Macrophages. **J. Immunol.** **173(10): 6319-26; 2004.**

Abbreviations

AIF – apoptosis inducing factor	LPS – lipopolysaccharides
AKT – see PKB	LTB4 – leukotriene B4
Apaf-1 – apoptotic protease activating factor	MAPK – mitogen-activated protein kinase
Bcl-2 – human B-cell lymphomas	MPO – myeloperoxidase
BH – Bcl-2 homology	NADPH – nicotinamide adenine dinucleotide phosphate
CARD – caspase recruitment domain	NF-kB – nuclear factor-kB
CSF – colony-stimulating factor	PAF – platelet-activating factor
DD – death domain	PECAM-1 – platelet/endothelial cell adhesion molecule 1 or CD31
DED – death effector domain	phosphatidylserine (PS)
DIABLO – direct IAP binding protein	PI3K – phosphatidyl inositol 3-kinase
DISC – death inducing signaling complex	PICD – phagocytosis-induced cell death
EndoG – endonuclease G	PKA – protein kinase A
ERK – extracellular signal-regulated kinase	PKB – protein kinase B or AKT
FADD – Fas-associated DD	PKC – protein kinase C
FasL – Fas ligand	PMA – phorbol myristate acetate
FcγRI – high-affinity receptor for IgG (CD64)	ROS – reactive oxygen species
FimH – type 1 fimbrial adhesin	SH2 – Src homology domain 2
FMLP – formyl-methionyl-leucyl-phenylalanine or fMLF	SHP-1 – SH2-containing tyrosine phosphatase
G-CSF – granulocyte-CSF	SipB – Salmonella invasion protein B
GM-CSF – granulocyte- macrophage-CSF	SMAC – second mitochondrial activator of caspases
HSP – heat shock proteins	TGF-β – transforming growth factor β
IAP – inhibitors of apoptosis	TLR – toll-like receptor
IFN-γ – gamma-interferon	TNF – tumor necrosis factor
IL-8 – interleukin-8	TRAIL – TNF-related apoptosis-inducing ligand
LFA-1 – leukocyte function antigen-1 or CD11a	TTSS – type-III secretion system
LMP – lysosomal membrane permeabilization	UPEC – uropathogenic <i>E. coli</i>
	UTI – urinary tract infection

Background

Immediate response to infection

When opportunistic or pathogenic bacteria colonize and invade the cells lining the epithelium of the lung (*Mycobacteria*), intestines (*Salmonella*), urinary tract (*E. coli*), or skin (*S. aureus*), the bacteria are recognized by different innate receptor molecules and inflammation is induced. It is not only the direct effect of released bacterial products (LPS and formylated peptides etc.), but also the generation and release of inflammatory mediators that lead to the more immediate reactions seen at the foci of infection (*Foreman, et al., 1994*). Infected epithelial cells produce and secrete chemokines such as interleukin-8 (IL-8) (*Agace, et al., 1993*), a major neutrophil chemoattractant. Inflamed endothelium further produce and immobilizes IL-8 and other chemoattractants such as platelet-activating factor (PAF) and leukotriene B4 (LTB4) on their surface to identify the “entry-points” for the neutrophils. In addition, the endothelium upregulates adhesion molecules (selectins and intercellular adhesion molecule-1 (ICAM1)), molecules that are required for the loose attachment and the firm adhesion process leading to sequestration of neutrophils to the inflamed site. In postcapillary venules or pulmonary capillaries at the site of inflammation, the slow flow rate is further reduced by vessel dilation, facilitating the transient adhesion and rolling of leukocytes along the endothelium. (*Springer, 1994*)

Adhesion and migration

Polymorphonuclear leukocytes, and in particular neutrophil granulocytes, play a key role in cellular innate defense against microorganisms (*Haslett, et al., 1989*). Neutrophils are the first leukocytes to be recruited to the site of infections, hours before monocytes and lymphocytes, thereby forming the first line of defense against bacterial and fungal infections (*Witko-Sarsat, et al., 2000*). In the absence of inflammation neutrophils circulate in the peripheral blood with a half-life of approximately 8-20 hr before being cleared in the liver, spleen, or lung. The physiological retention of neutrophils, mainly in pulmonary capillaries, appears to be mechanical rather than involving cell adhesion (*Yoder, et al., 1990, Yamaguchi, et al., 1997*).

Sequestration of neutrophils during inflammation is however highly dependent on cell-cell adhesion (Figure 1). P-selectin glycoprotein ligand-1 (PSGL-1 or CD162) (Moore, et al., 1994) and possibly L-selectin (CD62L) (Picker, et al., 1991) expressed on the surface of neutrophils mediate the loose attachment to newly expressed endothelial P- and E-selectins initiating rolling of the leukocyte on the vessel wall. Endothelial-displayed chemoattractants together with selectin ligation then leads to an “inside-out signaling” activation of β 2-integrins (CD11a, b, or c/CD18) on neutrophils. The firmer attachment between β 2-integrins and ICAM1 facilitates transmigration of the neutrophils through the vessel wall and into the tissue, a process called extravasation. Neutrophils are able to squeeze between adjacent cells and follow the gradient of chemoattractants bound to the extracellular matrix. Different sensitivity towards “end target-derived” chemoattractants (formylated peptides and complement C5a) and “regulatory cell-derived” attractants (LTB4 and IL-8) will guide the neutrophils away from the endothelial agonist source, and toward their final target within the infected tissue (Kitayama, et al., 1997). (Witko-Sarsat, et al., 2000)

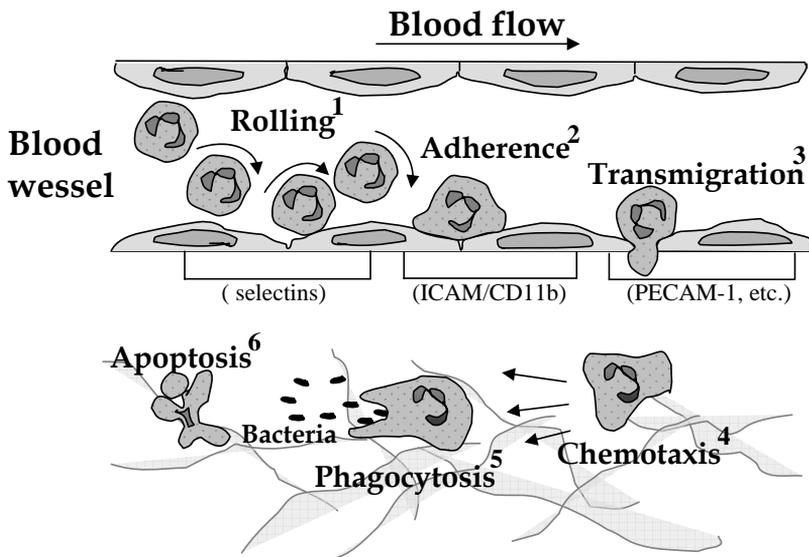


Figure 1. Schematic diagram summarizing the functions of the neutrophil in the inflammatory response.

Phagocytosis and intracellular killing

During the recruitment process, and before reaching the intruder, the neutrophil upregulates or activates cell surface receptors, increase their metabolic rate and acquire a state of alertness referred to as the priming. Upon contact with the bacteria several recognition mechanisms are operative. If the bacteria are opsonized by either complement (C3bi) or antibodies (IgG) they will bind to immunoreceptors such as the CR3 (also called MAC-1 or CD11b/CD18) or Fc γ -R, respectively expressed on neutrophils. Neutrophil also recognize microbes or microbial products directly via the family of toll-like receptors (TLRs) (*Medzhitov, et al., 1997*). TLRs mediate responses to pathogen-associated molecular patterns (PAMPs) shared by many microorganisms, where TLR4 can recognize lipopolysaccharides (LPS) and TLR2 can recognize peptidoglycan, respectively expressed by gram negative and gram positive bacteria. Finally recognition leads to engulfment of the bacteria, or phagocytosis. Compartmentalization of the ingested prey within a membrane-enclosed vesicle, the phagosome, allows neutralization and killing of the prey, thereby protecting the cell and the host. The antimicrobial efficiency of human neutrophils depends on two concurrent events: (1) the generation of reactive oxygen species (ROS) by assembly and activation of the NADPH oxidase at the phagosomal membrane, and (2) the release of enzymatic or antimicrobial proteins from granules by fusion with the phagosome. Activation of NADPH oxidase generates superoxide anion (O₂⁻) that is further converted to vast assortment of reactive oxidants. After superoxide anion dismutation into hydrogen peroxide, the heme protein myeloperoxidase (MPO) amplifies the toxic potential of hydrogen peroxide by producing reactive intermediates such as hypochlorous acid and chloramines (*Klebanoff, 1999*). Of the four morphologically distinct populations of granules, the azurophilic granules are considered the true microbicidal compartment, containing myeloperoxidase, serine proteases, small antibiotic peptides and other antimicrobial proteins (*Fouret, et al., 1989, Witko-Sarsat, et al., 2000*). Specific granules also contain antimicrobial molecules such as lactoferrin, cathelicidin, phospholipase A2 and lysozyme, that are destined predominantly for extracellular release. Other components of the specific granules are the metalloproteinases collagenase and gelatinase that are important for migration through tissues (*Weiss and Peppin, 1986*). The suggested order for exocytosis of

neutrophil compartments is secretory vesicles, gelatinase granules, specific granules and lastly azurophilic granules (*Sengelov, et al., 1993*).

Neutrophils and inflammation

Neutrophils are key actors in acute inflammatory reactions in response to pathogens. Arrival and accumulation of neutrophils in the tissue is part of inflammatory events, such as the appearance of oedema and monocyte migration (*Wedmore and Williams, 1981, Doherty, et al., 1988*). When reaching the injured tissue, the monocytes differentiate into macrophages that clear the effete neutrophils, remove residual debris and stimulate tissue repair (*Henson and Hume, 2006*).

Neutrophils contain a variety of agents with the capacity not only to damage tissue, but also cleave matrix proteins into chemotactic fragments that are able to amplify inflammation (*Vartio, et al., 1981*). Activated neutrophils can damage tissue by releasing oxygen free radicals, chlorinated oxidants, proteases from granules and other pro-inflammatory mediators. The serine proteinases elastase and proteinase 3 are regarded as major contributors to neutrophil-mediated damage, since they are capable of cleaving a variety of matrix proteins, including fibronectin, laminin, vitronectin and collagen type IV (*Kam, et al., 1992, Rao, et al., 1991*). In addition to local tissue-injury mediated effects, oxygen free radicals can also oxidize low-density lipoprotein into pro-atherogenic products, suggesting a link between neutrophil derived oxygen radicals and heart disease (*Jordan, et al., 1999*). It seems the beneficial effects of the inflammatory response can easily be lost if neutrophils are not kept under rigorous control. However, the neutrophil is not only an effector, but also a regulator of inflammation. Neutrophils can produce and secrete both pro- and anti-inflammatory cytokines, as well as their antagonist, indicating that their functions include initiation, amplification and resolution of inflammation (*Cassatella, 1999*). Also, secreted neutrophil proteinases such as elastase and collagenase exerts immunomodulatory effects by cleaving monocyte CD14 leading to inhibited LPS-mediated cell activation (*Le-Barillec, et al., 1999*), and by shedding of neutrophil Fc γ -RIIIB receptor (*Middelhoven, et al., 1997*). The main physiological protection against elastase and proteinase 3 is plasma α 1-antitrypsin (α 1-AT) and α 2-macroglobulin (*Mason, et al., 1991, Travis and Salvesen, 1983*). Neutrophils also synthesize α 1-AT, and this broad-spectrum

inhibitor of serine proteases coexists within the same granule population (*Mason, et al., 1991*).

Elimination of neutrophils

Most acute inflammatory responses resolve spontaneously due to endogenous regulatory mechanisms limiting the destruction of host tissues. Termination of neutrophil emigration from the blood results from; (1) changes in the cytokine/anticytokine and inflammatory/anti-inflammatory cytokines secreted by tissue cells and infiltrated leukocytes, (2) the return of endothelial cells to their resting state by shedding or internalizing of adhesion molecules and displayed chemokines, and (3) inactivation of chemoattractants by specific enzymes or via receptor-mediated endocytosis (*Ayesh, et al., 1995, Cao, et al., 1998, Hoffman and Specks, 1998*). The tissue-damaging potential of the neutrophils is further limited by mechanisms that inactivate neutrophils such as tachyphylaxis in response to proinflammatory mediators and apoptosis (*Witko-Sarsat, et al., 2000*). Neutrophils are an excellent example of cells in which ageing is equivalent to programmed cell death, or apoptosis. Apoptotic neutrophils show impaired responsiveness to fMLP, and an inability to phagocytose opsonized zymosan. In contrast, superoxide anion production in response to the receptor-independent stimulus PMA was intact in apoptotic neutrophils (*Whyte, et al., 1993a*). Apoptosis is therefore suggested to play an essential role in the resolution of inflammation, in that it profoundly reduces the capacity to generate and release histotoxic products to the surrounding milieu. Alterations in surface markers contribute in the recognition and subsequent removal of apoptotic cells from the site of inflammation by macrophages, and other cells (*Savill, et al., 1989, Fadok, et al., 1992, Henson and Hume, 2006*). Participation of other cells in this clearing process includes semi- and nonprofessional phagocytes such as dendritic cells, fibroblasts, epithelial cells and high endothelial venules (*Ip and Lau, 2004, Hall, et al., 1994, Wyllie, et al., 1980, Hess, et al., 1997*).

Apoptosis

Programmed cell death, or apoptosis, and its manifestation is a conserved physiological pathway central in the development of several tissues and organs (*Lockshin and Williams, 1965, Kerr, et al., 1972*). Apoptosis is also essential in adult animals to maintain normal cellular homeostasis. Epithelial cells in the gastrointestinal lining are constantly shed and replaced, and up to 98% of the T-cell produced in the bone marrow never make it through the selection process in the thymus (*Krammer, 2000*). Dysregulation of apoptosis affects many pathological conditions. Accelerated apoptosis is evident in acute and chronic degenerative diseases, immunodeficiency and infertility, whereas insufficient apoptosis can cause cancer or autoimmunity (*Fadeel, et al., 1999*).

Morphology of apoptosis

Apoptosis is an ubiquitous, genetically encoded process that enables cells to undergo cell death in response to different pro-apoptotic signals. This is a highly regulated process that requires ATP as energy source. Apoptosis *in vivo* is associated with the death of isolated cells, rather than continuous patches or areas of tissue. There is no inflammatory infiltrate, and nuclear shrinkage occurs relatively early in this process, whereas changes to organelles and loss of membrane integrity are relatively late. Neighboring cells, rather than immigrant professional phagocytes, phagocytose the dying cells. The DNA is rapidly broken down into a characteristic ladder, because endonucleases gain access to the DNA in the internucleosomal regions (*Vaux, 1993*).

Necrosis, on the other hand, affects many adjoining cells. It is characterized by swelling, early loss of plasma-membrane integrity and major organelle changes, as well as swelling of the nucleus. Necrosis is accompanied by an inflammatory infiltrate of phagocytic cells. If DNA degradation occurs, it is a late event (*Vaux, 1993*) (Figure 2).

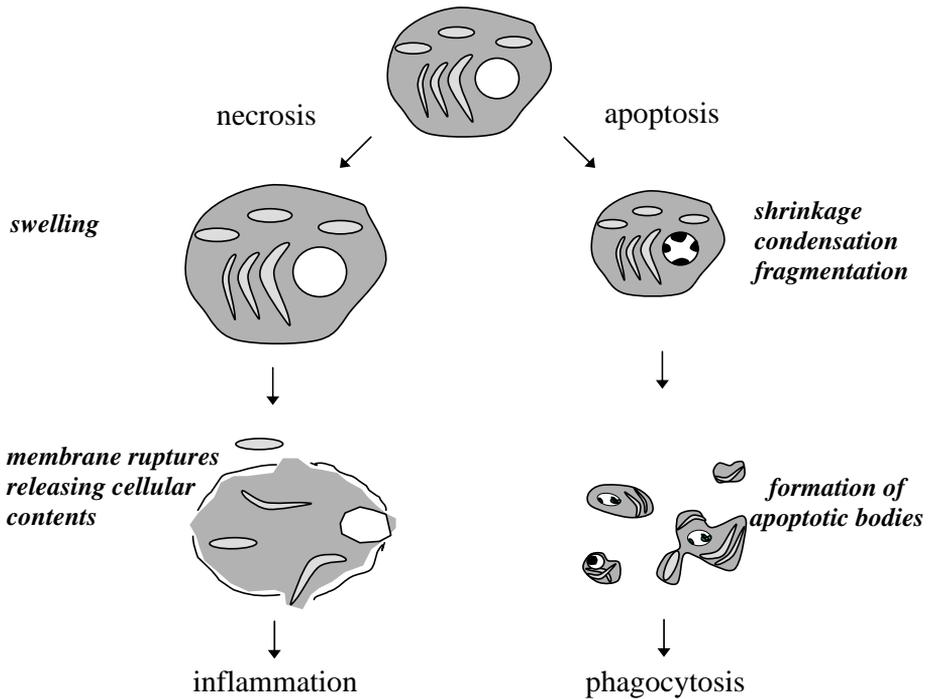


Figure 2. Apoptosis versus necrosis. Cells undergoing apoptosis display morphological changes such as shrinkage, condensation of the nucleus, DNA fragmentation and disintegration into apoptotic bodies. These are phagocytosed by the surrounding tissue or phagocytes. During necrosis the cell swells and ruptures, leading to release of its contents to the surrounding tissue, a process that may elicit an inflammatory response.

Caspases

Caspases are the central components of the apoptotic response (Shi, 2002), responsible for the morphology changes of apoptotic cells. They are a conserved family of enzymes that irreversibly commit a cell to die. The “c” in the term caspases indicates that they are cysteine proteases, and the “asp” refers to their ability to cleave after an aspartate (Asp) residue in their substrates (Alnemri, et al., 1996). Eleven caspases have been found in humans. Caspase-1, 4, 5, and 13 are involved in cytokine activation and inflammation but not in apoptosis. The other

so called apoptotic caspases are generally divided into two classes; the initiator caspases, which include caspase-2, 8, 9 and 10, and the effector caspases, which include caspase-3, 6 and 7. All caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. The initiator caspases are characterized by extended N-terminal prodomains that contains protein-protein interaction motifs, either the death effector domain (DED) in procaspase-8 and 10, or the caspase recruitment domain (CARD) in procaspase-2 and 9. DED and CARD, the death domain family members, provides the basis that enables the association with upstream adaptor molecules involved in procaspase activation as well as downstream caspase-cascade regulation (*Fan, et al., 2005*). Activation of effector caspses, which have short prodomains not allowing autoactivation of these enzymes, is carried out by an initiator caspase, through cleavage at specific internal Asp residues that separate the large (~p20) and small (~p10) subunits. The p20 and p10 subunits closely associate with each other to form a caspase heterodimer, and two heterodimers form an enzymatic active hetrotetramer. Once activated the effector caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets, as for the activation of both initiator and effector caspses, thereby amplifying the caspase cascade (*Nicholson, 1999*).

Substrates for caspases during apoptosis

Several proteins are potential targets for caspases (*Brockstedt, et al., 1998*), leading to cellular proteolysis and irreversible dismantling of the cell. The vast majority of the cleavage interrupts survival pathways in order to prevent counterproductive events from occurring simultaneously. For example, caspases are involved in the cleavage of poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK), which are two key proteins involved in DNA repair and homeostatic maintenance of genomic integrity (*Nicholson, 1996, Casciola-Rosen, et al., 1996*). Caspases are also involved in apoptosis through the inactivation of apoptosis inhibitors such as ICAD (inhibitor of caspase activated deoxyribonuclease) (*Enari, et al., 1998*). In normal cells, ICAD binds to CAD, forming an inactive complex, but upon ICAD cleavage CAD is liberated, thereby allowing the nuclease to cleave chromatin. Other caspase substrates include proteins involved in the regulation of the cytoskeleton such as gelsolin (*Kothakota, et al., 1997*) and fodrin (*Martin, et al., 1995a*), leading to loss of structural

integrity and membrane blebbing. In addition, caspases, in some systems, are involved in externalization of PS on the cell surface, thereby facilitating in the removal of dying cells (*Martin, et al., 1995b*). The functions of caspases can therefore be summarized; (1) to arrest the cell cycle and inactivate DNA repair, (2) to inactivate the inhibitors of apoptosis, (3) to mediate structural disassembly and morphological changes, and (4) to mark the dying cell for engulfment and disposal.

Bcl-2 family

Another important protein family involved in the regulation of cell survival and –death is the Bcl-2 family proteins. The Bcl-2 gene, first discovered in human B-cell lymphomas, is considered to be a proto-oncogene because it prolongs cell survival by inhibiting cell death (*Tsujimoto, et al., 1985*). At least 20 homologues of Bcl-2 have been identified in mammals. The Bcl-2 family proteins have at least one of the four Bcl-2 homology domains (BH1, BH2, BH3, and BH4). They are further classified into three groups; anti-apoptotic Bcl-2-like proteins (such as Bcl-2, Bcl-x_L, Mcl-1 or A1) displaying conservation in all four BH1-4 domains, pro-apoptotic “multidomain” proteins (such as Bax and Bak) lacking BH4 and pro-apoptotic BH3-only proteins (such as Bid, Bim, and Bad) only possessing a BH3 domain. Heterodimerization between individual members of the family is an important mechanism controlling their activity, thereby making the ratio of anti- and pro-apoptotic molecules such as Bcl-2/Bax a rheostat for setting the threshold of susceptibility towards apoptosis. For example, in the Bcl-x_L monomer its BH1, BH2 and BH3 domains create a hydrophobic pocket, which can accommodate a BH3 domain of a pro-apoptotic member (*Muchmore, et al., 1996*).

Initiation of apoptosis

In mammalian cells, the apoptotic machinery is triggered by a wide array of intracellular and extracellular signals, and depending on the origin of the death stimuli apoptosis proceed through two main routes, the intrinsic pathway (stress- or mitochondrial pathway) or the extrinsic pathway (death-receptor pathway) (Figure 3).

The intrinsic pathway is activated inside the cell and mediated by mitochondria. In response to apoptotic stimuli such as DNA damage or cytotoxic drugs several proteins are released from the intermembrane space of mitochondria into the cytoplasm. The liberation of mitochondrial proteins usually occurs after pro-apoptotic members of the Bcl-2 family bind to and neutralize the protective effect of anti-apoptotic Bcl-2 proteins. Some of the well-characterized mitochondrial proteins include cytochrome c, SMAC (second mitochondria-derived activator of caspases)/DIABLO (direct inhibitor of apoptosis (IAP)-binding protein with low pI), AIF (apoptosis-inducing factor), EndoG (endonuclease G) and OMI/HTRA2 (high-temperature-requirement protein A2). Perhaps the most intriguing one of these pro-apoptotic proteins is cytochrome c, which binds to and activates apoptotic protease activating factor-1 (Apaf-1) in the cytoplasm. The binding of cytochrome c to Apaf-1 induces a conformational change allowing Apaf-1 to bind to ATP/dATP. Cytochrome c, Apaf-1, ATP and recruited pro-caspase-9 form a complex called apoptosome, which converts this precursor molecule to active caspase-9. This in turn leads to processing of pro-caspase-3 and 7, thereby initiating the execution of apoptosis (Riedl and Shi, 2004).

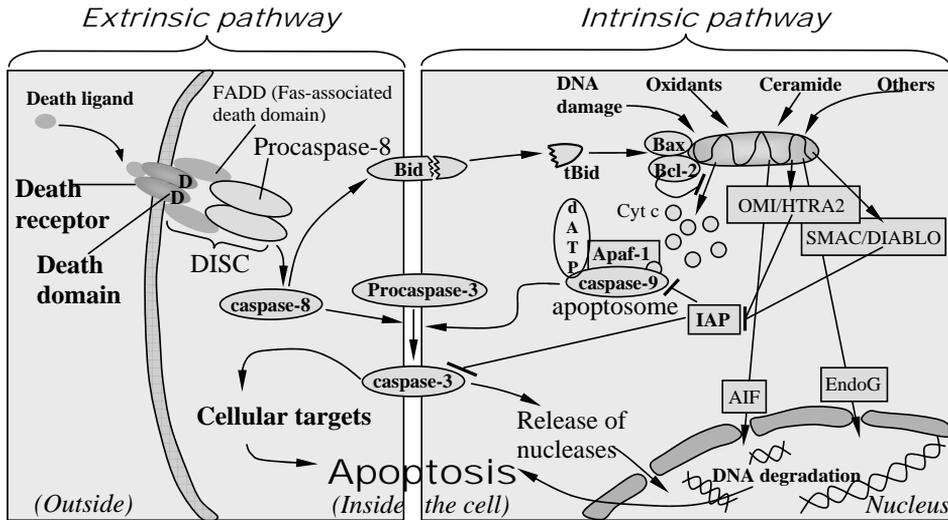


Figure 3. Two routes of apoptosis: the intrinsic pathway (stress- or mitochondrial pathway) and the extrinsic pathway (death-receptor pathway) are illustrated.

The extrinsic pathway is initiated by binding of an extracellular death ligand, such as Fas ligand (FasL), to its cell-surface death receptor Fas. The death receptor family includes CD95/Fas/Apo1, TNFR1, DR3/wsl-1/Tramp, DR4/TRAIL-R1, DR5/TRAIL-R2/TRICK2/Killer and DR6 (Dgeterev, et al., 2003). Death receptors have two distinguished features: multiple cysteine-rich repeats in the extracellular domain, and a protein-protein motif known as the death domain (DD) in the cytoplasmic tail. Binding of the constitutively homotrimeric death ligands to their receptor leads to the formation of a homotrimeric ligand-receptor complex that recruits further cytosolic factors, such as Fas-associated DD (FADD) and caspase-8, forming an oligomeric death-inducing signaling complex (DISC). It is the aggregation of FADD with its exposed DEDs that interacts with the DEDs in the prodomain of procaspase-8, which will induce the oligomerization of procaspase-8 localized on the cytoplasmic side of the plasma membrane. In the DISC, two subunits of procaspase-8 compact to each other followed by procaspase-8 autoactivation to caspase-8. The activation of downstream pathways of caspase-8 varies with different cell types. In Type I cells (cells of some lymphoid cell lines), caspase-8 is vigorously activated and can directly activate downstream effector caspases-3 and 7. In Type II cells (other than Type I cells), caspase-8 is only mildly activated and unable to

activate procaspase-3 directly. In those cells, however, caspase-8 can activate the mitochondrial-dependent pathway by cleaving/truncating the pro-apoptotic Bcl-2 family member Bid into its active form tBid. Translocation of tBid to the mitochondria and oligomerization with the pro-apoptotic Bcl-2 family members Bax and Bak leads to release of cytochrome c. The liberated cytochrome c induces formation of the apoptosome complex and activates the intrinsic pathway. This crosstalk between the receptor and mitochondria-mediated pathway can thereby amplify caspase activation necessary for apoptosis (*Riedl and Shi, 2004, Fan, et al., 2005*).

Other pathways

There are also multiple proteases, distinct from Caspases, that are involved in apoptosis. These include granzyme A and B, calpains, proteasome, lysosomal and granular enzymes, the cathepsins. Although apoptosis involving these proteases is often called caspase-independent cell death, this is not entirely true. For example, in granzyme B-mediated killing of virus-infected cells by cytotoxic T lymphocytes, it was initially shown that granzyme B was involved in the direct activation of procaspase-3 (*Darmon, et al., 1995*). However the full activation of caspase-3 was later shown to require release of pro-apoptotic mitochondrial proteins mediated through granzyme B-dependent Bid cleavage (*Pinkoski, et al., 2001*). Furthermore, in cells exposed to endoplasmic reticulum (ER)-stress, the Ca^{2+} -activated cysteine proteinase m-calpain leads to the activation of caspase-12 (*Wang, et al., 2005*) and phosphorylation of the BH3-only protein Bim, which in turn is critical for Bax-dependent cytochrome c release (*Lei and Davis, 2003*). It is thus possible that the above mentioned proteases provide additional entry points into apoptosis and participate in propagating proteolytic cascades and cleaving the same proteins as the caspases.

The lysosomal pathway for induction of cell death is often referred to as caspase independent. The lysosomes represent the major compartment for degradation of macromolecules by utilizing an array of acid-dependent hydrolases such as proteases, nucleases, phosphatases, lipases and glucosides, of which the most powerful proteolytic enzymes are the cathepsins. Although the first studies by De Duve half a century ago suggested that lysosomes are “suicide-bags” causing necrosis, later studies on the stability of lysosomes have shown that the release of these proteases (mainly cathepsin B, D, and L) trigger

apoptosis (Brunk and Svensson, 1999, Foghsgaard, et al., 2001, Guicciardi, et al., 2000, Mathiasen, et al., 2001, Monney, et al., 1998, Neuzil, et al., 1999, Ohsawa, et al., 1998, Roberg, et al., 1999, Roberg and Ollinger, 1998, Roberts, et al., 1997, Terman, et al., 2002, Werneburg, et al., 2002, Yuan, et al., 2002, Ishisaka, et al., 1999). Indeed, depending on the cell type or stimuli, cathepsins have shown to activate caspase-3 (cathepsin L) (Ishisaka, et al., 1999), and pro-apoptotic Bcl-2 proteins such as Bid (cathepsin B) and Bax (cathepsin D) resulting in mitochondria-dependent cell death (Bidere, et al., 2003, Stoka, et al., 2001, Boya, et al., 2003). This clearly indicates that lysosomal membrane permeabilization is an additional route for induction of apoptosis. Extracellular H₂O₂, diffusing into target cells, bring about a rapid and direct effect on the lysosomal membrane through a Fenton-like reaction causing peroxidative damage to the lipids in the membrane (Antunes, et al., 2001). This suggests that intracellular ROS production can be an important trigger for lysosomal membrane permeabilization and apoptosis. Cells in which lysosomal-dependent apoptosis have been studied include fibroblasts (Brunk, et al., 1997, Roberg, et al., 1999, Kagedal, et al., 2001), neuroblastoma cells (Brunk, et al., 1997), T lymphocytes (Bidere, et al., 2003), astrocytoma cells (Dare, et al., 2001), hepatocytes (Guicciardi, et al., 2000) and macrophages (Yuan, et al., 1997, Yu, et al., 2003).

Regulation of apoptosis

Since inappropriate activation of the apoptotic cascade can have devastating cellular consequences, the enzymes that control apoptosis must be tightly regulated.

Caspase activation and –activity can be regulated by a family of proteins known as inhibitors of apoptosis (IAP). Four of these members, termed c-IAP1, c-IAP2, XIAP and survivin, function as intrinsic regulators of the caspase cascade. IAPs are the only known endogenous proteins that can regulate the activity of both the initiator caspase-9 and the effector caspase-3 and 7 (Liston, et al., 2003). XIAP binding of caspase-9 prevents homodimerization of this procaspase, whereas inhibition of caspase-3 and 7 is brought about by steric hindrance. Moreover, XIAP and c-IAP2 trigger ubiquitination of caspases-3 and 7, suggesting that targeting of caspases to the proteasome is one of the anti-apoptotic mechanisms of IAPs. It was also observed that XIAP, c-IAP1 and c-IAP2 ubiquitinate SMAC/DIABLO and/or OMI, which are antagonists of IAP

functions. In response to various apoptotic stimuli SMAC/DIABLO and HTRA2/OMI are released from the intermembrane space of the mitochondria and binds IAPs, thereby liberating caspases from their IAP blockage (*Liston, et al., 2003*).

Apoptosis can also be regulated by Bcl-2 family-member heterodimerization and/or homodimerization leading to their neutralization or activation. Bcl-2 binding to Bax-like proteins prevents mitochondrial pore formation and cytochrome release, and Bax homodimerization or Bax:Bak heterodimerization leads to activation. Besides the ability to regulate each other, Bcl-x_L has been shown to inhibit the Apaf-1:pro-caspase-9 complex formation (*Hu, et al., 1998*), suggesting that anti-apoptotic Bcl-2 proteins can regulate caspase activity by altering the ability of procaspases to recruit scaffolding proteins. With the possible exception of Bid, the BH3-only proteins are thought to act by binding and neutralizing their pro-survival relatives. The BH3-only proteins can not induce apoptosis in the absence of Bax and/or Bak. These proteins are regulated by divers mechanisms, such as binding to dynein (Bim and Bmf), phosphorylation and subsequent binding to scaffold proteins (Bad), whereas Bid remain relatively inactive until proteolytically cleaved (for review see (*Cory and Adams, 2002*)).

Tyrosin phosphorylation is an important regulator of apoptosis (*Sweeney, et al., 1998*). The tyrosine kinase Lyn phosphorylates extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K), two central molecules involved in anti-apoptotic signaling (*Chang and Karin, 2001, Cantley, 2002, Klein, et al., 2000*). The downstream target of PI3K that is best characterized is the serine/threonine kinase protein kinase B (PKB, also known as AKT). PI3K/AKT-dependent phosphorylation of caspase-9 is the only known example of phosphorylation that directly regulates caspase activity (*Cardone, et al., 1998*). Phosphorylation of caspase-9 leads to its inactivation, preventing cleavage of caspase-3 and apoptosis.

Apoptosis in neutrophils

Since neutrophils can amplify the inflammatory response by the production of cytokines, these cells can be considered as both inflammatory effectors and immunoregulatory cells. To resolve the inflammation, the accumulated neutrophils need to be safely removed. In the absence of defined exogenous signals, neutrophils undergo constitutive apoptosis. Delayed neutrophil apoptosis has been associated with several acute and chronic inflammatory diseases and appears to be largely mediated by excessive production of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Dibbert, *et al.*, 1999). Induction of neutrophil apoptosis during the resolution of an inflammatory response can be mimicked *in vitro* by incubating the cells in the absence of sufficient concentrations of survival factors, a process called spontaneous apoptosis.

Death receptors

Apoptosis can be induced in response to specific ligands that engage so-called ‘death receptors’ of the tumor necrosis factor (TNF)/ nerve growth factor (NGF) receptor superfamily. Neutrophils express functional Fas receptors (CD95, APO-1) and undergo apoptosis in response to anti-Fas receptor activating antibodies (Daigle and Simon, 2001). Although neutrophils express both Fas receptors and Fas ligands, studies using anti-Fas ligand blocking antibodies and soluble recombinant Fas receptor molecules do not support the idea that spontaneous neutrophil apoptosis is the consequence of autocrine or paracrine Fas ligand/Fas receptor interactions in purified cell populations (Daigle and Simon, 2001). Clustering of CD95 in lipid rafts in the absence of receptor ligation was, however, shown to initiate spontaneous neutrophil apoptosis by activating caspase-8 and 3 (Scheel-Toellner, *et al.*, 2004), suggesting a role for endogenous FAS-receptors also in the modulation of spontaneous apoptosis.

Studies on the role of TNF- α in the induction of neutrophil apoptosis have yielded conflicting results. This could in part be explained by the observation that prolonged incubation (> 12 h) of human neutrophils with TNF- α decreases apoptosis, while this cytokine induces apoptosis in a sub-population of cells at earlier times of incubation (< 8 h) (Murray, *et al.*, 1997). One explanation for the

effect of TNF- α was provided by Cowburn *et al.* (Cowburn, *et al.*, 2002). They found that the biphasic effect of TNF- α on neutrophil apoptosis correlated with increase in Bad mRNA levels at 4 hrs followed by a decrease in Bad mRNA at 20 hrs. Apart from the inhibition of Bad mRNA levels they also showed that the survival effect of TNF- α is caused by a PI3-kinase-dependent phosphorylation and cytosolic translocation of pre-existing Bad.

Caspases

Several studies suggest a critical role for caspase-3 and caspase-8 in both spontaneous, and Fas or TNF receptor-triggered apoptosis in neutrophils (Pongracz, *et al.*, 1999, Khwaja and Tatton, 1999, Scheel-Toellner, *et al.*, 2004, Daigle and Simon, 2001, Yamashita, *et al.*, 1999). Caspase-1-deficient neutrophils have delayed spontaneous apoptosis but are fully susceptible to Fas receptor-mediated apoptosis (Rowe, *et al.*, 2002). In addition to caspase-3, 8 and 1, inactivation of caspase-9 also resulted in blocking neutrophils apoptosis (Daigle and Simon, 2001), suggesting that mitochondria play an important role in apoptosis in neutrophils. Even though human neutrophils are supposed to have few mitochondria, they seem to have a restricted role in induction of apoptosis (Marianski, *et al.*, 2004a).

Reactive oxygen species (ROS)

Chronic granulomatous disease (CGD)-patients with inherited dysfunction in the NADPH oxidase can not generate ROS, and, as a consequence, their neutrophils are unable to kill most ingested bacteria (Curnutte, 1992). Neutrophils from these patients were Fas resistant and showed delayed spontaneous apoptosis (Kasahara, *et al.*, 1997). Moreover treatment of these abnormal neutrophils with H₂O₂ induced apoptosis, whereas addition of catalase delayed apoptosis of normal neutrophils. These data indicate that ROS are major mediators of the apoptosis in neutrophils.

Bcl-2 family members

Neutrophils contain high levels of pro-apoptotic Bcl-2 proteins, such as Bax and Bak, which could largely contribute to the short life span of those terminally differentiated cells (Akgul, et al., 2001). Neutrophil apoptosis is associated with translocation of Bax to the outer mitochondrial membrane, cytochrome c release and caspase-3 activation, all of which are inhibited by G-CSF (Maianski, et al., 2002). The trigger for Bax translocation and/or activation can involve caspase-8 and cleavage of Bid (Maianski, et al., 2004b). Thus, caspase-8, Bid, Bax and Bak appear to be important in pro-apoptotic mitochondrial activation. Neutrophils may also express other BH3-only members of the Bcl-2 family, such as Bim and Bad. Bim^{-/-} mice promoted neutrophil accumulation *in vivo* and prolonged neutrophil survival *in vitro* (Bouillet, et al., 1999, Villunger, et al., 2003). Bad phosphorylation, by for instance GM-CSF, render this pro-apoptotic protein unable to bind Bcl-2 and Bcl-x_L, and as a consequence, Bcl-2 and Bcl-x_L can block Bax-like proteins and inhibit apoptosis (Downward, 1999).

Neutrophils also express anti-apoptotic members of the Bcl-2 family. Neutrophils have been reported to express Mcl-1, A1 and Bcl-x_L (Akgul, et al., 2001). The increased expression of anti-apoptotic proteins such as Mcl-1, in response to survival signals, may at least partly explain their anti-apoptotic effects (Moulding, et al., 1998). However, Bcl-2 itself appears to have no role in delaying apoptosis of mature neutrophils (Dibbert, et al., 1999, Akgul, et al., 2001).

Mechanisms for neutrophil survival

Besides G-CSF and GM-CSF, many other pro-inflammatory mediators have been proposed as neutrophil survival factors. LPS (Lee, et al., 1993), C5a (Lee, et al., 1993), fMLP (Lee, et al., 1993), ATP (Gasmi, et al., 1996), leukotriene B4 (Lee, et al., 1999), several interleukins (Colotta, et al., 1992, Girard, et al., 1996) and gamma-interferon (IFN- γ) (Klebanoff, et al., 1992, Daigle, et al., 2002) can all delay neutrophil apoptosis. Although the intracellular signaling pathways that control these processes are largely unknown, it is clear that phosphorylation cascades are important. For instance, GM-CSF triggered elevation in cAMP and delayed neutrophil apoptosis is regulated by protein kinase A (PKA) (Parvathani, et al., 1998). Among the different protein kinase C (PKC)-isoenzymes so far identified, several have found to be involved in apoptosis regulation. PKC-theta, PKC-epsilon and PKC-

alpha rescues T cells from Fas-triggered apoptosis via the mitogen-activated protein kinase (MAPK) cascade leading to phosphorylation of Bad (*Bertolotto, et al., 2000*). However, the existence of such a pathway has not yet been found in neutrophils. Furthermore, during spontaneous neutrophils apoptosis the PKC-delta isoenzyme was specifically involved in DNA-fragmentation and apoptosis (*Pongracz, et al., 1999*), whereas this isoenzyme showed anti-apoptotic signaling capacity in neutrophils stimulated with TNF- α (*Kilpatrick, et al., 2002*).

Tyrosine phosphorylation is important in anti-apoptotic signaling in neutrophils. For example, Lyn was identified as an important tyrosine kinase responsible for mediating GM-CSF survival (*Wei, et al., 1996*). In addition to Jak2 phosphorylation and involvement of STAT proteins (*Al-Shami, et al., 1998*), tyrosine kinase activation also leads to activation of PI3K and MAPK pathways (Figure 4).

MAPKs mediate signal transduction pathways through different cell surface receptors, where a role for two of these, p38MAPK and ERK, has been described in neutrophil apoptosis. Upon cellular stress such as UV exposure, hyperosmolarity or bacterial infection, p38MAPK activation is associated with death signaling (*Frasch, et al., 1998, Lundqvist-Gustafsson, et al., 2001, Aleman, et al., 2004*). Spontaneous apoptosis, however, was shown to involve both activated (*Aoshiba, et al., 1999*) and inactivated (*Alvarado-Kristensson, et al., 2002*) p38MAPK, while others have shown that spontaneous neutrophil apoptosis is independent of p38MAPK activity (*Frasch, et al., 1998, Aleman, et al., 2004*). Even though the involvement of p38MAPK in the control of neutrophil apoptosis seems unclear, divergent signals generated downstream of this kinase might help explain the different effects. Rane *et al.* (2001) have for example, recently shown that MK2, which is a direct target of p38MAPK, is the unknown PDK2 in neutrophils that activates the anti-apoptotic kinase PKB.

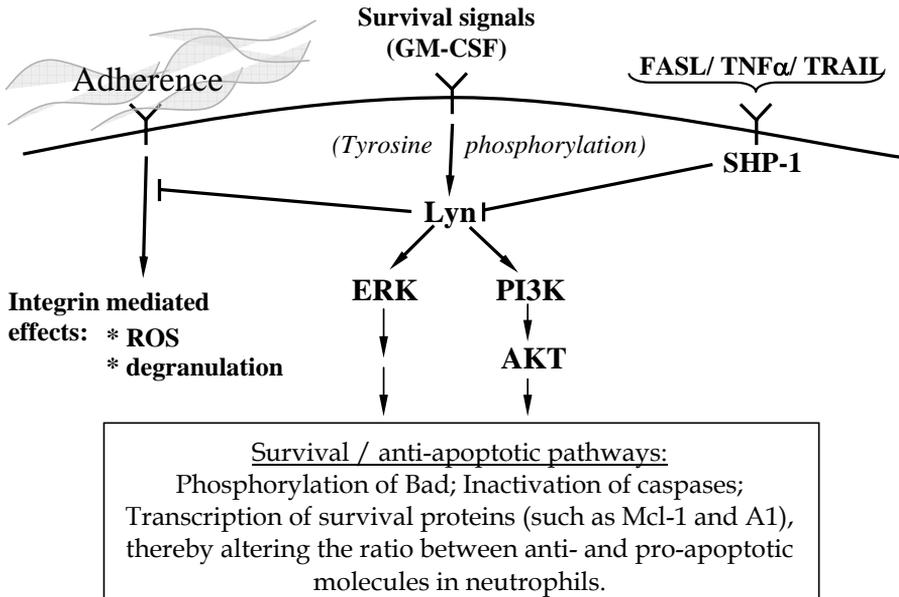


Figure 4. The role for ERK and PI3K/AKT in regulating neutrophil apoptosis, and the involvement of Lyn and SHP-1.

Activation of ERK, on the other hand, generates anti-apoptotic signals. LPS, LTB₄, GM-CSF and IL-8 do all delay neutrophil apoptosis by stimulating ERK activation (Klein, *et al.*, 2001, Petrin, *et al.*, 2006, Klein, *et al.*, 2000). Inasmuch as activation of β 2-integrins, in response to pro-apoptotic stimuli such as TNF- α and FAS ligand, is known to enhance apoptosis, Whitlock *et al.* (2000) further showed that clustered, inactivated β 2-integrins was capable of stimulating both ERK and AKT-activation. Additionally several “pro-inflammatory” cytokines such as GM-CSF, can activate PI3K, a kinase that together with one of its downstream targets, AKT, also is associated with generation of survival signals. In neutrophils the PI3K/AKT-pathway can relay its anti-apoptotic effects either through AKT-dependent phosphorylation of Bad (Klein, *et al.*, 2000), thereby reducing its pro-apoptotic effect, or by triggered upregulation of the anti-apoptotic protein Mcl-1 and downregulation of the pro-apoptotic protein Bax (Petrin, *et al.*, 2006).

Mechanisms limiting anti-apoptosis in neutrophils

Apoptosis play an important role in eliminating neutrophils from the inflamed tissue, thereby controlling the duration and intensity of an inflammatory response. Mechanisms controlling the accumulation or survival of neutrophils are likely to involve events that limit the synthesis of neutrophil survival factors, but also mechanisms directly activated in response to cell activation.

Src homology domain 2 (SH2)-containing tyrosine phosphatase (SHP-1)-deficient neutrophils exhibited reduced inhibition of GM-CSF-mediated survival upon simultaneous activation of Fas receptors, indicating a functional role for SHP-1 as an inhibitory phosphatase that limits anti-apoptotic signals (*Daigle, et al., 2002*). A role for SHP-1 in regulating neutrophil numbers was further supported by the observation that this phosphates is overexpressed in patients with severe neutropenia (*Tidow, et al., 1999*). Following Fas, TNF- α and TRAIL receptor activation, SHP-1 interaction and dephosphorylation of Lyn is one way by which SHP-1 negatively regulate survival signals (*Daigle, et al., 2002*) (Figure 4). Besides death receptor activation, the β -chain of the G-CSF receptor itself might activate SHP-1, thereby limiting, but not preventing, anti-apoptotic signals when additional SHP-1 activating stimulus is absent (*Yi, et al., 1993*).

Apoptosis modulation by microbes

Host pathogen interaction results in a variety of responses including phagocytosis of the pathogen, release of cytokines, secretion of toxins, as well as production of reactive oxygen species. Pathogens use different strategies to subvert normal host defense responses. Pathogens can modulate apoptosis by utilizing an array of virulence determinants that can interact with key components of the cell death pathway of the host or interfere with the regulation of transcription factors monitoring cell survival. Modulation of host cell apoptosis is one way for bacteria to eliminate key immune cells or evade host defenses that act to limit infection. Alternatively, suppression of apoptosis may facilitate the proliferation of intracellular pathogens (*Gao and Kwaik, 2000*). Virulence factors that induce or modulate cell death act by a variety of mechanisms including; (1) pore-forming toxins, which interact with the host cell membrane and leads to leakage of cellular components, (2) other toxins that express their enzymatic activity in the host cytosol, (3) effector proteins delivered directly into the host cell by a highly specialized type-III secretion system, and (4) other modulators of host cell apoptosis. (*Weinrauch and Zychlinsky, 1999*)

Toxins

The pore-forming toxin leukotoxin produced by the gram-negative bacterium *Actinobacillus actinomycetemcomitans*, specifically targets the β 2-integrin LFA-1 (leukocyte function antigen-1 or CD11a) predominantly found on lymphocytes, neutrophils, monocytes and macrophages (*Lally, et al., 1997*). LFA-1 binding results in apoptosis and elimination of immune cells. *E. coli* produced alpha-hemolysin also mediate cell death of human immune cells via LFA-1 (*Lally, et al., 1997*), but unlike *A. actinomycetemcomitans* leukotoxin, alpha-hemolysin is toxic to a broader range of cells. Another mechanism for induction of apoptosis by bacterial pathogens includes the inhibition of host cell protein synthesis by bacterial A-B toxins (*Kochi and Collier, 1993*). Opposed to the pore-forming toxins, these toxins have to be internalized in order to execute their full effect. The B subunit of the A-B toxin mediates host receptor attachment and facilitates delivery of the catalytic active A subunit to the host cytoplasm. The

bacterial toxins diphtheria toxin (Dtx) (Chang, et al., 1989), Shiga and Shiga-like toxins (Stx or verotoxin) (Tesh and O'Brien, 1991), produced by *Corynebacterium diphtheriae*, *Shigella dysenteriae* and Enterohemorrhagic *Escherichia coli* are examples of toxins that inhibit the protein synthesis machinery, and thereby kill the target cell.

Type-III secretion system

The type-III secretion system (TTSS) is a highly adapted secretory machinery used by certain gram-negative pathogens such as *Shigella* spp., *Salmonella* spp., and *Yersinia* spp.. The injection of effector proteins by this secretory apparatus is a way for bacterial pathogens to alter host cell signal transduction. Using different effector proteins, *Shigella* can trigger invasion and escape from the phagosome of macrophages (Zychlinsky, et al., 1992), whereas *Salmonella* trigger its own internalization into a membrane-bound vacuole from which it cannot escape. After uptake by macrophages, *Salmonella* finds itself in an environment where nutrients are limited, osmolarity is high and pH is low, resulting in a lag phase during which little bacterial growth occurs (Bajaj, et al., 1996). During this lag phase gene expression of *Salmonella* is changed, rendering the bacterium more resistant to the adverse condition and enabling the bacterium to replicate within the macrophage (Bajaj, et al., 1996). Both *Shigella* and *Salmonella* induce macrophage apoptosis, although the time for triggering apoptosis differs. *Shigella* triggers apoptosis first after escape from the phagosome, whereas *Salmonella* trigger apoptosis as part of the invasion process. However, there appears to be an extensive sequence homology as well as functional similarity in the cytotoxicity of their effectors responsible for triggering apoptosis. Expression and secretion of the invasion plasmid antigen B (IpaB, from *Shigella*) and *Salmonella* invasion protein B (SipB) cause caspase-1-dependent apoptosis in macrophages (Hilbi, et al., 1998, Hersh, et al., 1999). Unlike *Shigella* spp., which needs to be internalized in order to induce cell death, *Yersinia* spp. are able to induce apoptosis from the outside of the host cell (Mills, et al., 1997, Monack, et al., 1997). *Yersinia*-delivered effector proteins interfere with different host cellular processes, including alteration of the cytoskeleton, inhibition of phagocytosis and inhibition of the oxidative burst triggered by secondary infection with IgG-opsonized bacteria (Andersson, et al., 1996, Bliska and Black, 1995).

Yersinia also inhibits or modulates the cytokine response of the host, and thereby impinge on an important part of the immune response to infection. The release of cytokines such as TNF- α and IFN- γ is essential for combating *Yersinia* infection *in vivo* (Nakajima and Brubaker, 1993, Autenrieth and Heesemann, 1992). In *Yersinae*-infected macrophages, *in vitro*, the impairment of the normal TNF- α release correlated to decreased MAPK activity as well as an inhibition of NF- κ B activation (Schesser, et al., 1998, Palmer, et al., 1998). Inhibition in NF- κ B-mediated signaling not only abrogates the inducible cytokine expression, but also inhibits anti-apoptotic effects mediated by this transcription factor (Baichwal and Baeuerle, 1997).

Phagocytosis

Other pathogens that evade the killing by macrophages are *Legionella pneumophila* and *Mycobacterium tuberculosis* (Gao and Kwaik, 2000). These bacteria modulate the phagosomal maturation process so that the phagosome in which they reside is not acidified and fails to fuse with primary and secondary lysosomes. The bacteria continue to grow until their host macrophages are lysed, allowing the infection to spread. Although it is not clear whether host or mycobacterial factors are associated with apoptosis *in vivo*, the increase in apoptotic alveolar macrophages in bronchoalveolar lavages from patients with active tuberculosis, suggest that apoptosis plays an important role in clinical tuberculosis (Klingler, et al., 1997). It has also been shown that apoptosis of mycobacteria-infected macrophages was instrumental for the activation of bystander macrophages and resulted in significant growth inhibition of the microorganism. When apoptosis was prevented, the bacteria grew in an unlimited fashion within the infected macrophage, and these cells could not trigger activation of newly recruited macrophages (Fratazzi, et al., 1997). Besides apoptosis, activation of infected macrophages by neighboring cells may account for some of the bactericidal effect. For instance, IFN- γ , mainly produced by T cells and NK cells, triggers activation of macrophages to produce reactive oxygen species and reactive nitrogen species (Collins and Kaufmann, 2001, Gatfield and Pieters, 2000, Hu, et al., 2000), both of which are essential in the antimycobacterial defense. IFN- γ also increases MHC II presentation and phagolysosomal fusion, and mediates upregulation of TNF- α production in the infected cell (Schluger,

2001), altogether aiding in the clearing of infection. However, infection with live virulent *Mycobacterium tuberculosis* can inhibit macrophage responses to IFN- γ (Banaiee, et al., 2006).

The ability of phagocytes, such as neutrophils and macrophages, to kill invading bacteria is crucial for host defense in that it is immediate and not dependent on previous pathogen exposure. Compared to neutrophils, however, macrophages are more readily infected by many bacterial pathogens (DeLeo, 2004). The reason for macrophages being more prone to infection may relate to their physiological and functional differences. For instance, macrophages are more long-lived, making them a better choice for intracellular pathogens, and compared to neutrophils they have a reduced capacity to produce ROS (Johansson and Dahlgren, 1992), which could account for their limited bactericidal capacity. On the other hand, neutrophils are superbly adapted to kill microorganisms, and only the intracellular pathogens *Anaplasma phagocytophilum* and *Chlamydia pneumoniae* have conclusively been shown to delay apoptosis and subvert neutrophil killing mechanisms in order to survive and replicate within this cell (Scaife, et al., 2003, van Zandbergen, et al., 2004). Depending on the pathogen and stage of infection, host-pathogen interaction leading to macrophage apoptosis or inhibition of neutrophil apoptosis is generally considered to diminish the innate immune response to infection.

Neutrophils undergo rapid apoptosis. During bacterial infection, however, host-response mediated release of pro-inflammatory cytokines, such as IL-1 β , TNF- α , G-CSF, GM-CSF, IFN- γ and bacteria-derived products such as LPS, lipoteichoic acid and a number of bacterial toxins, delay spontaneous neutrophil apoptosis (Colotta, et al., 1992, Lotz, et al., 2004, DeLeo, 2004). This suggests that enhanced neutrophil survival is desirable during early stages of inflammation to promote the clearance of bacterial pathogens. Importantly, Watson et al. (1996a) and English et al. (2001) demonstrated that bacteria-induced apoptosis overrides any delay in cell fate imparted by factors such as LPS or GM-CSF. However, ingestion of pathogenic bacteria such as *Escherichia coli*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Burkholderia cepacia*,

Borrelia hermsii and *Listeria monocytogenes* significantly accelerate the rate of neutrophil apoptosis (reviewed in (DeLeo, 2004)). It is clear that apoptosis has a direct role in many infectious diseases, and that many microbial pathogens exploits and drives the apoptotic signaling in the host and not *vice versa* (Gao and Kwaik, 2000). Although accelerated bacteria-induced inactivation of neutrophils can be seen as beneficial for the bacterium, as these professional phagocytes are the most dangerous cells for the bacterium, host pathogen-induced neutrophil apoptosis can be interpreted differently. It was found that complement (C3)-opsonized paraffin oil droplets (Coxon, et al., 1996) and IgG-coated erythrocytes (Gamberale, et al., 1998) mediated phagocytosis-induced neutrophil apoptosis, indicating that phagocytosis *per se*, without the contribution of effectors from bacterial pathogen, can trigger the phagocytosis-induced cell death (PICD). These studies and those using serum-opsonized *E. coli* (Watson, et al., 1996b) or *Mycobacterium tuberculosis* (Perskvist, et al., 2002), indicate that NADPH-oxidase generated ROS is one important determinant needed for triggering PICD, since the inhibition of this enzyme or the scavenging of ROS inhibited apoptosis. In addition, heat-killed bacteria or those readily killed following neutrophil phagocytosis may accelerate apoptosis (Watson, et al., 1996b, DeLeo, 2004, Matsuda, et al., 1999, Perskvist, et al., 2002, Lundqvist-Gustafsson, et al., 2001). This suggests that once the bacteria are killed, apoptosis is accelerated with subsequent removal of the apoptotic cells. This could be beneficial for the host since the overall production or secretion of pro-inflammatory components from neutrophils decreases during apoptosis.

Identifying genes regulated during the onset of neutrophil apoptosis, occurring after phagocytosis, using an oligonucleotide microarray approach, has corroborated previous functional studies and extended the role of phagocytosis-induced apoptosis in a broader perspective termed apoptosis-differentiation program (Kobayashi, et al., 2003a). The changes in gene expression during apoptosis are proposed to be a part of an apoptosis-differentiation program constituting a final stage of transcriptionally regulated neutrophil maturation that is significantly accelerated during phagocytosis and the production of ROS (Kobayashi, et al., 2003a, Kobayashi, et al., 2004). The apoptosis-differentiation program in neutrophils was also shown to down-regulate the pro-inflammatory capacity

of neutrophils, a process critical for the resolution of inflammation (Kobayashi, et al., 2003b). When screening a diverse group of bacterial pathogens, it was found that those succumbing to neutrophil killing triggered up-regulation of genes encoding pro-apoptosis factors and down-regulation of genes encoding anti-apoptosis proteins (Kobayashi, et al., 2003c). Furthermore, phagocytosis of bacterial pathogens potentiated the innate immune response by inducing genes encoding proteins involved in activation and recruitment of immune effector cells, including macrophage inflammatory protein 2 (MIP-2 α), MIP-2 β , MIP-3 α , vascular endothelial growth factor and oncostatin M. In parallel, phagocytosis lead to a down-regulation of genes encoding key surface molecules, impairing further chemotaxis and recruitment. The common pathogen-induced transcription profile that included 305 up-regulated and 297 down-regulated genes increased with time (3-6 h). In the same study, however, *Streptococcus pyogenes* actively altered 50% of these common genes, of which many were apoptosis/cell fate-related genes, already after 90 min, thereby altering or accelerating the apoptosis-differentiation program. In an other study *Anaplasma phagocytophilum* did not trigger ROS, possibly due to uptake though endocytosis rather than phagocytosis, and did not induce the neutrophil apoptosis-differentiation program (Borjesson, et al., 2005). Although pro-inflammatory genes were eventually upregulated (24 h), this delayed pro-inflammatory response following infection with *A. phagocytophilum* may in part underlie intracellular survival and represent another pathogen immune evasion strategy.

In conclusion, as in macrophages, there seems to be at least two fundamental outcomes for the interaction of bacterial pathogens in neutrophils; (1) phagocytosis-induced apoptosis contributing to resolution of bacterial infection, or (2) phagocytosis or interaction of microorganisms altering the apoptosis program in neutrophils, resulting in pathogen survival and infection.

Clearance of apoptotic cells

Cell surface changes and recognition

Apoptosis is closely connected with changes in the expression of cell-surface molecules on the dying cell. Neutrophil apoptosis is accompanied by down-regulation of members of the immunoglobulin superfamily, including CD31 (PECAM-1), CD50 (ICAM-3), CD66 and CD87 (UPA receptor), as well as cell surface receptors such as CD15s (Sialyl Lewis X), CD11a (LFA-1/ α L integrin), CD16 (FcRIIIa and b), CD32 (FcRII), CD35 (CR1), CD88 (C5a Receptor), CD120b (TNF Receptor), CD62L (L-Selectin/LECAM-1) and CD43 (Leukosialin) (Akgul, et al., 2001, Dransfield, et al., 1995). Moreover, apoptotic neutrophils show an up-regulation of adhesion molecules like CD53, CD63 (granule membrane protein present in azurophilic granules), CD11b (CR3/ α M integrin) and CD11c (α X integrin) (Dransfield, et al., 1995, Beinert, et al., 2000). The general shift in cell-surface molecule expression on dying cells, leading to changed recognition-pattern by scavenger cells (Figure 5), such as macrophages, has led to the so called “don’t eat me” or “eat me”-signals. Self-recognition markers that transfer “don’t eat me”-signals to scavenger cells include CD47 (Oldenborg, et al., 2000) and CD31 (Brown, et al., 2002). The loss of CD47 (integrin-associated protein) on the surface of the dying cell removes the inhibitory signal of phagocytosis otherwise mediated by SIRP α on the scavenger cell, whereas CD31, although still exposed on apoptotic cells, loses its intracellular signal domain/mechanisms involved in disassociation and repulsion from the phagocyte surface. Cell surface changes such as modifications in the glycosylation pattern (specifically loss of sialic acids) together with oxidation of membrane proteins and lipids, can themselves be strong inducers for recognition and engulfment of apoptotic cells, but are also implicated in modifying expressed apoptosis markers (Azuma, et al., 2000, Watanabe, et al., 2002, Sambrano and Steinberg, 1995, Bird, et al., 1999, Medzhitov and Janeway, 2002). Externalization of PS, the most extensively studied and recognized marker on apoptotic cells (Fadok, et al., 1992, Fadok, et al., 2000, Henson, et al., 2001), was shown to be both necessary and sufficient for macrophage recognition and clearance (Kagan, et al., 2002). (See (de Almeida and Linden, 2005) for a more detailed summary on surface markers and receptors involved in clearance of apoptotic cells.) However, oxidation of PS

(PS-OX) through NADPH-oxidase dependent apoptosis in neutrophils and PS-OX containing liposomes was shown to clearly potentiate this clearance as compared to nonoxidized PS (Kagan, et al., 2002, Arroyo, et al., 2002). Oxidation of PS is suggested to be an integral part of the apoptosis program as it is not restricted to oxidative-stress induced apoptosis (Kagan, et al., 2000).

Cofactors for recognition

In addition to apoptotic-cell-associated ligands and phagocyte receptors an increasing variety of soluble intermediate factors are emerging, whose role is to opsonize apoptotic cells and/or creating molecular bridges between components of the apoptotic-cell and the phagocyte surface. Some bridging molecules and their interaction partners leading to enhanced engulfment of apoptotic bodies are; (1) the first component of complement, C1q, as well as its collectin relatives surfactant proteins SP-A and -B, and mannose-binding lectins, which links to the apoptotic-cell surface with the phagocyte receptor complex CD91/calreticulin, (2) the secreted glycoprotein, milk fat globule EGF factor 8 (MFG-E8) bridges via the vitronectin receptor ($\alpha_v\beta_3$ integrin) on macrophages and the exposed PS on apoptotic cells and (3) recognition apoptotic bodies by a complex formed by CD36 (a scavenger receptor that recognize PS), the $\alpha_v\beta_3$ integrin and the secreted bridging molecule thrombospondin. Other bridging molecules cited in the literature include IgM, and other opsonins present in serum such as C-reactive protein, β_2 -glycoprotein I, serum amyloid P component (SAP) and serum derived protein S (de Almeida and Linden, 2005, Gregory and Devitt, 2004). In that apoptosis is a dynamic, active process, and the cell surface continuously changes, distinct signals for recognition by phagocytes are exposed at different moments of the apoptotic process. Phagocytes in turn, display various molecules that are engaged in complexes of recognition in a time-dependent manner, according to the degeneration stage of the target cell. For example, nonactivated macrophages secrete thrombospondin, whereas activated macrophages secrete MFG-E8 (Hanayama, et al., 2002). Further, in cells undergoing apoptosis, annexin I was found to be recruited from the cytosol and colocalize with PS on the surface (Arur, et al., 2003), and recognition of the apoptotic cell through the specific PS receptor (PSR) was reported to occur via annexin I (Fadok, et al., 2000). Besides PSR, there are various molecules that are possible

candidate receptors for PS (CD68, LOX-1, SRA, SRBI and CD36) (Ramprasad, et al., 1995, Oka, et al., 1998, Platt, et al., 1996, Fukasawa, et al., 1996, Tait and Smith, 1999), indicating both that PS recognition is crucial but also that different expression of bridging molecules form the apoptotic cell or the surrounding foci of inflammation necessitates alternative recognition mechanisms. It is likely that cells committed to apoptosis express different markers depending on the level of apoptosis, but also reflecting the insult ultimately responsible for inducing apoptosis.

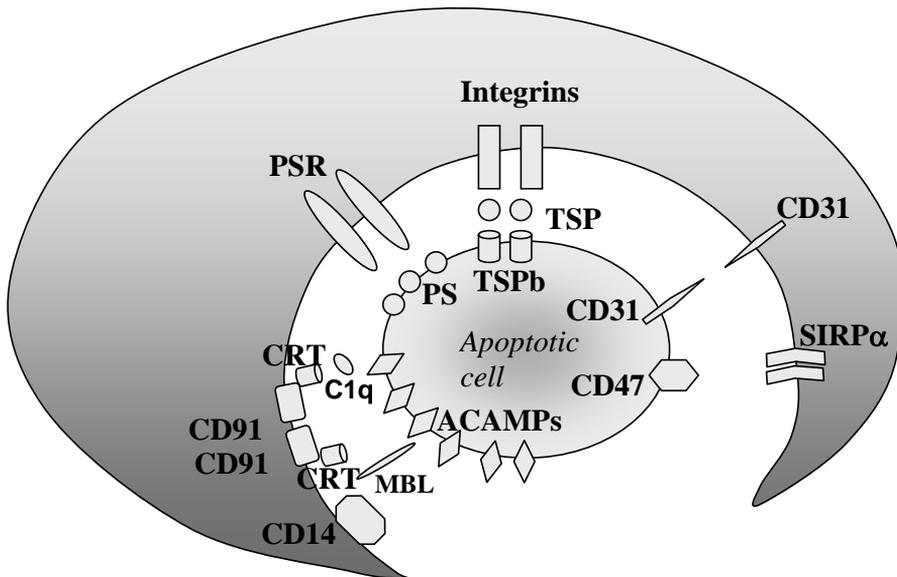


Figure 5. Positive and negative signals that regulate the recognition and engulfment of apoptotic cells. Phagocytosis of apoptotic cells is stimulated by various molecules displayed on the surface of both cell types. Some molecules present in viable cells avoid recognition by phagocytes, and are lost (CD47) or disabled (CD31) during apoptosis, allowing clearance of degenerating cells. Several components of the innate immune system, including CD14, β_2 -integrins (which can bind the opsonic complement fragment inactivated C3b, iC3b), the CD91-calreticulin (CRT) complex (which can bind the first component of complement, C1q, and collectins such as mannose-binding lectin (MBL)), recognizes apoptotic-cell-associated molecular patterns (ACAMPs) (Gregory and Devitt, 2004). Exposure of phosphatidylserine (PS) on the surface of apoptotic cells is a key “eat-me”-flag. It is recognized by the specific PS-receptor (PSR), and by other candidates such as scavenger receptors. SIRP α , signal regulatory protein α ; TSP, trombospondin; TSPb, trombospondin binding site.

Heat shock proteins as immunoregulatory molecules

Heat shock proteins (HSP), are commonly referred to as molecular chaperones due to their involvement in basic cellular processes such as folding of nascent polypeptides, translocation of polypeptides across membranes, and the assembly of macromolecule structures. The low levels of HSP expressed under normal physiological conditions are known to increase markedly in response to a wide variety of stressful stimuli including pathological conditions such as viral, bacterial, parasitic infections or fever, inflammation, malignancy or autoimmunity (Lindquist and Craig, 1988). Bacteria-induced HSP expression has for example been identified in liver and spleen cells of *Listeria monocytogenes* infected mice (increased HSP60 was localized to the plasma membrane) (Belles, et al., 1999), and in neutrophils from patients suffering severe sepsis (HSP27, HSP60, HSP70, HSP90 was increased) (Hashiguchi, et al., 2001). Recently, it has been shown that certain members of the HSP family (e.g., HSP60, HSP70, and HSP90) can directly stimulate cells of the innate immune system and thereby act as danger-signaling molecules (Srivastava, 2002, Asea, et al., 2000, Wallin, et al., 2002, Moseley, 2000). Proteins proposed to act as specific receptors for HSP include CD14 (Asea, et al., 2000), TLR2 and 4 (Asea, et al., 2002), CD40 (Becker, et al., 2002) and several scavenger receptors such as CD91 (Basu, et al., 2001), LOX-1 (Delneste, et al., 2002) and macrophage scavenger receptor 1 (Berwin, et al., 2003). This, together with the finding that HSP has the ability to bind PS (Arispe, et al., 2004) or function as an antigen carrier (Moseley, 2000), further signifies HSP as an immunoregulatory molecule. Also, there is evidence supporting a role for HSP (HSP70) in promoting clearance of virus-infected cells from the brain (Oglesbee, et al., 2002).

Consequences of apoptotic cells clearance

A hallmark of the phagocytic removal of apoptotic cells *in vivo* is the failure to generate an associated inflammatory response (Savill, et al., 1993, Savill, 1997). Uptake of apoptotic neutrophils has been shown to inhibit the production of pro-inflammatory mediators in macrophages by secretion of anti-inflammatory cytokines such as transforming growth factor β (TGF- β) (Savill, et al., 2002, Fadok, et al., 1998, Ren, et al., 2001, Huynh, et al., 2002, Fadok, et al., 2001a, Byrne and Reen, 2002). This active anti-inflammatory response in macrophages represents a mechanism for safe clearance of apoptotic cells under physiological conditions and serves as a

key step in the resolution phase of inflammation (Fadok, et al., 2001a, Savill, et al., 2002). However, in the cited studies, neutrophil apoptosis was induced by age or irradiation, thus the results may not apply to the very early phase of infection when many neutrophils undergo pathogen-induced apoptosis. We previously observed that pro-inflammatory responses in macrophages are triggered by uptake of Mycobacteria-induced apoptotic neutrophils, but not by ingestion of uninfected apoptotic neutrophils (Perskvist, et al., 2002). Other investigators have shown that interactions between macrophages and apoptotic inflammatory neutrophils prevent the growth of *Leishmania major* both *in vitro* and *in vivo*, whereas phagocytosis of uninfected apoptotic cells by macrophages promotes the intracellular growth of *Trypanosoma cruzi* (Ribeiro-Gomes, et al., 2004, Lopes, et al., 2000). This suggests that host-defense mechanisms against invading microorganisms do not end with the induction of phagocyte apoptosis. The above findings (and those presented in Paper IV) imply that interaction with apoptotic neutrophils primes macrophages so that they are not simply anti-inflammatory actors, as previously suggested, but also respond to danger signals in a more complex way that may play a crucial role in host defense. It is not yet known how macrophages distinguish between, and react differently to, uninfected or inflammatory apoptotic neutrophils.

Phagocytosis and ROS play an important role in modulating neutrophil apoptosis, and although significant progress has been made in the field of phagocyte apoptosis and pathogen-specific immune subversion, many questions remain unanswered. A better understanding for the mechanisms of pathogen- or host modulated apoptosis and its contribution to the inflammatory process is necessary for uncovering new therapeutical intervention targets that can help improve treatment of several infectious diseases.

Aim of the dissertation

Increasing numbers of pathogens have been found to modulate host cell apoptosis and thereby influence the progression of disease. Induction of host cell apoptosis may, however, not only represent a pathogenic strategy to eliminate key immune cells, but may also constitute a host defense mechanism against invading microorganisms. The aim of this thesis was therefore to elucidate the balance between pathogen-inflicted and host-associated measures leading to apoptosis or anti-apoptosis signaling. Consequences of different routes of pathogen entry into phagocytes were studied. The aim was also to clarify the role of bacteria-induced apoptotic neutrophils in macrophage activation and its contribution to inflammation.

The specific aims were to elucidate:

- How *Salmonella* invasion and receptor-mediated phagocytosis trigger pro- and anti-apoptotic signaling.
- The role for the type 1 fimbrial adhesin of uropathogenic *E. coli* in neutrophil apoptosis.
- How intraphagosomal versus nonphagosomal production of ROS affects neutrophil apoptosis.
- The effect of pathogen-induced apoptotic neutrophils on macrophage activation, and the potential role for heat shock proteins as apoptotic cell-derived danger signals.

Methods

Some of the methods are given here. The rest of the methods used are found in paper I-IV.

Separation of human neutrophils

Human neutrophils were isolated from heparinized whole blood from healthy donors by gradient centrifugation essentially as described by Böyum (*Boyum, 1968*). Briefly, neutrophils in the interphase between Polymorph Prep™ and Lymphoprep™ were collected and washed in phosphate-buffered saline (PBS), contaminating erythrocytes were removed by a brief hypotonic lysis and neutrophils were washed in Krebs-Ringer phosphate buffer (KRG) without Ca^{2+} . Neutrophils of about 98% purity were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine (RPMI-medium).

Human monocyte-derived macrophages

Human monocytes were prepared from peripheral blood monocytes (PBMC) (*Perskvist, et al., 2002*). Buffy-coat from healthy blood donors diluted 1:2 in 0.9 % NaCl were put on Lymphoprep™ and centrifuged at 450xg, for 30 min at room temperature (RT). The monocyte-containing bands were washed three times in PBS-heparin and twice in KRG without Ca^{2+} . After the final wash the cells were resuspended in DMEM culture medium, supplemented with penicillin and streptomycin, and plated at 4×10^6 /well in 24-well plates and allowed to adhere for 1 hour at 37°C in 5 % CO_2 . Non-adherent cells, mainly lymphocytes, were removed by repeated washes with KRG and the adherent monocytes were then cultured in DMEM containing 10 % human AB serum supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM L-glutamine. Macrophages were cultured 6-9 days and medium was changed every 3 days, yielding $\sim 10^6$ macrophages/well at time of assay. Before used in experiments the medium was replaced with DMEM without serum.

Culturing of U937 cells

Human monocytic U937 cells were grown in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml

penicillin and 100 U/ml streptomycin. Cells were split when the cell concentration reached $1.2-1.6 \times 10^6$ cell/ml, usually twice a week, and the cells were used at passage 5-30. Before experiments the cells were serum-starved for 2 h in RPMI medium without antibiotics at 37°C.

Phagocytosis

For bacterial phagocytosis or adhesion to neutrophils, bacteria were FITC labeled and the interaction was analyzed by both microscopy and flow cytometry (Heinzelmann, *et al.*, 1999). Bacterial aliquots were resuspended in FITC-solution (0.1mg FITC/mL in 0.1M carbonate buffer pH 9.6), incubated for 1 h at RT under rotation, were washed and then resuspended in RPMI-medium. Where indicated, these bacteria were subsequently opsonized with serum. For microscopy analysis, neutrophils (1×10^5) were allowed to adhere to glass slides for 15 min at 37°C in a moist chamber. Non-adhered neutrophils were removed with warm KRG and FITC-bacteria were added at the ratio 40 bacteria per neutrophil for 1 and 2 h at 37°C. The glass slides were then washed briefly in cold KRG and analyzed after adding ethidium bromide (EtBr).

A fluorescence microscope (Axioscope, Zeiss, Germany) was used to count green intracellular and red extracellular bacteria. Flow cytometry analysis was used to assay bacteria interaction with cells in suspension as follows; 1×10^6 cells were mixed with FITC-bacteria at the ratio of 40 bacteria per neutrophil in RPMI-medium and incubated at 37°C for 1 h. Reactions were terminated by adding cold KRG and unbound bacteria were washed off with cold KRG (300 g, 4°C, 7 min). The binding or phagocytosis of FITC-labeled bacteria (FL-1) was measured by flow cytometry (FACS-Calibur; BD Biosciences) and analyzed using the CellQuest software. EtBr (50µg/ml) was added to distinguish between intra- and extracellular bacteria.

Invasion and intracellular growth

For bacterial invasion of U937 cells, unstained bacteria were used in a gentamicin-protection assay as previously described (Lee and Falkow, 1990). After exposure to bacteria for 30 min in 37°C, cells were washed and incubated in RPMI medium supplemented with 50 µg/ml gentamicin for 1 h at 37°C. Thereafter the cells were washed in PBS and subsequently lysed in 1% Triton X-100 in PBS for 10 min. Viable bacteria released from U937 cells were

quantified by culturing serial dilution of the mixtures on LB agar plates and then performing colony-forming unit (CFU) counts. To assess the intracellular growth of bacteria, a medium containing 10 µg/ml gentamicin was used.

ROS production

The respiratory burst in neutrophils was measured by a luminol-amplified chemiluminescence (CL) assay in a six-channel Bioluminat LB9505 (Berthold Co. Wiblad, Germany), using disposable 4 ml polypropene tubes. Neutrophils (1×10^6), luminol (20 µM) and HRP (4 U/ml) in KRG were prewarmed for 10 min at 37°C, and then the light emission was recorded continuously. After establishing a baseline, prewarmed bacteria were added at the indicated bacteria to cell ratios. To distinguish between extra- and intracellular generation of ROS, neutrophils were preincubated with superoxide dismutase (400 U/ml) and catalase (4000 U/ml). These scavengers respectively remove extracellular superoxide anion and hydrogen peroxide.

Lysosomal membrane stability

Acridine orange (AO), a lysosomotropic weak base (pKa = 10.3), is retained in its charged form (AOH⁺) by proton trapping inside the acidic vacuolar compartment. At the end of the experiments, cells were incubated with RPMI-medium supplemented with AO (5 µg/ml for 15 min at 37°C), and cells with a

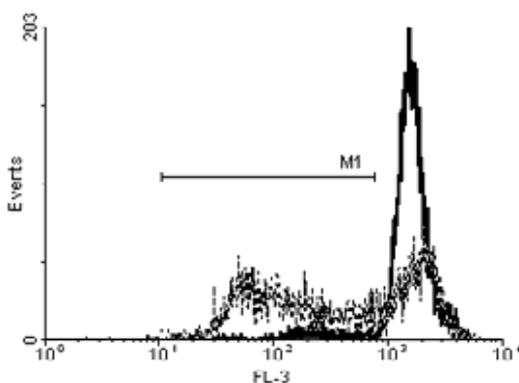


Figure 6. AO uptake method. AO-fluorescence profile of nonapoptotic (bold) and apoptotic (hatched) neutrophils. M1 is set to evaluate the percentage of cells that have lost their capacity to accumulate AO.

reduced number of intact AO-accumulating lysosomes or “pale cells” were analyzed as cells with decreased red fluorescence (the AO uptake method) (Zhao,

et al., 2000, *Yu, et al.*, 2003) (Figure 6). The AO relocation method (*Antunes, et al.*, 2001), with cells preloaded with AO before stimulation, was also used to monitor membrane permeabilization resulting in increased green cytoplasmic fluorescence. Green (FL-1 channel) and red fluorescence (FL-3 channel) was measured by flow cytometry (FACS-Calibur; BD Biosciences) and analyzed using the CellQuest software.

An alternative way to determine the level of lysosomal membrane damage was to assay the activity of the 250 kDa protein β -N-acetylglucosaminidase (NAG) in digitonin-extracted cytosols (*Bidere, et al.*, 2003). In the end of experiments cells were treated with an extraction buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1 mM pepablock, pH 7.5) containing 20 μ g/ml digitonin (or 100 μ g/ml digitonin for total cellular NAG activity) for 10 min on ice with inverting tubes every 2 min. NAG activity in different cytosol extracts (1×10^6 cells/sample) was estimated by adding three volumes of 0.2 M sodium citrate buffer, pH 4.5, containing 300 μ g/ml 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside. The liberation of methylumbelliferyl (excitation, 360/40 nm; emission, 460/40 nm) was measured for 40 min at 37°C with a Synergy HT multi-detection microplate reader from BIO-TEK Instruments, Inc. USA, and the V_{max} was calculated.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by tetramethylrhodamine ethyl ester (TMRE), which accumulates in the mitochondrial matrix. Decreased $\Delta\Psi_m$ was indicated by a reduction in the TMRE-induced red fluorescence intensity using flow cytometry-analysis in end of experiments. Samples were collected and loaded with RPMI-medium supplemented with 1 μ M TMRE for 15 minutes in the dark at 37°C. Neutrophils were then subjected to flow cytometry by activation with the 488-nm wavelength, and the red fluorescence was monitored in the FL-3 channel (650 LP filter)(Figure 7).

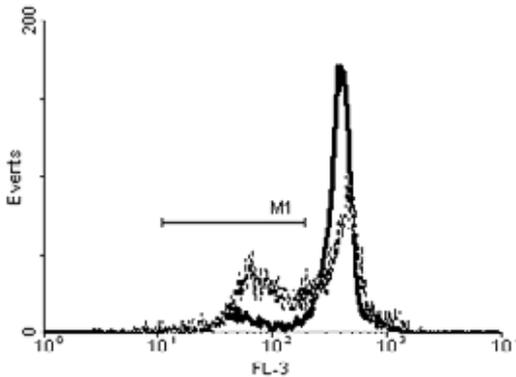


Figure 7. Mitochondrial membrane potential measurement. TMRE-fluorescence profile of nonapoptotic (**bold**) and apoptotic (*hatched*) neutrophils. M1 is set to evaluate the percentage of cells that have lost their potential.

Apoptosis

As an indicator of early neutrophil or U937 cell apoptosis, we analyzed the exposure of phosphatidylserine (PS) on the surface of apoptotic cells by staining

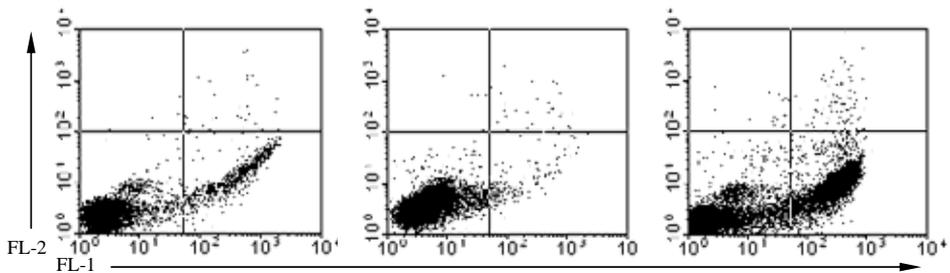


Figure 8. Apoptosis measurement of neutrophils. FITC-annexin V (FL-1, green) and PI (FL-2, red) labeling of live neutrophils. From left: unstimulated, anti-apoptotic, or pro-apoptotic stimuli. Lower right quadrant show apoptotic cells.

with FITC-conjugated annexin V according to the protocol from the manufacturer (R&D Systems) (Figure 8). Cells were washed once in binding buffer (150 mM NaCl, 5 mM KCl, 1mM MgCl₂, 1.8mM CaCl₂, 10mM HEPES, pH 7.4), and the specific binding of annexin V was achieved by incubating 10⁶ neutrophils in 60 μ l binding buffer containing a saturated concentration of FITC-annexin V for 15 min at 4°C in the dark. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with annexin V and propidium iodide (PI) before analysis. The binding of FITC-annexin V (FL-

1) and PI (FL-2) to the cells was measured by FACS and analyzed using the CellQuest software program (Perskvist, et al., 2002). Cells with increased FITC fluorescence (FITC⁺/PI), corresponding to an increased exposure of PS, were considered as early apoptotic, while cells with an increase in both FITC- and PI-fluorescence were considered late apoptotic or necrotic (Vermes, et al., 1995). At least 10,000 cells were counted in each sample and a gate based on forward and side scatter was set to exclude cell debris.

DNA fragmentation analysis was performed by resuspending $\sim 10^6$ cells in lysis buffer (0.1 % sodium citrate and 0.1 % Triton X-100) containing 50 $\mu\text{g}/\text{ml}$ of PI for evaluation of internucleosomal DNA fragmentation (Nicoletti, et al., 1991). The lysed cells were incubated 3-24 hours in the dark at 4°C. Occurrence of particles with low red (FL-3 channel) was measured by flow cytometry and analyzed using the CellQuest software (Figure 9).

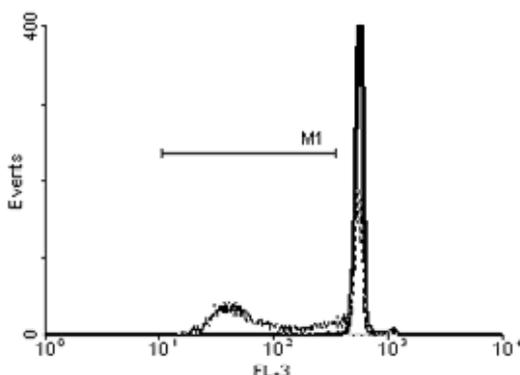


Figure 9. Apoptosis measurement of neutrophils. PI-labeling (FL-3, far red) of Triton-X-premeabilized cells. Histograms show nonapoptotic (bold) and apoptotic (hatched) cells, where M1 is set evaluate the percentage that have fragmented DNA.

Morphological analysis of apoptosis was also performed on cytocentrifuged neutrophils stained with Türck's reagent (crystal violet), where the characteristics of neutrophil apoptosis with condensed nuclei was examined under a light microscope. Trypan blue dye exclusion using microscopical evaluation was performed in parallel to further distinguish viable or apoptotic cells from necrotic cells. Cells with intact cell membrane do not take up the dye, whereas necrotic cells become blue.

Antisense transfection

The cDNAs for Akt1, Akt2 and Akt3, which contain the entire coding sequence, were generated by reverse transcription PCR from human fetal RNA, and each cDNA was subcloned in its antisense orientation into EcoRI site of the mammalian expression vector pCR3; Dr. Hiroyasu Emusi (National Cancer Center Research Institute East, Chiba, Japan) kindly provided these constructs (*Izuishi, et al., 2000*). After amplification of the antisense construct an EndoFree plasmid maxi kit (Qiagen, Valencia, CA) was used to purify the plasmid DNAs, and restriction analysis with subsequent gel electrophoresis was performed to verify insert size and restriction cleavage pattern. Control vector or antisense expression vectors (25 µg) were transfected into 10^7 U937 cells by electroporation using a Gene-Pulser (Bio-Rad, Hercules, CA). After the recovery culture of 48 h, transfectants were selected by treatment with 0.5 mg/ml G418. G418-resistant clones were pooled after 3 weeks of selection and then grown as a mixture that was used within 3 weeks.

Results & Discussion

Invasion and pro-apoptotic signaling in phagocytes (paper I and II)

Well-adapted pathogens such as *Salmonella typhimurium* (*S. typhimurium*) have developed specific mechanisms to modulate the fate of host cells. It is well established that *Salmonella* bacteria induce macrophage apoptosis both *in vivo* and *in vitro* (Chen, et al., 1996, Lindgren, et al., 1996, Monack, et al., 1996). Although the SPI-1 encoded protein SipB induces macrophage apoptosis by binding to caspase-1 (Hersh, et al., 1999), little is known about the relative contribution of other signaling pathways involved in *Salmonella*-induced apoptosis. We studied induction of apoptosis in U937 cell using the invasive wild type *S. typhimurium* strain SL1344, and its isogenic mutants *hilA*⁻, *prgH*⁻ or *sipC*⁻. These mutants were defective in the SPI-1 encoded type-III secretion system, and failed to enter the U937 cells. In order to facilitate their internalization into U937 cells, the mutants were opsonized, leading to receptor-mediated phagocytosis. This system was used to compare invasion of *S. typhimurium* utilizing its type-III secretion system with receptor-mediated phagocytosis.

We found that invasion by *S. typhimurium* induced apoptosis as depicted by an increased surface exposure of phosphatidylserine, cell shrinkage and DNA fragmentation. In contrast, receptor-mediated phagocytosis, or activation by its opsonized isogenic mutants (*hilA*⁻, *prgH*⁻ or *sipC*⁻), had only minimal effect on apoptosis, despite similar intracellular survival 18 h post infection. This indicates that although the different mutations in the type-III secretion system rendered the mutants unable to invade the cells, other host-adaptive properties protected the bacteria despite different pathway of entry. Since earlier findings are conflicting as to whether salmonella must be internalized in order to exert a cytotoxic effect on murine macrophages (Chen, et al., 1996, Monack, et al., 1996), we examined the impact of cytochalasin D (which disrupts actin polymerization) on apoptosis. Pre-treatment with cytochalasin D completely blocked the invasion and the SL1344-induced apoptosis without affecting the binding of the bacteria to the U937 cells. Taken together, these results show that entry through

invasion is required in order for wild type *S. typhimurium* to induce apoptosis in U937 cells.

Salmonella activates a number of signaling pathways that have been associated with apoptosis, including Rho GTPases, JNK, tyrosine kinases, PI3-kinase and AKT (Procyk, et al., 1999, Chen, et al., 1999, Jesenberger, et al., 2001, Steele-Mortimer, et al., 2000, Galan and Zhou, 2000). Rho GTPases are central actors in actin cytoskeletal rearrangements (Chimini and Chavrier, 2000). By using inhibitors that prevented targeting of Rho GTPases to the membrane and by using TAT-mediated delivery of dominant negative Rac1 and Cdc42, we concluded that inhibition of Rac1 and Cdc42 geranylgeranylation, as well as introduction of dominant negative Rac1 and Cdc42, decreased invasion of Salmonella and apoptosis. Furthermore, inhibition of tyrosine kinases and PI3K did not affect invasion and apoptosis, nor did they affect the activation of the Rho GTPases. In contrast, receptor-mediated phagocytosis as well as the associated activation of Rac1 and Cdc42 was efficiently blocked using either the tyrosine or the PI3-kinase inhibitor. Thus, receptor-mediated phagocytosis and invasion of *S. typhimurium* trigger different signaling pathways within macrophages.

We studied the interaction between type 1 fimbriated uropathogenic *E. coli* (UPEC) and human neutrophils. Type 1 fimbriae (or mannose-sensitive fimbriae) is the most commonly expressed virulence factor on *E. coli*-isolates from urinary tract infection (UTI)-patients (Foxman, et al., 1995). In addition to promoting avid bacterial adherence to the uroepithelium and facilitating colonization, the FimH adhesin of these organelles trigger invasion of UPEC into bladder epithelial cells, suggested to be a key event in the establishment and persistence of UTIs (Martinez, et al., 2000). Binding of type 1 fimbriated UPEC strains trigger a FimH dependent cytokine release (IL-8) from epithelial cells and enhance the recruitment of neutrophils to the urinary tract (Agace, et al., 1993, Godaly, et al., 1998). Although previous observation have shown that type 1 fimbriae bearing bacteria or purified FimH subunits trigger activation of the respiratory burst in neutrophils (Ohman, et al., 1982, Tewari, et al., 1993), no information regarding the effect of such an interaction on apoptosis has been reported.

We found that type 1 fimbriated bacteria bound firmly to neutrophils. The FimH negative isogenic mutant did not bind to neutrophils, and neither did the fimbriated bacteria in presence of D-mannose, which is in accordance with the characterization of this adhesin (Korhonen, *et al.*, 1981). This interaction did not lead to ingestion of the fimbriated bacteria by neutrophils, whereas opsonized FimH negative bacteria were ingested by 80% of the neutrophils. The fimbria-bearing *E. coli* induced a dose-dependent apoptosis, as depicted by increased surface exposure of phosphatidylserine with retained membrane integrity (i.e. no necrosis), and DNA fragmentation (data not shown), whereas the isogenic FimH negative mutant protected against apoptosis in an LPS-dependent manner (discussed below) regardless of the bacteria ratio used. By increasing the bacteria to cell ratio of the fimbriated bacteria, the apoptosis was shown to correlate with, and depend on, an increased production of intracellular ROS. Since heat-inactivated bacteria had similar effect on apoptosis as live bacteria, and blocking of the FimH adhesin completely abrogated the induced neutrophil apoptosis, we clearly show that expression of type 1 fimbriae on uropathogenic *E. coli*, but not soluble factors, injected effector proteins or toxins released from the bacteria, is essential for the induction of neutrophil apoptosis.

Although the FimH adhesin was sufficient for neutrophil binding, bacteria-associated LPS was required to mount an intracellular ROS production and to induce apoptosis. Involvement of LPS in type 1 fimbria-mediated interaction has been reported previously (Hedlund, *et al.*, 2001). However, in contrast to our study where LPS was essential for the activation of neutrophils, they found that the type 1 fimbriae alone was sufficient to mount a moderate cytokine production from uroepithelial cells, and that LPS augmented this response in a TLR4-dependent way (Hedlund, *et al.*, 2001).

Our results show that depending on the pathogen, invasion (*Salmonella*) or binding (*E. coli*) is required for inducing apoptosis of their respective target cells (Figure 10). However, the ability for these pathogens to survive, or modulate/induce apoptosis, seems to be cell specific. Interaction between FimH-expressing *E. coli* and macrophages, for example, resulted in lectinophagocytosis, attenuation of intracellular ROS production and survival of

the bacteria (Baorto, et al., 1997). This indicates that *E. coli*, which traditionally is regarded as an extracellular pathogen, can utilize different strategies depending on its virulence attributes (Tesh and O'Brien, 1991, Blomgran, et al., 2004), and which cell it encounters (Blomgran, et al., 2004, Baorto, et al., 1997). We have also recently observed that, although *S. typhimurium* has the capacity to survive inside macrophages (Lindgren, et al., 1996), ingestion/invasion of wild type *S. typhimurium* (SL1344) in the absence of opsonins resulted in an 80-90% killing of the intracellular bacteria already after 2 h in human neutrophils (Blomgran et al. unpublished observation).

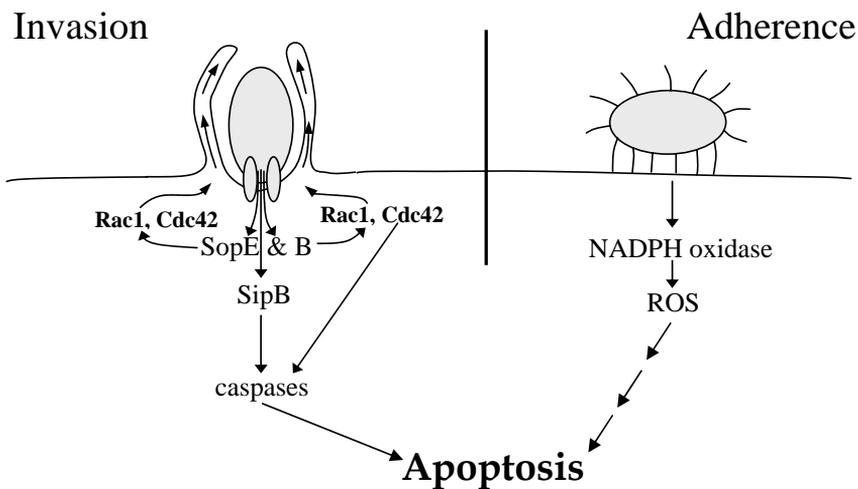


Figure 10. Two modes for pathogens to induce apoptosis in phagocytic cells are illustrated.

Phagocytosis and anti-apoptotic signaling in phagocytes (paper I and II)

The different signaling requirement for tyrosine and PI3-kinase activity during receptor-mediated phagocytosis of, or invasion by, *S. typhimurium* in U937 cells prompted us to study one of the major targets of those kinases, namely AKT. The proto-oncogene serine/threonine kinase AKT has a key role in cell proliferation, transcription, migration and promoting survival in response to a number of stimuli (Brazil and Hemmings, 2001, Coffey, et al., 1998, Klein, et al., 2000, Whitlock, et al., 2000). Although invasion and receptor-mediated phagocytosis both triggered

phosphorylation of this kinase, receptor-mediated phagocytosis resulted in a more prolonged, PI3K-dependent, increase in the AKT activity. The activity of AKT was measured by using glycogen synthase kinase-3 (GSK-3), a downstream target of AKT that becomes phosphorylated upon its activation (Frame and Cohen, 2001). To further substantiate the notion that AKT could be a potential candidate protecting the cells from apoptosis during receptor-mediated phagocytosis, we performed antisense transfection on AKT1, 2 and 3. Silencing AKT in those cells only marginally increased apoptosis in AKT1 and AKT2 transfectants infected with the SL1344 strain, whereas the most notable shift in apoptosis was seen with opsonized hila⁻ bacteria. We observed that infection with the opsonized mutant markedly increased apoptosis in the AKT1 and AKT2 transfectants, indicating that a concomitant activation of AKT during receptor-mediated phagocytosis is involved in protecting cells from apoptosis.

FimH⁻ *E. coli*, which did not physically interact with neutrophils, exerted an anti-apoptotic effect by the release of LPS. At high doses, bacteria-released LPS, in contrast to purified LPS, did not require serum to induce anti-apoptosis, thereby showing that bacteria released LPS and purified LPS differ in their dependence on cofactors. Release of LPS is also suggested to be the mechanism for anti-apoptosis seen at low concentrations of the FimH⁺ strain or when binding of the FimH⁺ strain is inhibited with D-mannose. Since neutralization of LPS rendered the type 1 fimbriated bacteria incapable of inducing apoptosis as well as anti-apoptosis, LPS has a pivotal role in modulation of apoptosis. Serum-opsonized mutants triggered an extensive ROS production, but caused only a modest increase in neutrophil apoptosis. The NADPH oxidase inhibitor DPI lowered the apoptotic rate induced by the opsonized mutant to that for the unopsonized mutant, indicating that phagocytosis without ROS production is not enough to trigger apoptosis, and that anti-apoptotic mechanisms are involved in preventing apoptosis from accelerating during receptor-mediated phagocytosis in neutrophils. In line with our work, Zhang *et al.* (2003) showed that complement-mediated phagocytosis of yeast particles by neutrophils generates a NADPH-oxidase dependent apoptosis, which is attenuated by a parallel β 2-integrin-activated ERK survival pathway. Given that clustered inactive as well as activated β 2-integrins, can regulate neutrophil apoptosis through AKT

(Whitlock, et al., 2000), it is reasonable that AKT provides protection during phagocytosis-induced apoptosis also in neutrophils.

The results from paper I and II illustrates that during receptor-mediated phagocytosis both pro- and anti-apoptotic signaling mechanisms are activated (Figure 11). Since many pathogens induce phagocytosis-dependent cell death, although the mechanisms involved are not entirely elucidated (Watson, et al., 1996b, Kobayashi, et al., 2003c, DeLeo, 2004), it is safe to suggest that the mechanism of entry, the properties of different bacteria, as well as the level of counter-balancing host mechanisms will determine the fate of the phagocyte.

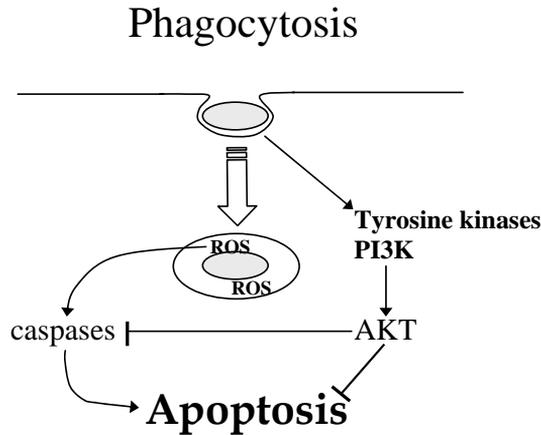


Figure 11. Pro- and anti-apoptotic pathways activated during receptor-mediated phagocytosis.

Role for lysosomes (azurophilic granules) in neutrophil apoptosis (paper III)

Reactive oxygen species have been implicated as a common mediator of apoptosis in a variety of systems (Buttke and Sandstrom, 1994), including phagocytosis-induced cell death in neutrophils (Watson, et al., 1996b, Blomgran, et al., 2004, Kobayashi, et al., 2004). It should be emphasized, however, that certain stimuli such as soluble immune complexes, the calcium ionophore A23187 and fMLP activate the respiratory burst, but do not trigger (Haslett, et al., 1991, Colotta, et al., 1992, Watson, et al., 1996b) or delay (Gamberale, et al., 1998, Whyte, et al., 1993b) neutrophil

apoptosis. We hypothesize that these contrasting effects of ROS on neutrophil apoptosis depend on the location of such an activation (extracellular, intraphagosomal, or intracellular but not intraphagosomal), but also on the duration and magnitude of the activity. All the above-mentioned criteria can influence the activation of the redox-sensitive caspases (Ueda, *et al.*, 2002). It was shown, for example, that phagocytosis with ROS targeted to the phagosomal vacuoles triggers caspase-8 activation and onset of apoptosis (Zhang, *et al.*, 2003), whereas extensive intracellular ROS production in the absence of phagocytosis (PMA-stimulated), inhibits caspase activity, but yet induces apoptosis (Fadeel, *et al.*, 1998). These results necessitate a novel oxidant-dependent pathway for neutrophil apoptosis. The results in paper III provide mechanistic data for an oxidant-dependent pathway that is triggered during adhesion of the 1 fimbriated UPEC.

Despite the fact that similar ROS production was triggered in neutrophils upon phagocytosis of, and adhesion by, UPEC, only adhesion by the fimbriated strain markedly increased apoptosis compared to spontaneous apoptosis (paper II). As exogenous hydrogen peroxide is known to diffuse into cells and initiate the lysosomal pathway to apoptosis (Antunes, *et al.*, 2001), we compared intraphagosomal versus nonphagosomal-coupled ROS production to explore the role of lysosomes in neutrophil apoptosis. The closest resemblance to primary lysosomes in neutrophils is the azurophilic granules, containing lysosomal enzymes including cathepsins, other hydrolases and MPO. Since azurophilic granules are devoid of the membrane marker for lysosomes, i.e. lysosome-associated membrane proteins (LAMPs), they may not strictly be defined as lysosomes (Cieutat, *et al.*, 1998). However, as cathepsins are implicated in lysosomal membrane permeabilization (LMP)-dependent apoptosis in other cells (Roberg, *et al.*, 1999, Guicciardi, *et al.*, 2000, Bidere, *et al.*, 2003), we found it appropriate to use the term lysosomes also for azurophilic granules in neutrophils.

Time-dependent increase in apoptosis induced by the type 1 fimbriated *E. coli* strain was preceded by permeabilization and release of lysosomal enzymes to the cytosol. This LPM was dependent on NADPH-oxidase derived ROS, but not on the activity of caspases or the lysosomal enzymes *per se*. Bcl-2 family

proteins are involved in regulating apoptosis, where the ratio of pro- and anti-apoptotic molecules within this family sets the threshold of susceptibility for apoptosis in neutrophils (Weinmann, *et al.*, 1999, Perskvist, *et al.*, 2002). The FimH⁺ bacteria induced generation of the pro-apoptotic Bid-cleavage product (t-Bid) already after 30 min of stimulation, suggesting that Bid-cleavage could be an important link in ROS-induced LPM. We also detected a time-dependent decrease in the expression of the anti-apoptotic Bcl-2 protein homologue Mcl-1. The balance or ratio of tBid and Mcl-1 showed that the fimbriated bacteria had induced a shift towards apoptosis visible already after 30 min and significant after 60 min. Westernblott technique could not detect any changes in the amount of Bax, unless Bax was first immunoprecipitated. Immunoprecipitation of Bax further revealed a decreased heterodimerization of Bax to Mcl-1, indicating that intracellular (nonphagosomal) ROS production and LMP not only increases Bax expression, but also liberates it from its anti-apoptotic sequestration to Mcl-1. Bid is suggested to be the only Bcl-2 protein that functions as an agonist of the pro-apoptotic Bcl-2 proteins Bax and Bak (Wang, *et al.*, 1996). Bid-induced conformational changes in Bax was found responsible for mitochondrial cytochrome c release during apoptosis (Desagher, *et al.*, 1999), a process also involving Bid induced oligomerization and insertion of Bax into the outer mitochondrial membrane (Eskes, *et al.*, 2000).

Cathepsins D is the only reported cathepsin that has a direct effect on Bax for its induction of apoptosis (Bidere, *et al.*, 2003, Stoka, *et al.*, 2001). On the other hand, using specific inhibitors, we observed that such a direct effect of cathepsins on Bax could not be the mechanisms for how NADPH-oxidase provoked LMP induced apoptosis in neutrophils. We found that the specific cathepsin D inhibitor did not reduce the mitochondrial damage or apoptosis. By using the cysteine cathepsin inhibitor EST (also called E64d, mainly for cathepsin B and L) we observed that the induction of mitochondrial damage and apoptosis was an indirect effect through the cleavage of Bid. The cathepsin B and L inhibitor protected Bid from cleavage and reduced the mitochondrial damage and apoptosis to a similar level as the NADPH oxidase inhibitor. As the caspase inhibitor suppressed apoptosis even in the presence of both lysosomal and mitochondrial membrane permeabilization, we concluded that the function of cathepsins as downstream

executioner proteases, as seen in other cells (Foghsgaard, et al., 2001, Roberts, et al., 1997), does not apply to our system. Rather, we found that cathepsins operate upstream of mitochondria in neutrophils. The caspases involved in this type 1 fimbriae-induced apoptosis are those activated downstream of mitochondria, such as caspase-9 and 3. Caspase-8, an upstream initiator caspase known to cleave Bid (Li, et al., 1998, Yin, et al., 1999), was only activated when DPI (the NADPH oxidase inhibitor) was used (data not shown), which is in line with the redox sensitive nature of caspases shown by Fadeel *et al.* (1998).

The complement-opsonized FimH⁻ mutant, which caused a massive intra-phagosomal ROS production, did not induce any major effect on the lysosomes. Rather, both the opsonized as well as the unopsonized FimH⁻ mutant protected against both lysosomal and mitochondrial damage. The strict correlation between lysosomal- and mitochondrial damage and the level of apoptosis, indicates that the level of lysosomal damage (permeabilization) is also a suitable, alternative, marker for apoptosis in neutrophils. The reason for the attenuated apoptosis during complement-mediated production of ROS could be the consequence of the confinement of ROS to the phagosomal vacuoles and the simultaneous activation of survival pathways. Spontaneous apoptosis in neutrophils have shown to involve caspase-8 activation that is indirectly regulated by an accumulation of ROS (Scheel-Toellner, et al., 2004). We showed that NADPH-oxidase generated ROS is not the trigger for the delayed LMP and apoptosis in neutrophils. It is however possible that mitochondria-produced ROS could be part of a positive feed-back loop between LMP and mitochondrial damage, where leakage of mitochondrial proteins can target the lysosomes, which in turn further aggravates mitochondrial damage ultimately leading to apoptosis.

In conclusion the findings in paper III suggest a close correlation between the involvement of ROS in the initiation of LMP and the release of proteases, which gives rise to a cysteine cathepsin-dependent cleavage of the Bcl-2 family protein Bid, subsequent mitochondrial damage and apoptosis in human neutrophils (Figure 12). During immune receptor-mediated phagocytosis of bacteria, the formation of a phagolysosome and compartmentalization of ROS protects

against ROS-inflicted damage (on both lysosomes and mitochondria) and apoptosis. However, during activation of phagocytes by certain stimuli, intracellular ROS not confounded in the phagosome, can trigger apoptosis by targeting the lysosomal granules. This may be a strategy by which certain pathogens evade the innate immune response.

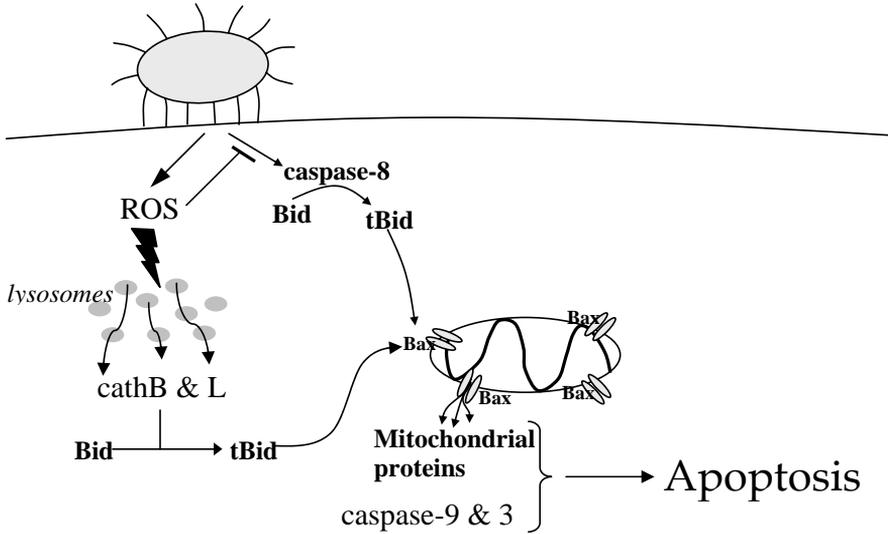


Figure 12. Lysosomal-dependent apoptosis in neutrophils. Intracellular generated ROS induces lysosomal permeabilization and the release of cathepsins (cath), triggering apoptosis through the mitochondrial pathway by the cleavage of Bid.

Modulation of macrophage activation by apoptotic neutrophils (paper IV)

Bacteria-induced apoptotic neutrophils are probably the first cells to be cleared by macrophages in an infected tissue. Still this process has not been studied with the same intensity as the sequestration of aged apoptotic cells (Savill, *et al.*, 1989, Gregory and Devitt, 2004, de Almeida and Linden, 2005, Henson and Hume, 2006). Hence, we studied this interaction and activation of macrophages to clarify the modulation of inflammation during the very early phase of infection.

Since apoptotic and necrotic cells have different effects on activation of macrophages (Savill, *et al.*, 2002, Fadok, *et al.*, 2001b, Cocco and Ucker, 2001), we first

established that the neutrophils encountering bacteria were in the early stage of apoptosis with preserved membrane integrity (~50 % annexin V positive, and <4% positive for both annexin V and propidium iodide). Both bacteria-induced (*E. coli* strain ATCC 25922 or *Staphylococcus aureus* strain Wood46 were used) and UV-irradiated apoptotic neutrophils were ingested to similar extent by the peripheral-blood monocyte-derived macrophages. Approximately 40% of the macrophages stained positive for the neutrophil marker MPO. In agreement with previous reports (Fadok, et al., 1998, Huynh, et al., 2002), macrophages that had ingested UV-induced apoptotic neutrophils displayed inhibited production of the pro-inflammatory cytokine TNF- α , whereas they produced increased levels of the anti-inflammatory cytokine TGF- β 1. In contrast, macrophages that had phagocytosed bacteria-induced apoptotic neutrophils showed markedly increased levels of TNF- α and a slightly decreased amount of TGF- β 1. To further test the hypothesis that macrophages are activated by bacteria-induced apoptotic neutrophils the expression of Fc γ RI (CD64, high-affinity receptor for IgG) was examined. Enhanced expression of Fc γ RI is a hallmark for inflammatory macrophages (van de Winkel and Capel, 1993, Deo, et al., 1997). Fc γ RI was constitutively expressed on the surface of macrophages and was not affected by ingestion of uninfected apoptotic neutrophil. In contrast, the Fc γ RI expression on macrophages that had ingested bacteria-induced apoptotic neutrophils increased by ~60%. We noted that the direct effect of live bacteria had little influence on macrophage activation, indicating that the bacteria-induced apoptotic neutrophils, rather than bacteria determinants, were responsible for the pro-inflammatory response. However, the pattern recognition receptors involved in binding apoptotic cells overlap with those that recognize pathogens (Fadok, et al., 2001a, Savill, et al., 2002). For example, CD14 on macrophages are involved in both LPS-binding, mediating a pro-inflammatory signal, as well as mediating recognition and internalization of apoptotic cells. Hence, we determined whether or not bacteria associated with the inflammatory neutrophils undergoing apoptosis were responsible for the activation of macrophages. Apoptosis of neutrophils exposed to formalin-fixed bacteria was enhanced by irradiation, yielding similar degree of apoptosis and clearance by macrophages as live bacteria-induced apoptotic neutrophils. Interaction with those neutrophils still displayed much lower production of TNF- α than macrophages incubated with neutrophils undergoing apoptosis induced by viable bacteria. Also incubating

macrophages with LPS, the same concentration as found in macrophages that had ingested live *E. coli*-induced apoptotic neutrophils, only triggered the macrophages to produce ~10% of the TNF- α level observed by macrophages exposed to viable *E. coli*-induced apoptotic neutrophils.

Since our results clearly indicate that very little of the pro-inflammatory activation of macrophages is caused directly by bacteria associated with neutrophils, the apoptotic neutrophils must generate some other signals to be sensed by macrophages. It has been hypothesized that the immune system is more concerned by damage than with foreignness (*Medzhitov and Janeway, 2002, Matzinger, 2002*), and accordingly the immune system is called into action and initiate protective immune responses by “danger molecules” released from the tissue during infection or stress (*Matzinger, 2002*). To test the contribution of neutrophil-derived danger signals in the activation of macrophages, we analyzed the expression and effect of heat shock proteins (HSP). After 3 h of culture, spontaneous and UV-irradiated neutrophils exhibited little or no increase in HSP, whereas apoptosis induced by bacteria or by mild heat (43°C for 30 min) stimulated an increased expression of HSP60 and HSP70. No difference in expression of HSP90 could be detected. Uptake of heat-stressed apoptotic neutrophils stimulated a substantial production of TNF- α by macrophages that were not affected by the addition of LPS. The fact that purified HSP60 and HSP70 were unable to provoke an pro-inflammatory response *per se*, suggests that HSP together with distinct apoptotic markers on bacteria-induced apoptotic neutrophil are needed for activation of macrophages, HSP perhaps functioning as an endogenous bridging molecule. However, purified HSP was able to markedly potentiate the LPS-response in macrophages showing of the versatile nature of HSP as an immunoregulatory molecule. To further confirm that HSP from apoptotic neutrophils are involved in activation of macrophages, we inhibited HSP expression. Pretreatment of neutrophils with cycloheximide almost completely blocked the increased expression of HSP60 and HSP70, induced by exposure to viable *E. coli* bacteria or heat stress, and subsequently lowered the TNF- α production in macrophages.

Aged (Ren, et al., 2001), UV-irradiated (Fadok, et al., 1998, Huynh, et al., 2002, Fadok, et al., 2001b) or FAS-induced (Serinkan, et al., 2005) apoptosis have been part of the basis confirming the immunosuppressive nature of apoptotic cells on macrophages. We additionally show that pathogen-induced apoptotic neutrophils have the capacity to trigger macrophage activation (Figure 13).

Pro- and anti-inflammatory apoptosis

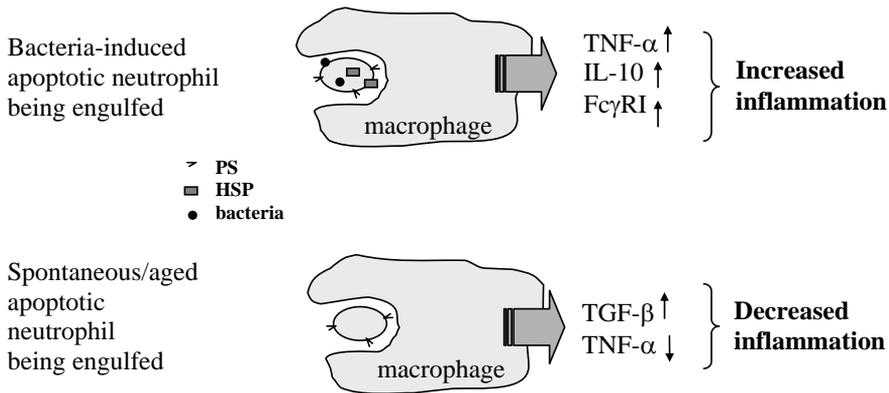


Figure 13. Regulation of macrophage activation and inflammation by apoptotic neutrophils.

We have previously shown that such an indirect activation of macrophages by apoptotic neutrophils also applies for the intracellular pathogen *Mycobacterium tuberculosis* (Perskvist, et al., 2002). Thus, our results demonstrate that during the initial stage of infection, pathogen-induced neutrophil apoptosis will facilitate local macrophages to gain control over the microbes. Even though no single cytokine is effective, the pro-inflammatory mediator TNF- α induces an autocrine activation and differentiation of macrophages, helps to reestablish intracellular killing capacity of infected macrophages, increases the expression of phagocytic receptors on neutrophils, as well as accelerating phagocytosis-induced apoptosis and recruits more leukocytes to the area of infection (Dick, et al., 2004, Chan, et al., 1992, Forsberg, et al., 2001, Zhang, et al., 2003). These findings suggest that bacteria-induced neutrophil apoptosis represents a novel link between innate and adaptive immune responses through the activation of macrophages. Once the bacteria are cleared from the site of infection,

phagocytosis of excess uninfected apoptotic neutrophils will inhibit macrophage activation, resulting in rapid resolution of inflammatory responses, thereby avoiding undesirable tissue damage.

Conclusions

- Mechanism of entry and bacterial effectors regulate apoptosis in phagocytic cells. Invasion by *S. typhimurium* triggers apoptosis in U937 cells in a Rho-GTPase-dependent way, whereas receptor-mediated phagocytosis triggers both pro- and anti-apoptotic signaling, where the activation of AKT prevents accelerated apoptosis.
- Intracellular NADPH oxidase-dependent ROS production generated in response to adherence by, or receptor-mediated phagocytosis of, *E. coli* triggers apoptosis in neutrophils. Phagocytosis with inhibited production of intraphagosomal ROS revealed that phagocytosis in neutrophils also triggers anti-apoptotic pathways. LPS is essential for *E. coli* to exert both pro- and anti-apoptotic effects, where the difference in presentation, i.e. with or without fimbriae, determines the outcome.
- Bacteria-triggered LMP represents a novel pathway for neutrophil apoptosis. NADPH oxidase-produced ROS, not linked to phagosomal vacuoles, triggers permeabilization and release of lysosomal proteases. Cysteine-cathepsin dependent cleavage of the Bcl-2 family protein Bid, together with liberation of Bax (from its anti-apoptotic sequestration by Mcl-1), facilitates apoptosis through mitochondrial membrane permeabilization and subsequent caspase activation.
- Pathogen-induced apoptotic neutrophils, in contrast to uninfected apoptotic neutrophils, activate macrophages to produce large quantities of the pro-inflammatory cytokine TNF- α . This demonstrates that during the initial phase of infection, pathogen-induced neutrophil apoptosis will help local macrophages to gain control over the microbes. Moreover, HSP represent a stress signal that enables macrophages to distinguish between, and react differently to, uninfected and inflammatory apoptotic neutrophils.

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