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Phagocyte apoptosis in innate immunity: Initiation by bacterial phagocytosis and modulation by B-cell lymphoma-2 (Bcl-2) family members

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Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. Christian Becker
Prüfer der Dissertation:	1. Univ.-Prof. Dr. Michael Groll
	2. Univ.-Prof. Dr. Georg Alexander Häcker

Die Dissertation wurde am 05.08.2008 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 22.09.2008 angenommen.

Index of Abbreviations

A1	B-cell leukemia/lymphoma 2 related protein A1a
aa	Amino acids
Apaf-1	Apoptotic protease-activating factor
ATM	Ataxia telangiectasia mutated homolog
vATPase	Vacuolar adenosine triphosphatase
Bad	Bcl-2-antagonist of cell death
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-xl	BCL2-like 1
Bcl-w	BCL2-like 2
BH domain	Bcl-2 homolgy domain
Bid	BH3-interacting-domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting mediator of cell death
Bok	BCL2-related ovarian killer
Boo	BCL2-like 10
BMDM	Bone marrow-derived macrophage
Bmf	Bcl-2-modifying factor
BSA	Bovine serum albumin
CARD	Caspase-recruitment domain
CFDA-SE	Carboxyfluorescein diacetate, succinimidyl ester
CHO	Chinese hamster ovarian
CHX	Cycloheximide
CSF	Cerebrospinal fluid
Cy5	Indodicarbocyanine-5
dATP	Deoxyadenosine triphosphate
DED	Death effector domain
DISC	Death inducing signaling complex
DHR	123-dihydrorhodamine
DLC1	Dynein light chain 1
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EEA1	Eary endosome antigen 1
EGFP	Enhanced green fluorescent protein

ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Fas (TNFRSF6)-associated via death domain
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanate
FLIP	FLICE/caspase-8 inhibitory protein
FOXO3a	Forkhead transcription factor
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GDF	GDI displacement factor
GDI	Guanosine diphosphate dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GTP	Guanosine triphosphate
Gr1	Lymphocyte antigen 6 complex, locus G
GRASP65	Golgi reassembly stacking protein 1, 65kDa
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hox	Homeobox
Hrk	Harakiri
HRP	Horse radish peroxidase
IL	Interleukin
IFN	Interferon
ITPG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kDA	kiloDalton
LPS	Lipopolysaccharide
Mcl-1	Myeloid cell leukemia sequence 1
MEF	Mouse embryonic fibroblasts
MHC	Major histocompatibility complex
Myd88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
NO	Nitric oxide
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
OmpA	Outer membrane protein 1

ORF	Open reading frame
p53	Transformation related protein 53
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PI	Propidium iodide
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
PS	Phosphatidyl serine
Puma	p53-upregulated modulator of apoptosis
Rab	Ras-related in brain
Rab5CA	Rab5 constitutively active mutant
Rab5DN	Rab5 dominant-negative mutant
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment receptor
shRNA	Small hairpin RNA
snRNP	Small nuclear ribonucleoprotein
TEMED	N,N.N'N'-Tetraethylmethylendiamine
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tom5	Translocase of the mitochondrial outer membrane 5
TRADD	TNFRSF1A-associated via death domain
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
Tris	Tris(hydroxymethyl)-aminomethan
U	International units
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WT	Wild-type
xg	x-multiples of earth gravity acceleration
zVAD	(N-CBZ-Val-Aal-Asp(O-Me) fluoromethyl ketone)

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1 Introduction

This work investigated a number of aspects of phagocyte apoptosis in the context of responses of the innate immune system to bacterial infections. The first part oft the introduction into this subject gives an overview over cell death induction by apoptosis in general, describes the two major cellular signal transduction pathways that can initiate apoptosis, and details the current state of knowledge about the control of these pathways by the Bcl-2 family of proteins. The second part describes how innate immune responses develop during infection, how macrophages and neutrophils combat pathogens, and how apoptosis of these phagocytes impacts on the initiation, course and termination of immune responses.

1.1 Apoptosis

1.1.1 Physiological relevance

Cell death is an integral part of the life of complex multicellular organisms, and serves as a means to safely and rapidely dispose of superfluous, damaged, infected, or otherwise dangerous cells. The first report of this process dates from the 19th century, where it was noticed that cells disappear during the normal development of toads (Vogt et al., 1842). In 1972, Kerr and his co-workers realised that a certain form of active cell-autonomous death, which they termed “apoptosis”, is a consequence of a conserved mechanism that deletes cells during embryonic development, that mediates cellular turnover in adult tissues, and that occurs in pathological situations (Kerr et al., 1972). The role of apoptosis during development is perhaps best exemplified in the nematode worm *Caenorhabditis elegans*, which has to dispose of exactly 131 out of his 1090 cells to reach the adult stage (Sulston and Horvitz, 1977). In more complex adult organisms, the total cell number in rapidly self-renewing tissues such as the gut lining has to be maintained constant, which is achieved through apoptosis of old or damaged cells. This process is tightly controlled, as too much cell death during homeostasis can contribute to degenerate conditions, and inhibition or malfunctions in apoptosis have been recognised as one major cause for tumorigenesis (Cory et al., 2003).

Apoptosis also assumes vital roles in the immune system. During lymphopoiesis, apoptosis prevents the survival of autoreactive lymphocytes that could provoke autoimmune disease (Strasser, 2005). The natural homeostasis of short-lived neutrophils in healthy man requires the removal of ~10^{11} cells each day (Dancey et al., 1976). In the termination phase of

immune responses to pathogens or other antigens, the vast majority of recruited neutrophils, or of the pool of expanded antigen-reactive T and B cell clones formed during an adaptive response, also die by apoptosis (Marsden and Strasser, 2003). Furthermore, apoptosis can be triggered in virally infected cells (Clem et al., 1993), which has promted viruses to acquire genes coding for proteins that mimick the function of cellular anti-apoptotic proteins, in order to promote cell survival (White, 2006). These widespread implications of apoptosis emphasize its importance in the life of higher multicellular organisms.

1.1.2 Morphological changes

Apoptosis is characterised by distinct morphological features (Kerr et al., 1972). Cells first detach from the extracellular matrix, round up, and protrusions from the plasma membrane – so-called "blebs" – appear. The chromatin starts to condense and is cleaved at the internucleosomal linkers to form DNA fragments multiples of ~180 bp in length (Wyllie, 1980). The whole cell shrinks, and the nucleus dissembles into several condensed fragments in a process termed pyknosis. All cytosol, organelles, and parts of the condensed nucleus are finally compartmentalised into several membrane-bound "apoptotic bodies" (Kerr et al., 1972). In *C. elegans*, these cellular remnants are taken up by neighbouring cells (Ellis et al., 1991), and in mammals cleared by macrophages or epithelial cells (Kerr et al., 1972). Once engulfed, the material is broken down through a lysosomal mechanism (Bursch et al., 1985).

Apoptosis typically does not induce inflammation or damage of the neighbouring tissue, and probably for this reason has evolved as a ubiquitous and convenient means to dispose of unwanted cells. Upon genetic prevention or experimental inhibition of apoptosis, cells receiving death signals revert to a form of cell death termed necrosis (Chautan et al., 1999; Vercammen et al., 1998). Necrosis is characterised by cytoplasmic swelling and organelle dissolution, followed by membrane rupture and the release of noxious cellular components that can damage neighbouring cells (Russell et al., 1972). Certain death stimuli can, however, induce a mixed morphological phenotype – the distinction between apoptosis and necrosis is not always clear-cut.

1.1.3 Molecular signal transduction pathways

Apoptosis is induced and executed by a specialised signalling pathway common to all mammalian cells. Due to a strong evolutionary conservation, homologues of the key

components of this pathway are also found in *C. elegans* and the fly *Drosophila*, and are even exchangable to some extent between species (the anti-apoptotic human Bcl-2 can mimick the protective effect of the anti-apoptotic CED-9 in the worm (Hengartner and Horwitz, 1994)).

1.1.3.1 The apoptotic death machinery

The apoptotic signal transduction pathway leads to a group of cysteine proteases termed caspases, that cleave the carboxyl side of aspartate residues (Thornberry and Lazenbik, 1998). Caspases execute cellular death by cleaving several proteins with vital functions including U1 70 kDa snRNP (mRNA splicing), GRASP65 (Golgi reassembly), PARP and ATM (DNA repair), cytokeratin-18 (intermediate filaments), and lamin (nuclear envelope) (Fischer et al., 2003). They also activate other enzymes involved in cell demolition, such as caspase-activated DNase (CAD) responsible for DNA fragmentation, which is liberated upon caspase cleavage of its inhibitor ICAD (Tang and Kidd, 1998).

In healthy cells, caspases are present as inactive pro-enzymes (zymogens) comprising an N-terminal pro-domain, and a large and a small subunit separated by a linker (Denault and Salvesen, 2002). They can be classified into two groups according to their mechanism of activation. The “effector” caspases-3, -6 and -7 are activated through cleavage at the linker and subsequent removal of the (short) pro-domain. This allows dimerisation of the two subunits, and two dimers associate in turn to form the active enyzme possessing two catalytic sites. Effector caspase processing is brought about predominantly by already active “initiator” caspases, which form the upper level of the so-called “caspase cascade”. The initiator caspases-8 and -9 become activated in response to extracellular death signals that are relayed over the extrinsic pathway, and by intracellular stress signals that come over the intrinsic pathway, respectively (see Figure 1, page 5). Caspase activation and activity can also be regulated by interactions with inhibitor of apoptosis proteins (IAPs), which are capable of inhibiting caspase-3, -7, and -9 (Deveraux and Reed, 1999). IAPs are overexpressed in certain tumours (Ambrosini et al., 1997), but their physiological role is not yet fully understood.

1.1.3.2 The death receptor or extrinsic pathway

The extrinsic pathway is initiated by binding of extracellular “death ligands” to death receptors including Fas, TNFR1 and TRAIL that belong to the tumor necrosis factor (TNF) receptor superfamily (Ashkenazi, 1998). Cell death induced by death ligands is particularly

important in the killing of virus-infected cells by cytotoxic T cells and natural killer cells (Russell and Ley, 2002). Upon ligation, death receptors trimerise and bind adaptor proteins (FADD for Fas or TRAIL, and TRADD for TNFR1) to their cytoplasmic regions (Boldin et al., 1995; Chinnaiyan et al., 1995). These adaptors recruit caspase-8 via interactions of the death effector domains (DED) present in the adaptor and the long pro-domain of the protease (Muzio et al., 1995), generating a so-called death inducing signalling complex (DISC) (Kischkel et al. 1995). Even as zymogen, caspase-8 retains about 1% activity (of the mature form) (Salvesen and Dixit, 1999) and the proximity with other caspase molecules within the DISC is thought to lead to dimerisation, autocatalytic processing and conversion to the active tetrameric form (Muzio et al., 1998; Boatright et al., 2003). Active caspase-8 then processes the effector caspases-3 and -7 (Muzio et al., 1997; Srinivasula et al., 1996). Death receptor signalling can be inhibited by the expression of a cellular or viral form of FLICE-inhibitory protein (FLIP) (Irmler et al., 1997; Thome et al., 1997). FLIP contains death domains and a catalytically dead caspase-like domain, and can thus block procaspase-8 recruitment sites at the DISC, or inhibit procaspase-8 processing via direct binding (Krueger et al., 2001).

Caspase-8 can also engage the mitochondrial pathway via cleavage of the pro-apoptotic BH3-only protein Bid to its active form tBid (Li et al., 1998; Luo, 1998). In type I cells that include lymphocytes, large quantities of caspase-8 are generated that autonomously process caspase-3 and cause rapid cell death execution. In type II cells such as hepatocytes, however, sufficient processing activity is only produced by additional activation of the mitochondrial pathway (Scaffidi et al., 1998), and inhibition of this amplification loop abolishes apoptosis induction (Lacronique et al., 1996; Rodriguez et al., 1996).

1.1.3.3 The mitochondrial or intrinsic pathway

The intrinsic pathway is activated in response to a variety of intracellular stress signals including DNA damage, ER stress, drug-induced cytoskeletal perturbations, and some extracellular-derived signals such as cytokine or growth factor deprivation (Cory et al., 2003; Ferri and Kroemer, 2001). It is controlled by three subgroups of the Bcl-2 family of proteins. The pro-apoptotic BH3-only proteins serve as stress sensors or “initiators”, and by either direct or indirect means activate the pro-apoptotic Bax-like “effector” proteins that induce permeabilisation of the outer mitochondrial membrane. The third group, the Bcl2-like proteins, can be seen as “guardians” of this pathway as they provide an anti-apoptotic counterbalance.

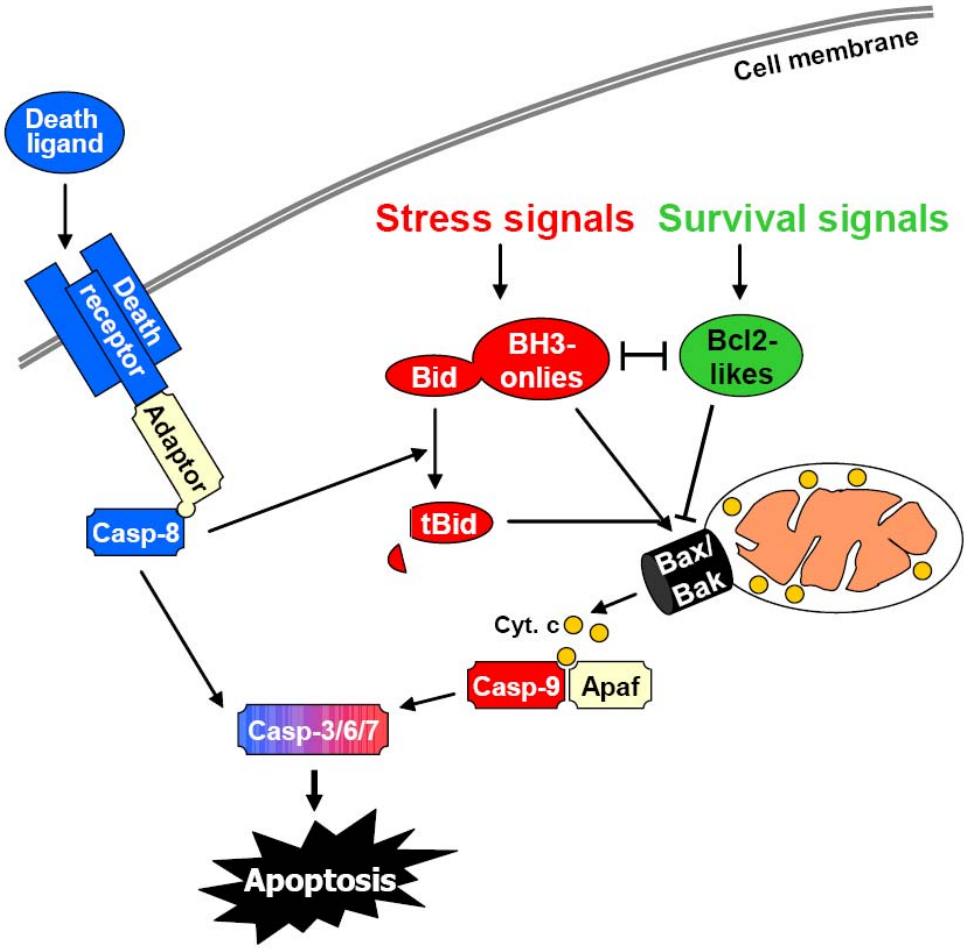


Fig. 1 Signal transduction through the extrinsic and intrinsic apoptosis pathway

The extrinsic pathway (in blue) is initiated by extracellular death ligands (such as FasL, TNF-α, or TRAIL) engaging their respective death receptor. Adaptor protein binding to the cytoplasmic domains of trimerised receptors leads to recruitment and processing of the initiator caspase-8, which activates the effector caspases-3/6/7 that via cleavage of vital cellular proteins primarily execute apoptosis. Caspase-8 can also activate the intrinsic pathway via cleavage of the BH3-only protein Bid to tBid. The intrinsic or mitochondrial pathway (in red) is triggered in response to stress signals (such as induced by growth factor deprivation, DNA damage, cellular detachment, etc.) that activate pro-apoptotic BH3-only proteins (Bim, Puma, Noxa, Bad, etc.). BH3-only proteins directly or indirectly activate pro-apoptotic Bax or Bak, which permeabilise the outer mitochondrial membrane, leading to the release of cytochrome c from the intermembrane space into the cytosol. Released cytochrome c forms a complex with the adaptor Apaf-1, triggering recruitment and processing of the initiator caspase-9. Caspase-9 then activates the effector caspases-3/6/7, causing cell death. The activation of pro-apoptotic BH3-only and Bax-like proteins is inhibited by anti-apoptotic Bcl2-like proteins (Bcl-2, Bcl-xl, A1, etc.), that can be upregulated in response to survival signals (mostly induced by growth factors and cytokines). BH3-only and Bcl2-like proteins inhibit the function of each other through direct interactions.

Once sufficient amounts of Bax-like proteins have become activated, a point of no return is reached. The predominant view is that Bax or Bak oligomerise to form pores in the outer mitochondrial membrane, through which molecules from the intermembrane space are released into the cytoplasm (Degli Esposti et al., 2003; Sharpe et al., 2004). One of these molecules, cytochrome *c*, usually shuttles electrons from complex III to complex IV in the respiratory chain. In the cytosol, however, it binds to Apaf-1 and induces a conformational change that promotes binding of dATP (Jiang and Wang, 2000; Li et al., 1997) and subsequent oligomerisation of Apaf-1 into a heptameric complex called the apoptosome (Acehan et al., 2002; Zou et al., 1999). Via interactions between the caspase recruitment domain (CARD) present in Apaf-1 and in the prodomain of caspase-9, the apoptosome can recruit multiple procaspase-9 molecules that become activated through proximity-induced dimerisation and autocatalysis (Qin et al., 1999; Srinivasula et al., 1998; Stennicke et al., 1999). Active caspase-9 then processes the effector caspases-3, -6 and -7 (Li et al., 1997; Srinivasula et al., 1998). Figure 1 summerises how signal transduction through the extrinsic and intrinsic pathway leads to apoptosis induction.

1.1.4 The Bcl-2 protein family

The B-cell leukaemia (Bcl-2) family of proteins control the activation of the mitochondrial apoptosis pathway in a manner that involves the integration of various pro-death and pro-survival signals. Bcl-2 proteins contain one to four different Bcl-2 homology (BH) domains, and according to their function and topology can be classified into three groups (Figure 2). The anti-apoptotic “Bcl2-like” members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and Boo) contain all four BH domains (except A1). The pro-apoptotic “Bax-like” members (Bax, Bak, and Bok) contain the BH domains 1-3, and the pro-apoptotic “BH3-only” members (Bid, Bim, Puma, Noxa, Bad, Bmf, Bik, and Hrk) only possess the BH3 domain. Murine Noxa is special in that it contains two BH3 domains. Many Bcl-2 proteins also have a C-terminal transmembrane domain that allows targeting to intracellular membranes.

The anti-apoptotic Bcl2-like subgroup has a well conserved α-helical fold with 2 central hydrophobic helices flanked on either side by 2 or 3 amphipathic helices (Fesik, 2000). Intriguingly, Bax-like proteins, and one member of the BH3-only subgroup, Bid, are structurally homologous despite their pro-apoptotic function (Chou et al., 1999; Moldoveanu

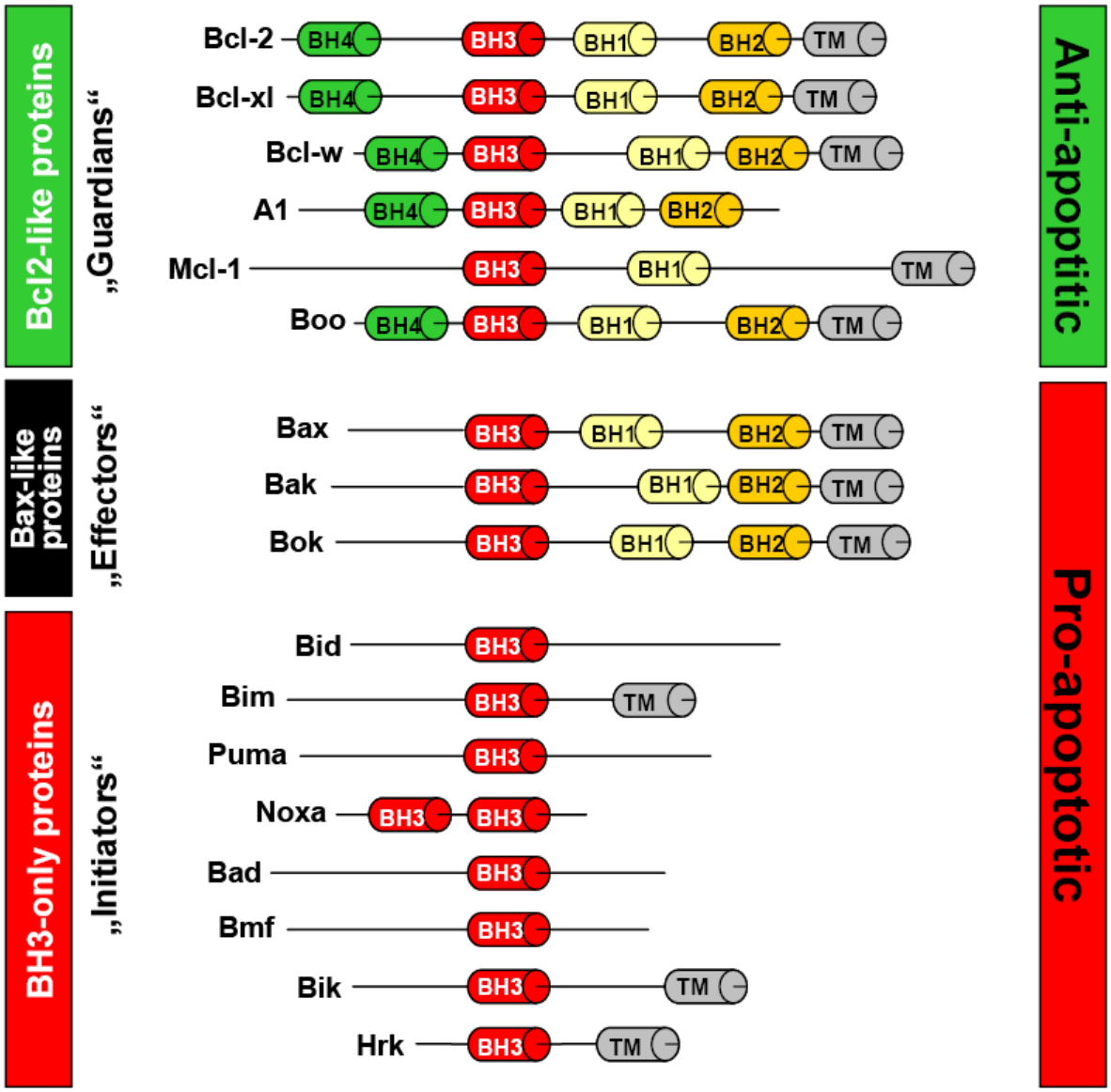


Fig. 2 Domain structure of mammalian Bcl-2 family members

Bcl-2 family proteins interact with each other predominantly through their Bcl-2 homology (BH) domains. According to the number of BH domains they possess, they can be subclassified into anti-apoptotic Bcl2-like proteins (BH1-4), pro-apoptotic Bax-like proteins (BH1-3), and pro-apoptotic BH3-only proteins (BH3). Mcl-1 is unusual in that it lacks the BH2 domain, and human Noxa only contains one BH3 domain. The mitochondrial apoptosis pathway is “triggered” by the BH3-only proteins that sense stress signals, and subsequently activate the Bax-like “effectors” which mediate cytochrome c release from mitochondria. Bcl2-like proteins “guard” this pathway by providing an anti-apoptotic function. Modified from Strasser, 2005.

et al., 2006; Suzuki et al., 2000). Based on secondary structure predictions, the other BH3-only proteins do not assume this fold (Aouacheria et al., 2005). Purified Bim, Bad, and Bmf (lacking the C-terminus) even appear to adopt substantial structure only in the presence of Bcl2-like proteins (Hinds et al., 2007), and this intrinsic plasticidity may allow BH3-only proteins to heterodimerise with various anti-apoptotic partners.

Bcl2-like proteins interact with pro-apoptotic proteins via a hydrophobic cleft that is formed by the BH1-3 domains (Fesik, 2000), and that can accommodate the hydrophobic face of the amphipathic α-helical BH3 domain of BH3-only and Bax-like proteins (Liu et al., 2003; Muchmore et al., 1996; Petros et al., 2000; Sattler et al., 1997). An intact BH3 domain is essential for binding and apoptosis induction by BH3-only proteins (Chittenden et al., 1995; Desagher et al., 1999), and co-immunoprecipitation and yeast-two-hybrid assays have revealed different binding preferences for Bcl2-like proteins (Boyd et al., 1995; Chen et al., 2005; Hsu et al., 1998a; O'Connor et al., 1998; Oda et al., 2000; Opferman et al., 2003; Wang et al., 1996; Yang et al., 1995). These binding preferences appear to be governed predominantly by the sequence of the conserved LXXXGDE motif in the BH3 domain, as they are largely reproducable with BH3 domain-derived synthetic peptides (~25-mers) and because they can be altered or abolished by amino acid substitutions within this motif (Chen et al., 2005; Certo et al., 2006). Bid, Bim and Puma are capable of interacting with all Bcl2-like proteins, whereas the other BH3-only proteins show more selective binding (Figure 3A). These interactions or "mutual sequestrations" constitute one layer of regulation for the activity of BH3-only (and Bcl2-like) proteins. Others regulatory mechanims that have been described are upregulation (Puma, Noxa, Hrk) (Imaizumi et al., 1997; Nakano and Vousden, 2001; Oda et al., 2000), phosphorylation (Bik) (Verma et al., 2001), or dephosphorylation (Bad) (Zha et al., 1996), and yet unidentified post-translational modifications (Bim, Puma). How the Bax-like proteins interact with the other Bcl-2 family members is still a matter of debate and will be discussed below.

1.1.4.1 Activation models for Bax and Bak

In the intrinsic pathway, mitochondrial permeabilisation and cytochrome *c* release is mediated directly by Bax-like proteins. The initiators of this pathway – the BH3-only proteins – lie upstream of the latter, because direct overexpression of BH3-only proteins fails to induce apoptosis in the combined absence of Bax and Bak (Cheng et al., 2001). The third subfamily

member, Bok, appears to assume important functions only in reproductive tissues (Inohara et al., 1998; Hsu and Hsueh, 1998).

In healthy cells, Bax is monomeric and predominantly found in the cytosol (with some loosely attached to the outer mitochondrial membrane) (Gross et al., 1998; Hsu et al., 1998; Wolter et al., 1997). During apoptosis induction, Bax undergoes conformational changes that expose the N-terminus, as well as the C-terminus harbouring the transmembrane anchor (Desagher et al., 1999; Nechushtan et al., 1999). What exactly causes these changes is not clear, but they are associated with the translocation of the protein to mitochondria, where it inserts into the outer membrane and forms homo-oligomeric complex (Antonsson et al., 2001; Hsu et al., 1997; Wolter et al., 1997). Bak, by contrast, already resides at the mitochondria in a stably membrane-inserted form (Griffiths et al., 1999; Wei et al., 2000). Its activation also involves a conformational change at the N-terminus, which leads to homo-oligomerisation (Griffiths et al., 1999; Mikhailov et al., 2003; Nechushtan et al., 2001). How the formation of Bax or Bak oligomers causes permeablisation of the mitochondrial membrane exactly is not known. Based on structural homology to pore-forming diphteria toxin (Kagan et al., 1981; Muchmore et al., 1996; Suzuki et al., 2000) it has been proposed that oligomerised Bax-like proteins constitute pores themselves. Alternatively, they may alter membrane curvature and induce the formation of larger pores incorporating lipids (Basanez et al., 1999; 2001).

Concerning the activation of Bax-like proteins, two specific models have received much attention. The displacement model postulates that only the sequestration of Bax-like proteins by anti-apoptotic Bcl2-like proteins holds the mitochondrial pathway in check. In this model, BH3-only proteins promote death by binding to the anti-apoptotic proteins and displacing them, which de-inhibits Bax or Bak, causing their autoactivation, oligomerisation and cytochrome *c* release (Chen et al., 2005; Willis and Adams, 2005). The direct binding model proposes that certain "activator" BH3-only proteins – the cleaved form of Bid (tBid), Bim and possibly Puma – can directly activate Bax or Bak, without the need for displacement of Bcl2-like proteins (Letai et al., 2002; Certo et al., 2006). BH3-only proteins such as Noxa or Bad, which on their own cannot activate Bax-like proteins in most cell types (Certo et al., 2006; Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002), would in this model act as "sensitisers" by displacing the activator BH3-only proteins from their sequestration by anti-apoptotic Bcl2-like proteins (Letai et al., 2002) (Figure 3B).

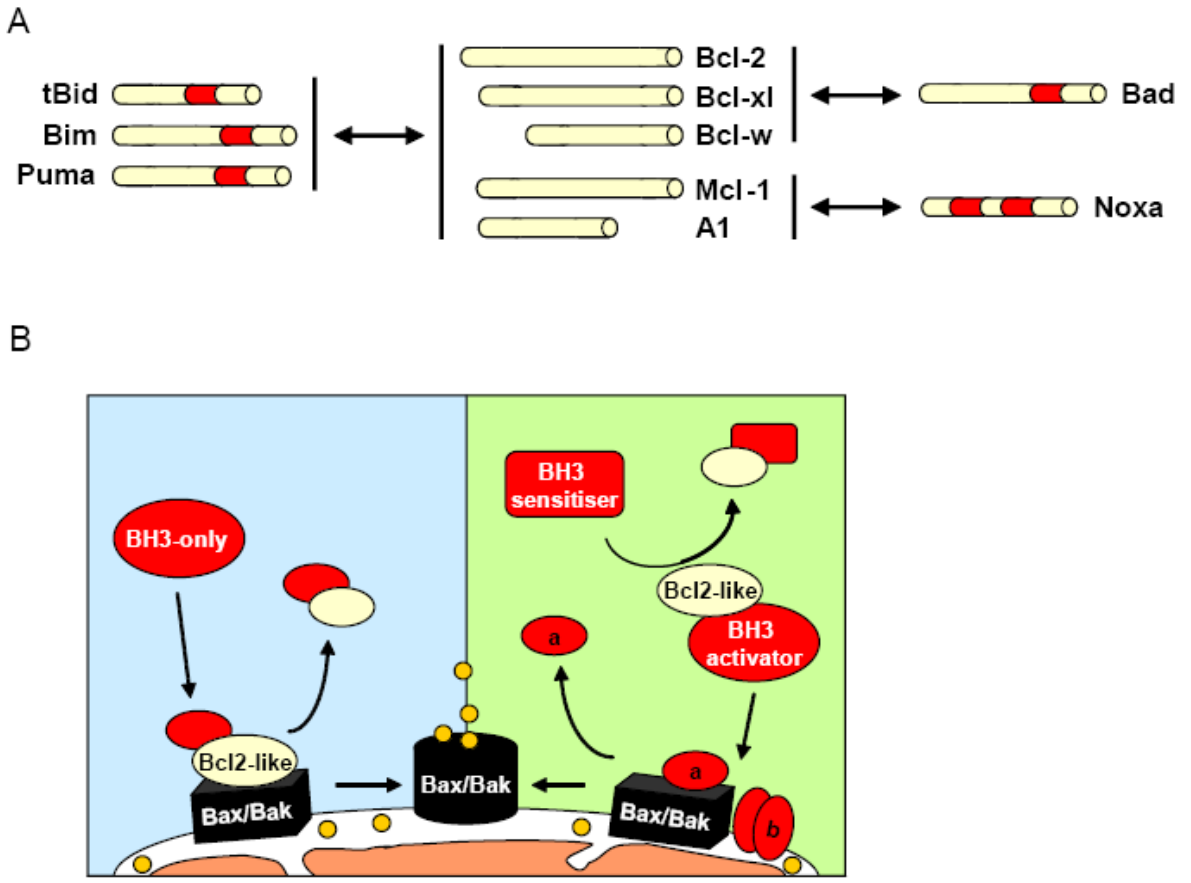


Fig. 3 Selective interactions amongst Bcl-2 proteins and activation models for Bax/Bak

A. BH3-only proteins (BH3 domain in red) show selectivities regarding their Bcl2-like binding partners (in the middle). tBid only binds weakly to Bcl-2, but avidly to the other anti-apoptotic proteins. Modified from Youle and Strasser, 2008. **B**. In the displacement model (blue), Bax/Bak autoactivate once inhibitory Bcl2-like proteins have been displaced by BH3-only proteins. In the direct binding model (green), Bax/Bak have to become activated by BH3-only "activator" proteins (tBid, Bim and possibly Puma). Both transient interactions with Bax/Bak (a) or activatory changes to membrane topology (b) have been considered. "Sensitiser" BH3-only proteins (Noxa, Bad, Bmf) can indirectly cause activation by liberating BH3-only activators from bound Bcl2-like proteins. Note that in both models the pro-apoptotic activity of BH3-only proteins is inhibited by interactions with anti-apoptotic proteins, and may be enhanced by upregulation (higher protein levels can no longer be neutralised by Bcl2-like proteins), and post-translational modifications (may reduce the affinity for Bcl2-like proteins).

The absolute requirement for activator BH3-only proteins has however been questioned in two recent studies. Cells deprived of tBid, Bim, and Puma by genetic means and siRNA-mediated protein knock-down, can still be driven into apoptosis by the co-overexpression of the sensitisers Noxa and Bad (Kim et al., 2006), or by overexpression of Noxa combined with application of ABT-737 (Willis et al., 2007), a synthetic peptide functionally mimicking the

BH3 domain of Bad (Oltersdorf et al., 2005). Noxa and Bad can bind to Mcl-1, and Bcl-xl, respectively, both of which are constitutively associated with Bak (Willis et al., 2005). But although their removal is required for Bak activation (Cuconati et al., 2003; Willis et al., 2005), the observation that a Bak mutant that fails to bind Bcl-xl and Mcl-1 does not autoactivate but still responds to apoptotic stimuli indicates that it is not sufficient (Kim et al., 2006). In the displacement model, this finding can only be explained by the presence of a yet unidentified additional inhibitory protein that keeps Bak inactive.

With regard to Bax, a sequestration by Bcl2-like proteins could only occur after a semi-activated Bax has translocated to the outer mitochondrial membrane, because in cytosolic form the protein is monomeric (Hsu et al., 1998; Wolter et al., 1997). However, cross-linking or co-immunoprecipitation studies have not revealed any detectable assocation with Bcl-2 or Bcl-xl even after Bax membrane insertion in apoptotic cells, or upon protection conferred by Bcl-2 overexpression (Hsu et al., 1998; Mikhailov et al., 2001). Also, Bax mutants that do no longer bind Mcl-1, Bcl-xl and Bcl-2 do not autoactivate yet still permit apoptosis induction (Kim et al., 2006).

The displacement model may thus hold true concerning the activation of Bak by "sensitiser" BH3-only proteins. By contrast, displacement does not appear to be required for the direct activators: mutants of tBid or Bim that no longer bind to Bcl-xl, Mcl-1 and Bcl-2 can still induce Bax insertion into the mitochondrial outer membrane, cytochrome *c* release and apoptosis when overexpressed (Eskes et al., 2000; Kim et al., 2006; Marani et al., 2002; Weber et al., 2007). Indeed, in reconstituted systems, the presence of any additional proteins seems to be dispensable as tBid, Bim, or their respective BH3 peptides alone can induce the insertion of (monomeric) Bax into bilayer liposomes, leading to membrane permeabilisation (Kuwana et al., 2002; 2005). Furthermore, the overexpression of Bim enhances Bax-induced mitochondrial dysfunction in yeast, which does not possess Bcl-2 family homologues (Weber et al., 2007).

Whether direct activation also implies direct binding, however, is not clear. If this is the case, interactions between Bax-like and BH3-only activators have to be very transient. To date, direct binding to Bax has only been detectable in studies using a modified Bid peptide with a chemically stabilised α-helical structure in the BH3 domain (Walensky et al., 2006). In order to account for this potential lack of interaction, heterodimerisation-independent mechanisms for the direct model that involve membrane remodelling have been suggested (Degli Esposti and Dive, 2003). Supporting this idea are studies showing that tBid also

enhances the permeabilisation of liposomes already containing pre-activated and oligomerised Bax, possibly by inducing membrane curvature stress and a redistribution of lipids between monolayers (Terrones et al., 2004; Degli Esposti et al., 2002).

1.1.4.2 Functions of Bcl-2 family proteins in the immune system

The founding member of the Bcl-2 family of proteins has been identified through a chromosomal translocation in certain B-cell lymphomas, which caused the overexpression of Bcl-2 (Tsujimoto et al., 1989). Experimental gene-targeting in mice has been performed for all Bcl-2 proteins except Boo by now, and in many but not all cases has produced phenotypes with regard to homeostasis and functionality in the immune system.

The overexpression of Bcl-2 leads to an abnormal accumulation of most cell types of haematopoietic origin, and promotes the survival of progenitor cells and autoreactive lymphocytes (Martinou et al., 1994; Ogilvy et al., 1999; Strasser et al., 1991a; 1991b). *In vitro*, increased Bcl-2 levels protect against a range of apoptotic stimuli including growth factor withdrawal, γ-irradiation, and treatment with glucocorticoids and various cytotoxic drugs (Huang et al., 1997).

A deficiency in Bcl2-like proteins, by contrast, can lead to early embryonical or post-natal death by causing severe abnormalities such as the total loss of haematopoietic stem cells (Mcl-1), increased death of immature lymphocytes (Bcl-xl), or the loss of mature lymphocytes (Bcl-2) (Motoyama et al., 1995; Rinkenberger et al., 2000; Veis et al., 1993). The relevance of A1 is difficult to analyse since mice bear four *a1* genes (Hatakeyama et al., 1998), but the loss of *a1a* causes accelerated granulocyte and mast cell apoptosis (Hamasaki et al., 1998; Xiang et al., 2001). Mice deficient in Bcl-w have no apparent immune phenotype (Print et al., 1998).

With regard to pro-apoptotic proteins, the deficiency in Bax causes a mild accumulation of lymphocytes, and Bak-deficient mice have increased levels of platelets (Lindsten et al., 2000; Knudson et al., 1995). The combined loss of Bax and Bak, however, leads to highly increased numbers of myeloid and lymphoid cells and partial embryonic death (Lindsten et al., 2000). The fact that double- but not single-deficient cells are resistant against most forms of stress-induced apoptosis indicates a strong functional redundancy between the two proteins (Wei et al., 2001; Zong et al., 2001).

The severity of the knock-out phenotype for BH3-only proteins correlates well with their ability to activate Bax-like proteins. Lack of the “sensititisers” Bik, Hrk, Bmf, or Bad

causes no significant abnormalities in the haematopoietic system (Coultas et al., 2005; 2007; Labi et al., 2008; Ranger et al., 2003), and neither does that of the "activator" Bid (Kaufmann et al., 2007; Yin et al., 1999), possibly owing to its restriction to apoptosis induction in type II cells. Mice deficient in the sensitiser Noxa do not have an obvious phenotype either (Villunger et al., 2003a), but fibroblasts and keratinocytes are highly resistant against UV irradiation-induced DNA damage (Naik et al., 2007). DNA damage usually activates the tumour suppressor p53 (Lakin and Jackson, 1999), and it has been found that p53 transcriptionally upregulates Noxa expression (Oda et al., 2000). Induction of Noxa, in turn, displaces Mcl-1 from Bak and promotes its proteasomal degradation (Nijhawan et al., 2003; Willis et al., 2005). Noxa may also be upregulated independently of p53 in response to type-1-interferon-signalling (Oda et al., 2000).

Loss of Puma also does not generate obvious abnormalities in the haematopoietic compartment with respect to cellular homeostasis or development (Villunger et al., 2003a). However, Puma contributes to apoptosis of myeloid progenitor cells, thymocytes and mast cells induced by growth factor withdrawal (Ekoff et al., 2007; Jeffers et al., 2003; Han et al., 2001; Villunger et al., 2003a), and to the death of activated T lymphocytes during shutdown of adaptive immune responses (Bauer et al., 2006; Fischer et al., 2008). Expression of the protein upon growth factor deprivation is driven by the transcription factor Foxo3a (You et al., 2006), and *puma* has also been found to be a transcriptional target of p53 (Han et al., 2002; Nakano and Vousden, 2001; Yu et al., 2001). In consequence, thymocytes and fibroblasts deficient in Puma are resistant to γ-irradiation and other DNA-damage inducing agents, and resistance was found to be higher in *puma*-/- compared to *noxa*-/- cells (Villunger et al., 2003a; Jeffers et al., 2003). This more dominant role of Puma in p53-mediated apoptosis may be explained by its promiscous binding to all Bcl2-like proteins, and its potential ability to activate Bax-like proteins directly.

Of all BH3-only proteins, the loss of Bim produces the most severe phenotype, with partial embryonic lethality. *Bim* knock-out mice accumulate neutrophils, monocytes, and mature B and T lymphocytes, indicating that homeostasis in the haematopoietic system is strongly perturbed (Bouillet et al., 1999). In addition, Bim is required for macrophage apoptosis following bacterial clearance during innate immune responses (Kirschnek et al., 2005), for the termination of adaptive immunity mediated by T cells (Bauer et al., 2006; Pellegrini et al., 2003), and for the deletion of low-affinity memory B cells and autoreactive lymphocytes (Bouillet et al., 2002; Enders et al., 2003; Fischer et al., 2007). *In vitro*, Bim

deficiency renders thymocytes and neutrophils resistant to cytokine withdrawal and various cytotoxic drugs (Bouillet et al., 1999; Villunger et al., 2003b).

Bim transcripts are subject to alternative splicing, giving rise to the three isoforms BimEL, BimL, and BimS (O'Connor et al., 1998) (Figure 4). In most cell types, the two long forms, BimEL and BimL are readily detectable, whereas the short BimS form is usually expressed in very low amounts (O'Reilly et al., 2000). Like *puma*, *bim* expression is upregulated in response to cytokine withdrawal by the transcription factor Foxo3a (Dijkers et al., 2000), but increased protein levels alone are not always associated with apoptosis induction (Bauer et al., 2007; Kirschnek et al., 2005). Also, although all Bim isoforms contain the BH3 domain and induce apoptosis upon transient overexpression, cytotoxicity decreases in the order BimS > BimL > BimEL, suggesting attenuation of the longer isoforms by post-translational modifications (O'Connor et al., 1998).

As one regulatory mechanism, sequestration of the protein to microtubules has been proposed. In healthy cells, most BimEL and BimL (but not BimS) can be co-immunoprecipitated with the microtubular dynein motor subunit dynein light chain-1 (DLC1), and upon apoptosis induction, Bim-DLC1 complexes translocate to mitochondria

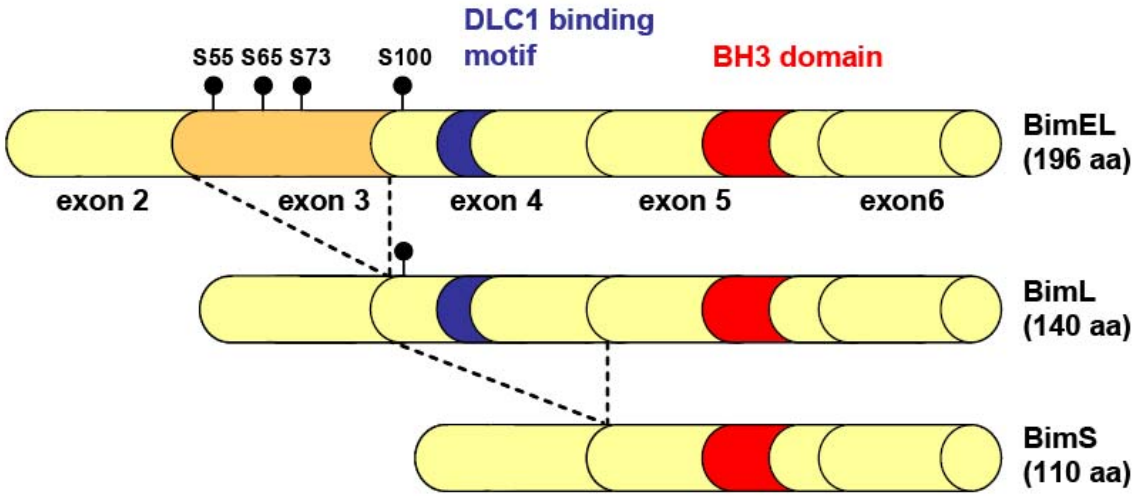


Fig. 4 Alternative splicing and functional motifs in the BH3-only protein Bim

The full-length *bim* transcript encoding the BimEL isoform is constitutively spliced down to produce the smaller isoforms BimL and BimS. The DLC1 binding site has been reported to sequester BimEL and BimL to microtubular dynein motor complexes. The black pins indicate serine phosphorylation sites. Protein lengths and residue numbers refer to mouse Bim. Modified from Hubner et al., 2008

(Puthalakath et al., 1999). However, in certain cell types such as lymphocytes, there is already considerable constitutive association of BimEL and BimL with mitochondria (Gomez-Bougie et al., 2005; Yamaguchi et al., 2002; Zhu et al., 2004). The phosphorylation of BimEL or BimL on serine residues by extracellular-regulated kinase (ERK1/2) has been shown to promote its proteasomal degradation, but phosphorylation site mutants only possess a slightly increased pro-apoptotic activity *in vitro* and *in vivo* (Hacker et al., 2006; Hubner et al., 2008; Ley et al., 2003, 2005; Luciano et al., 2003). Other post-translational modifications of Bim proteins have so far not been identified.

1.2 The innate immune system

1.2.1 Initiation of the innate immune response

All higher multicellular organisms have an immune system that combats invasive pathogens and prevents the host from being parasitised or killed. In vertebrates, additional "adaptive" immune mechanisms have evolved that endow the immune system with an immunological memory.

In mammals, innate immunity is largely dependent on cells of the myeloid lineage that includes granulocytes (>90% neutrophils, remainder eosinophils and basophils), monocytes/macrophages, and dendritic cells (Beutler, 2004). Monocytes are blood-borne cells that constantly enter all tissues of the body, where they differentiate into macrophages. Pathogens in tissues are usually detected first by these cells, which engulf them and initiate an immune response through the production of a range of pro-inflammatory cytokines. Interleukin (IL)-1β, tumor necrosis factor (TNF)-α and prostaglandins activate the endothelium of local blood vessels, causing the expression of adhesion molecules and a vascular dilation that allows the rapid extravasation of blood-borne granulocytes into the tissue, followed later by monocytes that differentiate into inflammatory macrophages (Carlos and Harlan, 1994; Medzhitov, 2007; Williams et al., 1977). Chemokines and chemotactic lipids attract and direct the infiltrating cells to the site of infection (Rot and von Andrian, 2004). In addition to mediating recruitment, pro-inflammatory cytokines stimulate cellular microbicidal functions, and prolong the lifespan of short-lived immune cells such as neutrophils (Colotta et al., 1992; Coxon et al., 1999).

Pathogens are killed mainly through engulfment (phagocytosis) and intracellular destruction by phagocytes (macrophages and neutrophils), but also through toxic components of the humoral system including antimicrobial peptides (secreted by neutrophils and epithelial cells) (Ganz and Lehrer, 1998), lysozyme and complement, a set of proteolytic enzymes present in body fluids. Initiation of the complement cascade creates a lytic "membrane attack complex" on the surface of microorganisms, produces opsonising peptides that enhance phagocytosis, and generates pro-inflammatory and chemotactic peptides that activate and attract immune cells (Carroll and Fischer, 1997). Small numbers of pathogens can be dealt with locally, but in case of persistant or spreading infections, additional immune mechanisms become activated. Systemic levels of pro-inflammatory cytokines above a certain threshold activate the bone marrow epithelium to release neutrophils, and stimulate hepatocytes to secret acute phase proteins, which have an opsonising function and activate complement (Steel and Whitehead, 1994). The innate immune system can also detect cells infected by bacteria, parasites or viruses. Such infections often induce "overexpressed self" or "missing self", i.e. alterations in the levels of host proteins presented on the cell surface in form of peptide fragments bound to major histocompability (MHC) class I molecules. These changes can be recognised by natural killer cells, which eliminate infected cells by inducing apoptosis (Raulet, 2004).

The bridge to adaptive immunity is formed by myeloid dendritic cells (Hoebe et al., 2004). Dendritic cells are able to present processed protein constituents from phagocytosed microorganisms on their surface, in form of antigenic peptides complexed to MHC molecules. After cellular activation by a pathogen encounter, dendritic cells mature and migrate to lymph nodes or the spleen, where they can present antigens to naïve $CD4^+$ T lymphocytes (Guermonprez et al., 2002). T cells recognise MHC-peptide complexes via their antigen-specific T-cell receptor, and receptor ligation in combination with co-stimulatory signals from dendritic cells induces proliferation and differentiation into different subsets of $CD4^+$ T helper cells, namely T_H1, T_H2, or the recently discovered T_H17 cells (Reinhardt et al., 2006). T helper cells support the innate immune system in several ways by producing particular cytokines that enhance the microbicidal functions of macrophages (T_H1), that stimulate B lymphocytes to secret antigen-specific antibodies which opsonise microorganisms and activate complement (T_H1/T_H2), or that mobilises neutrophils from the bone-marrow into the bloodstream via production of IL-17 (T_H17). What particular T_H subset predominates is decided by the array of co-stimulatory receptors and the cytokine mix present during antigen

presentation and the clonal expansion phase. The composition of the cytokine mix in turn is governed by the nature of the pathogen, which engages specific sets of so-called pattern-recognition receptors on the dendritic cell (Medzhitov, 2007).

Pattern-recognition receptors (PRRs) have evolved to bind highly conserved surface lipids, sugars and proteins shared by many microorganisms such as lipopolysaccharide on gram-negative bacteria, and bacterial or viral nucleic acids (Janeway et al., 1989). The Toll-like receptor (TLR) family of PRRs comprises 12 members, which are expressed on various immune cells including macrophages, dendritic cells, and (more selectively though) neutrophils (Takeda and Akira, 2005; Parker et al., 2005). They recognise microbial and viral ligands at the cell surface or in endosomal compartments, and are upregulated in response to pathogens and pro-inflammatory cytokines (O'Mahony et al., 2008). Toll-like receptors can signal through four cytosolic adaptor proteins, the most important of which is Myd88 (Medzhitov et al., 1998; Kawai et al., 1999). Myd88 signalling leads to activation of the transciption factors NFκB or of members of the interferon response factor (IRF) family, and/or of MAP kinases, which induce the expression of pro-inflammatory cytokine genes in response to bacterial components, or transcription of type I interferon genes in response to viruses (secreted interferons induce the expression of antiviral proteins via autocrine or paracrine signalling) (Uematsu and Akira, 2006). As TLRs appear not to able to detect pathogens that have already invaded the cytosol, many cells also have a cytoplasmic sensor systems for bacteria and viruses such as the NOD-LRR family and RNA helicases, which are also able to induce pro-inflammatory cytokines through NFκB (Akira et al., 2006).

1.2.2 Phagocytosis and phagosomal elimination of pathogens

The phagocytic response of professional phagocytes (macrophages, neutrophils and dendritic cells) to extracellular microorganisms is a sophisticated anti-microbial mechanism of the immune system. It does not only neutralise or kill, but also removes the bulk of the dead pathogen through intracellular degradation.

Phagocytosis is initiated by the engagement of pattern-recognition receptors (lectins, scavenger receptors) and/or receptors that bind opsonins (Fc, complement, and integrin receptors). Several signalling pathways involving Rho GTPases, phospholipase C, PI3-kinase and protein kinase C then orchestrate rearrangements of the actin cytoskeleton that cause the plasma membrane to invaginate, wrap around and close behind the bound target, trapping it

inside the cell in a so-called phagosome (Underhill and Ozinsky, 2002). In order to gain bactericidal and digestive properties, nascent phagosomes mature to lytic phagolysosomes within 30-60 minutes (Vieira et al., 2002). This maturation requires a series of sequential fusion events with early endosomes, late endosomes and lysosomes (Desjardins et al., 1997; Jahraus et al., 1998) that deliver acid hydrolases such as the proteolytic cathepsins responsible for particle degradation (Claus et al., 1998; Oh et al., 1996), and membrane components like the vacuolar-type ATPase (vATPase), which mediates acidification by transferring protons inside the phagosome (Lukacs et al., 1990; Pitt et al., 1992). In macrophages, the intraphagosomal pH gradually drops to about 4.5 - 5.5 during maturation (Veira et al., 2002), thereby passing through optimal pH conditions for a variety of hydrolases (Claus 1998, Oda 1991, Oh 1996).

This marked drop in pH is not observed in neutrophils and dendritic cells, however. In neutrophils, intraphagosomal killing depends not such much on the exchange with late endosomes and lysosomes, but rather on a very early fusion with granules containing broad specificity proteases such as elastase, cathepsin G and proteinase 3 in very high concentrations (Borregaard and Cowland, 1997; Reeves et al., 2002). These enzymes require a slightly above neutral pH and elevated potassium levels for their activity (Reeves et al., 2002), both of which is achieved by recruitment of NADPH oxidases to the membrane of nascent phagosomes. By transferring electrons into the phagosomal lumen, these enyzmes cause an elevation of the pH via the generation of superoxide that consumes protons (Cross and Segal, 2004), and induce the influx of K^+ as additional charge compensation (Reeves et al., 2002). This activation of NADPH oxidases, also referred to as “respiratory burst”, is very powerful but also transient in neutrophils. Dendritic cells maintain this burst for hours, keeping their phagosomes at neutral pH (Watts, 2006). Together with already lower constitutive levels of lysosomal acid proteases, this ensures a slower and less complete breakdown of microbial proteins, which is important for antigen presentation (Delamarre et al., 2005; Savina et al., 2006).

1.2.3 Modulation of phagosome maturation by Rab proteins

Phagosome maturation involves multiple fusion events, and their regulation has been shown to involve members of the Ras-related in brain (Rab) family of small GTPases (Vieira et al., 2002). There are more than 60 Rab proteins in mammals, which associate selectively with

vesicles and assemble SNARE proteins that drive membrane fusion by promoting the tight tethering to specific target organelles (Stenmark and Olkkonen, 2001; Ungermann and Langosch, 2003). Rab proteins become posttranslationally modified by the attachment of prenyl groups to their C-terminus (Anant et al., 1998). This membrane anchor can be masked by GDP dissociation inhibitor (GDI) that keeps the protein in a soluble form (Zerial and McBride, 2001). GDI displacement by GDI displacement factors (GDFs) that are present on specific vesicles are thought to govern the association of Rab proteins with specific membranes (Dirac-Svejstrup et al., 2001; Pfeffer and Aivazain, 2004). After membrane targeting, Rabs have to become activated by exchanging their bound GDP for GTP, which is catalysed by guanine exchange factors that are also thought to reside on particular vesicles (Munro, 2004; Ullrich et al., 1994). The inactivation of Rabs by GTP hydrolysis requires GTPase-activating proteins (GAPs), which stimulate the weak intrinsic GTPase activity of

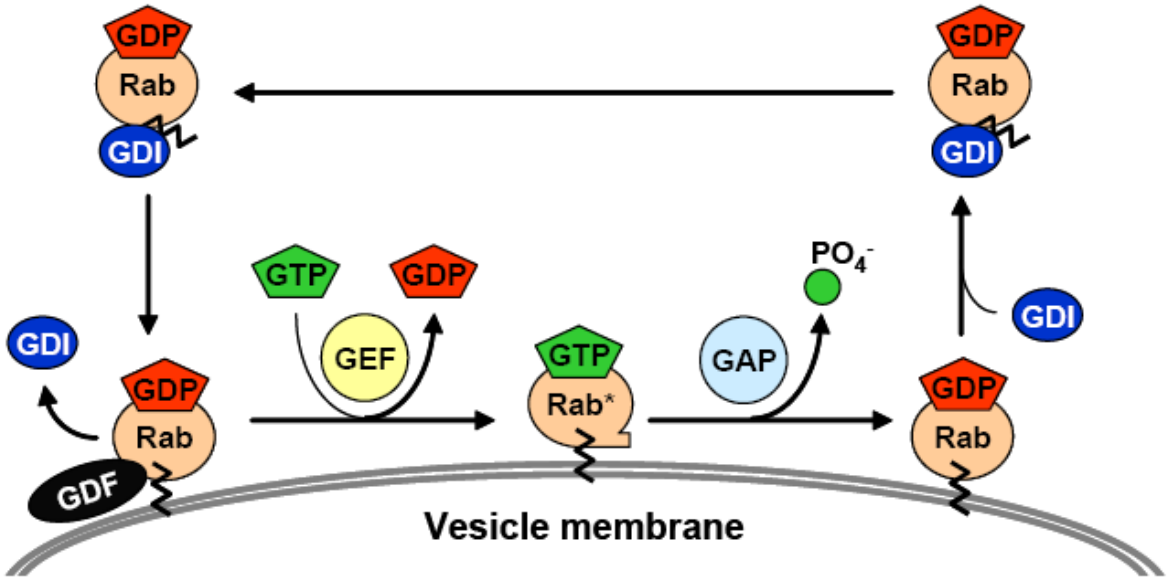


Fig. 5 The activity cycle of Rab proteins

The cytosolic Rab:GDP dissociation inhibitor (GDI) complex is targeted to organelle membranes probably by recruitment via vesicle-specific GDI-displacement factors (GDFs). These displace GDI, allowing membrane anchorage of Rab through its prenylated C-terminus. Guanine exchange factors (GEFs) replace GDP for GTP, thereby activating the Rab protein. Activated Rab assembles SNARE proteins that drive membrane fusion by promoting the tight tethering to specific target organelles. Rab protein activity is sustained until a GTPase-activating protein (GAP) activates its intrinsic GTPase, causing the hydrolysis of GTP back to GDP. Inactive Rab-GDP can be re-extracted from the membrane by GDI.

Rab proteins (Liu et al., 1998). Inactive Rab-GDP can then be re-extracted from the membrane by GDI to complete the cycle (Ullrich et al., 1993) (Figure 5).

The Rab family member Rab5a has first been identified as key regulator in early endosome traffic (Bucci et al., 1992), but has been shown to also become recruited to latex bead phagosomes (Desjardins et al., 1994, 1997) and phagosomes harbouring *E. coli* (Roberts et al., 2000). In cell-free assays, the exogenous addition of Rab5a stimulates the fusion of phagosomes with early as well as late endosomes and lysosomes (Jahraus et al., 1998), while its removal from phagosomal membranes inhibits fusion (Funato et al., 1997). The expression of mutant forms of Rab5a has also supported a role for the protein in the regulation of phagosome maturation. Alvarez-Dominguez *et al.* showed that the overexpression of constitutively active (GTPase-defective) Rab5a enhances fusion of phagosomes containing heat-killed *Listeria monocytogenes* with endosomes. Expression of a dominant-negative form (GTP-binding defective), on the other hand, inhibits fusion (Alvarez-Dominguez et al., 1996).

1.2.4 Phagocyte apoptosis during the immune response

Phagocyte death is commonly observed in the course of innate immune responses to pathogens. Several bacteria infect macrophages and can kill their host cell after intracellular replication. *Salmonella* that infect pre-activated macrophages translocate a virulence factor into the host cytosol that activates caspase-1 (Hersh et al., 1999). Caspase-1 processes the precursor forms of IL-1β and IL-18 (Kuida et al., 1995; Li et al., 1995), which causes secretion of IL-1β, and can also kill the phagocyte (Monack et al., 1996; 2001; Richter-Dahlfors et al., 1997). *Shigella* also translocate a virulence factor into the host cytosol that activates caspase-1 (Hilbi et al., 1998), and infected macrophages produce IL-1β (Zychlinksy et al., 1994, 1996). The release of the pro-inflammatory cytokine induces strong inflammation and ultimately clearance of the infection, but the pathogen may benefit from this by being shed from the host into the environment via inflammatory diarrhoea (Navarre and Zychlinsky, 2000).

Macrophage apoptosis can also be induced by the uptake of pyogenic extracellular bacteria, such as *Streptococcus pneumonia (S. pneumonia)*, *Staphylococcus aureus (S. aureus)*, and pathogenic strains of *Escherichia coli* (*E. coli*) (Ali et al., 2002; Baran et al., 1996; Bastiani et al., 2005; Dockrell et al., 2001; Rodrigues et al., 1999). Low-virulence strains of *E. coli* have also been shown to induce macrophage apoptosis (Hacker et al., 2002).

This death is marked by engagement of the mitochondrial pathway, as it involves activation of caspases-9 and -3, and can be inhibited by overexpression of Bcl-2.

Dendritic cells appear to be rarely targeted by bacteria, although *Listeria* have been reported to induce apoptosis upon infection (Guzman et al., 1996). Neutrophils are largely unsuitable for infection due to their very potent microbicidal functions, although *Chlamydia pneumoniae* and *Ehrlichia* bacteria have been reported to be able to (at least temporarly) survive and replicate within these cells (Chen et al., 2000; Van Zandbergen et al., 2004). Neutrophil death is, however, readily modulated by the uptake of a variety of extracellular bacteria. *In vitro*, the exposure to LPS, low doses of *E. coli*, heat-inactivated *S. pneumonia*, or opsonised *S. aureus* actually inhibit apoptosis (Colotta et al., 2002; Matsuda et al., 1999; Watson et al., 1996), while high bacterial concentrations of low-virulence *E. coli*, or exposure to live *S. pneumonia* can cause necrosis (Watson et al., 1996; Zysk et al., 2000). *In vivo*, an initial prolongation of the short lifespan of neutrophils is beneficial for combatting pathogens. Neutrophil necrosis resulting from excessive numbers of bacteria or virulent strains can have different impacts on the immune response. On the one hand, liberated active proteases from neutrophil granules stimulate the secretion of pro-inflammatory cytokines from macrophages (Fadok et al., 2001), which can amplify the immune response. On the other hand, these proteases are toxic to host tissue, and breakdown of the extracellular matrix as well as pronounced inflammatory vasodilation can also promote the dissemation of the pathogen (DeLeo, 2004).

The later stages of inflammation, however, are usually associated with apoptosis of neutrophils in inflamed tissues and their subsequent removal by macrophages (Grigg et al., 1991; Savill et al., 1989, 1991, 1992), which prevents the release of noxious contents from dying neutrophils (Savill et al., 1989). Apoptotic cells are recognised by changes in cell surface structure, and one important and generic event is the extrusion of the membrane lipid phosphatidyl serine (PS) from the inner to the outer leaflet of the plasma membrane (Martin et al., 1995). This translocation may result from the downregulation of aminophospholipid translocase normally shuttling PS lipids from the outer to the inner membrane layer, and from upregulation of a phospholipid "scramblase" that mediates bidirectional movement of all classes of phospholipids across the membrane (Verhoven et al., 1995, Wolfs et al., 2005). Macrophages express a receptor binding to phosphatidyl serine, which alongside other receptors such as the thrombospondin receptor, scavenger receptors, and the vitronectin receptor is involved in the recognition of apoptotic cells (Krysko et al., 2006).

Contrary to bacterial phagocytosis, the uptake of apoptotic neutrophils does not result in the production of pro-inflammatory cytokines and chemokines (Meagher et al., 1992). Instead, contact with apoptotic cells usually triggers the production of anti-inflammatory transforming growth factor (TGF)-β by macrophages and monocytes. Via auto- and paracrine stimulation, TGF-β suppresses the secretion of IL-1β, TNF-α or IL-12 (Byrne et al., 2002; Fadok et al., 1998; Kim et al., 2004), can downregulate the expression of Toll-like receptors (Cartney-Francis et al., 2004), and reduces the expression of the adhesion receptor L-selectin on granulocytes, thereby impairing their ability to enter inflammed tissues (Malipiero et al., 2006; Tedder et al., 1995). In addition, the ingestion of apoptotic cells can also inhibit the mobilisation of neutrophils from the bone marrow by reducing the levels of macrophage IL-23 and subsequently T lymphocyte IL-17 production (Stark et al., 2005). Furthermore, the increased production of anti-inflammatory lipid mediators such as lipoxins, resolvins or protectins (Serhan, 2007) at the late stage of inflammation reduces vascular permeability and neutrophil infiltration (Levy et al., 2001; Takano et al., 1998) and stimulates the uptake of apoptotic neutrophils (Godson et al., 2000).

The importance of neutrophil apoptosis, their engulfment, and the production of TGF-β for the resolution of inflammation has been demonstrated in several experimental *in vivo* models (Cox et al., 1995; Huynh et al., 2002, Rossi et al., 2006). Accordingly, genetic defects in humans resulting in impaired clearance of apoptotic neutrophils have been linked to chronic inflammatory conditions such as cystic fibrosis and chronic obstructive pulmonary disease (Hodge et al., 2003; Vandivier et al., 2002), or autoimmune diseases such as systemic lupus erythematosus (Herrmann et al., 1998; Ren et al., 2003). Also, delays in the apoptosis of circulating neutrophils induced by excessive pro-inflammatory cytokine production contribute to the inflammatory injury observed in patients suffering from sepsis, the systemic inflammatory response syndrome, or inflammatory bowel disease (Ertel et al., 1998; Ina et al., 1999; Jimenez et al., 1997).

1.3 Aim of this work

The present study investigated a number of aspects of phagocyte apoptosis. Its aim was to clarify the relevance of bacterial phagocytosis and degradation in the induction of macrophage death, to analyse the role of apoptosis in the shutdown of neutrophil effector functions, and to identify Bcl-2 family members involved in neutrophil apoptosis.

Previous work had indicated that phagocytosis-associated death of macrophages is dependent on the internalisation of *Escherichia coli* bacteria, but it remained unclear whether their intracellular breakdown is also required for apoptosis induction. The first part of this work analysed the relation between bacterial uptake and cell death on a quantitative level, and examined whether the subsequent intracellular killing and breakdown of bacteria was connected to the induction of apoptosis.

The second part was concerned with neutrophil apoptosis in the context of inflammation. Several studies have indicated that prolonged neutrophil lifespan can be detrimental to the host but have not provided clear evidence that directly connects this effect to a harmful preservance (and continued execution) of neutrophil immune functions – rather than to the absence of an anti-inflammatory clearance of neutrophils. Therefore, it was analysed how the prevention of neutrophil apoptosis by the overexpression of anti-apoptotic Bcl-2 would affect their ability to exert effector functions, and the uptake of these kept-alive cells by macrophages. The experiments were conducted with neutrophils derived from immortalised progenitor cells by use of the recently developed Hoxb8 system, which also required the initial establishment of appropriate culture and differentiation conditions.

The final part of this study was devoted to analysing the role of Bcl-2 family proteins in spontaneous neutrophil apoptosis, and in cell death induced by the withdrawal of cytokines. A role for the BH3-only protein Bim had been described previously, and the BH3-only protein Puma has been implicated in growth factor deprivation-induced cell death in other cell types. In order to clarify the role of Puma, to measure the effect of its absence in relation to that produced by the lack of Bim, and to identify other Bcl-2 family members involved, neutrophil apoptosis was analysed in a panel of Hoxb8 neutrophil lines deficient in specific BH3-only proteins.

A more detailed knowledge of these processes is important to understanding under what conditions phagocyte death takes place in the macrophage effector arm of the innate immune system during infections by pyogenic bacteria, and how the resolution of

inflammation and the termination of the immune response is modulated by the functional shutdown of the neutrophil effector arm. In particular the latter has important implications for anti-inflammatory therapy of patients suffering from chronic or acute inflammatory diseases.

2 Material and Methods

2.1 Material

2.1.1 Buffers

Name	Constituents	pH*
Annealing buffer (10x)	100 mM Tris, 1M NaCl, 10 mM EDTA	7.5
AnnexinV buffer	10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$	7.4
$CaCl_2$ buffer	2.5 M $CaCl_2$	-
Carbonate buffer	0.1 M Na_2CO_3	9.2
Hepes-buffered saline (HBS) (2x)	250 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 40 mM Hepes, 0.2% (w/v) Glucose	7.1
Hepes extraction buffer	50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton-X100	7.5
Giemsa buffer	80 mM Na_2HPO_4, 35 mM KH_2PO_4	7.2
KCM buffer (5x)	2.5 M KCl, 1.5 M $CaCl_2$, 2.5 M $MgCl_2$	-
Laemmli buffer (6x)	0.35 M Tris, 10% (w/v) SDS, 30% (v/v) Glycerol, 9.3% (w/v) DTT	6.8
Resolving gel buffer (4x)	1.5 M Tris, 0.4% (w/v) SDS	8.8
Stacking gel buffer (4x)	0.5 M Tris, 0.4% (w/v) SDS	6.8
Tris-buffered saline (TBS) (+Tween 20) (TBST)	200 mM Tris, 1.4 M NaCl 0.05% (v/v)	7.6
Tris-EDTA (TE) buffer	10 mM Tris, 1 mM EDTA	8.0
Tris extraction buffer	50 mM Tris, 150 mM NacCl, 1 mM EDTA, 1% (v/v) Triton-X100	7.5
Tris-glycine buffer	250 mM Tris, 1.9 M Glycine	-

*adjusted to:

2.1.2 Blocking solutions, primary and secondary antibodies

Blocking solutions

Agent	Application	(w/v)	Supplier
BSA	Western blot	5% in PBS	Sigma, München, Germany
Semi-skinned milk	Western blot	5% in TBST	Sigma
Donkey serum	Microscopy	5% (v/v) in 0.5% BSA-PBS	Dianova, Hamburg, Germany

Primary antibodies

Antigen	Application	Block (w/v), Dilution	Supplier
A1	Western blot	5% BSA, 1:250	New England Biolabs, Frankfurt/M, Germany
Actin (β)	Western blot	5% Milk, 1:3000	Sigma
Bak	Western blot	5% Milk, 1:1000	Upstate, Lake Placid, US
Bcl-2 (mouse)	Western blot	5% Milk, 1:1000	BD Pharmingen, Heidelberg, Germany
Bcl-2 (human)	Western blot	5% Milk, 1:1000	BD Pharmingen
Bcl-xl	Western blot	5% Milk, 1:1000	New England Biolabs
Bim	Western blot	5% Milk, 1:1000	New England Biolabs
Bim	Microscopy	1:100	Stressgene, Victoria, Canada
Bim	Microscopy	1:100	Clone 3C5, A. Strasser, Melbourne, Australia
Caspase-3	Flow cytometry	1:500	BD Pharmingen
CD11b-PE	Flow cytometry	1:200	eBiosciences, Breda, Germany
E.coli	Flow cytometry, Microscopy	1:300 1:300	MoBiTec, Göttingen, Germany
EEA1	Microscopy	1:100	Abcam, Cambridge, UK
GAPDH	Western blot	1:3000	BD Pharmingen
Gr1-FITC	Flow cytometry	1:100	BD Pharmingen
Mcl-1	Western blot	5% Milk, 1:2000	Rockland, Gilbertsville, US
OmpA	Western blot	1:2000	S. Paschen, München, Germany
Puma	Western blot	5% BSA, 1:5000	New England Biolabs
Rab5a	Western blot	5% Milk, 1:1000	M. Zerial, Dresden, Germany
Tubulin	Western blot, Microscopy	1:3000 1:100	Sigma Cytoskeleton, Denver, US

Secondary antibodies

Antigen/Conjugate	Application	Dilution	Supplier
Donkey anti-rabbit-Cy5	Flow cytometry, Microscopy	1:150 1:150	Dianova
Donkey anti-rabbit-FITC	Flow cytometry	1:200	Dianova
Donkey anti-rat-Alexa488	Microscopy	1:200	Invitrogen, Karlsruhe, Germany

Donkey anti-sheep-Alexa568	Microscopy	1:200	Invitrogen
Goat anti-hamster-HRP	Western blot	1:5000	Sigma
Goat anti-mouse-HRP	Western blot	1:5000	Sigma
Goat anti-rabbit-HRP	Western blot	1:5000	Sigma

2.1.3 Plasmids, primers and synthetic oligos

Plasmids

Name	Backbone	Host	Resistance	Supplier
pcDNA4/TO/*myc*-HisA	-	*E. coli*	Ampicillin	Invitrogen
pcDNA4/TO-BimS	pcDNA4/TO/*myc*-HisA	*E. coli*	Ampicillin	own
pcDNA4/TO-BimL	pcDNA4/TO/*myc*-HisA	*E. coli*	Ampicillin	own
pcDNA4/TO-BimEL	pcDNA4/TO/*myc*-HisA	*E. coli*	Ampicillin	own
pEGFP-C1	-	*E. coli*	Kanamycin	Clontech, St-Germain-en-Laye, France
pEGFP-C1-Rab5Q79L	pEGFP-C1	*E. coli*	Kanamycin	B. Knoll, Houston, US
pEGFP-C1-Rab5S34N	pEGFP-C1	*E. coli*	Kanamycin	B. Knoll, Houston, US
pLVTHM	pLVTH	*E. coli*	Ampicillin	Addgene, Cambridge, US
pLVTHM-Rab5	pLVTHM	*E. coli*	Ampicillin	own
pLVTHM-shLuciferase	pLVTHM	*E. coli*	Ampicillin	own
psPAX2	psPAX2	*E. coli*	Ampicillin	Addgene
pMD2.G	pMD2.G	*E. coli*	Ampicillin	Addgene
pMito-GFP	pEGFP-C1	*E. coli*	Kanamycin	own
pITPG-RFP		*E. coli*	Chlor-amphenicol	K. Trülzsch, München, Germany

Primers and synthetic oligos

Name	Sequence	Supplier
pLVTHM_seq	5'-TGT CGC TAT GTG TTC TGG-3'	Metabion
shLuc_fwd	5'-GCG GTG CCC GTG CGC TGC TGG TGC CAA CTT CAA GAG AGT TGG CAC CAG CGC ACT TTT TGG AAA T-3'	Metabion, München, Germany
shLuc_rev	5'-CGA TTT CCA AAA AGT GCG CTG CTG GTG CCA ACT CTC TTG AAG TTG GCA CCA GCA CAC GGG GA-3'	Metabion
shRab5_fwd	5'-CGC GTC CCC AAG CAC AGT CCT ATG CAG ATG TTC AAG AGA CAT CTG CAT AGG ACT GTG CTT TTT TTG GAA AT-3'	Metabion
shRab5_rev	5'-CGA TTT CCA AAA AAA GCA CAG TCC TAT GCA GAT GTC TCT TGA ACA TCT GCA TAG GAC TGT GCT TGG GGA-3'	Metabion

2.1.4 Cell lines and culture media

Cell line	Supplier	Medium	Supplier
293FT	Invitrogen	DMEM	PAA, Pasching, Austria
B16-GM-CSF	H. Häcker, St. Jude's Hospital, Memphis, US	RMPI-Low Endotoxin	PAA
CHO-SCF	H. Häcker	Optimen	Invitrogen
HeLa-TRex	ATCC	DMEM	PAA
HeLa-TRex-BimS	own	DMEM	PAA
HeLa-TRex-BimS	own	DMEM	PAA
HeLa-TRex-BimS	own	DMEM	PAA
Hoxb8 macrophages	H. Häcker	RPMI-Low Endotoxin	PAA
Hoxb8 neutrophils	H. Häcker	Optimem	Invitrogen
RAW 264.7	ATCC	RPMI-Low Endotoxin	PAA
-	-	Accutase	PAA
-	-	FCS-Low Endotoxin	PAA
-	-	FCS-Tetracyclin-free	PAA
-	-	PBS	PAA

2.1.5 Bacteria and culture media

Organism	Strain	Resistance	Supplier
Escherichia coli (wt)	DH5α	-	own
E. coli – RFP	DH5α	Chloramphenicol	own
Streptococcus pneumoniae	D39 (serotype 2)	-	K. Kirschning, München, Germany

Media constituents:

Escherichia coli growth medium: Lysogeny broth (LB) medium

0.5% w/v yeast extract, 1% w/v tryptone, 1% w/v sodium chloride, pH 7.0

Streptococcus pneumonia growth medium: Trypticase soy yeast extract (TSY) medium

3% w/v trypticase soy broth, 0,3% w/v yeast extract, pH 7.0

2.2 Methods

2.2.1 Genetics

2.2.1.1 Cloning of Rab5a and Luciferase shRNA into lentiviral pLVTHM vector

Three µg of the lentiviral vector pLVTHM were digested with the restriction enzymes ClaI (18 U) and MluI (32 U) (both Fermentas, St. Leon-Rot, Germany) for 4 hours at 37°C in 1x Tango buffer (Fermentas) in a final volume of 50 µl. During the last hour, generated overhangs were dephosphorylated by addition of 1 µl shrimp alkaline phosphatase (Fermentas). The cut vector was run on a 0.7% agarose gel, excised using a sterile scalpel, extracted with the Qiaex II gel extraction kit (Qiagen, Hilden, Germany) and purified using the Wizard Plus SV Miniprep kit (Promega, Mannheim, Germany) according to the manufacturer's instructions. DNA was eluted in TE buffer and its concentration measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany).

In order to generate the insert, 4 ng each of the sense and antisense synthetic oligonucleotides for Luciferase, and Rab5a, respectively, were combined in 1x annealing buffer in a final volume of 10 µl. The solution was heated at 98°C in a waterbath for 10

minutes to destroy all oligonucleotide intrastrand secondary structure, and annealing was performed by slowly cooling down to room-temperature (RT). Successful annealing was verified on a 2% agarose gel. In order to regenerate the phosphate groups at the overhangs that may have been lost by hydrolysis, 10 U of T4 polynuclease kinase (Fermentas) were added, and solutions were incubated for 1 hour at 37°C followed by heat-inactivation of the kinase at 75°C for 15 minutes. Immediately prior to ligation, annealed oligonucleotides were diluted in TE buffer to yield a stoichiometric ratio of the insert to the vector of 5:1.

Ligation was performed using 150 ng of vector DNA and 4.7 ng of insert DNA in 1x ligation buffer (Fermentas) with 5 U T4 ligase (Fermentas) in a final volume of 20 µl. The solution was incubated at 22°C for 16 hours. Three µl of the ligation products were diluted in KCM buffer to 100 µl, and 100 µl of *E. coli* thawed on ice added. Bacteria were made permeable by heat-shock at 42°C for 2 minutes, cooled on ice, and incubated with 500 µl LB medium at 37°C for 45 minutes while rotating. 100 µl of the suspensions were spread out on LB-ampicillin (100 µg/ml) plates, and incubated overnight at 37°C. The following day, colonies were picked and cultured in 5 ml LB medium with 100 µg/ml ampicillin overnight. Vector DNA was purified using the Wizard Plus SV Miniprep kit (Promega) according to the manufacturer's instructions. The correct insert sequence of the cloned products (pLVTHM-shRab5 or pLVTHM-shLuciferase) was verified by sequencing with the pLVTHM_seq primer (Medigenomix, München, Germany), and the vector was re-transformed into *E. coli*. Picked colonies were pre-cultured in 5 ml LB medium with ampicillin, followed by an overnight culture in 100 ml LB medium with ampicillin, and vector DNA was purified with the Qiagen MaxiPrep Kit (Qiagen) according to the manufacturer's specifications.

2.2.1.2 Production of Rab5a and Luciferase shRNA-containing lentivirus

293FT cells were seeded overnight at 2 x 10^6/10 cm culture plate, and medium (DMEM) was changed the next morning. The cells were transiently transfected with plasmids encoding the viral packaging machinery, the envelope, and the lentiviral vector encoding the shRNA against Rab5a or Luciferase by calcium phosphate precipitation: 20 µg pLVHTM-shRab5a/Luciferase, 15 µg PAX2 (packaging) and 6 µg MD2.G (envelope) were mixed and diluted to 500 µl with dH_2O, and 500 µl of 2x HBS were added. Upon addition of 50 µl 2.5 M $CaCl_2$ a precipitate formed, and after 20 minutes incubation at RT the complete solution was applied drop-wise to the 293FT cells (with medium). The precipitate was removed after 6 hours, and 6 ml/plate of fresh medium were added. Cells were left to produce virus for 40

hours, then the supernatant was cleared of cells and debris by centrifugation at 1600 xg for 15 minutes at RT, followed by filtration through a 0.22 µm disposable filter.

2.2.1.3 Generation of RFP-expressing *Escherichia coli* (strain DH5α)

Escherichia coli transgenic for a plasmid encoding ITPG-inducible red fluorescent protein (RFP) (pITPG-RFP) were cultured overnight in 100 ml LB medium with chloramphenicol (170 µg/ml), and plasmid DNA was purified using the Qiagen MaxiPrep kit (Qiagen) according to the manufacturer's instructions. Three µg of the plasmid were diluted in KCM buffer to 100 µl, and 100 µl of *Escherichia coli* strain DH5α thawed on ice added. Bacteria were heat-shocked at 42°C for 2 minutes, cooled on ice, and incubated with 500 µl LB medium at 37°C for 45 minutes while rotating. 100 µl of the suspension was spread out on LB-chloramphenicol (170 µg/ml) plates, and incubated overnight at 37°C. The following day, one colony was picked, pre-cultured in 5 ml LB medium with chloramphenicol, followed by an overnight culture in 100 ml LB medium with chloramphenicol. Plasmid DNA was purified with the Qiagen MaxiPrep kit (Qiagen) according to the manufacturer's specifications.

2.2.2 Protein biochemistry

2.2.2.1 Determination of protein concentrations

Protein concentrations were determined by the Bradford reaction, using the Bio-Rad Bradford kit (Biorad, München, Germany). Bovine serum albumin (Sigma) dissolved in the respective lysis buffer (Hepes or Tris) was used as standard (0.5 µg/ml – 0.05 µg/ml). Standards and samples (1 µl) were added to 200 µl Bradford reagent in 96-well plates, and after 5 minutes protein concentrations were measured in triplicate by absorption at 595 nm in a Sunrise spectrophotometer (Tecan, Männedorf, Germany).

2.2.2.2 SDS-PAGE and Western blotting

The resolving gel was prepared in resolving gel buffer at 12.5% bis-polyacrylamide (Sigma). Polymerisation was initiated by addition of ammonium persulfate (0.02% (w/v), Sigma) and TEMED (0.1% (v/v), Sigma), the solution cast into a gel chamber, and overlayed with isopropanol. After polymerisation, the alcohol was washed away and the prepared stacking gel cast on top (in stacking gel buffer, at 3.6% bis-polyacrylamide, ammonium persulfate and

TEMED as before). Loaded samples were run through the stacking gel at 130 V, and through the resolving gel at 180 V, followed by transferral of the proteins onto a nitrocellulose membrane (Wattman, Dassel, Germany) by blotting in Tris-glycine buffer in 20% methanol at 100 V for 90 minutes at 4°C. The membranes were blocked in 5% semi-skinned milk (Sigma) in Tris-buffered saline + Tween-20 (TBST) for 1 hour at RT, and incubated with primary antibodies in 5% milk-TBST overnight at 4°C (or in 5% BSA-TBST after rinsing with TBST), rinsed twice and washed three times for 5-10 minutes at RT in TBST. After blocking in 5% milk-TBST for 20 minutes, secondary antibody incubations were performed in 5% milk-TBST for 1 hour at RT. Membranes were rinsed twice and washed three times for 5 minutes in TBST, followed by two washes in TBS. After draining, membranes were overlayed with EC Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK), drained after 2 minutes, and subjected to film exposure. Exposed films were developped using a Curix 60 machine (Agfa, Köln, Germany).

2.2.3 Cell biology

2.2.3.1 RAW macrophages

2.2.3.1.1 Generation of stable transfectants for mutant Rab5a proteins

All RAW macrophage culture was carried out in petri dishes with RPMI medium unless indicated otherwise. To generate RAW macrophages stably expressing EGFP, or mutant Rab5a proteins N-terminally tagged with EGFP, 5 x 10^6 cells were resuspended in 400 µl medium supplemented with 25% FCS (without antibiotics), and 10 µg of plasmid DNA (vector pEGFP-C1, pEGFP-C1-Rab5Q79L, or pEGFP-C1-Rab5S34N) was added. Cells were made permeable to exogenous DNA through electroporation, left at RT for 5 minutes, centrifuged at 250 xg for 5 minutes, resuspended in normal medium and distributed over two 15 cm culture plates. After 48 hours, medium was replaced with fresh medium containing 0.6 mg/ml G418 for selection of cells that have stably integrated the construct with the included resistance gene into their genome. Selection was continued for 7-10 days with medium changes every 2-3 days. Once individual cells had formed colonies of appropriate size, culture plates were washed three times with PBS to remove non-adherent cells. Then colonies (or parts thereof) were picked and transferred into 12-well plates without G418. After 4-6 days culturing, the derived cell populations were analysed by flow cytometry with respect to the

expression level of the EGFP-tagged mutant protein, and also to the proportion of EGFP-positive cells, which gives an indication of the clonicity of the cell population. Only macrophage populations with a significant expression level and >90% positivity were chosen for further experimentation.

2.2.3.1.2 Lentiviral introduction of Rab5a or Luciferase shRNA and cell sort

RAW macrophages were seeded out overnight in 6-well plates (5 x 10^5 cells). The next day, medium was removed, cells were incubated for 24 hours with 1 ml of lentiviral supernatant of the pLVTHM-Rab5 or pLVTHM-Luciferase constructs (addition of 1 ml fresh medium after 8 hours). Residual virus was then removed by washing with PBS, and cells were detached in 1 ml accutase for 20 minutes. After addition of 5 ml medium and centrifugation, cells were resuspended in 6 ml medium, and split into two 6-plate wells. After 48 hours culture was continued in the presence of 5 μg/ml puromycin for selection, cells were transferred into petri dishes after 72 hours, and then grown for further 6 days in the presence of puromycin. Flow cytometry analysis revealed that about 2% of macrophages were EGFP-positive, i.e. stably expressing from an integrated lentiviral vector. Culture was continued for another 4 days in the absence of puromycin. In order to isolate the EGFP-positive cells from the whole population, 10^8 macrophages were centrifuged at 400 xg for 5 minutes, resuspended in 10 ml medium-50% FCS, and then subjected to a flow cytometry sort. Sorted macrophages were first cultured in 6-well plates and then transferred to petri dishes. Flow cytometry analysis showed that about 70% of macrophages in the shRab5a and shLuciferase population were EGFP-positive.

2.2.3.2 Hoxb8 progenitor macrophages and neutrophils

2.2.3.2.1 Production of SCF and GM-CSF supernatants

Supernatant containing granulocyte-macrophage colony stimulating factor was produced from B16 cells carrying a transgene encoding the growth factor. Cells were seeded out on 15 cm culture dishes in RPMI medium, and at about 70% confluency split to 10 culture plates. From the fourth day onwards (for 3 consecutive) days, medium containing the secreted growth factor was replaced with fresh medium. The removed medium was centrifuged at 1600 xg for 5 minutes to pellet residual cells, and the supernatant frozen at -20°C. When all supernatant had been collected and pooled, it was filtered through a 0.22 μm disposable filter, aliquoted

and then stored at -20°C. Supernatant containing stem-cell factor was produced in the same way, by use of transgenic CHO cells cultured in Optimem medium. The optimal factor concentration for later use in cell culture was determined by analysing cell survival and growth rates of Hoxb8 macrophages or neutrophils in dependency of supernatant concentration.

2.2.3.2.2 Culture and differentiation of progenitor cells

Myeloid progenitor cells were isolated from the bone-marrow of C57BL/6 mice and retrovirally transduced with Hoxb8, and polyclonal populations selected by the group of H. Häcker (Memphis, US) (method described in Wang et al., 2006). Hoxb8 macrophage progenitor cells were cultured in RPMI medium in 6-well plates (3 ml/well) in the presence of 1 μM oestrogen, and 2% GM-CSF. For the induction of differentiation by oestrogen removal, cells were centrifuged at 180 xg for 5 min, resuspended in 10 ml PBS/10% FCS supplemented with 1% GM-CSF, and washed once again. Progenitor cells were resuspended in medium containing 2% GM-CSF at a density of 5 x 10^5/10 ml, and differentiated in petri dishes for 6 days. For use in experiments, the differentiated (now adherent) macrophages were washed once with PBS, incubated in 3 ml accutase for 30 minutes for detachment, and after addition of 5 ml medium centrifuged at 400 xg for 5 minutes and resuspended at the appropriate density in the required medium.

Hoxb8 neutrophil progenitors were cultured in Optimem medium in the presence of 1 μM oestrogen, and 4% SCF. Oestrogen was removed as described above, with 2% SCF present in the wash medium. Progenitors were resuspended in medium containing 2% SCF at a density of 1 x 10^5/ml, and differentiated in 6-well plates (3 ml/well) for 4 days. For use in experiments, cells (in majority in suspension) were simply taken off, or pipetted off very gently.

2.2.3.2.3 Macrophage and neutrophil differentiation markers

Hoxb8 macrophages differentiated for 6 days and progenitor cells were centrifuged at 250 xg, resuspended in RPMI medium and incubated at 37°C/5%CO_2 for 30 minutes to allow recycling of surface markers cut off by treatment with accutase for cell detachment. Cells (1 x 10^6) were then centrifuged at 250 xg at 4°C for 5 minutes, resuspended in cold PBS/10% FCS and two equal volumes were transferred into 96-well plates (bottom V-shape). After re-

pelleting at 4°C, cells were stained with an antibody against CD11b conjugated with the fluochrome PE at 4°C for 20 minutes (1:100 in PBS/10% FCS). After two washes and resuspension in PBS/10% FCS, samples were put on ice and analysed by flow cytometry.

The cell differentiation status in Hoxb8 neutrophils was analysed by Giemsa stain and surface expression of Gr1. For Giemsa staining, 2 x 10^5 neutrophils differentiated for various lengths of time, or progenitor cells, were centrifuged at 400 xg for 5 minutes, and resuspended in 150 µl Optimem medium. Neutrophils were transferred onto glass slides by cytospin (removes majority of liquid and pellets cells onto the slide as a monolayer) at 400 xg for 5 minutes, and fixed in methanol for 5 minutes. After drying, cells were stained in 4.5% Giemsa (Sigma) in Giemsa buffer for 20 minutes, rinsed, dryed, and examined under a light microscope. For the analysis of Gr1 expression, 1 x 10^6 neutrophils were centrifuged at 400 xg for 5 minutes, and then processed as described for macrophage surface-staining above. Here, a FITC-conjugated antibody against the cell surface marker Gr1 (1:100) was used. Cells were analysed by flow cytometry.

2.2.3.2.4 Transient transfection of macrophages

Hoxb8 macrophages were transiently transfected with plasmids encoding EGFP, or the EGFP-tagged dominant-negative or constitutively active Rab5a by use of the Macrophage Nucleofector kit from Amaxxa. Differentiated macrophages (4 x 10^6) were centrifuged at 90 xg for 7 minutes, resuspended in 100 µl nucleofector solution, and 2 µg of plasmid DNA (pEGFP-C1, pEGFP-C1-Rab5Q79L, or pEGFP-C1-Rab5S34N) was added. Suspensions were then subjected to electroporation, and transferred into petri dishes containing normal RPMI medium. Transfected macrophages were detached from dishes 24 hours later with accutase as described under 2.2.3.2.2, and used for experiments.

2.2.3.3 Culture, preparation and staining of bacteria

Escherichia coli were grown in 30 ml LB medium overnight at 37°C, for induction of RFP expression in transgenic bacteria isopropyl-β-D-thiogalactopyranoside (ITPG) (Sigma) was added to 1 mM. Bacteria were centrifuged at 2800 xg at 4°C for 8 minutes, washed twice with cold PBS, and passed through a 5 µm disposable filter. Afterwards the bacterial suspension was adjusted to an optical density at 600 nm (OD_{600}) of 2.0 ± 0.05 by dilution with PBS (with an OD_{600} of 2.0 corresponding to 2 x 10^9 bacteria/ml).

Streptococcus pneumoniae were grown in 100 ml TSY medium overnight at 37°C, and pelleted at 1600 xg at 4°C for 10 minutes. After washing twice with PBS, OD_{600} was adjusted to 1.0 ± 0.05 with PBS (corresponding to 1 x 10^9 bacteria/ml). For staining of pneumococci with fluorescein isothiocyanate (FITC, Sigma), bacterial cultures were centrifuged at RT and washed once in carbonate buffer. After resuspension in 1 ml carbonate buffer, FITC was added to 20 µg/ml and pneumococci incubated at 37°C for one hour. Bacteria were then washed twice with PBS at 4°C, and OD_{600} adjusted to 1.0 ± 0.05 with PBS.

2.2.3.4 Phagocytosis assays

2.2.3.4.1 Uptake of *Escherichia coli* and *Streptococcus pneumoniae*

Escherichia coli-RFP grown in the presence of ITPG (to induce red-fluorescence) and prepared as described under 2.2.3.3 were centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium to yield the appropriate concentration (in most experiments 4 x 10^7/250 µl for a bacteria-to-cell ratio of 200:1). RAW macrophages stably transfected with EGFP, constitutively active or dominant-negative Rab5a-EGFP (see 2.2.3.1.1), or lentivirally transduced with pLVTHM-Luciferase or pLVTHM-Rab5a (see 2.2.3.1.2) were centrifuged at 400 xg for 5 minutes and resuspended in RPMI medium at 2 x 10^5/250 µl. Macropage and bacterial suspensions (250 µl each) were combined into Eppendorf tubes and incubated for 30 minutes at 37°C while rotating. The rotation-induced mixing significantly enhances phagocytosis by increasing the number of physical contacts between cells and bacteria. Samples were then put on ice, and immediately prior to flow cytometry analysis pipetted up and down vigorously in order to dislodge non-engulfed bacteria weakly adhering to the cellular surface.

Phagocytic macrophages can be identified by the red-fluorescence produced by internalised bacteria. As the cellular morphological changes associated with phagocytosis already induce a small non-specific increase in red-fluorescence, macrophages incubated with *E. coli-RFP* that had not been induced to fluoresce with IPTG were used as controls. Phagocytic indices were calculated by multiplying the percentage of phagocytic (red-fluorescent) macrophages within the sample population with the value of their average red-fluorescent intensity. Because the latter value depends critically on the mean RFP expression level in the bacteria, and can vary significantly between bacterial cultures and therefore also experiments, phagocytic indices were normalised. In Figure 8B, the values of the phagocytic indices obtained for the “12h 200:1” samples in separate experiments (e_n) were normalised to

their mean value (e_m), and the resulting normalisation factor (e_m/e_n) multiplied with the phagocytic index of all other samples in the respective experiment. In Figure 12, the same calculations were performed using the "WT + E.coli-RFP" (A), "EGFP-negative" (B), and "shLuciferase + E.coli-RFP" (C) samples as normaliser. In Figure 16E, samples "WT + DMSO + E.coli-RFP" were used as normaliser. In some experiments, RAW macrophages were pre-treated with 25 nM bafilomycin A_1, 25 mM ammonium chloride, or DMSO for 30 minutes. These agents were also included at the same concentration in the media used for resuspending bacteria.

For the measurement of phagocytosis of *Streptococcus pneumoniae* by Hoxb8 neutrophils, FITC-stained pneumococci (see 2.2.3.3) were centrifuged at 1600 xg at 4°C for 5 minutes and resuspended in Optimem medium at 4 x 10^7/250 µl. Neutrophils were centrifuged at 250 xg and resuspended in Optimem medium at 4 x 10^5/250 µl. Neutrophil and bacterial suspensions (250 µl each) were combined into Eppendorf tubes and incubated for 1 hour at 37°C while shaking. Afterwards, samples were put on ice and analysed by flow cytometry.

2.2.3.4.2 Staining of intracellular *Escherichia coli*

In Hoxb8 macrophages transiently transfected with EGFP, constitutively active or dominant-negative Rab5a-EGFP (see 2.2.3.2.4), bacterial phagocytosis was not analysed by use of RFP-expressing *E. coli* but by staining of ingested bacteria after uptake. This alternative approach was used because transient transfections usually produce a very broad range of expression levels of the transgene, and thus here in this case, EGFP-positivity. The high-energy part of the light spectrum emitted from EGFP also to some extent non-specifically excites RFP. In cases where the range of the intracellular EGFP expression level is very narrow (as in stably transfected RAW macrophages) and therefore the non-specific excitation of RFP remains rather constant, this can be compensated for. With a very wide range of EGFP expression, compensation is very inaccurate and produces unreliable results. To circumvent this problem, macrophage phagocytosis was analysed by marking ingested bacteria with an antibody-coupled blue fluorescent dye that cannot be excited non-specifically by the EGFP emission.

WT *E. coli* were prepared and co-incubated with macrophages under the same conditions as described under 2.2.3.4.1 for 4 hours. Then suspensions were then centrifuged through a PBS/30% sucrose cushion at 250 xg at 4°C for 8 minutes in order to remove non-engulfed bacteria that during fixation (in 3.7% formaldehyde for 10 minutes at RT) would

otherwise become non-specifically attached to cells. Fixed cells were transferred to 96-well plates (bottom V-shape), centrifuged at 400 xg at 4°C for 5 minutes, and resuspended in PBS. After re-pelleting, cells were permeabilised with 0.1% Triton X-100 for 4 minutes, centrifuged, and resuspended in PBS/0.5% BSA containing *E. coli* BioParticles opsonising reagent at 1:300. After a staining period of 40 minutes, cells were washed twice in PBS/0.5% BSA, and resuspended in donkey anti-rabbit-Cy5 antibody at 1:150 in PBS/0.5% BSA. After 30 minutes, cells were washed three times in PBS/0.5% BSA, and subjected to flow cytometry analysis. As macrophages were only ~20% positive for expression of the transgene (as indicated by their green-fluorescence) within a sample, the cells negative for expression (not fluorescent) were used as "internal" control. Phagocytic macrophages within both the positive and negative population were identified by their blue-fluorescence, and phagocytic indices were calculated and normalised as described above (see 2.2.3.4.1).

2.2.3.4.3 Uptake of apoptotic neutrophils by macrophages

RAW macrophages were centrifuged at 400 xg for 5 minutes, washed once in PBS and 5 x 10^6 cells resuspended in 50 µl of diluent C (PK-H26 kit, Sigma). Fifty µl of diluent C containing 2% of the dye PK-H26 (stains cellular membranes) were added and the suspension mixed thoroughly. After 5 minutes, residual dye was quenched by addition of 1 ml sterile-filtered PBS/1% BSA and 5 ml RPMI medium. After one minute, macrophages were pelleted and washed twice with PBS before resuspension in RPMI and seeding out into 24-well plates at 2 x 10^5/well overnight.

The next day, immediately prior to co-incubation with red-stained macrophages, neutrophils that had been rendered apoptotic by deprivation of stem cell factor for various lengths of time as described under 2.2.3.10.2 were stained green with CFDA-SE (Invitrogen). CFDA-SE is a cell-permeable dye that is converted by intracellular esterases into an amine-reactive dye emitting fluorescence. Part of the apoptotic neutrophils was stained with AnnexinV-FITC/propidium iodide (see 2.2.3.10.4), while 10^7 cells were centrifuged at 400 xg for 5 minutes, washed once with PBS and resuspended in 1 ml PBS containing 1 µM CFDA-SE. After 10 minutes, stained cells were pelleted and residual dye quenched by resuspension in Optimem medium and incubation at 37°C/5% CO_2 for 30 minutes. After re-pelleting, neutrophils were resuspended in RPMI at 1 x 10^6/500 µl, and added to macrophage monolayers (addition of 500 µl/well after removal of old medium). Cells were co-cultured for

4 hours, and macrophages then rinsed once with PBS and detached in 300 µl/well accutase for 20 minutes. Cellular suspensions were put on ice and subjected to flow cytometry analysis.

2.2.3.5 Measurement of nitrix oxide production

Bacteria were prepared as described under 2.2.3.3, centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium at 4 x 10^7/250 µl. RAW or Hoxb8 Macrophages were centrifuged at 400 xg for 5 minutes, resuspended in RPMI medium at 2 x 10^5/250 µl with DMSO, bafilomycin A_1 (RAW 25 µM, Hoxb8 5 nM) or ammonium chloride (25 mM), and after 30 minutes co-incubated with bacteria (250 µl each) in Eppendorf tubes. Samples were rotated at 37°C for 30 minutes and transferred into 12-well plates. Five hundred µl medium/well were added, and incubation was continued for 20 hours at 37°C/5%CO_2. DMSO, bafilomycin A_1 or ammomiun chloride were also included at the same concentration in the media used for resuspending bacteria and the medium added later. After the incubation period the sample medium was removed, cleared off cells and bacteria by centrifugation at 4000 xg for 10 minutes, and the supernatant was frozen at -80°C.

Fifty µl of supernatants and standards ($NaNO_2$ diluted in medium to yield a concentration range of 5 – 0.05 µM) were transferred into a 96-well plate, and 50 µl Gries reagent was added (60 mM sulfanilamide, 0.4 mM N-ethylenediamine dihydrochloride, 2% (w/v) phosphorous acid). After 5 minutes, absorbance was measured in triplicate at 540 nm (690 nM reference) in a Sunrise spectrophotometer (Tecan, Männedorf, Germany).

2.2.3.6 Measurement of reactive oxygen species production

The production of reactive oxygen species by Hoxb8 neutrophils was measured using 123-dihydrorhodamine (Sigma), a cell-permeable dye that fluoresces green when oxidised. Neutrophils were centrifuged at 400 xg for 5 minutes and resuspended in Optimem medium at 4 x 10^5/250 µl. *Streptococcus pneumoniae* were pelleted at 1600 xg at 4°C for 10 minutes, resuspended at 4 x 10^7/250 µl, and both suspensions (each 250 µl) combined in Eppendorf tubes and incubated at 37°C while shaking. After 30 minutes, 123-dihydrorhodamine was added to 2 µM and co-incubation was continued for another 30 minutes. Cells were centrifuged at 400 xg at 4°C for 5 minutes, washed once with cold PBS, put on ice and analysed by flow cytometry.

2.2.3.7 Measurement of IL-1β production

Streptococcus pneumoniae were prepared as described under 2.2.3.3, centrifuged at 1600 xg at 4°C for 10 minutes and resuspended in 800 µl Optimem medium at 1.5 x 10^6/200 µl. Hoxb8 neutrophils were centrifuged at 250 xg for 5 minutes, and resuspended in Optimem medium at 1.5 x 10^6/400 µl. Bacteria (200 µl) and neutrophils (400 µl) were combined in Eppendorf tubes and rotated at 37°C for 30 minutes. Twice 200 µl of each sample (5 x 10^5 neutrophils) were then transferred into 96-well culture plates. Following 7.5 hours of incubation at 37°C/5% CO_2, supernatants were taken off and cleared off cells and bacteria by centrifugation at 16000 xg, at 4°C for 5 minutes. Supernatants were frozen at -80°C. IL-1β in the undiluted supernatants was measured by ELISA using the Quantikine Mouse IL-1β Assay kit (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions.

2.2.3.8 Measurement of intracellular bacterial killing

Intracellular bactericidal activity against *Escherichia coli* was analysed in RAW macrophages cultured in the absence of antibiotics for at least 48 hours prior to experiments. Bacteria were prepared as described under 2.2.3.3, centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium containing 25 µM bafilomycin or DMSO at 2.5 x 10^6/250 µl. Macrophages were centrifuged at 400 xg for 5 minutes, resuspended in RPMI medium containing 25 µM bafilomycin or DMSO at 5 x 10^5/250 µl, and incubated at 37°C/5%CO_2 for 30 minutes for 30 minutes. From both suspensions 250 µl were combined in Eppendorf tubes and incubated at 37°C for 30 minutes while rotating. Then, suspensions were put on ice and underlayed carefully with 1 ml sterile-filtered cold PBS/30% sucrose, and centrifuged at 250 xg for 8 minutes. The sucrose cushion allows passage of cells, but non-engulfed bacteria are retained at the interface due to their low density. This removal is important because otherwise extracellular bacteria would add non-specifically to the count of surviving bacteria. Supernatant was removed, and cells were resuspended in 500 µl cold PBS/10% FCS and underlayed once again with 1 ml sucrose. After centrifugation, suspensions were resuspended in 500 µl cold PBS. Of these samples, 100 µl were lysed directly, while another 100 µl were cultured for further 2 hours. For direct lysis (t0), 100 µl were transferred into tubes containing 900 µl ddH_2O (this induces osmotic swelling of cells followed by cell rupture). After 10 minutes on ice, the lysate containing released intracellular bacteria was diluted 10-fold in

PBS, and 100 µl were spread out on LB plates. This set of half samples gives the number of phagocytosed but not yet killed bacteria. For culture continuance (t+2), 100 µl were added to 400 µl RPMI medium in suspension tubes containing 31 µM bafilomycin A_1 or DMSO, plus 63 µg/ml gentamycin. Tubes were incubated at 37°C/5% CO_2 for 2 hours, with vortexing every 30 minutes, centrifuged at 250 xg at 4°C for 8 minutes, and cells resuspended in 1 ml ddH_2O/10% PBS. Lysates were then treated and applied to LB plates as described above. This set of samples gives the number of surviving bacteria after an intracellular killing period of 2 hours. LB plates were incubated for 16 hours at 37°C, and colonies formed were scored. The percentage of surviving *E. coli* calculated by relating the respective t0 and t+2 counts.

2.2.3.9 Measurement of intracellular bacterial degradation

For the analysis of intracellular bacteria digestion in a pulse-chase experimental setting, *Escherichia coli* were centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium containing 25 µM bafilomycin or DMSO at 1.0, 0.8, or 0.6 x 10^7/250 µl depending on the RAW macrophage clone used. Macrophages were pelleted at 400 xg for 5 minutes, resuspended in RPMI medium containing 25 µM bafilomycin or DMSO at 1 x 10^6/250 µl, and incubated at 37°C/5%CO_2 for 30 minutes. Bacteria and macrophages were co-incubated and extracellular bacteria removed by centrifugation through sucrose as described under 2.2.3.8. Cells were then resuspended in 1 ml cold PBS, samples split in two, and centrifuged at 250 xg at 4°C for 8 minutes. One half was lysed directly while the other was incubated for further 8 hours. For direct lysis, cells were resuspended in 50 µl Hepes extraction buffer and held on ice for 30 minutes followed by freezing at -20°C. The other half of the sample was resuspended in 2 ml RPMI medium containing 25 µM bafilomycin or DMSO and incubated in 6-well plates at 37°C/5%CO_2 for 8 hours. Then, plates were put on ice, wells rinsed once with cold PBS, and macrophages lysed in 50 µl Hepes extraction buffer for 30 minutes. Cellular debris was pipetted and scraped off the well bottom, the lysate collected and frozen at -20°C.

For the measurement of intracellular bacterial degradation under the “steady state” conditions (i.e. continuous uptake and digestion of large quantities of bacteria) used for apoptosis induction (see 2.2.3.10.1), *E. coli* were centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium containing 25 µM bafilomycin or DMSO at 4 or 8 x 10^7/250 µl. Macrophages were pelleted at 400 xg for 5 minutes, resuspended in RPMI

medium containing 25 µM bafilomycin or DMSO at 2 x 10^5/250 µl, and incubated at 37°C/5%CO_2 for 30 minutes. Bacteria and macrophages (each 250 µl) were co-incubated at 37°C while rotating for 30 minutes, and suspensions then transferred to 12-well plates, and 500 µl medium with bafilomycin/DMSO was added. After an incubation time of 12 hours at 37°C/5%CO_2, plates were put on ice and extracellular bacteria were removed by rinsing adherent macrophages three times with cold PBS. Then, cells were lysed in 50 µl Hepes extraction buffer for 30 minutes on ice. Lysates from duplicate samples were collected by pipetting and scraping, pooled and frozen at -20°C. Protein content in samples was measured by the Bradford test (see 2.2.2.1), and proteins were reduced by addition of Laemmli buffer and heating at 95°C for 5 minutes.

In order to probe macrophage lysates for their bacterial content, 20 µg of protein were run out on a 12.5% SDS-PAGE gel and transferred to membranes by Western blotting (see 2.2.2.2). Membranes were probed with an antibody recognising bacterial OmpA at 1:2000 at 4°C overnight, followed by detection with secondary anti-mouse-HRP at 1:5000 for 1 hour at RT.

2.2.3.10 Apoptosis induction and apoptosis assays

2.2.3.10.1 Induction of macrophage apoptosis by *Escherichia coli*

Bacteria were prepared as described under 2.2.3.3, centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium at 4 x 10^7/250 µl. RAW or Hoxb8 Macrophages were centrifuged at 400 xg for 5 minutes, resuspended in RPMI medium at 2 x 10^5/250 µl, and co-incubated with bacteria (250 µl each) in Eppendorf tubes. Samples were rotated at 37°C for 30 minutes and transferred into 12-well plates. Five hundred µl medium/well were added, and incubation was continued for 20-24 hours at 37°C/5%CO_2. In some experiments, macrophages were pre-treated with bafilomycin A_1 (RAW macrophages 25 nM, Hoxb8 macrophages 5 nM), 25 mM ammonium chloride, or DMSO for 30 minutes. These agents were also included at the same concentration in the media used for resuspending bacteria and the medium added later.

For the analysis of the effects of bafilomycin A_1 on apoptosis in macrophages already loaded with bacteria, cells and bacteria were co-incubated as described for 12 hours, and bacteria were then washed away by rinsing wells three times with PBS. Bafilomycin A_1

or DMSO was then added with 1 ml of fresh RPMI medium at the concentrations mentioned above, and culture continued for further 12 hours.

2.2.3.10.2 Induction of spontaneous neutrophil apoptosis

Hoxb8 neutrophils were induced to undergo spontaneous apoptosis by ageing either in the presence or absence of stem cell factor. For ageing in the presence of SCF, neutrophils were centrifuged at 250 xg for 5 min, and resuspended in 10 ml 10% FCS-PBS supplemented with 2% SCF. After re-pelleting, cells were resuspended in Optimem medium with 2% SCF at a density of 2 x 10^6/ml, and culture continued in 12-well plates (1 ml/well) for various periods of time. For ageing in the absence of SCF, neutrophils were treated as above, but with no stem cell factor present in wash and culture medium.

2.2.3.10.3 Hoechst staining

Cellular nuclei were stained by addition of 1 µg/ml Hoechst dye and incubation at 37°C/5%CO_2 for 20 minutes. Cells were centrifuged at 3500 xg at 4° for 5 minutes, and resuspended in 10 µl PBS. The sample was applied to glass slides, and the number of "healthy" and apoptotic nuclei present in randomly chosen fields of vision were scored under a fluorescence microscope. A total of 300-400 cells were examined per sample.

2.2.3.10.4 AnnexinV-propidium iodide staining

Cells (5 x 10^5) were centrifuged at 400 xg for 5 minutes and resuspended in 400 µl cold AnnexinV buffer. Twice 200 µl were transferred into 96-well plates (bottom V-shape) and cells were pelleted again at 4°C. Cells were then resuspended in 35 µl AnnexinV buffer containing AnnexinV-FITC (1:50, BD Pharmingen) and 2 µg/ml propidium iodide (Sigma) and put at 4°C in the dark for 20 minutes. After addition of 100 µl AnnexinV buffer, cells were analysed by flow cytometry. In some experiments, cells were only stained with propidium iodide.

2.2.3.10.5 Active Caspase-3 staining

Cells (1 x 10^6) were centrifuged at 400 xg for 5 minutes, resuspended in cold PBS and two equal volumes were transferred into 96-well plates (bottom V-shape). After re-pelleting at 4°C, cells were fixed in 100 µl PBS/3.7% formaldehyde at 4°C for 10 minutes. After addition of 100 µl PBS, repelleting and one wash with PBS, cells were permeabilised in 200 µl

PBS/0.5% BSA/1% (w/v) saponin (Sigma) for 4 minutes, and after centrifugation stained with an anti-active Caspase-3 antibody (1:500) in 35 cold µl PBS/0.5% BSA at 4°C for 30 minutes. After addition of 165 µl cold PBS/0.5% BSA and one wash in PBS/0.5% BSA, cells were stained with anti-rabbit-FITC antibody (1:200) in 35 µl PBS/0.5% BSA at 4°C for 20 minutes. Cells were washed twice with and resuspended in PBS/0.5% BSA, samples put on ice and subjected to flow cytometry analysis.

2.2.3.11 Protein extraction from healthy and apoptotic neutrophils

Extracts were prepared from Hoxb8 neutrophils either after a differentiation period of 4 days, or after various lengths of time after washing and continued culturing in the absence of stem cell factor at the conditions described under 2.2.3.10.2. Cells were collected and centrifuged at 250 xg for 5 min at 4°C, followed by washing in 1 ml cold PBS and re-centrifugation. Pellets were then resuspended in ~10 µl Tris extraction buffer/1 x 10^6 cells, lysed 30 minutes on ice, and centrifuged at 16000 xg for 15 minutes at 4°C to pellet nuclei and large membrane debris. A small aliquot of the supernatant was directly frozen at -80°C for later protein concentration measurement, the remainder was boiled in Laemmli buffer for 5 minutes at 95°C, and then frozen at -80°C.

2.2.3.12 Immunofluorescence staining and microscopy

2.2.3.12.1 Induction of giant vacuoles in Rab5CA macrophage lines

RAW macrophages were seeded out overnight onto coverslips in 12-well plates at 2 x 10^5/well. *Escherichia coli* were prepared as described under 2.2.3.3 and resuspended in RPMI medium at 1 x 10^6/1 ml. The medium was removed from the plates and macrophages incubated in 1 ml of bacterial suspension/well. Alternatively, LPS (1 µg/ml) was added to the medium. After 24 hours, the proportion of cells harbouring one large vacuole was scored under a light microscope, 300-400 cells were counted per sample.

2.2.3.12.2 Detection of EEA1 in RAW macrophages

RPF-transgenic *Escherichia coli* grown in the presence of ITPG (to induce red-fluorescence) and prepared as described under 2.2.3.3 were centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium at 4 x 10^7/250 µl. RAW macrophages were centrifuged at 400 xg for 5 minutes, resuspended in RPMI medium at 2 x 10^5/250 µl and co-incubated with

bacteria (both 250 µl) in Eppendorf tubes for 30 minutes at 37°C while rotating. Then, cells were seeded onto coverslips resting in 12-well plates, and after 6 hours washed three times in PBS. Cells were fixed in 3.7 % formaldehyde followed by permeabilisation in 0.1 % Triton X-100, washed once in PBS, and blocked in PBS/0.5% BSA/5% heat-inactivated donkey serum (Dianova, Hamburg, Germany) for 15 minutes. After three washes in PBS/0.5% BSA, cells were incubated with rabbit anti-EEA1 antibody (1:100) in PBS/0.5% BSA for 40 minutes, followed by three washes and incubation in donkey anti-rabbit-Cy5 antibody (1:150) for 30 minutes in the dark. After three washes in PBS, coverslips were mounted onto glass slides in 3 µl Mowiol, sealed with nailvarnish, and stored at 4°C in the dark. Cells were examined with a Leica confocal microscope (TCS SP5, 63x/1.2 lens, Leica, Bensheim, Germany). Pinholes were set to scan layers < 1 µm, at a resolution of 1024 x 1024 pixels.

2.2.3.12.3 Detection of Bim in RAW macrophages

Escherichia coli prepared as described under 2.2.3.3 were centrifuged at 2800 xg at 4°C for 5 minutes and resuspended in RPMI medium to yield a concentration of 4 x 10^7/250 µl. RAW macrophages were centrifuged at 400 xg for 5 minutes and resuspended in RPMI medium at 2 x 10^5/250 µl. Macropage and bacterial suspensions (250 µl each) were combined into Eppendorf tubes and incubated for 30 minutes at 37°C while rotating. Cells were then seeded onto coverslips in 12-well plates, and 500 µl medium was added. For controls or LPS stimulation, mock-infected macrophages were seeded onto coverslips, and 500 µl medium without or with LPS (1µg/ml final) was added. After 24 hours, medium was removed and fresh medium added containing Mitotracker-Orange CMTMRos (Invitrogen) at 1:4000 for 30 minutes. Cells were then rinsed three times in PBS, fixed in 3.7 % formaldehyde followed by permeabilisation in 0.1 % Triton X-100, washed once in PBS and blocked in PBS/0.5% BSA/5% heat-inactivated donkey serum (Dianova) for 15 minutes. After three washes in PBS/0.5% BSA, cells were incubated with *E. coli* BioParticles opsonising reagent (1:300) in PBS/0.5% BSA for 40 minutes, followed by three washes and incubation with rat anti-Bim antibody (1:100, clone 3C5) for 40 minutes. After three washes, cells were incubated with secondary antibodies donkey anti-rabbit-Cy5 (1:150) and donkey anti-rat-Alexa488 (1:200) for 30 minutes in the dark. After three washes in PBS, coverslips were mounted onto glass slides in 3 µl Mowiol, sealed with nailvarnish, and stored at 4°C in the dark. Cells were

examined with a Leica confocal microscope (TCS SP5, 63x/1.2 lens, Leica, Bensheim, Germany). Pinholes were set to scan layers < 1 µm, at a resolution of 1024 x 1024 pixels.

2.2.3.12.4 Detection of Bim in HeLa cells

For transient transfection with mitochondrial-localised EGFP, 4 x 10^6 HeLa-TRex clones that stably express the tetracycline repressor from the pCDNA6/TR vector plus the tetracyclin-inducible vector pcDNA4/TO/*myc*-HisA with the ORF of either mBimS, hBimL, or hBimEL as insert (clones generated by B. Seiffert) were centrifuged at 400 xg for 5 minutes, resuspended in 400 µl DMEM medium-20% tetracyclin-free FCS without antibiotics, and after addition of 10 µg pMito-GFP transferred into electroporation cuvettes (Biorad, München, Germany). After electroporation cells were rested 10 minutes, transferred into 10 ml DMEM-0.5% FCS, centrifuged at 250 xg for 5 minutes, and resuspended in normal DMEM medium and seeded onto coverslips in 24-well plates at 8 x 10^4/well. After 24 hours, Bim expression was induced by addition of tetracyclin (1 µg/ml, Sigma, Germany) for 6 hours. Cells were rinsed three times in PBS, fixed in 3.7 % formaldehyde followed by permeabilisation in 0.1 % Triton X-100, washed once in PBS and blocked in PBS/0.5% BSA/5% heat-inactivated donkey serum (Dianova) for 15 minutes. After three washes in PBS/0.5% BSA, cells were incubated with sheep anti-microtubule antibody (1:100) in PBS/0.5% BSA for 40 minutes, followed by three washes and incubation with rabbit anti-Bim antibody (1:100, Stressgene) for 40 minutes. After three washes, cells were incubated with secondary antibodies donkey anti-sheep-Alexa568 (1:200) and donkey anti-rabbit-Cy5 (1:150) for 30 minutes in the dark. After three washes in PBS, coverslips were mounted onto glass slides in 3 µl Mowiol, sealed with nailvarnish, and stored at 4°C in the dark. Cells were examined with a Leica confocal microscope (TCS SP5, 63x/1.2 lens, Leica, Bensheim, Germany). Pinholes were set to scan layers < 1 µm, at a resolution of 1024 x 1024 pixels.

3 Results

3.1 Macrophage apoptosis in response to the uptake of pyogenic bacteria

Non-pathogenic *Escherichia coli* constitute the primary gram-negative species of Enterobacteriaceae in the gastrointestinal tract, where they do not cause harm and are tolerated by the immune system. Perforation of the intestinal mucosa though, i.e. from an ulcer, ruptured appendix or surgical error, leads to dissemation of these commensal bacteria into the abdomen, and *E. coli* are one of the most common causes of a number of localised and systemic infections (Lorber and Swenson, 1975; Hau, 1990).

In vitro, low-virulence *E. coli* have been demonstrated to induce apoptosis in murine bone marrow-derived macrophages and the macrophage line RAW upon phagocytosis (Hacker et al., 2002; Kirschnek et al., 2005). Although signalling through pattern recognition receptors may contribute to this form of apoptosis, it is clearly not sufficient. Heat-killed *E. coli*, which showed similar stimulatory potential but were taken up very poorly, failed to induce apoptosis, and macrophage death could be blocked by treatment of cells with cytochalasin D, a drug that inhibits the actin polymerisation required for phagosome formation and internalisation (Hacker et al., 2002; Rodrigues et al., 1999). Also, exposure to LPS did not induce cell death, and mice deficient in Myd88 were not protected from phagocytosis-induced apoptosis (Hacker et al., 2002). Together with the observation that intracellular breakdown of bacteria started already several hours before the manifestation of apoptosis, these results suggested a link between phagocytosis and intracellular digestion of bacteria, and the initiation of apoptosis. This hypothesis was tested in murine RAW and Hoxb8 macrophages in which phagocytosis and digestion of *E. coli* were modulated by the expression of mutant Rab5a proteins, or by the treatment with the phagosome acidification inhibitor bafilomcyin A_1.

3.1.1 Experimental setup for bacterial phagocytosis-induced apoptosis in RAW and Hoxb8 macrophages

In previous work, macrophage apoptosis had been induced by co-incubating bacteria and macrophages at a bacteria-to-cell ratio of 1000:1 for 1 hour. Bacteria were then (largely) removed and cells incubated for further 23 hours, during which time the majority of

macrophages underwent apoptosis. Finally, cellular nuclei were stained with Hoechst, a membrane-permeable DNA-intercalating fluorescent dye, and the proportion of cells containing apopotic (condensed) nuclei was scored using a fluorescence microscope. This method induced apoptosis in 70-80% of macrophages (Hacker et al., 2002; Kirschnek et al., 2005).

Because a relatively large number of *E. coli* were used, and the removal of bacteria was difficult to control quantitatively, it was decided to change the experimental setup to a continuous co-incubation for 24 hours without bacterial removal, and to use fewer bacteria. When titration experiments with bacteria-to-cell ratios ranging from 50:1 to 400:1 were performed, a ratio of 200:1 produced a comparable rate of apoptosis of ~60% in RAW macrophages (Figure 6). Reproducability with this experimental setup was very high.

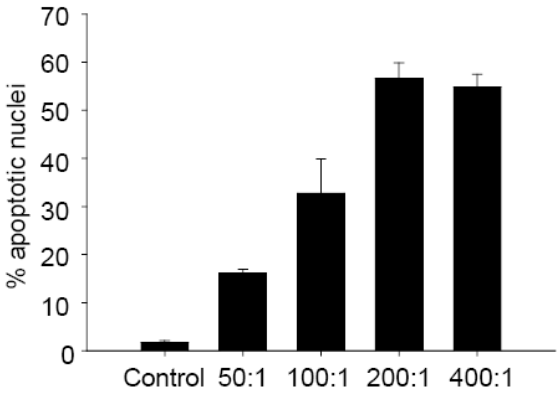


Fig. 6 Bacterial titration on RAW macrophage apoptosis

RAW cells were co-incubated for 24 hours with increasing concentrations of *E. coli* (bacteria-cell ratio indicated) and apoptosis was quantified by Hoechst staining. Error bars indicate the standard error of the mean (SEM) of three independent experiments.

Experiments were also performed using murine bone marrow-derived macrophages, which may be considered more similar to macrophages found *in vivo*. However, as it was not possible to reach sufficiently reproducible results from these cells, and also because of the low amount of primary macrophages gained by this method, it was decided to make use of the Hoxb8 system recently described by Wang et al. that allows the generation of "near-primary" macrophages (Wang et al., 2006).

In this system, the phagocytes are derived from myeloid progenitor cells isolated from the bone marrow of mice that have been immortalised by retroviral transfection with a transgene encoding Hoxb8. This protein belongs to the family of Class I Hox homeodomain transcription factors that promote the expansion of haematopoietic progenitor cells (Thorsteindottir et al., 2002). Upon ectopic expression, Hoxb8 blocks the differentiation of primary granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent

macrophage progenitors, or stem cell factor (SCF)-dependent neutrophil progenitors (Knoepfler et al., 2001). In the system used here, macrophage progenitor cells had been transduced with a version of Hoxb8 that has been fused N-terminally to the oestrogen-binding domain of the oestrogen receptor (Wang et al., 2006). As long as this domain is occupied by oestrogen, Hoxb8 remains in its active state. Thus, in the presence of oestrogen, Hoxb8 progenitors can be cultured and expanded in their undifferentiated state. Upon removal of oestrogen from the culture, Hox8 becomes deactivated, permitting differentiation into macrophages that do not divide further (Figure 7A). By all features tested, these cells functionally behave as primary macrophages (Wang et al., 2006).

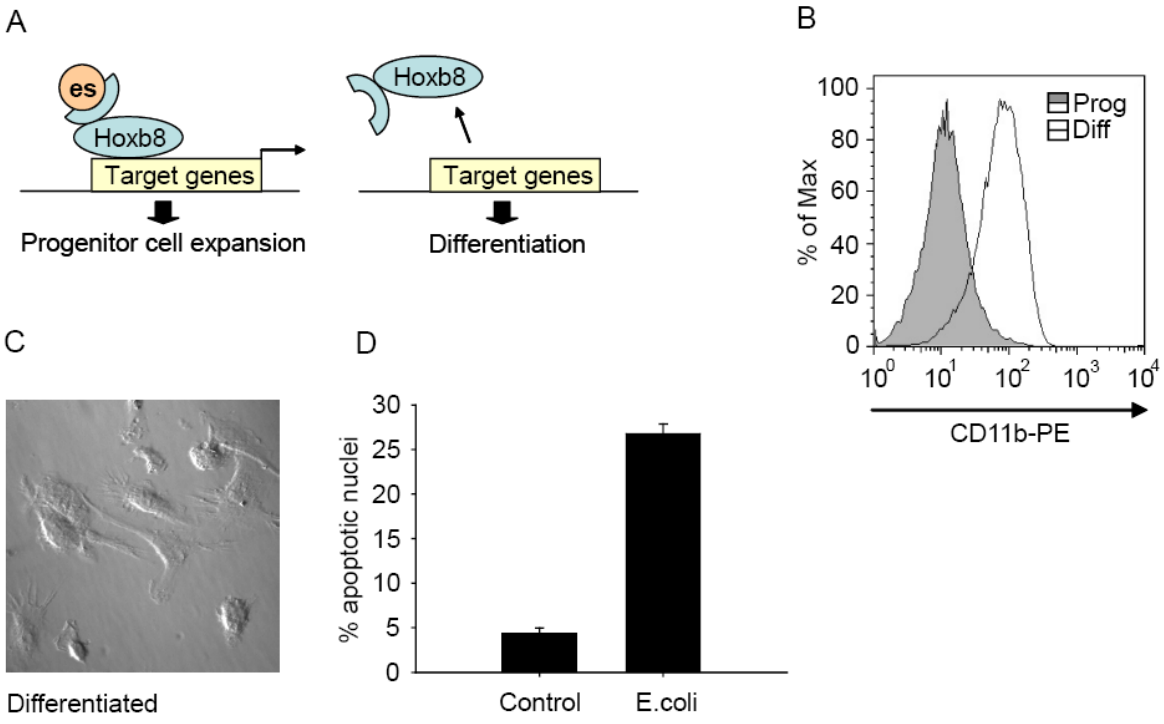


Fig. 7 Hoxb8 macrophage differentiation and apoptosis induction

A. Inactivation of the transcription factor Hoxb8 by oestrogen removal switches off gene transcription, allowing differentiation of progenitor cells into macrophages. **B**. Upregulation of the macrophage surface maturation marker CD11b after 6 days of differentiation. **C**. Appearance of differentiated macrophages in phase-contrast microscopy, note the typical spread-out morphology of the adherent cells. **D**. *E. coli* were co-incubated with macrophages at a ratio of 200:1 for 24 hours, and apoptosis was quantified by Hoechst staining. Error bars represent the SEM of three separate experiments.

A polyclonal line of progenitor cells was established that could be expanded fast, and that differentiated into macrophages within 6 days after oestrogen removal. Differentiated cells showed a clear increase in the expression of the differentiation marker CD11b on the cell surface (Figure 7B), were adherent, and adopted the typical macrophage-like morphology (Figure 7C). Apoptosis could reproducibly be induced in these cells upon phagocytosis of *E. coli*, albeit at a somewhat lower rate than in RAW macrophages (Figure 7D).

3.1.2 Quantitative link between bacterial phagocytosis and macrophage apoptosis

After establishment of the experimental procedures, the first experiments were aimed at analysing the correlation between apoptosis induction and the duration and quantity of the intracellular bacterial burden. In order to determine whether the induction of macrophage apoptosis would require only an initial, or a continuous phagocytic stimulus, *E. coli* were removed from the culture after 3 hours of co-incubation. Cell death was completely abolished and could also not be restored by addition of LPS after bacterial removal (Figure 8A).

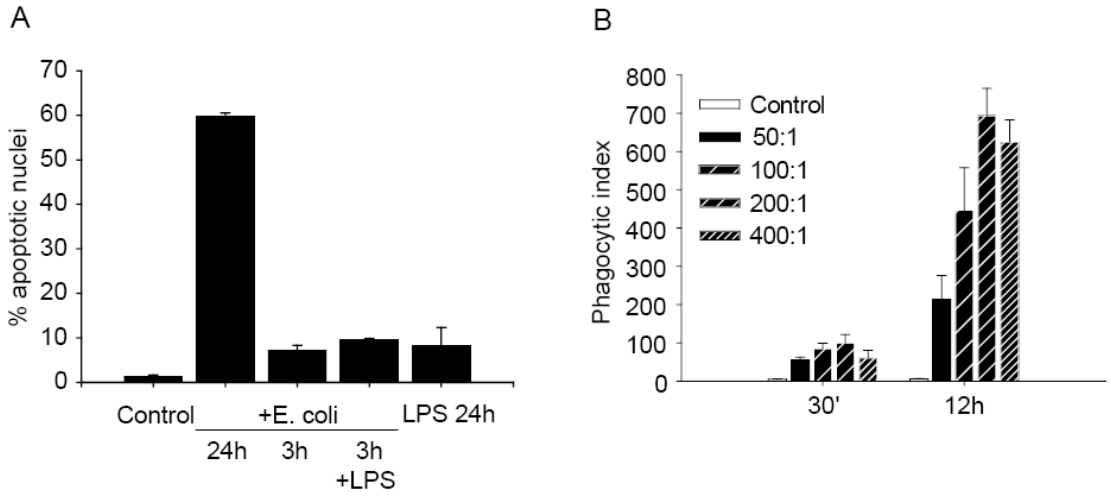


Fig. 8 Macrophage apoptosis requires prolonged bacterial concentration-dependent phagocytosis

A. RAW macrophages were co-incubated with *E. coli* at a bacteria-to-cell ratio of 200:1 or with LPS (1 µg/ml) for 24 hours, or co-culture was interrupted after 3 hours by removing bacteria (and adding LPS). Apoptosis was quantified after 24 hours by Hoechst staining. **B**. Red-fluorescent *E. coli* were co-incubated with RAW macrophages at the indicated ratios, and the bacterial load was quantified by flow cytometry analysis after 30 minutes, and 12 hours, respectively. Phagocytic indices were normalised (see Material and Methods, page 36). Error bars indicate the SEM of three individual experiments.

The removal of *E. coli* after only 12 hours still led to a decrease in the proportion of apoptotic macrophages to about 15-20% (see Figure 16F, page 64). This shows that periods longer than 12 hours of actual phagocytosis are necessary for apoptosis induction.

The titration experiment had already established that macrophage death increases with rising bacterial titres. In order to verify that higher titres also result in higher phagocytic activity, the intracellular bacterial load was measured by incubating macrophages with red-fluorescent *E. coli* for 30 minutes or 12 hours. The proportion of red-fluorescent macrophages (i.e. cells having ingested bacteria) and their mean-fluorescence was measured by flow cytometry, and these two values were multiplied to yield the phagocytic index. This index increased in line with the number of bacteria, until reaching a plateau at a bacteria-to-cell ratio of 200:1, indicating that macrophages come to the limit of their phagocytic capacity at this concentration of bacteria (Figure 8B). A similar maximum had been reached when measuring apoptosis induction in dependency of the bacterial titre (see Figure 6, page 48). It should also be mentioned that at a ratio of 200:1, residual bacteria remained in the culture, whereas at a ratio of 100:1, all bacteria were cleared away. These results indicate that macrophage apoptosis is quantitatively linked to the intracellular bacterial burden, and that RAW macrophages need to phagocytose between 100 and 200 *Escherichia coli* bacteria in the course of at least 12 hours in order to become apoptotic to a large extent.

3.1.3 Overexpression of mutant Rab5a proteins and Rab5a knock-down

Mutant forms of the small GTPase Rab5a have been reported to modulate the fusion of heat-killed *Listeria*-harbouring phagosomes with endosomes (Alvarez-Dominguez et al., 1996), and also to affect the uptake of latex beads (Duclos 2000). Rab5a might therefore also influence the phagocytosis of *Escherichia coli* and its intracellular degradation in phagosomes, both being processes which previous data had suggested to be connected to the induction of apoptosis. Thus, it was hypothesised that Rab5a function may impact on macrophage apoptosis, and to verify this, functional mutants of this protein were introduced into macrophages.

RAW macrophages were transfected with a constitutively active (Rab5CA), or dominant-negative (Rab5DN) version of Rab5a N-terminally tagged with EGFP. Such EGFP-tagged versions of Rab5a have been used previously (Stenmark 1994, Rosenfeld 2001), and the tag has been shown not to interfere with the function of this or other Rab family proteins

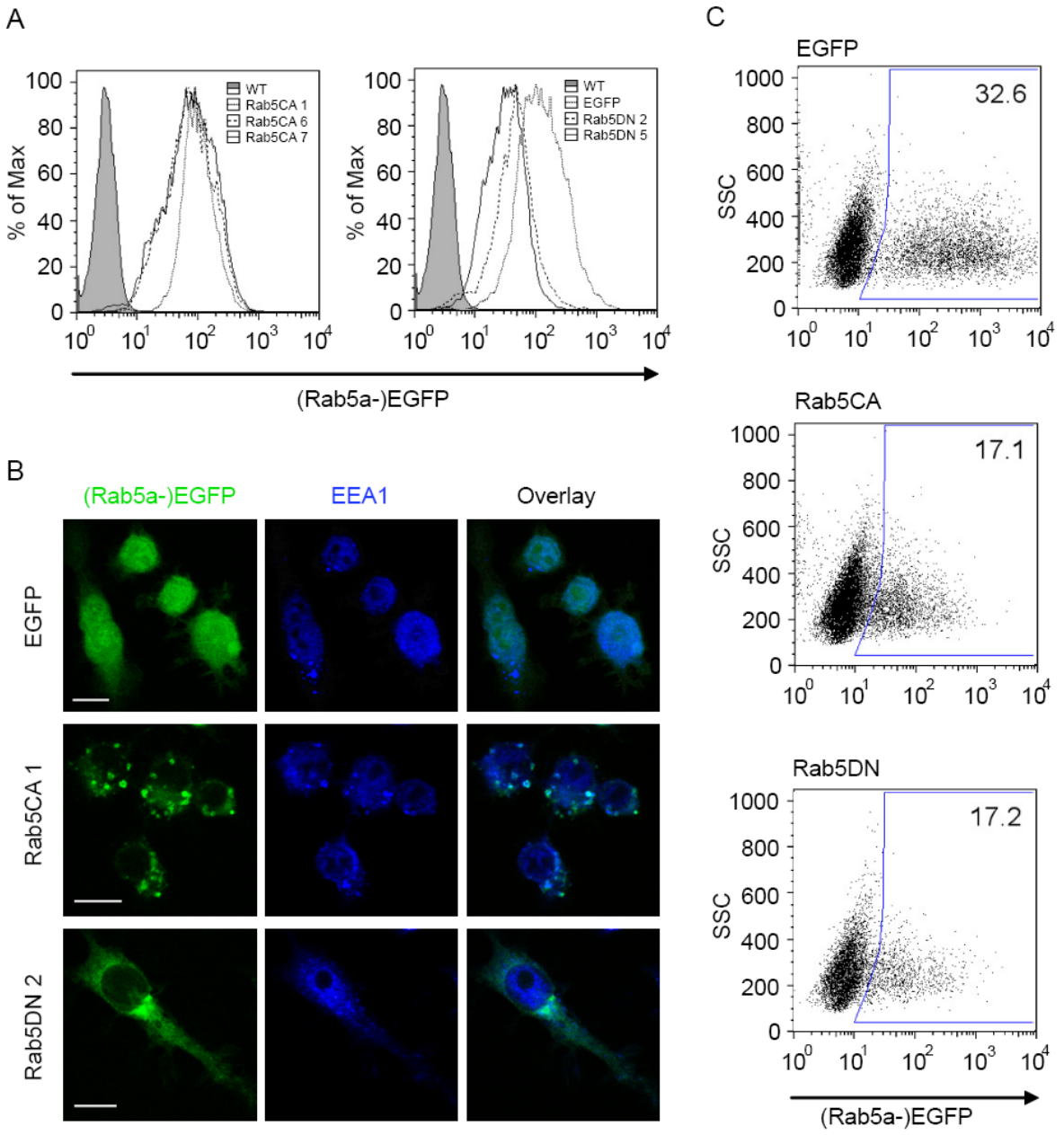


Fig. 9 Expression and localisation of mutant Rab5a proteins in RAW and Hoxb8 macrophages

A. Protein expression levels of EGFP-tagged constitutively active (Rab5CA, three cell lines) (left panel) and dominant negative (Rab5DN, two cell lines) Rab5a, or control EGFP (right panel) in stably transfected RAW macrophages. **B**. RAW macrophages expressing mutant Rab5a-EGFP (green) were stained for the Rab5a effector EEA1 (blue; weak nuclear staining is non-specific), and examined by immunofluorescence microscopy. Similar results were obtained in all three Rab5CA and the two Rab5DN clones. Scale bars indicate 10 µm. **C**. Expression of EGFP, or EGFP-tagged Rab5CA and Rab5DN in Hoxb8 macrophages 24 hours after transient transfection. The percentage of positive cells within the population is indicated. Data are representative of three separate experiments. SSC = side scatter.

(Bucci 2001, Ali 2004). After transfection, stable clones were generated by selection and subcloning, yielding monoclonal lines with a high constitutive expression level (Figure 9A). The functionality of both Rab5a mutants was verified by analysing their co-localisation with the effector protein early endosome antigen 1 (EEA1) by confocal microscopy. EEA1 only binds to the active, GTP-bound form of Rab5a (Simonsen et al., 1998), and thus interacts with Rab5CA but not with Rab5DN. As expected, in all macrophage lines overexpressing Rab5CA, a good co-localisation between the Rab5a protein and EEA1 could be observed (Figure 9B). In contrast, dominant-negative Rab5a did not co-localise with EEA1 but instead displayed a dispersed cytosolic staining pattern with partial concentration in a perinuclear region. Both staining patterns are typical of these two Rab5a mutants and have been reported before (Stenmark et al., 1994, Rosenfeld et al., 2001). The mutant Rab5a proteins were also overexpressed in Hoxb8 macrophages. Like primary macrophages, Hoxb8 macrophages are very difficult to transfect. A transfection kit from Amaxa produced the best results, giving a transfection rate of 15-30% (Figure 9C).

Finally, Rab5a was knocked-down by shRNA in RAW macrophages. In the shRNA approach, a 60-70 bp transcript forming a stem-loop structure through internal complementarity is expressed off a RNA polymerase III promoter. The double-stranded stem comprises 19-29 base pairs, with one strand being identical in sequence to the target mRNA, and both strands being linked via a 9-basepair loop sequence (Paddison et al., 2002). After export into the cytosol, the shRNA is cleaved into 21-basepair duplexes by Dicer, and one strand becomes incorporated into cytoplasmic RNA-induced silencing complexes (RISCs), enabling hybridisation to and degradation of target mRNAs (Scherr and Eder, 2007). Constructs containing shRNA specific for Rab5a, or for Luciferase as control, plus an EGFP marker gene were lentivirally delivered into RAW macrophages. As the infection rate was very low (~2%), macrophage populations were subsequently sorted for EGFP-positive cells by flow cytometry, which produced polyclonal lines that were positive for transgene expression by ~75% (Figure 10A). Lysates of these cells were probed for the level of residual Rab5a, showing that the expression of Rab5a shRNA led to a decrease in Rab5a protein level by about 50% (Figure 10B).

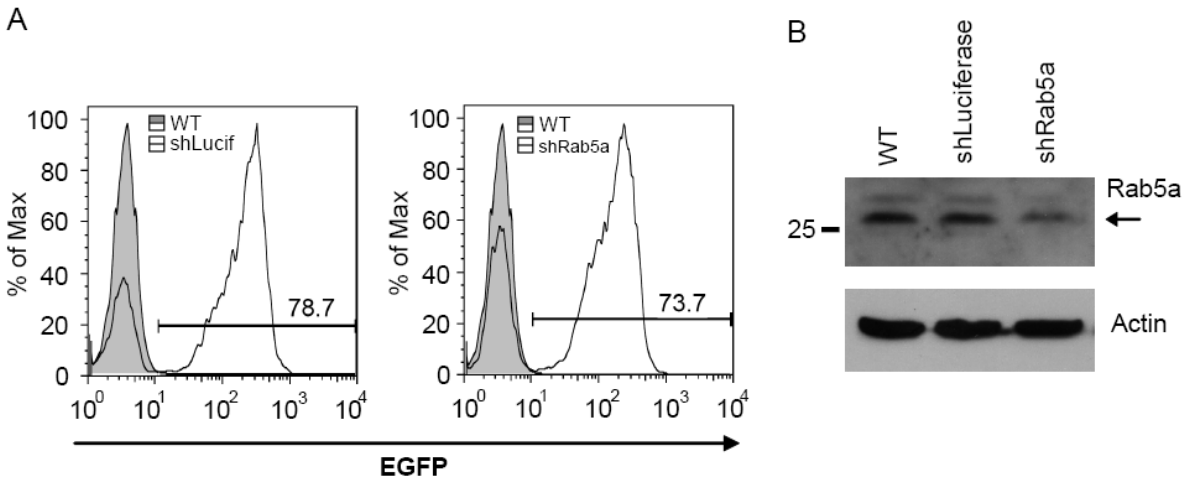


Fig. 10 shRNA-mediated knock-down of Rab5a in RAW macrophages

A. Expression of EGFP (shRNA is expressed off the same promoter) in RAW macrophages stably transfected with constructs encoding shRNA against Rab5a, or *firefly* luciferase (control). The percentage of positive cells within the transfected population is indicated. **B**. Western blot showing endogenous levels of Rab5a in RAW macrophages stably expressing shRNA against luciferase and Rab5a.

3.1.4 Formation of enlarged phagosomes in macrophages overexpressing constitutively active Rab5a

The overexpression of constitutively active Rab5a has also been reported to cause the appearence of considerably enlarged phagosomes in macrophages phagocytosing *Leishmania donovani* (Duclos et al., 2000). When the cellular morphology after uptake of *Escherichia coli* was analysed in the Rab5a macrophage lines, the formation of very large vacuoles could also be observed in all populations overexpressing constitutively active Rab5a, but not in control cells or macrophages expressing Rab5DN. After 24 hours, vacuoles were enlarged to such an extent that they could be clearly observed under a light microscope (Figure 11A). Intriguingly, these giant vesicles were also induced by stimulation of cells with LPS alone, although to a somewhat lower extent (Figure 11B).

In order to identify the enlarged vacuoles formed by phagocytosis of *E. coli* (with free LPS in the bacterial suspension) indeed as phagosomes rather than endosomes, mutant macrophages were incubated with red-fluorescent bacteria. Confocal microscopy confirmed that enlarged vesicles were bounded by Rab5CA-EGFP and had indeed accumulated a large

number of bacteria (Figure 11A, bottom row). Phagosomes in control cells or macrophages overexpressing Rab5DN, by contrast, contained many individual phagosomes harbouring many fewer bacteria. This indicates that the enhancement of Rab5a activity stimulates homotypic endosome-endosome fusion, and upon phagocytosis of *E. coli*. also phagosome-endosome and phagosome-phagosome fusion.

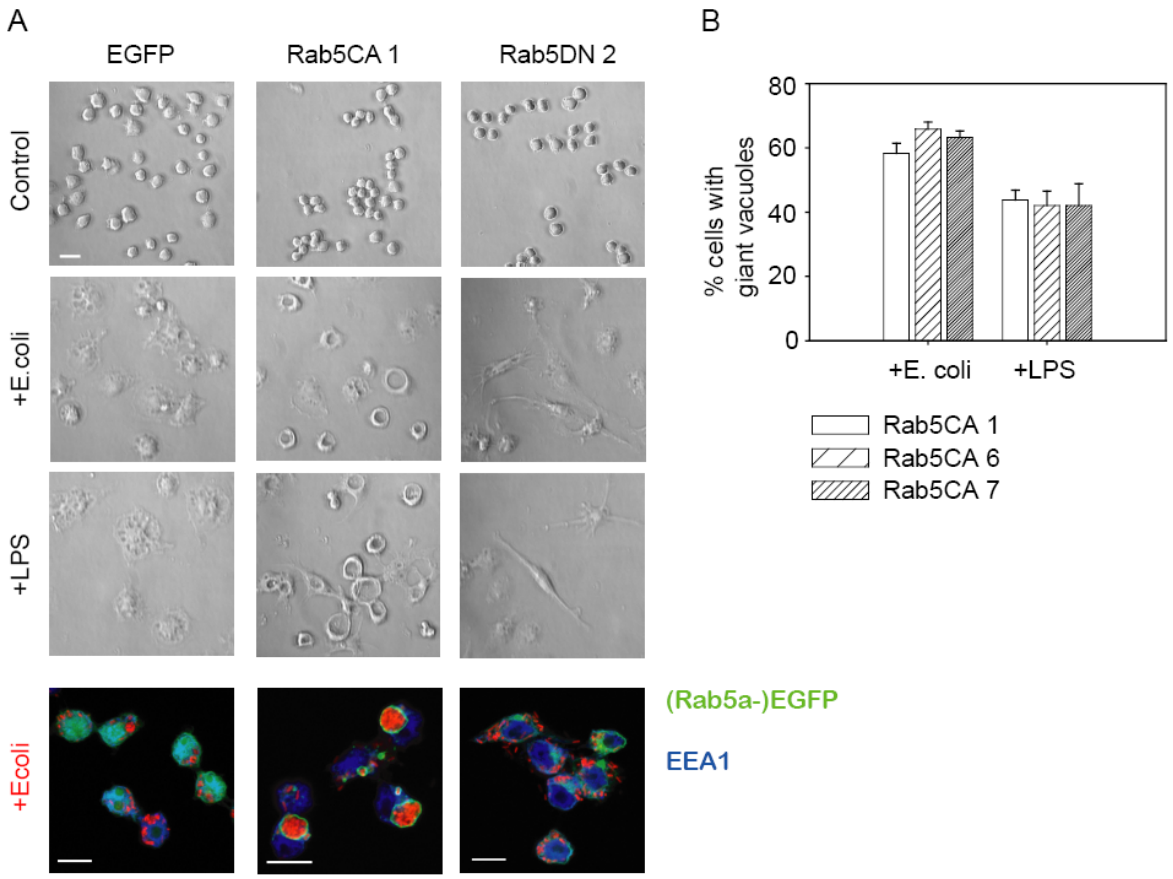


Fig. 11 Induction of giant vacuoles in macrophages overexpressing Rab5CA

A. RAW macrophages expressing mutant Rab5a-EGFP were stimulated with *E. coli* at a bacteria-to-cell ratio of 50:1, or with LPS (1 µg/ml) for 24 hours, and examined by phase-contrast microscopy (top panel). Mutant RAW macrophages were co-incubated with red-fluorescent *E. coli* at a bacteria-to-cell ratio of 200:1 for 6 hours and stained for the Rab5a effector EEA1 (blue) (bottom row). Scale bars indicate 10 µm. Data are representative of three independent experiments **B**. Quantification of giant vacuoles in mutant RAW macrophages stimulated with bacteria or LPS as in (A) for 24 hours. Error bars indicate the SEM of three individual experiments.

3.1.5 Stimulation of phagocytosis by overexpression of mutant Rab5a or by Rab5a knockdown

The capacity for bacterial phagocytosis was measured in all mutant macrophages by incubation with red-fluorescent (or otherwise marked) *Escherichia coli* for 30 minutes, followed by flow cytometry analysis. In RAW cells expressing constitutively active Rab5a, the phagocytic index was elevated 2.4 - 3.6 fold compared to the control lines (Figure 12A). A similar Rab5CA-induced increase has also been observed for the uptake of latex beads (Duclos et al., 2000). Surprisingly, the expression of dominant-negative Rab5a led to a similar stimulation of phagocytosis, with a 2.6 – 3.6 fold increase of the phagocytic index (Figure 12A).

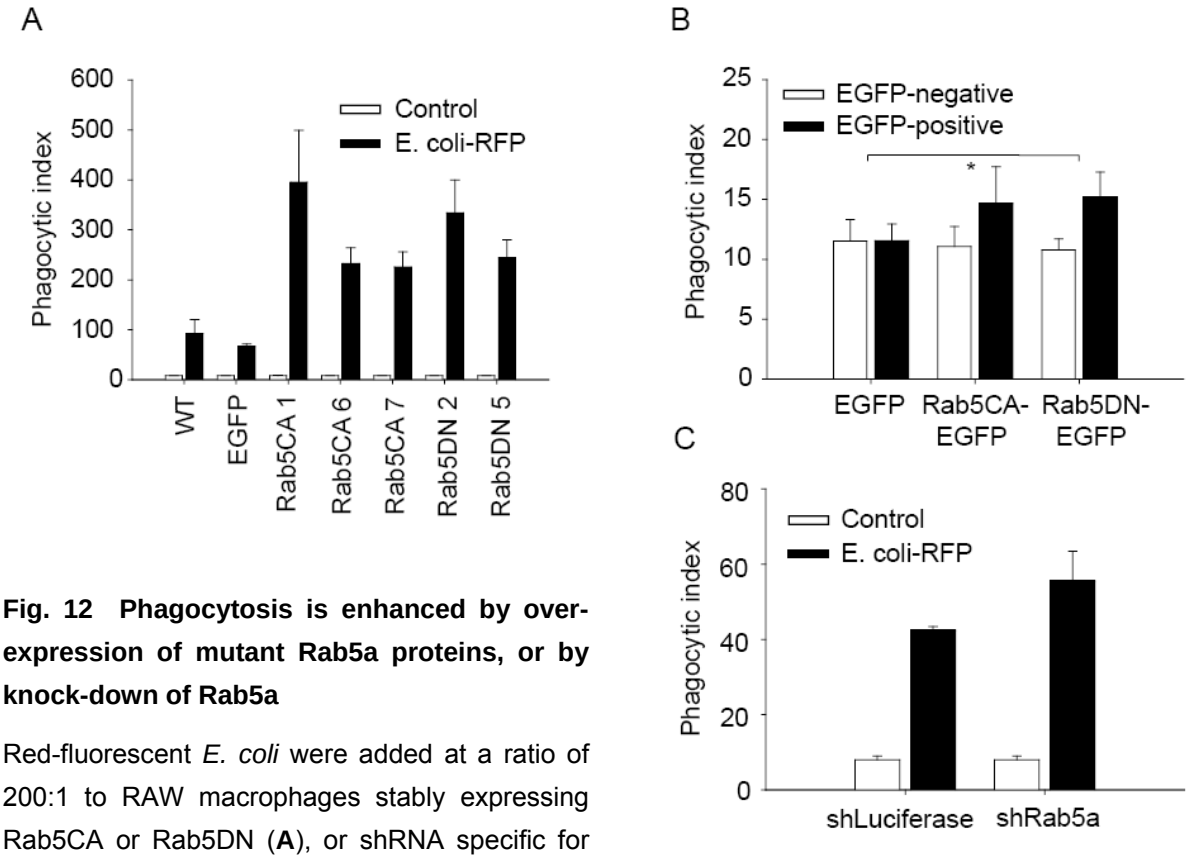


Fig. 12 Phagocytosis is enhanced by over-expression of mutant Rab5a proteins, or by knock-down of Rab5a

Red-fluorescent *E. coli* were added at a ratio of 200:1 to RAW macrophages stably expressing Rab5CA or Rab5DN (**A**), or shRNA specific for luciferase or Rab5a (**C**). Phagocytosis was quantified after 30 minutes by flow cytometry analysis. Error bars indicate the SEM of four (A) or three (C) independent experiments. **B**. Hoxb8 macrophages transiently transfected with constructs for EGFP, Rab5CA-EGFP, or Rab5DN-EGFP were co-incubated with bacteria as described in (A) for 4 hours, and then stained with an anti-serum against *E. coli*. Transfection efficiency was ~20%; the complete cell population was analysed and phagocytic indices were established for Rab5a-EGFP-negative and positive macrophages within the same sample. Error bars indicate the SEM of three separate experiments. *p=0.050, using Mann-Whitney Test. Phagocytic indices were normalised (see Material and Methods, page 36).

Bacterial uptake was also analysed in Hoxb8 macrophages 24 hours after transient transfection with EGFP, Rab5CA, or Rab5DN. Here, the phagocytic capacity was measured by staining intracellular bacteria that had been ingested over a period of 4 hours with an antibody-coupled blue fluorescence. Red-fluorescent bacteria could not be used because of a broader range of interfering Rab5a-EGFP induced green fluorescence in these cells (see Materials and Methods, page 37 for a more detailed explanation). Because only about 20% of cells were positive for the expression of mutant Rab5a (measurable by EGFP-positivity, see Figure 9C, page 52), it was possible to directly compare Rab5a-EGFP-positive cells with Rab5a-EGFP-negative cells within the same sample. Although the relative increase in phagocytic activity brought about by the transient expression of mutant Rab5a was much lower in Hoxb8 macrophages than in the mutant RAW macrophage lines, and only reached statistical significance for the Rab5DN transfectants, the tendency towards a stimulation of phagocytosis was clear (Figure 12B).

When phagocytosis was analysed in RAW macrophages expressing knock-down shRNA against Rab5a, a small increase in bacterial uptake could be seen, compared to control cells expressing shRNA against Luciferase (Figure 12C). The phagocytic index overall was reduced by about 40% when compared to that obtained with WT macrophages or the EGFP line; this effect may be due to the presence of dsRNA in the cytosol of these cells and subsequent cellular activation events.

These data show that both the overexpression of constitutively active Rab5a, and the expression of dominant-negative Rab5a, as well as a reduction in Rab5a protein levels, causes a stimulation in phagocytic capacity. The reason for these findings is unclear, one possible explanation is that of an indirect effect on pathways controlling phagocytosis, such as an altered sequestration pattern of regulatory proteins by the Rab5a mutants, and/or in the case of the Rab5a knockdown a quantitatively changed interaction.

3.1.6 Enhanced phagocytosis is associated with increased macrophage apoptosis

In the next set of experiments, the mutant macrophages were analysed for their sensitivity towards apoptosis induction following uptake of *Escherichia coli*. Bacteria and RAW macrophages were co-incubated at a ratio of 200:1, and the proportion of apoptotic nuclei was scored after 24 hours. Macrophage death increased from ~55% in the controls to ~85% in

macrophages overexpressing Rab5CA, and to ~80% in the Rab5DN lines (Figure 13A). No differences were observable in terms of cellular and nuclear morphology in dying mutant cells, and the death increase in the mutant macrophages was inhibitable by the broad caspase inhibitor zVAD to the same degree as in wt cells (Figure 13B), indicating apoptosis and not some other form of cell death. This strong elevation in apoptosis was specific to phagocytosis-associated events, as apoptosis decreased with lower bacterial titres (Figure 13C) and cells responded normally to other apoptotic stimuli such as UV irradiation or staurosporine treatment (Figure 13D).

In Hoxb8 macrophages, cell death analysis was very difficult to perform because of the stress exterted on cells during transient transfections, the low survival and transfection rate, and the leakage of Rab5a-EGFP from the transgene-positive population in the later stages of apoptosis. The best results were obtained by measuring the loss of cell membrane integrity as parameter for cell death. Macrophages were stained with propidium iodide (PI), a non-cell permeable dye that intercalates in dsDNA (when entering the cell and nucleus through holes in the membranes), immediately prior and 14 hours after co-incubation with bacteria, and were subjected to flow cytometry analysis. The increase in PI-positivity was calculated by dividing the proportion of PI-positive cells in the total cell population at 14h by that at 0h (Figure 13E). The experimental data did not show significant differences, but the small tendendy towards cell death enhancement by overexpression of Rab5CA or Rab5DN pointed in the same direction as the results obtained with RAW macrophages.

When phagocytosis-induced apoptosis was analysed (by Hoechst staining) in RAW macrophages expressing shRNA against Rab5a, a minor increase in macrophage death was seen, compared to the control line expressing shRNA against Luciferase (Figure 13F). No differences were observed concerning the sensitivity to staurosporine. The overall extent of apoptosis induction was reduced to 20-30%, but this was also true for the level of apoptosis induced by staurosporine, pointing towards a non-specific effect that may be related to cellular activation induced by the presence of cytosolic dsRNA.

In macrophages that overexpress mutant Rab5a, or have reduced levels of Rab5a, the stimulation in phagocytosis thus correlates with an increase in apoptosis. Conversely, a reduction in the bacterial titre reduces macrophage death. This quantitative relationship had also been observed in the bacterial titration experiments conducted with WT cells. However, in the mutant macrophages the contribution of an additional effect of altered Rab5a function on apoptosis – such as changes in bacterial killing and breakdown – cannot be excluded from

this data. Thus, microbicidal functions in the mutant cell lines were analysed in the following experiments.

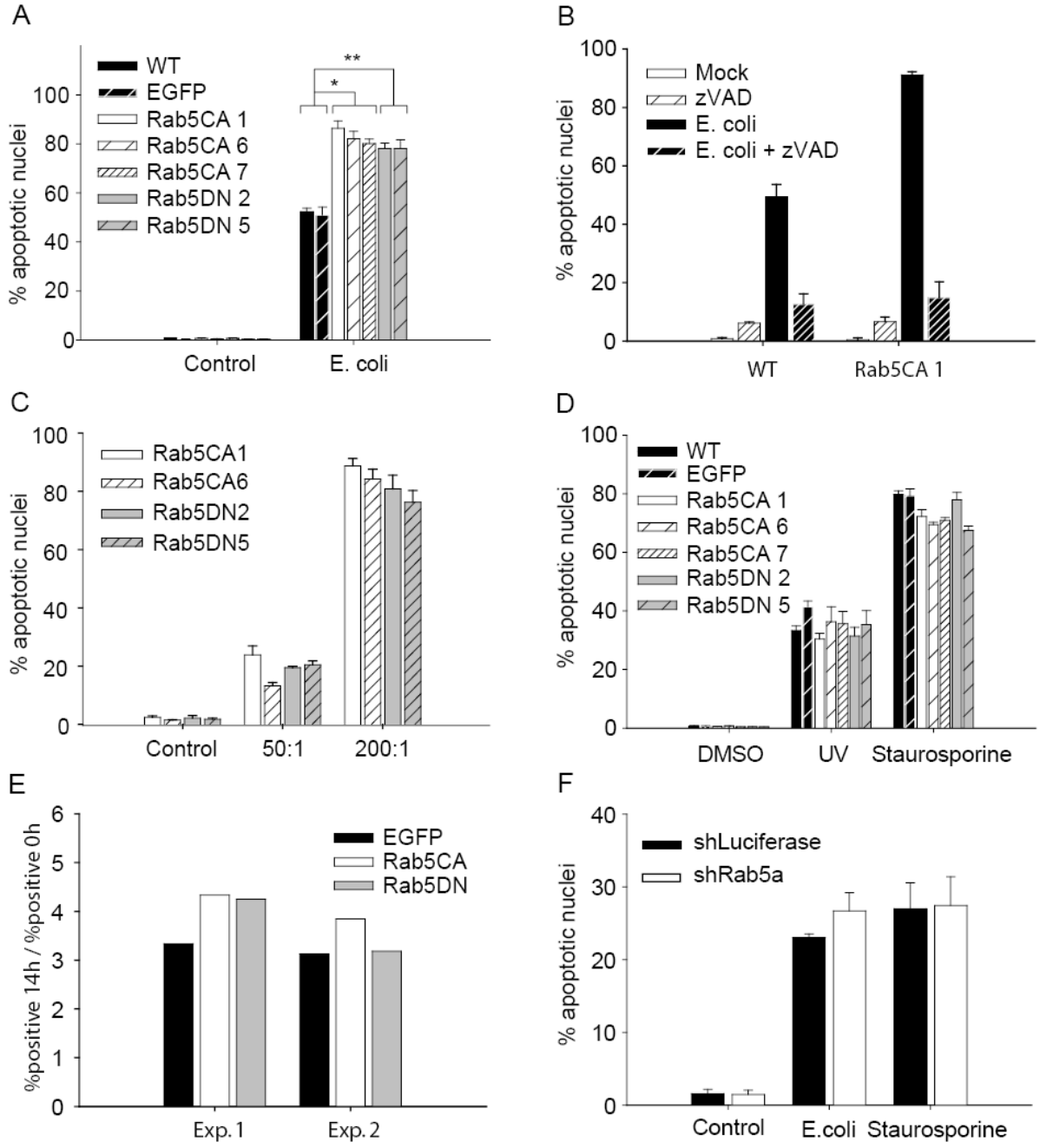


Fig. 13 Overexpression of Rab5CA or Rab5DN increases macrophage apoptosis induced by bacterial uptake

A-C. *E. coli* were co-incubated with RAW macrophages at a ratio of 200:1 (A+B) or additionally 50:1 (C), and apoptosis was quantified by Hoechst staining after 24 hours. zVAD was included in the culture at 50 µM were indicated. */**p<0.001, using Student's *t* test. **D**. RAW cells were irradiated with UV at 200 mJ/cm^2, or incubated with staurosporine at 1 µg/ml, and apoptosis was quantified by

Hoechst staining after 24, and 16 hours, respectively. **E**. Hoxb8 macrophages transiently transfected with EGFP, Rab5CA or Rab5DN were incubated with *E. coli* at a bacteria-to-cell ratio of 200:1, and apoptosis was quantified by Hoechst staining after 24 hours. Two separate experiments are shown. **F**. RAW cells expressing shRNA specific for luciferase or Rab5 were incubated with *E. coli* (bacteria-to-cell ratio 200:1) or treated with staurosporine (1 µg/ml), and apoptosis was quantified by Hoechst staining after 24, and 16 hours, respectively. Error bars represent the SEM of three independent experiments (except (B), standart deviation of two experiments).

3.1.7 Bafilomycin A_1 but not mutant Rab5a inhibits bacterial killing and degradation

Directly after phagocytosis, the nascent phagosome matures to gain microbicidal properties, and in this process also acquires vacuolar-type ATPases that mediate lumen acidification (Lukacs et al., 1990; Pitt et al., 1992). Phagosomal acidification in macrophages can be blocked experimentally by use of bafilomycin A_1 (Lukacs et al., 1990), an antibiotic reactive against gram-positive bacteria that has been isolated from *Streptomyces* spp. (Werner et al., 1984; Bowman et al., 1988). At nanomolar concentrations, bafilomycin A_1 specifically inhibits vacuolar-type ATPases, but does not affect the function of animal mitochondrial or plasma membrane ATPases, or of membrane ATPases from *Escherichia coli* (Bowman et al., 1988). In alveolar macrophages, treatment with this antibiotic has been shown to reduce intracellular killing of *Staphylococcus aureus* (Bidani et al., 2000). Treatment of WT macrophages with bafilomycin A_1 thus served as a "benchmark" against which putative inhibitory effects of mutant Rab5a or Rab5a knockdown on antimicrobial activity could be compared.

Bacterial killing was analysed by co-incubating *E. coli* and RAW macrophages pre-treated with bafilomycin A_1 or DMSO for 30 minutes, followed by centrifugation of cells through a sucrose cushion in order to remove residual extracellular bacteria (which become trapped at the interface). Half of the samples were directly lysed, while the other half was incubated for another 2 hours before lysis (in the presence of gentamycin to inhibit growth of any residual extracellular bacteria). Lysates were plated on LB plates, and formed bacterial colonies counted the next day. The comparison between the number of colonies directly after phagocytosis and after 2 hours of intracellular killing allows the calculation of the proportion of surviving bacteria. In these experiments a very low bacterial titre (5:1) was chosen for two reasons; large amounts of *E. coli* cannot be completely removed after co-incubation and

residual bacteria would thus confound results. Second, this bacteria-to-cell ratio is low enough to make it a limiting factor, i.e. during the 30 minute co-incubation period, the mutant Rab5a macrophages with their enhanced phagocytic capacity do not take up significantly more bacteria than the WT macrophages. In WT or EGFP-expressing macrophages, about 30% of *E. coli* survived intracellular killing (Figure 14). In macrophages treated with bafilomycin A_1, survival was increased to 70%, while the overexpressing of Rab5CA or Rab5DN had no effect.

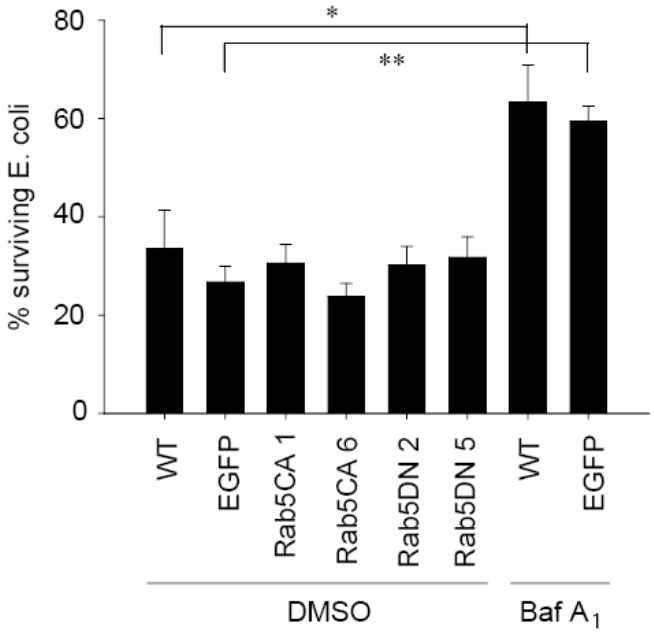


Fig. 14 Bafiloymcin A_1 but not overepxression of mutant Rab5a proteins reduces intracellular bacterial killing

RAW macrophages were pre-treated with 25 nM bafilomycin A_1 or DMSO for 30 minutes. *E. coli* were added to cells at a ratio of 5:1, and non-ingested bacteria were removed after a phagocytosis period of 30 minutes by centrifugation through a sucrose cushion. Cells were either lysed directly, or after an incubation period of 2 hours (in the presence of 50 µg/ml gentamycin). Lysates were plated on LB plates, and the number of colony forming units was scored after 16 hours. Error bars show the SEM of three separate experiments. *p=0.0518, **p=0.0019, using Student's *t* test.

For the analysis of bacterial digestion, macrophages were pre-treated with DMSO or bafilomycin A_1 for 30 minutes and co-incubated with *E. coli*. After 30 minutes of phagocytosis, bacteria were removed as described above, and half of the samples lysed immediately, or after 8 hours of incubation. Western blots of the lysates were then probed with an antibody against OmpA, an abundant bacterial pore protein that is present in the outer membrane (Chai and Foulds, 1977). OmpA is synthesised as a precursor that is processed upon crossing of the inner cytoplasmic membrane, and in SDS-PAGE therefore appears as a double band (Henning et al., 1979; Zimmermann and Wickner, 1983). The loss of OmpA signal over time was taken as marker for intracellular bacterial breakdown.

At a bacteria-to-cell ratio of 10:1, a stronger OmpA signal was present in all mutant Rab5a clones directly after phagocytosis, compared to control macrophages (Figure 15A), confirming the stimulation in bacterial uptake induced by mutant Rab5a.

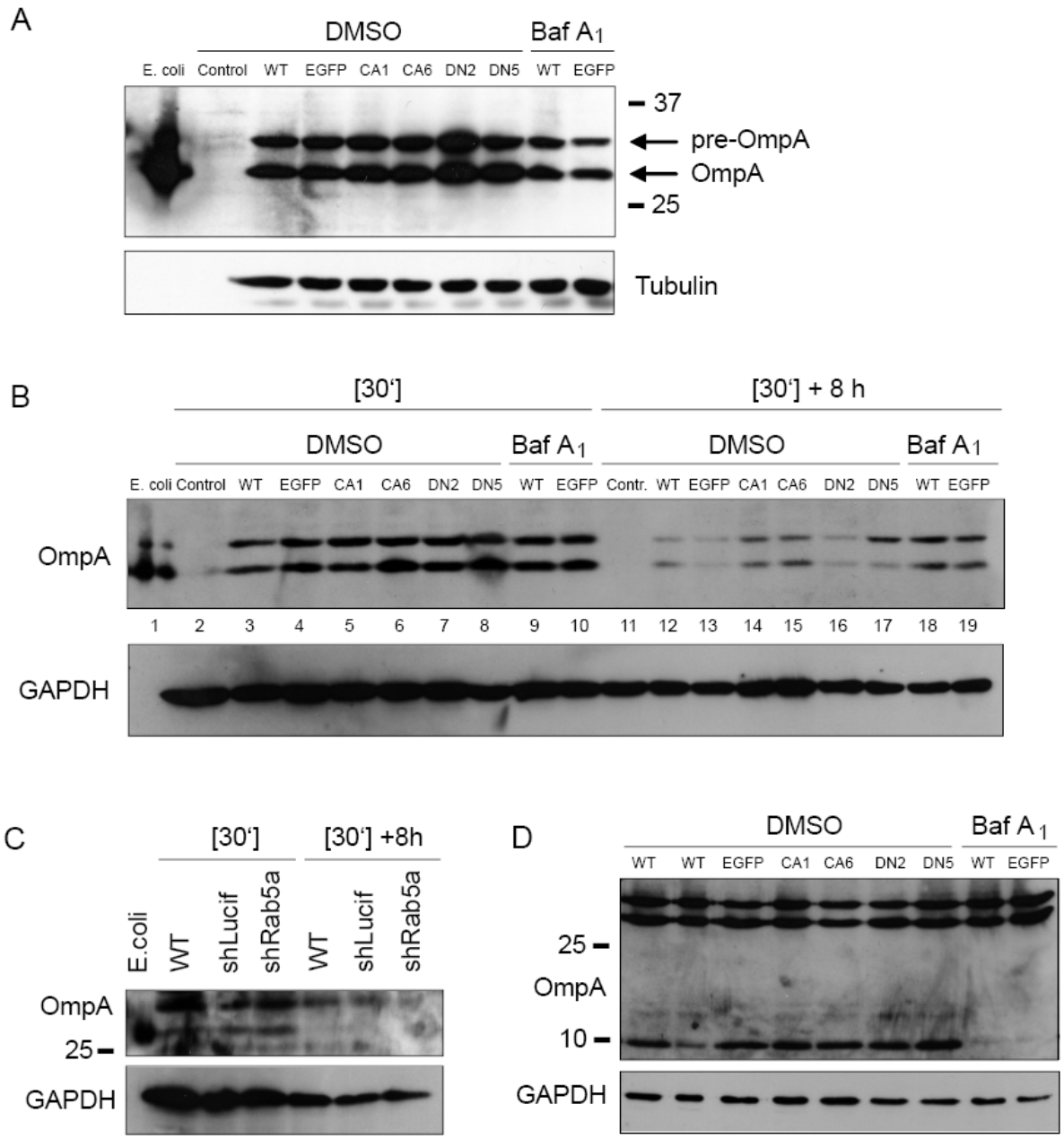


Fig. 15 Bafilomycin A_1 but not mutant Rab5a or Rab5a knockdown inhibits bacterial digestion

A. RAW macrophages were pre-treated with 25 nM bafilomycin A_1 or DMSO for 30 minutes. *E. coli* were added to cells at a ratio of 10:1, and non-ingested bacteria were removed after a phagocytosis period of 30 minutes by centrifugation through a sucrose cushion. Cells were lysed and extracts were probed with an antibody against the bacterial outer membrane protein OmpA. **B**. RAW macrophages

pre-treated as in (A) were co-incubated at a bacteria-to-cell ratio of 10:1 (WT and EGFP), 8:1 (Rab5CA6 and Rab5DN5), or 6:1 (Rab5CA1 and Rab5DN2), and after removal of bacteria cells were either lysed directly or after an incubation period of 8 hours. Lysates were probed for OmpA protein. **C.** *E. coli* and RAW cells expressing shRNA specific for luciferase or Rab5 were incubated at a ratio of 10:1, and samples were processed as in (A). **D**. *E. coli* and RAW cells were co-incubated at a ratio of 200:1 (except lane 2, 400:1) for 12 hours, non-ingested bacteria were washed away, and extracts were prepared and analysed as in (A). All data are representative of two (or three (B)) individual experiments.

When bacteria-to-cell ratios were adjusted between cell lines, i.e. lowered to 8:1 or 6:1 for the Rab5a clones, a similar initial bacterial load was obtained (Figure 15B, lanes 3-10). In WT and EGFP-expressing macrophages, the comparison of the signal obtained directly after uptake (lanes 3 and 4) with that after a digestion period of 8 hours (lanes 12 and 13) showed a clear bacterial breakdown. Treatment with bafilomycin A_1 significantly reduced bacterial digestion (compare lanes 12/13 with 18/19), but macrophages expressing the mutant Rab5a proteins (lanes 5-8 and 14-17) displayed a far less clear reduction in bacterial breakdown, or no reduction at all. In RAW macrophages expressing shRNA against Rab5a, bacterial digestion appeared to be enhanced to a very minor degree (Figure 15C).

Bacterial breakdown was also analysed in an experimental setup that more closely resembles the conditions used to induce macrophage apoptosis. Instead of "pulsing" macrophages with *E. coli*, cells and bacteria were co-incubated at a ratio of 200:1 for 12 hours, and lysates were prepared after removal of extracellular bacteria. In this "steady-state" conditions of bacterial phagocytosis and breakdown, in WT and control macrophages an OmpA band appeared around ~10kD, which must represent an intermediate breakdown product of this protein (Figure 15D). This band, however, was not present in macrophages treated with bafilomycin A_1, indicating inhibition of protein cleavage. No such inhibition was observed in the mutant Rab5a clones.

Taken together, these results demonstrate that a bafilomycin A_1-induced inhibition of phagosome acidification inhibits intracellular bacterial killing and breakdown in macrophages. By contrast, microbicidal or digestive capacities are not significantly affected by the expression of mutant Rab5a proteins or by Rab5a knockdown. This data makes it very likely that the increased apoptosis seen in macrophages overexpressing mutant Rab5a proteins can be attributed to the higher phagocytic capacity of these cells.

3.1.8 Phagocytosis-induced apoptosis is inhibited by bafilomycin A_1

Having established that bactericidal activity in macrophages can be effectively inhibited by treatment with bafilomycin A_1, this approach was used in the following to analyse the connection between bacterial killing and degradation, and macrophage apoptosis. WT and EGFP-expressing RAW macrophages were pre-treated with DMSO or the drug for 30 minutes, and co-incubated with *Escherichia coli* for 24 hours (ratio 200:1). Bafilomycin A_1 reduced cell death from ~60 to 30% (Figure 16A), but did not inhibit staurosporine-induced apoptosis (Figure 16B). In Hoxb8 macrophages, bafilomycin A_1 reduced apoptosis to a similar extent, from ~30 to 15% (Figure 16C). Macrophage death could also be decreased significantly by treatment with ammonium chloride (Figure 16D), a lysosomotropic amine that has been shown to inhibit phagosome-lysosome fusion (Hart and Young, 1991).

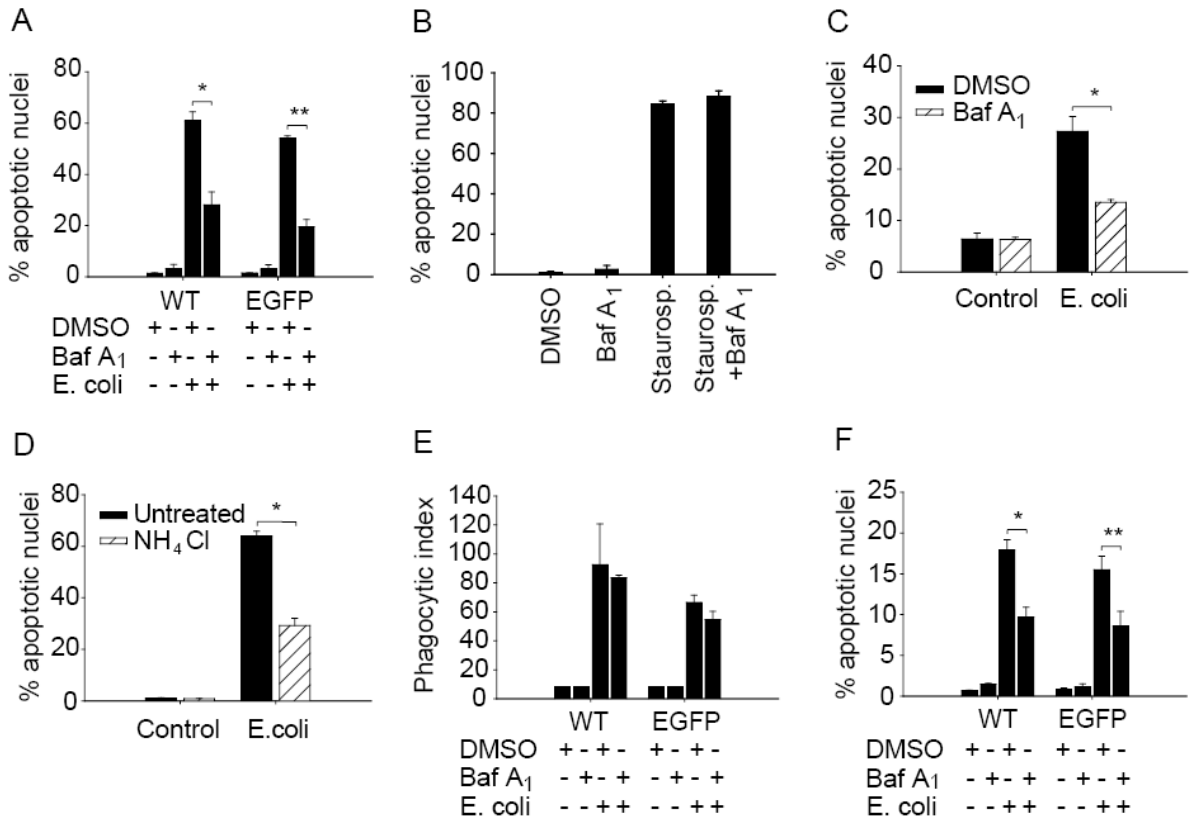


Fig. 16 Effects of bafilomycin A_1 on macrophage phagocytosis and apoptosis upon uptake of bacteria

A. RAW macrophages were pre-treated with 25 nM bafilomycin A_1 or DMSO for 30 minutes. *E. coli* were added at a ratio of 200:1, and samples were co-incubated for 24 hours. *p=0.0084, **p=0.0031. **B**. RAW macrophages pre-treated with bafilomycin A_1 as in (A) or left untreated were incubated with staurosporine for 16 hours. **C**. RAW macrophages were pre-treated with 25 mM ammonium chloride

(NH_4Cl) for 30 minutes, and co-cultured with bacteria as in (A). *p=0.001. **D**. Hoxb8 macrophages were pre-treated with 5 nM bafilomycin A_1 or DMSO for 30 minutes, and co-cultured with bacteria as in (A). *p=0.0376. **E**. RAW cells were pre-treated with 25 nM bafilomycin A_1 or DMSO, and red-fluorescent *E. coli* were added to cells at a ratio of 200:1. After 30 minutes co-incubation, samples were subjected to flow cytometry analysis. Phagocytic indices were normalised (see Material and Methods, page 36). **F**. *E. coli* and RAW cells were co-incubated at a ratio of 200:1 for 12 hours, cells were washed to remove non-ingested bacteria and incubated for further 12 hours in the presence of 25 nM bafilomycin A_1 or DMSO, followed by Hoechst staining. *p=0.0097, **p=0.0489. All apoptosis was quantified by Hoechst staining, all error bars represent the SEM of three independent experiments. All statistical analysis was performed using Student's *t* test.

The analysis of macrophage phagocytosis using red-fluorescent *E. coli* revealed that bafilomycin A_1 treatment reduced bacterial uptake to a minor degree during 30 minutes of co-incubation (Fig. 16E). Because of the quantitative relationship between phagocytosis and apoptosis induction, it was conceivable that this small decrease might produce significant effects at later time points. As a control, macrophage death was also measured when bafilomycin A_1 treatment was started after cells had been co-incubated with bacteria for 12 hours, and after residual bacteria had been removed. In this way, the drug cannot inhibit phagocytosis but only affect the killing and degradation of already internalised bacteria. Using these conditions, a similar reduction in macrophage apoptosis was observed (Figure 16F). The overall apoptotic rate was lower because of the early removal of bacteria. These results show that an inhibition of phagosomal acidification resulting in reduced bacterial killing and degradation protects macrophages from apoptosis induction.

The reason for this protective effect on the molecular level is not clear. Macrophage activation by LPS exposure or bacterial phagocytosis in the presence of IFN-γ or TNF-α leads to the induction of nitric oxide synthase (Cunha et al., 1993), and nitric oxide (NO)

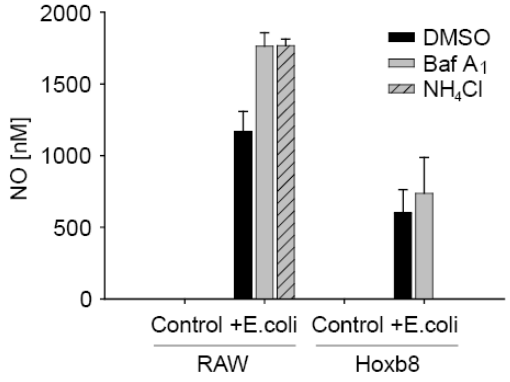


Fig. 17 Production of nitric oxide by RAW and Hoxb8 macrophages during bacterial phagocytosis

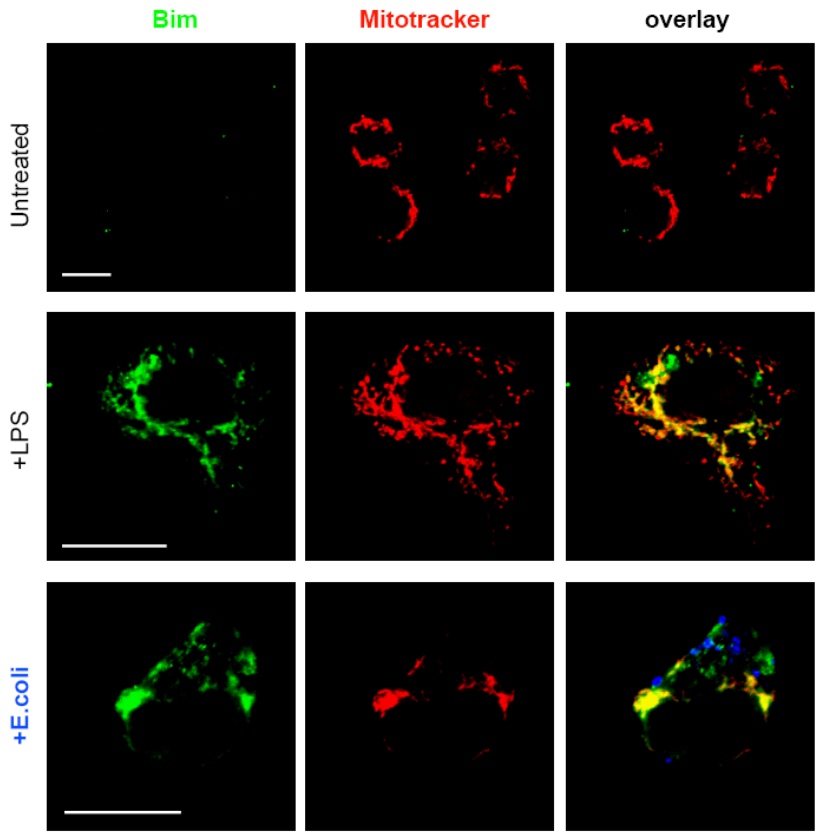
RAW or Hoxb8 macrophages were pre-treated with DMSO, bafilomycin A_1 (25 nM, RAW; 5 nM, Hoxb8) or ammonium chloride (25 mM) for 30 minutes, and incubated with *E. coli* at a bacteria-to-cell ratio of 200:1 for 20 hours. Supernatants were analysed for nitric oxide content. Error bars indicate the SEM of three separate experiments.

production has been reported to be associated with apoptosis induction (Albina et al., 1993). NO production was therefore measured in the supernatant of RAW and Hoxb8 macrophages that were pre-treated with DMSO, bafilomycin A_1 or ammonium chloride and then co-incubated with *E. coli* for 20 hours. Neither bafilomycin A_1 nor ammonium chloride inhibited the production of nitric oxide (Figure 17), making it unlikely that this compound plays an important role in macrophage death.

3.1.9 Bim accumulates at mitochondria during phagocytosis-induced cell death

Previous work had demonstrated that the pro-apoptotic BH3-only protein Bim is involved in macrophage apoptosis induced by phagocytosis of *Escherichia coli* (Kirschnek et al., 2005). The exposure to bacteria or a stimulation with LPS induced a Myd88-dependent upregulation of the two long isoforms of Bim, BimL and BimEL, and an accumulation of the protein in the low molecular weight fraction that includes mitochondria but also other organelles.

Fig. 18 Macrophage Bim upregulated by stimulation with LPS or bacteria accumulates at mitochondria



RAW macrophages were stimulated for 24 hours with LPS (1 µg/ml), or with *E. coli* at a bacteria-to-cell ratio of 200:1. Cells were then stained with mitotracker to identify mitochondria (red), an antibody against Bim (green) recognising all isoforms of the protein, and an antiserum against *E. coli* (blue), and examined by immunofluorescence microscopy. Note that Bim levels in untreated cells are below the detection limit. Scale bars indicate 10 µm. Data are representative of two individual experiments.

To verify whether induced Bim specifically translocates to mitochondria, RAW macrophages were co-incubated with bacteria or treated with LPS for 24 hours, stained for Bim and mitochondria and examined by confocal microscopy. In unstimulated cells, Bim could not be visualised due to an expression level below the detection limit (Figure 18). Bim protein upregulated by exposure to *E. coli* or LPS however was readily detectable, and co-localised extensively with mitochondria.

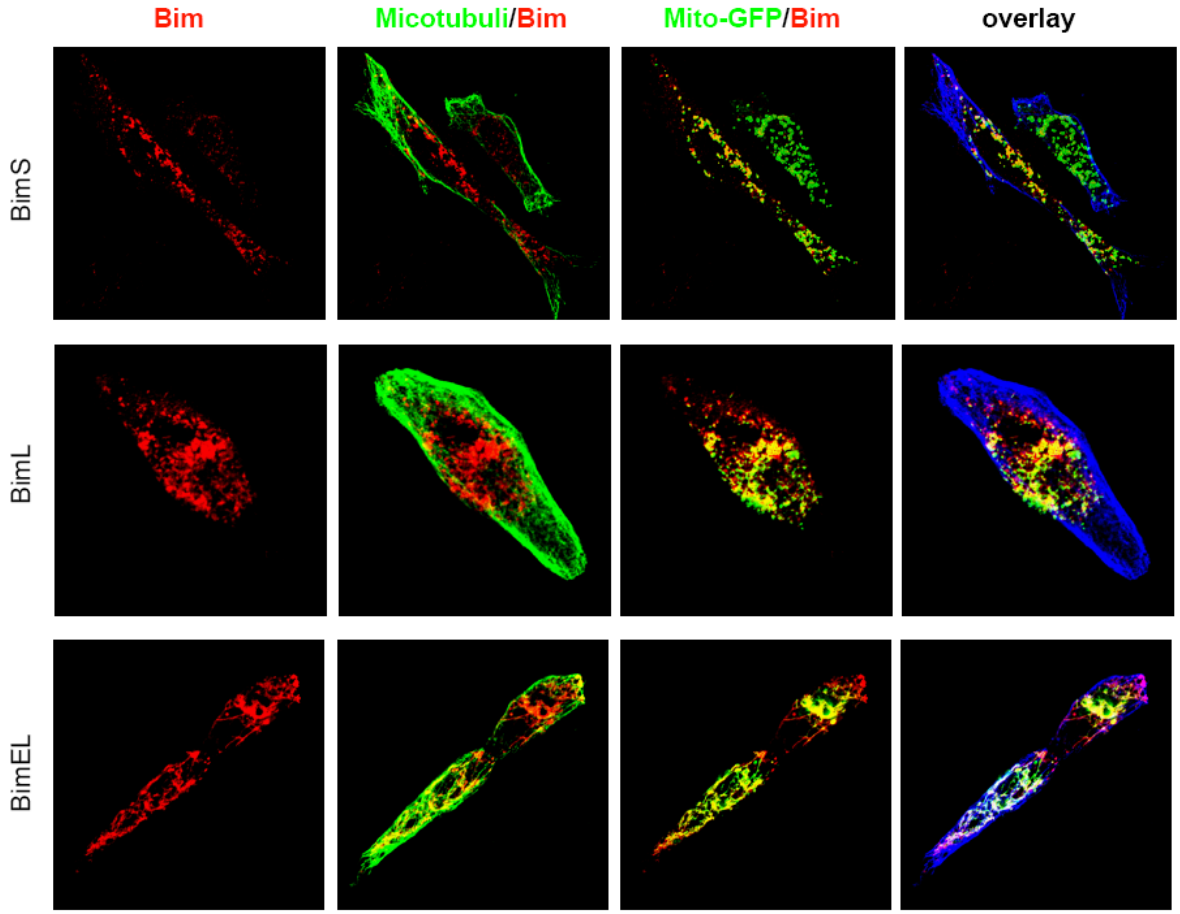


Fig. 19 Localisation of overexpressed isoforms of BimS, BimL, and BimEL in HeLa cells

HeLa-TRex clones that stably express the tetracycline repressor plus a tetracyclin-inducible vector coding for BimS, BimL, or BimEL were transiently transfected with GFP localising specifically to mitochondria (Mito-GFP, green), and after 24 hours treated with tetracyclin for 6 hours to induce Bim expression. Cells were stained with an antibody specific for Bim (red) and microtubules (green/blue), and examined by immunofluorescence microscopy. Note that the levels of endogenous Bim in uninduced (and therefore also induced) cells were below the detection limit. Data are representative of three independent experiments.

The antibody used to detect Bim could not discrimiate between the different Bim isoforms, and it was therefore decided to verify their localisation in HeLa TRex cells, where the expression of individual isoforms could be induced by tetracyclin. In these cells, upregulated BimS and BimL localised almost exclusively to mitochondria, whereas BimEL appeared partially mitochondrial and partially bound to microtubules (Figure 19).

3.2 The role of apoptosis in the shutdown of neutrophil immune functions

Immune responses require a coordinate termination to avoid the risk of immunopathology or autoimmune disease. Bacterial infections are usually first combatted by neutrophils and monocytes that infiltrate infectious sites from the bloodstream (Medzhitov, 2007), and once the pathogen is cleared, recruited neutrophils die by apoptosis and are removed by macrophages and inflammatory monocytes through phagocytosis (Savill, 1997). The removal of apoptotic neutrophils in this way ensures that toxic granule contents such as elastase and cathepsin G are not released in the course of secondary necrosis (Savill et al., 1989; Stern et al., 1996), and also promotes the resolution of inflammation by deactivating phagocytes (Huynh et al., 2002; Meagher et al., 1992). An experimental prolongation of neutrophil lifespan by injection of endotoxins, pro-inflammatory cytokines, or apoptosis-inhibiting peptides, has been shown to delay this resolution in inflammations of the lung, pleural cavity, and brain (Coxon et al., 1999; Parsey et al., 1999; Sawatzky et al., 2006). It is not known though to what extent apoptosis impacts on the functional shutdown of neutrophils, and therefore it remains unclear whether inflammation is sustained in consequence of prolonged neutrophil effector functions, or rather as a result of a diminished clearance of possibly already inactive neutrophils.

These questions were addressed by testing the functional capacity of murine Hoxb8 neutrophils that were kept alive by the overexpression of the anti-apoptotic protein Bcl-2, and by analysing engulfment of these cells by macrophages. The overexpression of Bcl-2 was chosen because – unlike pro-survival cytokines or anti-apoptotic peptides – it specifically inhibits the mitochondrial apoptosis pathway and does not activate other signal transduction pathways. The capacity of Bcl-2 to inhibit spontaneous neutrophil apoptosis has been demonstrated previously in primary neutrophils from *bcl2*-transgenic mice (Ogilvy et al.,

1999; Villunger et al., 2000). This *in vitro* study with Hoxb8 neutrophils was complemented by experiments conducted in wt and *bcl2*-transgenic mice *in vivo*, using a model of murine pneumococcal meningitis (carried out by the group of Uwe Koedel at the Ludwig-Maximilian-Universtät München).

3.2.1 Generation of neutrophils *in vitro* via the Hoxb8 system

Murine neutrophils used for *in vitro* experimentation are often isolated from blood, from peritoneal exudates, or from the bone marrow of mice. The use of such primary neutrophils can, however, be difficult due to experimental variation and low yield. Therefore, the Hoxb8

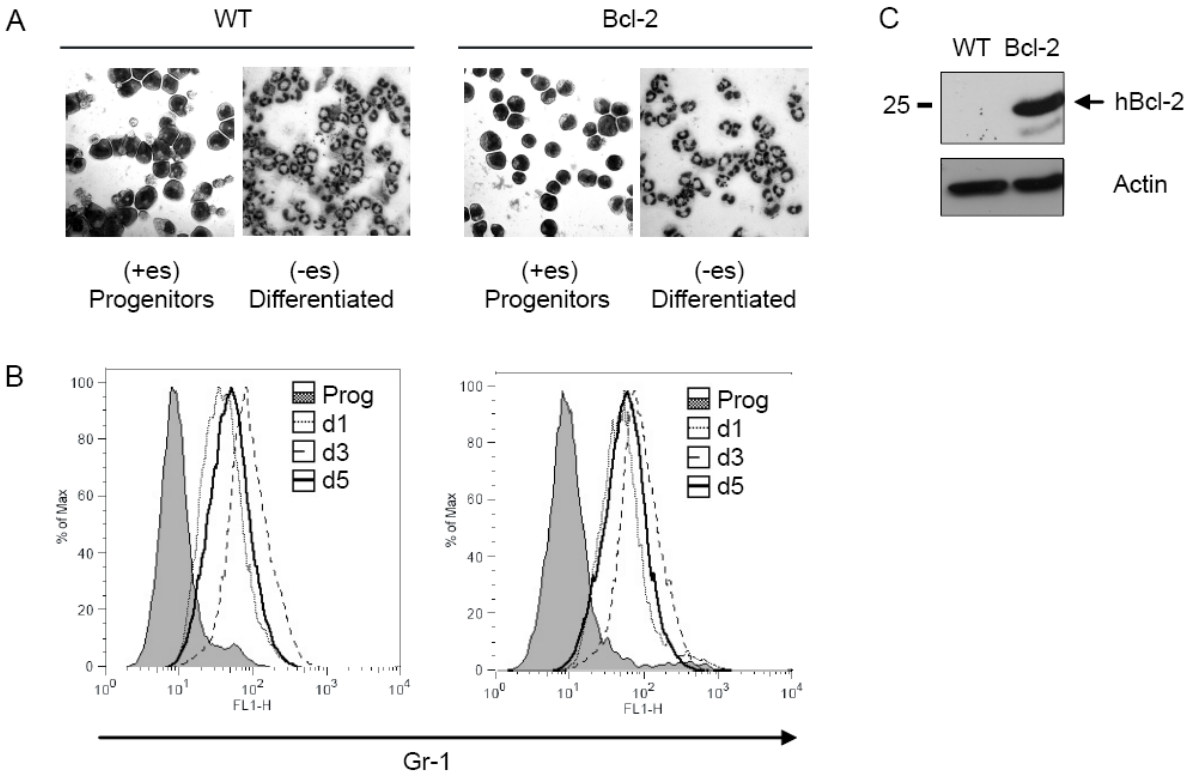


Fig. 20 Differentiation of wt and Bcl-2-overexpressing neutrophils from progenitor lines in vitro

A. WT or Bcl-2-overexpressing progenitors were cultured with stem cell factor in the presence or absence of oestrogen for 4 days. Cells were subjected to Giemsa stain to reveal their nuclear morphology. Note the typical segmentation and doughnut shape of mouse neutrophil nuclei. **B**. Differentiation of wt or *bcl2*-transgenic progenitors was induced by oestrogen withdrawal. Surface expression of the neutrophil marker Gr-1 was measured by flow cytometry on days 1, 3, and 5. The reduction in cell size at the later stage of differentiation may contribute to the slight reduction in Gr-1 expression after day 3. **C**. Western blot showing expression of human Bcl-2 in Hoxb8 neutrophils differentiated for 4 days.

system described already in detail on page 48 was used, as it also allows the generation of "near-primary" neutrophils behaving as primary cells by all means tested (Wang et al., 2006).

Polyclonal Hoxb8-immortalised neutrophil progenitor cells were generated from wt mice and *vav-bcl-2* transgenic mice overexpressing human Bcl-2 in the haematopoietic compartment. Both progenitor cell lines expanded equally well in the presence of oestrogen and stem cell factor, and upon removal of oestrogen differentiated into neutrophils in the course of 4 - 4.5 days. Fully differentiated cells adopted the doughnut-shaped nuclear morphology typical of neutrophils (Figure 20A). The expression profile of the neutrophil differentiation marker Gr-1 was virtually identical between wt and Bcl-2 overexpressing neutrophils, showing an upregulation of the surface protein until day 3, which was slightly decreased again at day 5 (Figure 20B). This reduction is very likely due to the shrinkage of cell size that accompagnies neutrophil development. Western blot analysis confirmed the overexpression of human Bcl-2 in differentiated neutrophils from the Bcl-2 cell line (Figure 20C).

3.2.2 Overexpression of Bcl-2 protects neutrophils from apoptosis induction

Neutrophils develop from progenitor cells of the myeloid lineage in the bone marrow, and expansion and survival of these cells *ex vivo* is enhanced by stem cell factor (SCF) (Metcalf and Nicola, 1991; McNiece et al., 1991; Heyworth et al., 1992). In the Hoxb8 system, the expansion of myeloid progenitor cells is also crucially dependent on SCF, and only the presence of this growth factor drives differentiation into neutrophils (as opposed to macrophage development in the presence of GM-SCF) (Wang et al., 2006). *In vivo*, neutrophils leaving the bone marrow and entering the blood dispose of only a very limited lifespan (8-20h) (Edwards, 1994), and *ex vivo* spontaneously undergo cell death even in the presence of serum, and absence of any extracellular pro-death stimuli (Savill et al., 1989). This "spontaneous death" exhibits typical features of apoptosis such as cell shrinkage and chromatin fragmentation (Savill et al., 1989). The initiator for such spontaneous apoptosis *in vivo* is not known, one contributing factor may be an altered growth factor and cytokine environment in the periphery providing less pro-survival signals.

In order to induce spontaneous neutrophil apoptosis *in vitro*, two experimental situations were analysed: either by "ageing" neutrophils in the presence of stem cell factor, or

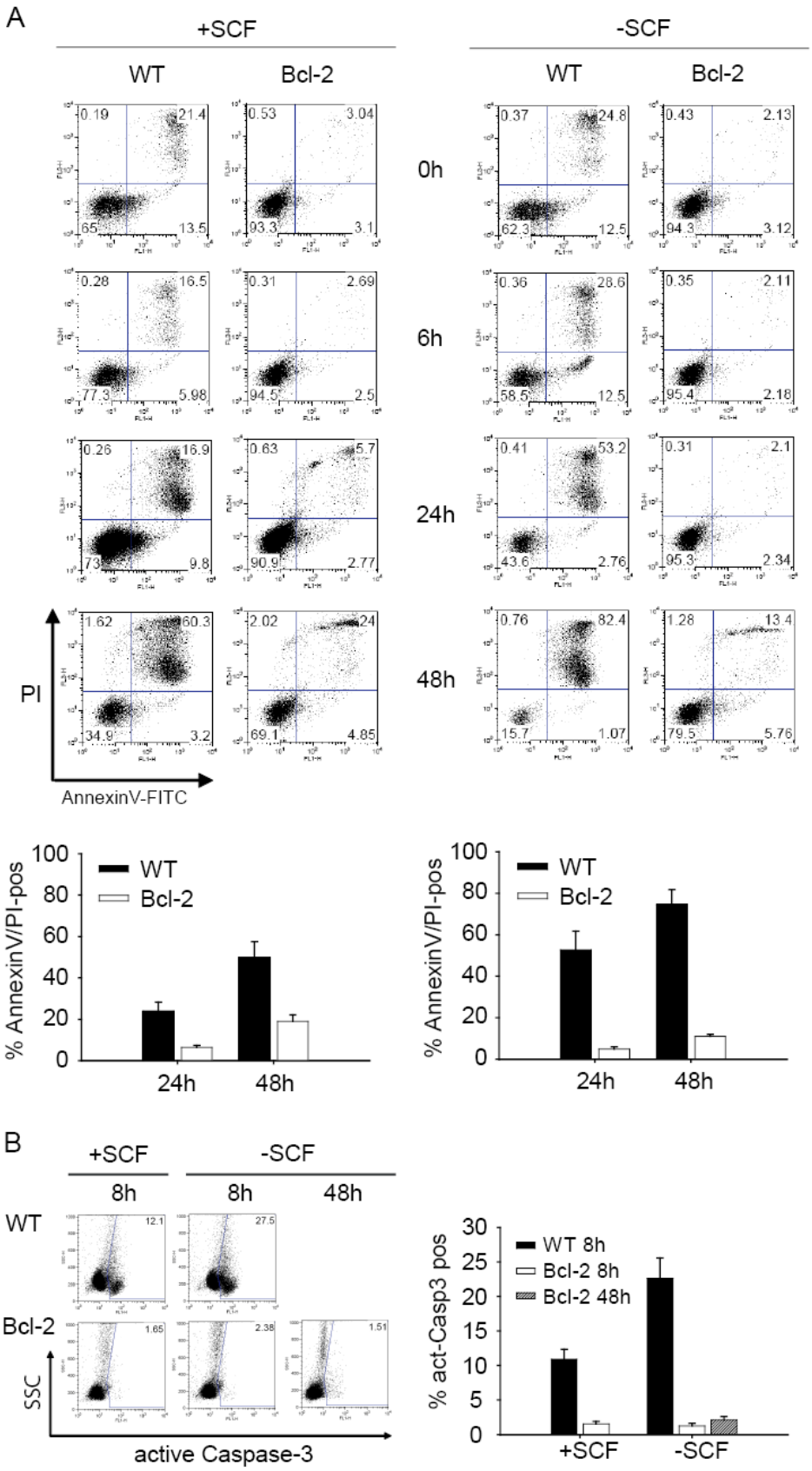
by withdrawal of the growth factor. For apoptosis induced in the presence of SCF, differentiated neutrophils were washed with PBS-10% FCS plus SCF, and culture was continued with fresh stem cell factor for various lengths of time. Alternatively, neutrophils were both washed and aged in the absence of SCF. Apoptosis was then quantified by measuring either the extent of AnnexinV-propidium iodide positivity, or the intracellular levels of active caspase-3.

The first method exploits the capability of AnnexinV to bind to phosphatidyl serine, which becomes extruded to the outer leaflet of the plasma membrane during apoptosis. The extent of phosphatidyl serine exposure can be quantified in flow cytometry measurements by use of AnnexinV conjugated to a fluochrome such as FITC. AnnexinV staining was performed in parallel to staining with propidium iodide (PI), a non-membrane permeable DNA-intercalating dye that is able to stain chromatin once the permeability of cellular membranes increases in the course of apoptosis.

Cultures of differentiated wt neutrophils were apoptotic to ~25% immediately after washing (and before the onset of ageing) (Figure 21A). This may be attributed to inadequacies of the *in vitro* culture system, and also to the fact that a polyclonal progenitor population is driven into differentiation. Therefore, differences in the expression levels of Hoxb8 may result in slight variations in the initial "true" progenitor status level, and in the speed of differentiation, i.e. a fraction of cells may develop faster and hence at time point zero already have started ageing. Wt neutrophils underwent ageing-associated apoptosis with different kinetics in the presence or absence of stem cell factor (Figure 21A). In the presence of SCF, the proportion of AnnexinV-PI positive cells reached ~60% only after 48 hours. Neutrophils deprived of SCF underwent apoptosis much faster, here cells had already become apoptotic to about 55% already after 24 hours, and to 80% after 48 hours.

In neutrophils overexpressing Bcl-2, on the other hand, there was hardly any apoptosis detectable immediately after differentiation, or after 24 hours both in the continued presence or in the absence of stem cell factor. Even after 48 hours of ageing, the proportion of apoptotic cells had increased to only ~25% in cultures supplemented with SCF, and to ~15% in cultures where SCF had been withdrawn. These results show that the overexpression of Bcl-2 confers an almost complete protection from spontaneous apoptosis, but that there is a small part of neutrophil death that cannot be blocked by Bcl-2. Similar results were obtained in subsequent experiments (Figure 21A, graph).

Fig. 21 Overexpression of Bcl-2 protects neutrophils from apoptosis induction



A. WT or Bcl2-overexpressing neutrophils differentiated for 4 days were cultured for the indicated lengths of time in the presence or absence of stem cell factor (SCF). Cells were then stained with AnnexinV-FITC and propidium iodide (PI) and analysed by flow cytometry. Graphs show the percentage of AnnexinV-PI double-positive cells. Error bars indicate the SEM of five independent experiments.

B. Neutrophils differentiated for 4 days were cultured for 8 or 48 hours in the presence or absence of SCF, and stained for active caspase-3. Error bars show the SEM or three independent experiments. SSC = side scatter.

Apoptosis induction was also quantified by staining for the active form of caspase-3. In wt neutrophils, rapid activation of the caspase could already be seen after 8 hours, and again, activation was more pronounced under conditions of SCF withdrawal (Figure 21B). The overexpression of Bcl-2, on the other hand, almost completely abolished caspase-3 activation. The overall proportion of cells positive for caspase activity is expected to be lower than that for AnnexinV-PI positivity, due to a higher number of wash steps during staining and subsequent loss of in the particular the more fragile, late apoptotic neutrophils, and due to leakage of caspase-3 protein out of apoptotic cells.

These results show that Hoxb8 neutrophils undergo spontaneous apoptosis to a large extent when ageing either in the presence or absence of stem cell factor, and that apoptosis is more pronounced in absence of the growth factor. The enforced overexpression of Bcl-2 almost completely blocks apoptosis induction, indicating that primarily the mitochondrial apoptosis pathway is initiated during spontaneous apoptosis.

3.2.3 Retainment of effector function in Bcl-2 overexpressing neutrophils

The very potent antimicrobial function of neutrophils resides in the ability to phagocytose, and rapidly kill phagosome-trapped pathogens by fusion with granules containing highly microbicidal proteases (Borregaard and Cowland, 1997) that become activated through the respiratory burst-induced elevation of phagosomal pH (Reeves et al., 2002). Apoptotic neutrophils have lost the ability to execute these immune functions (Whyte et al., 1993), however, shutdown of neutrophil function may also potentially be mediated by non-apoptotic signalling events associated with ageing. In the latter case, aged but “undead” Bcl-2 neutrophils would nevertheless loose their effector functions. Therefore, the immune functions of neutrophils between differentiated wt and Bcl-2 neutrophils were compared with that of Bcl-2 neutrophils deprived of stem cell factor for 48 hours.

It was first tested whether aged Bcl-2 neutrophils would retain the ability to ingest bacteria. Neutrophils were incubated with FITC-stained pneumococci for 1 hour, and then subjected to flow cytometry analysis. About half of the cells in the wt and Bcl-2 neutrophil populations phagocytosed pneumococci as indicated by their acquisition of green fluorescence. No difference was observable when using Bcl-2 neutrophils deprived of SCF (Figure 22A), indicating that phagocytic functions are fully preserved in these cells.

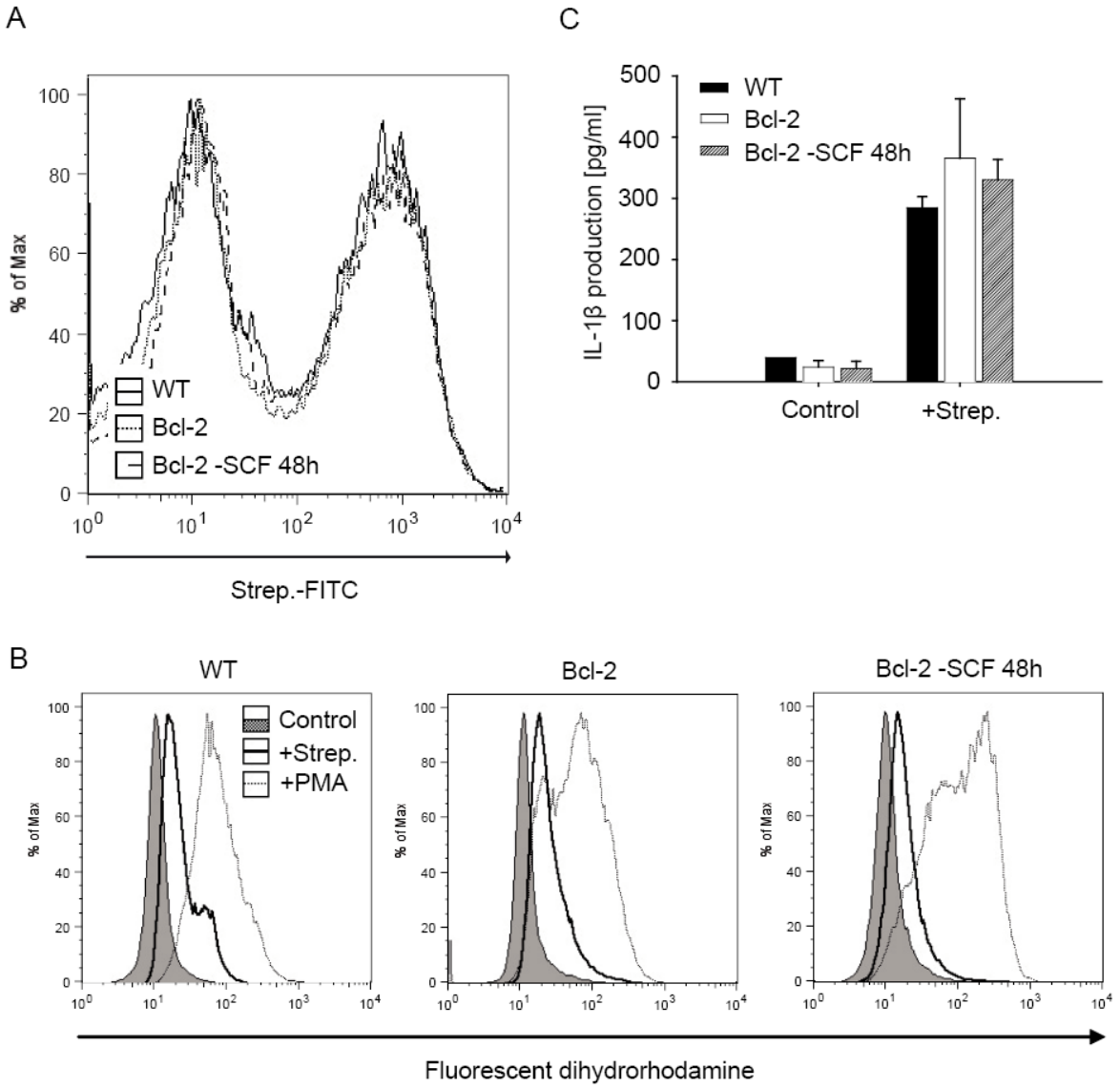


Fig. 22 Immune effector functions are preserved in aged neutrophils overexpressing Bcl-2

Effector functions of wt or Bcl-2-overexpressing neutrophils differentiated for 4 days were compared with that of Bcl-2 neutrophils differentiated for 4 days and then cultured in the absence of SCF for 48 hours. In all experiments, *Streptococcus pneumoniae* and neutrophils were co-incubated at a ratio of 100:1 **A**. Phagocytic uptake of FITC-labelled pneumococci was analysed by flow cytometry after 1 hour of co-incubation. Very similar results were seen in three independent experiments. **B**. The production of reactive oxygen species was measured as capacity of neutrophils to oxidise dihydrorhodamine into a fluorescent dye. Cells were incubated for one hour with pneumococci (bold line) or with PMA (5µg/ml, dotted line). Unstimulated cells are shown as shaded histogram. Very similar results were seen in three independent experiments. **C.** Secretion of IL-1β was measured in supernatants of neutrophils stimulated with pneumococci for 8 hours. Error bars show SEM of four independent experiments.

In the next experiment, the same set of neutrophil populations were analysed for their ability to produce the respiratory burst upon phagocytosis of pneumococci. Neutrophils were incubated with bacteria for half an hour, after which the membrane-permeable dye 123-dihydrorhodamine (DHR) was added and culture continued for another 30 minutes. When oxidised by intracellular reactive oxygen species (ROS) generated by the respiratory burst, DHR emits a green fluorescence that can be detected by flow cytometry. As a positive control, neutrophils were treated with phorbol myristate acetate (PMA), an agent that induces the production of ROS through stimulation of NADPH oxidase (Cox et al., 1987). No differences in terms of ROS production induced by the uptake of pneumococci, or by treatment with PMA, were observed between wt, Bcl-2, and Bcl-2 neutrophils deprived of stem cell factor for 48 hours (Figure 22B), demonstrating that the ability to produce an oxidative burst is also retained.

Neutrophils are not only very efficient phagocytes and bacterial killers, but can also be stimulated to produce pro-inflammatory cytokines including IL-1β, TNF-α, and (in humans) IL-8, which can have immunomodulatory effects (Cassatella, 1995; 1999). To measure the ability of differentiated and aged neutrophils to produce IL-1β, cells were incubated with pneumococci for 8 hours. The level of the cytokine secreted into the supernatant was then analysed by an ELISA. There were no significant differences between wt, Bcl-2, and Bcl-2 neutrophils deprived of SCF for 48 hours (Figure 22C), showing that aged Bcl-2 neutrophils do not loose the ability to produce the pro-inflammatory cytokine.

Taken together, these results demonstrate that aged neutrophils kept alive by the overexpression of Bcl-2 do not loose the ability to phagocytose bacteria, to activate their granule proteases through an respiratory burst, and to release pro-inflammatory IL-1β. Ageing on its own is therefore not sufficient to shut down the immune functions of neutrophils. Neutrophil deactivation in this context can only occur through apoptosis.

3.2.4 Aged neutrophils overexpressing Bcl-2 are not removed by macrophages

Aged neutrophils are mainly removed by macrophages, and it has been shown that apoptosis and the associated morphological changes at the cell surface are the crucial events that lead to recognition and ingestion by the phagocyte (Savill et al., 1989). It was therefore tested

whether the inhibition of apoptosis by overexpression of Bcl-2 would also block the ageing-associated uptake of neutrophils.

Differentiated neutrophils were aged in the absence of stem cell factor for various lengths of time, and part of the cells were then stained with AnnexinV-PI to control for apoptosis induction (Figure 23A, top panel). The remaining neutrophils were labelled with the green-fluorescent dye CFDA-SE and co-cultured with red-stained RAW macrophages at a cellular ratio of 5:1. After 4 hours, the macrophages were detached and the cell suspension was subjected to flow cytometry analysis. The cells in the upper right gates (Figure 23A,

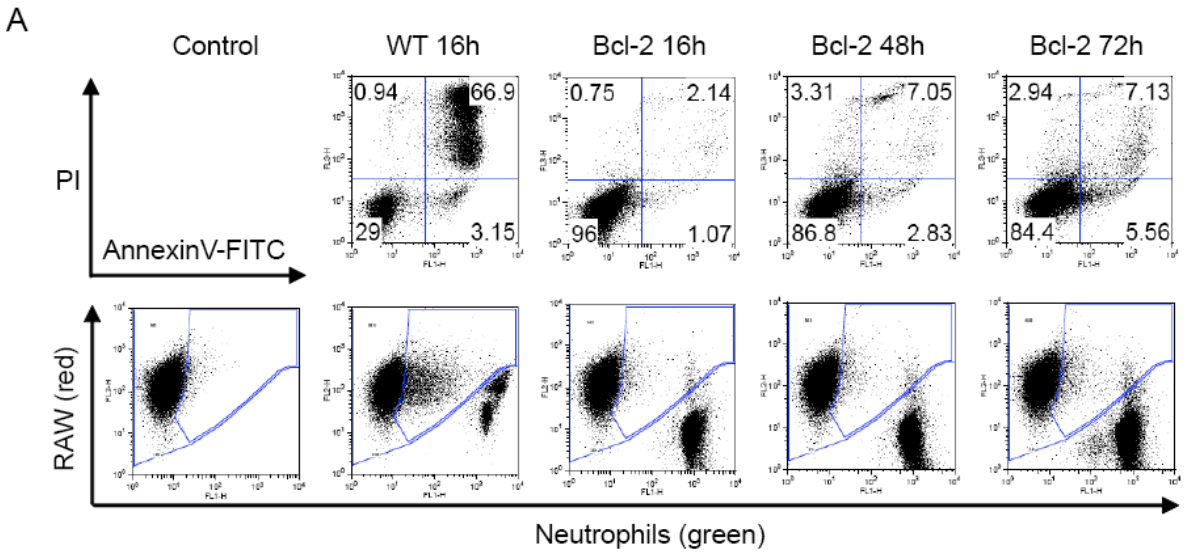


Fig. 23 Bcl-2 overexpression protects aged neutrophils from removal by macrophages

A. Neutrophils differentiated for 4 days were cultured in the absence of SCF for the indicated periods of time, and stained with AnnexinV-FITC and propidium iodide (PI) (upper panel) or CFDA-SE. Green-fluorescent (CFDA-SE-stained) neutrophils were then added to cultures of RAW macrophages stained with the red dye PKH26 at a neutrophil-to-macrophage ratio of 5:1. After 4 h of co-incubation, cultures were subjected to flow cytometry analysis. The phagocytic (red and green fluorescent) macrophage population is found in the upper right gate (lower panel). **B**. The graph shows the percentage of phagocytic cells within the macrophage population. Error bars indicate the SEM of three individual experiments.

bottom panel) are macrophages marked by red and green fluorescence, i.e. represent cells that have taken up CFDA-SE-labelled neutrophils. Wt neutrophil cultures aged for 16 hours underwent apoptosis to ~65%, and were clearly taken up by macrophages – about a quarter of the population became green-fluorescent (Figure 23B). Neutrophils overexpressing Bcl-2, by contrast, did not undergo significant apoptosis even after 72 hours of SCF-withdrawal, and were very poorly taken up by macrophages. Bcl-2 therefore not only blocks spontaneous neutrophil apoptosis, but also the uptake of aged neutrophils by macrophages.

3.3 Bcl-2 family proteins involved in neutrophil apoptosis

Signal transduction via the mitochondrial pathway constitutes one of the major pathways initiating spontaneous neutrophil apoptosis, because cell death is strongly inhibited *in vivo* and *in vitro* by the overexpression of Bcl-2 (Ogilvy et al., 1999; Villunger et al., 2000). One activator of this pathway is the BH3-only protein Bim. *Bim*-deficient mice have a 2-3 fold excess of granulocytes (Bouillet et al., 1999), and neutrophils lacking this protein show prolonged survival upon ageing *in vitro* (Villunger et al., 2003b). The fact that neutrophils overexpressing Bcl-2 are protected considerably better still (and are also completely protected in the Hoxb8 system), points towards the involvement of other BH3-only proteins though. One prominent candidate is Puma, which contributes to the death of myeloid progenitor cells, thymocytes and mast cells induced by growth factor withdrawal (Ekoff et al., 2007; Jeffers et al., 2003; Han et al., 2001; Villunger et al., 2003a).

To confirm and test, respectively, the involvement of Bim and Puma in apoptosis induction, spontaneous cell death was compared within a panel of neutrophil lines generated via the Hoxb8 system from wt, *bcl2*-transgenic, Bim knock-out (Bim-/-), and Bim-Puma double knock-out (Bim/Puma-/-) mice. The use of these cell lines, rather than primary neutrophils, allowed a very “clean” assessment of the relative contribution of Bim and Puma, since non-specific effects on granulopoiesis that may arise as a result of an altered survival of other cell types in the *bim(/puma)*-deficient or *bcl2*-transgenic mice are largely excluded in the Hoxb8 system. Also analysed were the expression kinetics of Bim and Puma, and that of several pro-survival Bcl2-like proteins during apoptosis induction.

3.3.1 Generation of Bim-/- and BimPuma-/- neutrophils via the Hoxb8 system

Polyclonal neutrophil progenitors lines deficient in the BH3-only proteins Bim or double-deficient in Bim and Puma were generated from Bim-/- or Bim/Puma-/- mice, via the Hoxb8 system described in detail on page 48. Progenitor cells expanded equally well as that of the wt or Bcl-2 overexpressing cell lines, and upon withdrawal of oestrogen differentiated in the

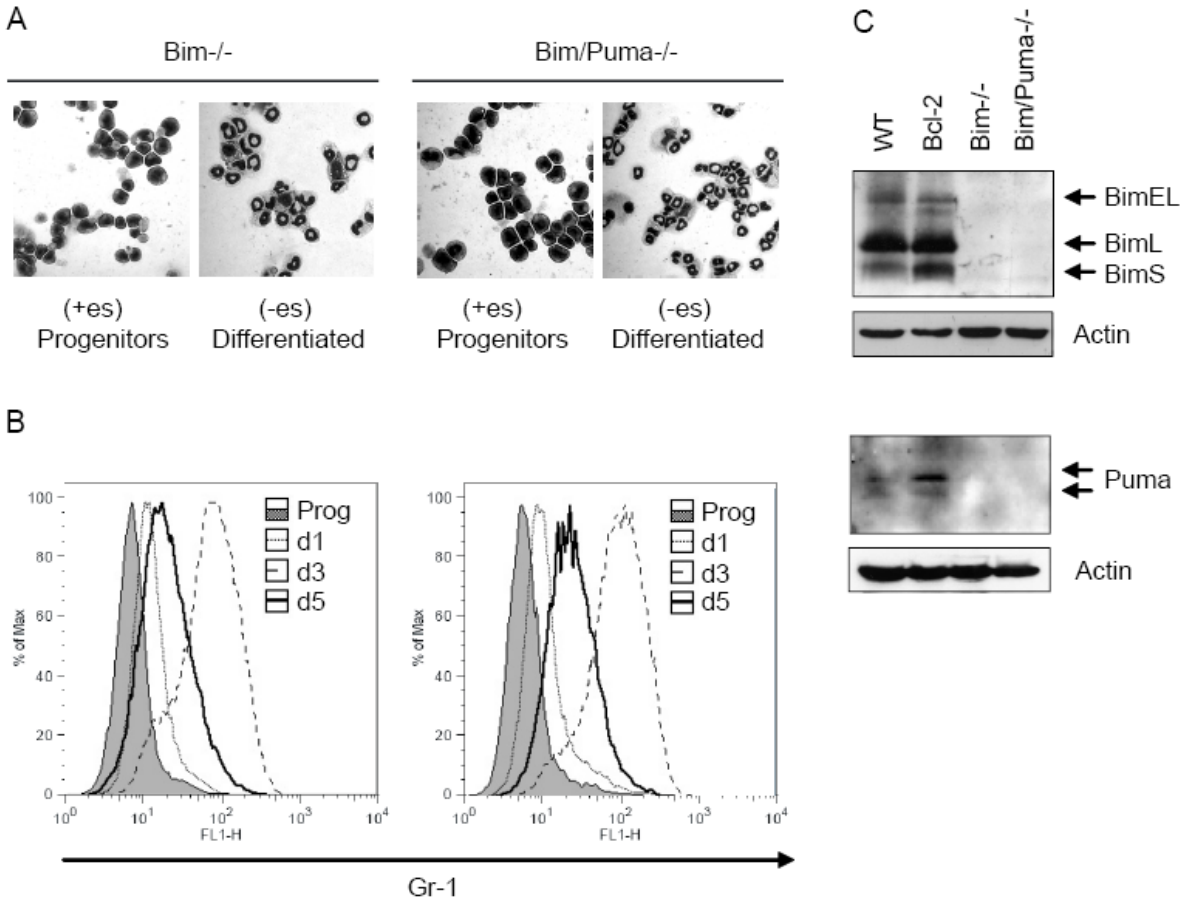


Fig. 24 Differentiation of neutrophils deficient in Bim or double-deficient in Bim and Puma from progenitor lines in vitro

A. Progenitor cells deficient in Bim, or Bim and Puma were cultured with stem cell factor in the presence or absence of oestrogen for 4 days. Cells were stained for nuclear morphology with Giemsa. **B**. Differentiation of Bim or Bim/Puma-deficient progenitors was induced by oestrogen withdrawal. Surface expression of the neutrophil marker Gr-1 was measured by flow cytometry on days 1, 3, and 5. **C**. Western blot showing absence of expression of Bim or both Bim and Puma in gene-deficient neutrophils differentiated for 4 days. Puma expression in Bim-/- neutrophils was very low, a faint double band was nevertheless visible on the film.

same time frame of 4 – 4.5 days into cells showing the typical nuclear morphology of neutrophils (Figure 24A). The expression profile of the differentiation marker Gr1 during cellular development was very similar to that obtained for wt and Bcl-2 neutrophils (Figure 24B, compare with Figure 20B, page 69). Western blot analysis confirmed the absence of all three isoforms of Bim in the Bim-/- and Bim/Puma-/- cell lines. Puma was expressed in two isoforms at very low levels (in Bim -/- barely visible), and absent in the Bim/Puma-/- line (Figure 24C).

3.3.2 Bim and Puma are the key but not sole mediators of neutrophil apoptosis

Spontaneous neutrophil apoptosis was induced by ageing differentiated neutrophils either in the presence, or absence of stem cell factor. At various time points extending up to 72 hours, apoptosis was measured by staining cells with AnnexinV-PI, or an antibody detecting active caspase-3.

Immediately after differentiation (and washing), wt neutrophils were AnnexinV-PI positive to about 35% (Figure 25A, left). This ratio was decreased to ~20% in Bim-/- or BimPuma-/- neutrophils, and still lower in neutrophils overexpressing Bcl-2 (about 10%). In the presence of stem cell factor, no significant apoptosis induction took place during the first 24 hours of ageing in all four neutrophil lines. After 48 hours however, wt neutrophils had become AnnexinV-PI positive to ~80%, while apoptosis in Bim-/- or Bim/Puma-/- (~35%), and Bcl-2 neutrophils (~20%) had also increased, but not reached near as high levels. After 72 hours, about 90% of wt neutrophils were dead, while the fraction of apoptotic cells in the other neutrophil lines had not substantially increased further. Apoptosis induction was also analysed by staining for active caspase-3. Very similar results were obtained, with Bim-/-, BimPuma-/- and Bcl-2 neutrophils already being less apoptotic at time point zero, and substantial apoptosis induction showing only after 48 hours of ageing (Figure 25B, left). Again, after 72 hours, cell death was very high in wt cells (~80% positive for active caspase-3), and much lower (10-35%) in the other neutrophil lines.

In the absence of stem cell factor, ageing-associated apoptosis proceeded with much faster kinetics and was more pronounced in all four neutrophil lines (Figure 25A, right). Already 8 hours after SCF withdrawal, wt neutrophils had become AnnexinV-PI positive to ~60%, and to almost 90% after 24 hours. At 24 hours, also the ratio of apoptotic cells in the

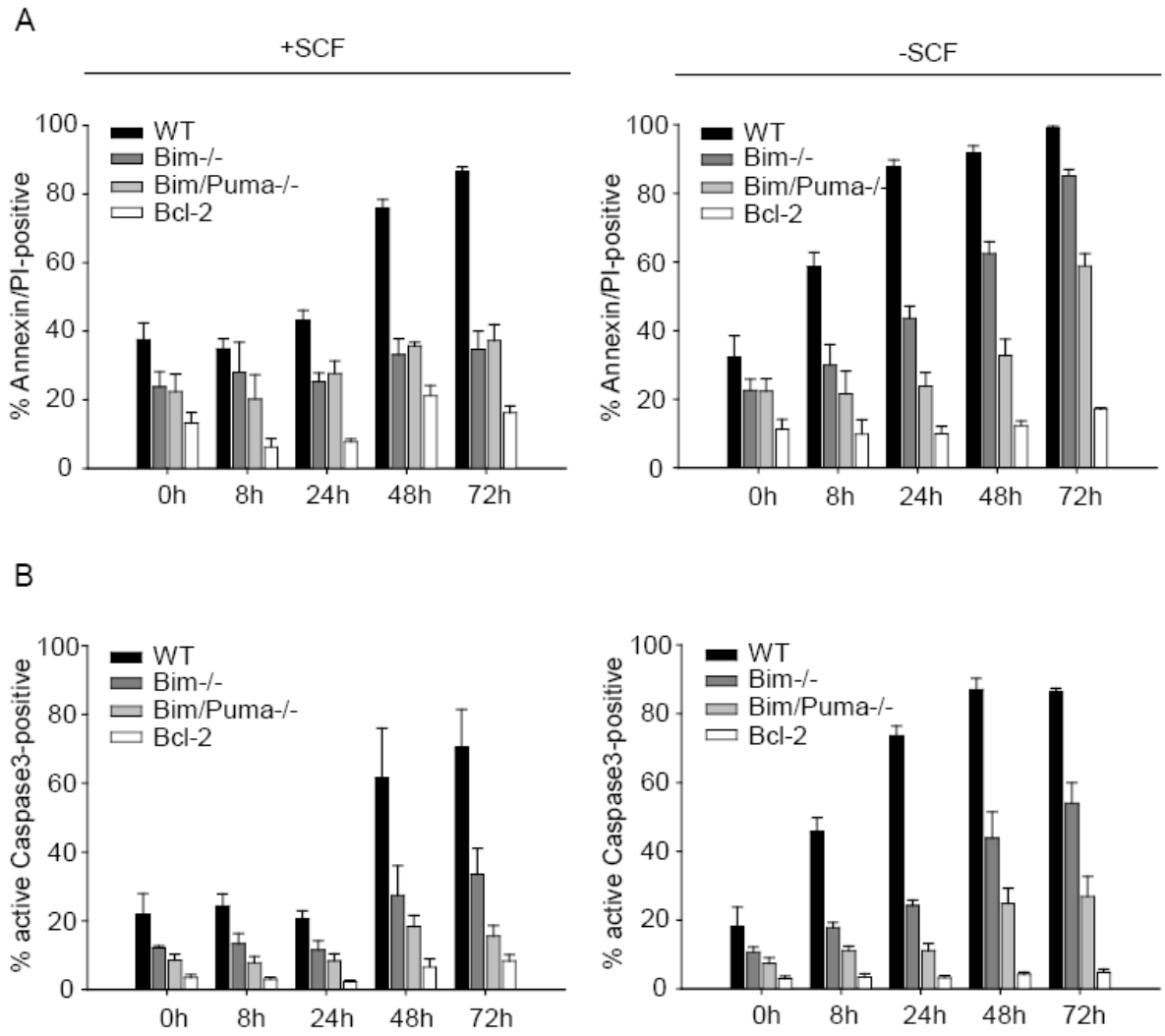


Fig. 25 Contribution of Bim and Puma to apoptosis induction

A panel of wt, Bcl2-overexpressing, Bim-/- or Bim/Puma-/- neutrophils differentiated for 4 days were cultured for the indicated periods of time in the presence or absence of SCF. **A**. Cells were then stained with AnnexinV-FITC and propidium iodide (PI) and analysed by flow cytometry. Graphs show the percentage of AnnexinV-PI double-positive cells. Error bars indicate the SEM of four independent experiments. **B**. Cells were stained for active caspase-3. Graphs show the percentage of positive cells. Error bars indicate the SEM or three separate experiments.

Bim-/- line had doubled to 40%, whereas no rise had occurred in Bim/Puma-/- or Bcl-2 neutrophils. After 48 hours, 90% of wt neutrophils were AnnexinV-PI positive, and over 60% of Bim-/- cells. The proportion of dead neutrophils in the Bim/Puma-/- line had increased by half to ~30%, but no change was observable in the Bcl-2 line. After 72 hours, almost no living cell could be detected any longer in the wt neutrophil population. Bim-/- cells were dead to more than 80%, and the ratio of apoptotic cells had doubled to 60% in the Bim/Puma-/- line.

Bcl-2 neutrophils, on the other hand, had not become substantially more apoptotic, with levels still below 20%. Very similar results were obtained when cell death was measured by active caspase-3 staining (Figure 25B, right).

From these data sets several conclusions can be drawn. Under both culture conditions, the loss of the pro-apoptotic protein Bim confers a substantial protection from spontaneous apoptosis induction, although it becomes weaker upon prolonged stem cell factor withdrawal. Intriguingly, the additional loss of Puma does not result in a better protection in the presence of SCF (the differences between Bim-/- and Bim/Puma-/- in Figure 25B are not significant at any time point), but clearly inhibits apoptosis further in the absence of SCF, suggesting the engagement of slightly different apoptosis-inducing pathways under these two conditions. Another finding was that regardless of the culture conditions, neutrophils overexpressing the anti-apoptotic protein Bcl-2 were still better protected than neutrophils having lost both Bim and Puma. This indicates that yet an additional factor must be involved in apoptosis induction, most probably either the activation of another pro-apoptotic BH3-only protein, or the loss of an anti-apoptotic Bcl2-like protein.

3.3.3 Upregulation and potential phosphorylation of Bim and Puma during apoptosis induction

The analysis of cell death induction in the neutrophil lines deficient in Bim and Puma had identified these two proteins as important mediators of spontaneous apoptosis. How the pro-apoptotic activity of Bim and Puma is controlled on the molecular level in neutrophils is not known, transcriptional upregulation as well as post-translational modifications may play a role. To detect changes in their expression levels associated with apoptosis induction upon stem cell factor withdrawal, differentiated wt and Bcl-2 overexpressing neutrophils were cultured in the absence of SCF, and lysates prepared at various time points up to 72 hours.

The constitutive expression of Bim was low in wt and Bcl-2 neutrophils, but all three isoforms, BimEL, BimL and BimS, were detectable (Figure 26, t0). Factor deprivation resulted in the upregulation of majorly the BimEL and BimL isoforms in both cell lines. Increased levels of the protein were already visible after 6 hours, peaking at 12 hours in wt, and at 48 hours in Bcl-2 neutrophils. For the BimEL isoform, an upper band in very low quantities could be detected already at t0 in wt and Bcl-2 neutrophils (asterix), sugesting some constitutive phosphorylation. The intensity of this band increased though after 6 hours of

ageing. With respect to BimL, after 24 hours of SCF deprivation a clear shift was observed in wt neutrophils, and in Bcl-2 neutrophils, a faint upper band appeared (double asterix), which may also indicate a phosphorylated form of the protein.

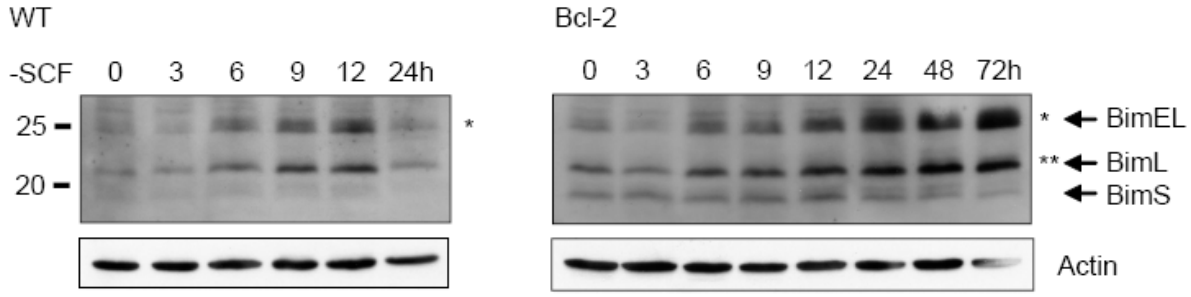


Fig. 26 Bim upregulation upon SCF withdrawal-induced apoptosis

WT and Bcl2-overexpressing neutrophils differentiated for 4 days were cultured for the indicated lengths of time in the absence of SCF. After each time point extracts were made and lysates were probed with an antibody against Bim. Asterixes indicate potentially phosphorylated forms of the protein. Data are representative of three individual experiments

Puma was expressed in wt, Bcl-2 and Bim-/- neutrophils at very low levels, but the two isoforms were detectable at t0 (Figure 27). The withdrawal of stem cell factor led to an slight upregulation of the larger isoform starting at 6 hours in wt neutrophils, that became more pronounced after 48 hours in the Bcl-2 and Bim-/- lines. In wt neutrophils, the appearance of a slightly higher molecular weight band of the smaller Puma isoform could be detected from the 6 hour time point onwards (asterix), suggesting phosphorylation.

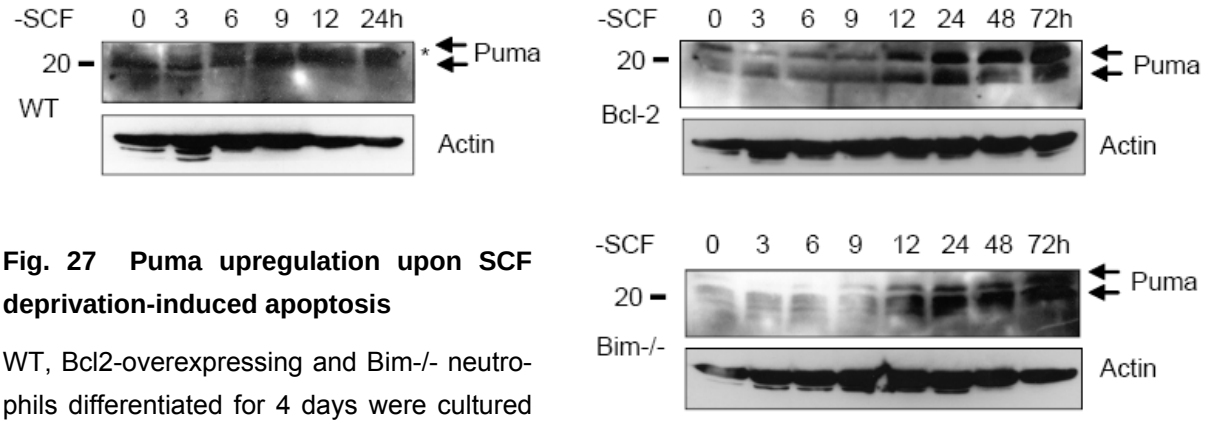


Fig. 27 Puma upregulation upon SCF deprivation-induced apoptosis

WT, Bcl2-overexpressing and Bim-/- neutrophils differentiated for 4 days were cultured for the indicated periods of time in the absence of SCF. After each time point extracts were made and lysates were probed with an antibody specific for Puma. The asterix indicates a potentially phosphorylated form of the protein.

3.3.4 Spontaneous neutrophil apoptosis is associated with a reduction in A1 but not Mcl-1 levels

Neutrophils express several anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-w, Mcl-1 and A1. In primary mouse neutrophils, Bcl-2, Bcl-xl and Bcl-w are expressed at very low levels, and their expression does not change during ageing-associated apoptosis (Moulding et al., 2001; O'Reilly et al., 2001; Villunger et al., 2000). Bcl-2, Bcl-xl, and Bcl-w were also expressed in barely detectable amounts in Hoxb8 neutrophils from the wt, Bim-/-, Bim/Puma-/-, and (human) Bcl-2 overexpressing lines, and protein levels did not change significantly during ageing for up to 72 hours in the presence or absence of stem cell factor (Figure 28). Loss of any of these three anti-apoptotic proteins therefore does not appear to play a role in apoptosis induction. In support of this, the deficiency in Bcl-2 or Bcl-w does not render mouse granulocytes more susceptible to apoptosis induced by cytokine withdrawal or treatment with cytotoxic agents (Villunger et al., 2003b), and granulopoiesis is not affected in knock-out mice for either Bcl-2, Bcl-xl, or Bcl-w (Print et al., 1995; Motoyama et al., 1998; Veis et al., 1993).

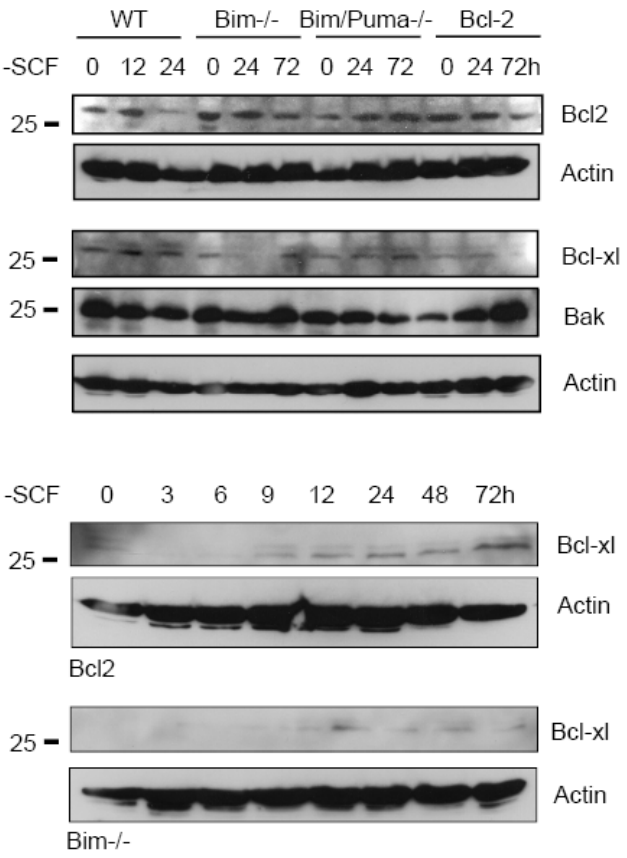


Fig. 28 Apoptosis induced by SCF withdrawal is not accompagnied by reduction in Bcl-2 or Bcl-xl levels

WT, Bcl2-overexpressing, Bim-/- and Bim/Puma-/- neutrophils differentiated for 4 days were cultured for the indicated lengths of time in the absence of SCF. After each time point extracts were produced and lysates were probed with antibodies for the presence of Bcl-2, Bcl-xl, and Bak. Data are representative of two independent experiments.

Mcl-1 is constitutively expressed in neutrophils, and because of its rapid synthesis and very short half-life (Moulding et al., 2001) is regarded as a highly regulable modulator of apoptosis. Partial loss of the protein has been reported during ageing-associated apoptosis (Moulding et al., 1998; Moulding et al., 2001). Neutrophils from conditional Mcl-1 knock-out mice die more rapidly in culture (Dzhagalov et al., 2007), and antisense nucleotide knock-down of Mcl-1 increases the death of human blood neutrophils (Leuenroth et al., 2000).

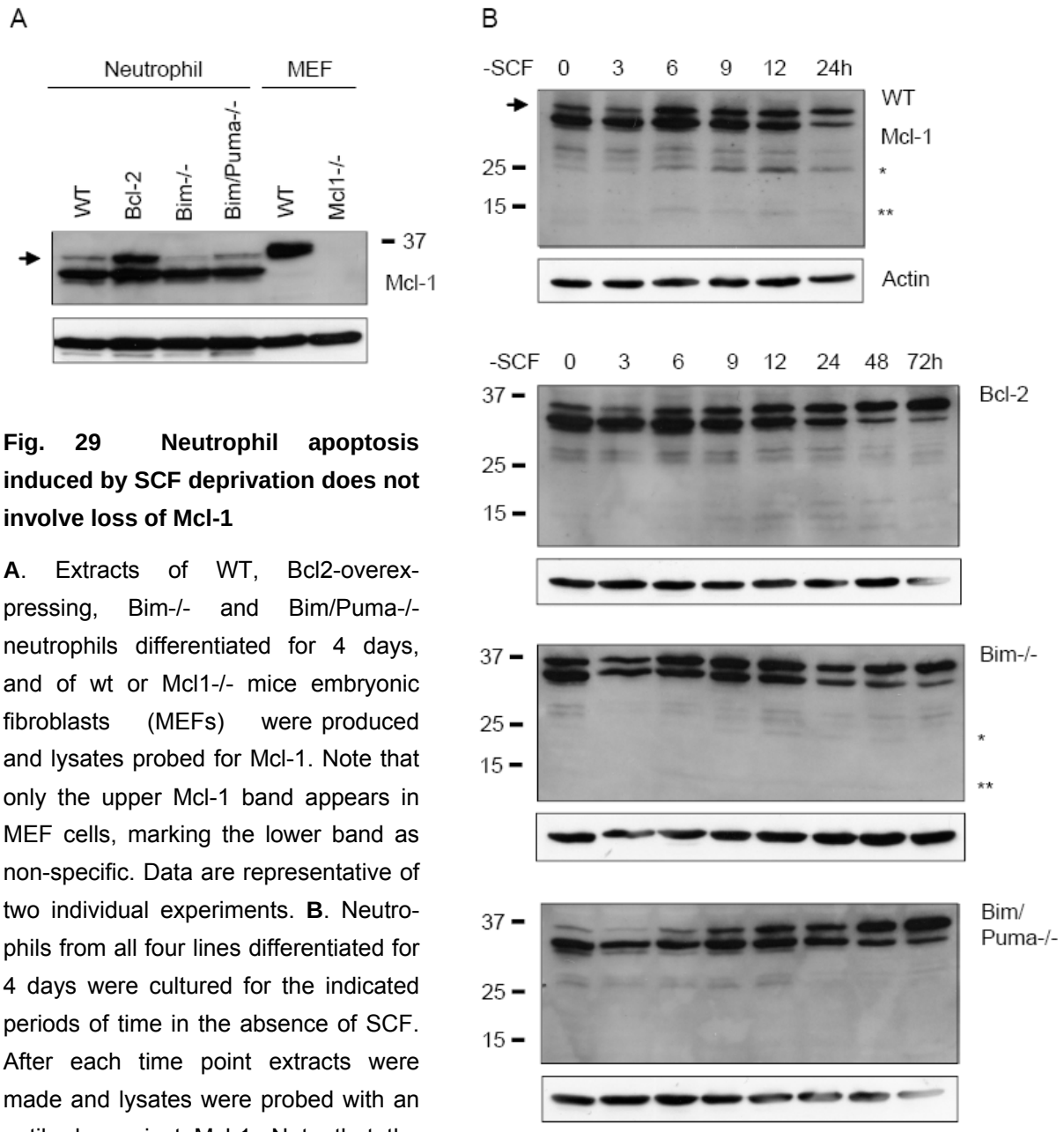


Fig. 29 Neutrophil apoptosis induced by SCF deprivation does not involve loss of Mcl-1

A. Extracts of WT, Bcl2-overexpressing, Bim-/- and Bim/Puma-/- neutrophils differentiated for 4 days, and of wt or Mcl1-/- mice embryonic fibroblasts (MEFs) were produced and lysates probed for Mcl-1. Note that only the upper Mcl-1 band appears in MEF cells, marking the lower band as non-specific. Data are representative of two individual experiments. **B**. Neutrophils from all four lines differentiated for 4 days were cultured for the indicated periods of time in the absence of SCF. After each time point extracts were made and lysates were probed with an antibody against Mcl-1. Note that the band indicated by the arrow represents full-length Mcl-1, the asterixes indicate apoptosis-associated lower molecular weight forms (~25 and ~15 kD) of the protein. Data are representative of three separate experiments.

In differentiated neutrophils of all four Hoxb8 lines, Mcl-1 was readily detectable by Western blotting, and appeared as a double band running at 36, and ~33 kD, respectively (Figure 29 A, lanes 1-4). Murine full-length Mcl-1 has a predicted molecular mass of 35.2 kD, and when extracts of wt and Mcl-1 knock-out mouse embryonic fibroblasts (MEFs) were tested, on height of the upper band in neutrophils a very closely spaced doublet appeared in wt MEFs, but was absent in Mcl1-/- MEFs. In neutrophils, Mcl-1 protein is therefore represented by the upper band, while the lower band derives from a non-specific cross-reaction of the antibody. The appearance of an unspecific lower Mcl-1 band has also been noted by other investigators using this antibody (Willis et al., 2005).

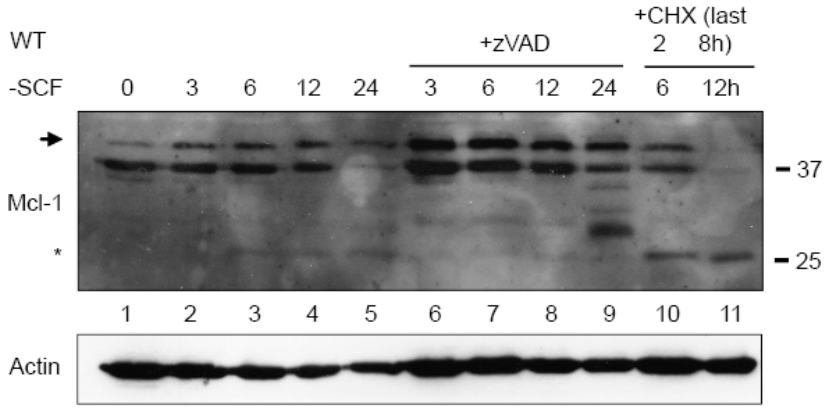
It was very surprising to find that there was no loss of Mcl-1 observable in neutrophils from any cell line upon ageing-associated apoptosis in the absence of stem cell factor (Figure 29 B). In ageing wt and Bim-/- cells deprived of SCF, Mcl-1 levels actually marginally increased after 12, and 48 hours, respectively, while there was a substantial increase of the protein over time in Bim/Puma-/- and Bcl-2 cells.

In ageing wt neutrophils deprived of stem cell factor, one band running at ~25 kD increased in intensity after 6 hours, and this band also appeared very weakly in Bim -/- neutrophils (Figure 29 B, asterix). Another band at ~15 kD was also visible, although much weaker (double asterix). Two pro-apoptotic splice variants of human Mcl-1 have been reported. Mcl-1_{Exon-1} is produced by macrophages in response to pneumoccoal infections (Marriott et al., 2005), but in its murine version would comprise 22 kD and therefore be too short to represent the 25 kD Mcl-1 band. Murine Mcl-1S, however, would be 26 kD large and fit in size.

If this lower molecular weight form was indeed a splice variant of Mcl-1, *de novo* synthesis of the protein should be blocked by treatment with the protein translation inhibitor cycloheximide. When wt neutrophils were aged in the absence of SCF and treated with the agent at a point in time before the appearance of the 25 kD band), full-length Mcl-1 had disappeared, but not the 25 kD band (asterix) (Figure 30, lanes 10 and 11). On the other hand, the band disappeared upon treatment of ageing neutrophils with the caspase-inhibitor zVAD (Figure 30, lanes 6-9), which would not affect *de novo* synthesis. All this points towards the 25 kD band representing a degradation product generated by ageing-associated caspase activation.

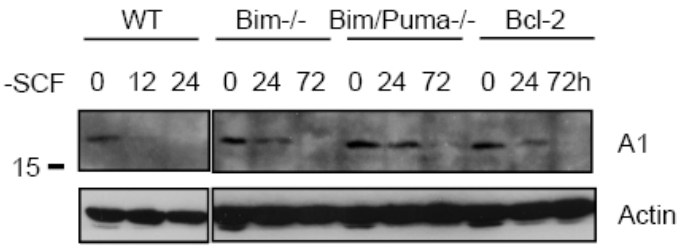
Fig. 30 The 25 kD form of Mcl-1 represent a breakdown product of the protein

WT neutrophils were differentiated for 4 days and cultured for the indicated periods of time in the absence of SCF (lanes 1-5), plus the addition of 50 µM zVAD (lanes 6-9), or plus the addition of 1 µg/ml cycloheximide (CHX) for the last indicated length of time of the whole period of factor withdrawal (lanes 10 and 11). Cells were then extracted and lysates were probed for Mcl-1 expression. Note that the 25 kD band (asterix) of Mcl-1 largely disappears in the presence of zVAD, but not in the presence of CHX. The intense lower molecular weight band appearing in lane 9 probably indicates a breakdown product of Mcl-1 produced by proteases becoming activated in the caspase-inhibited cells. Data are representative of two independent experiments.



Neutrophils from both humans and mice also constitutively express A1 (Chuang et al., 1998; Orlofsky et al., 1999). Although neutrophils from mice deficient in the *a1a* gene develop normally, their neutrophils show an accelerated spontaneous apoptosis when cultured *in vitro* (Hamasaki et al., 1998). When Hoxb8 neutrophils were aged in the absence of stem cell factor, a loss in the protein level of A1 could clearly be observed in the wt and mutant cell lines (Figure 31). This loss was already seen after 12 hours in wt, and after 24 hours in Bim-/-, BimPuma-/-, and Bcl2-overexpressing neutrophils. Taken together, the results relating to the expression of anti-apoptotic Bcl2-like proteins indicate that A1 may play a role in the induction of spontaneous neutrophil death.

Fig. 31 Stem cell factor withdrawal-induced apoptosis involves loss of A1



WT, Bcl2-overexpressing, Bim-/- and Bim/Puma-/- neutrophils differentiated for 4 days were cultured for the indicated lengths of time in the absence of SCF. After each time point extracts were made and lysates probed with an antibody specific for A1. Data are representative of two separate experiments.

4 Discussion

Phagocytes are essential to the function of the innate immune system as they combine the ability to initiate immune responses against invading microorganisms, to kill and remove pathogens through phagocytosis, and to promote the resolution of inflammation and termination of the immune response. The present work investigated how phagocytosis and the intracellular destruction of bacteria – the most important effector function of phagocytes – can modulate macrophage apoptosis, to what extent apoptosis governs the shutdown of neutrophil effector functions during the termination of immune responses, and which Bcl2-family proteins are involved in the induction of neutrophil apoptosis in this phase.

4.1 The induction of macrophage apoptosis by bacterial phagocytosis

Phagocytosis and the subsequent maturation of the phagosome to a lytic compartment enabling killing and degradation of trapped pathogens constitute the principal anti-bacterial defence mechanism of the innate immune system. A number of pathogens have acquired the ability to subvert phagocyte killing by escape from the phagosome (*Listeria*) (Gaillard et al., 1987), or by actively inhibiting or delaying phagosome maturation (*Legionella, Mycobacteria, Toxoplasma, Salmonella*) (Alpuche Aranda et al., 1992; Horwitz and Maxfield, 1984; Sibley et al., 1985; Sturgill-Koszycki et al., 1994). Although extracellular bacteria that do not possess virulence factors allowing intracellular survival, such as *Staphylococci*, *Streptococci*, or *Escherichia coli* (*E. coli*), are successfully killed and degraded by this mechanism, the death of phagocytes upon exposure to these bacteria has been observed in a number of studies (Ali et al., 2003; Baran et al., 1996; Bastiani et al., 2005; Dockrell et al., 2001; Hacker et al., 2002; Rodriguez et al., 1999).

Previous work on low-virulence *E. coli* had suggested that macrophage death is dependent on the intracellular breakdown of bacteria (Hacker et al., 2002). The results from the present work now showed that the inhibition of phagosome maturation by application of bafilomycin A_1 markedly reduced intraphagosomal killing and degradation of *E. coli* bacteria, and prevented phagocytosis-associated apoptosis to a significant degree. The expression of both constitutively active and dominant-negative functional mutants of Rab5a, surprisingly,

had no discernible effects on bacterial killing and digestion, but instead substantially enhanced phagocytosis and apoptosis.

4.1.1 Reduction in macrophage apoptosis upon inhibition of bacterial killing and degradation

Macrophages treated with bafilomycin A_1 show an reduction in their microbicidal activity towards phagocytosed *Staphylococcus aureus* and *Leishmania donovani* (Bidani et al., 2000; Duclos et al., 2000), and the same was observed with respect to the intracellular killing of *E. coli*. Bafilomycin A_1 inhibits the acidification of phagosomes (Lukacs et al., 1990), thereby reducing the acquisition of acid hydrolases, the processing of zymogens such as cathepsin D to their active form, and generally diminishing enzyme activity (Claus et al., 1998; Duclos et al., 2000; Oda et al., 1991). Accordingly, treatment of cells with pepstatin A, an inhibitor of cathepsin D, abolishes their ability to kill intraphagosomal *Listeria monocytogenes* (Prada-Delgado et al., 2005), and mycobacteria modify their host phagosomes in a way precluding the acquisition of the mature form of the enyzme (Ullrich et al., 1999)

Impaired bacterial killing may also be partly due to a reduction in the production of toxic reactive oxygen species (ROS) that has been reported in bafilomycin A_1-treated alveolar macrophages (Bidani and Heming, 1995; Bidani et al., 2000). However, although the production of ROS has been associated with the induction of apoptosis in neutrophil phagocytes (Lundqvist-Gustafsson and Bengtsson, 1999; Yamamoto et al., 2002), it is highly unlikely that a bafilomycin A_1 or ammonium chloride-induced reduction in ROS levels is also responsible for the observed reduction in macrophage apoptosis, for several reasons. Unlike granulocytes, stimulated macrophages only generate a very weak respiratory burst because they do not possess a pool of granule-localised NADPH oxidase membrane subunits (Johansson et al., 1995; Yagisawa et al., 1996). This weak burst may still contribute to some extent to the induction of macrophage apoptosis, but on its own it is clearly not sufficient: the exposure to heat-inactivated *E. coli* – which are taken up very poorly – produces an oxidative burst similar to that induced by live bacteria, but does not cause cell death (Hacker et al., 2002). Also ruled out as substantial modulator of apoptosis can be the reactive nitrogen species reported to be associated with apoptosis induction in macrophages stimulated with LPS plus IFN-γ (Albina et al., 1993), as neither bafilomycin A_1 nor ammonium chloride reduced nitric oxide production in RAW or Hoxb8 macrophages exposed to bacteria.

It thus appears that the induction of macrophage apoptosis is specifically linked to the intraphagosomal degradation of bacteria, and this hypothesis is further strengthened by the effects of Rab5a mutants described in this work.

4.1.2 Intraphagosomal destruction of extracellular bacteria does not depend on Rab5a

Several studies have demonstrated that experimental perturbations of the function of the small GTPase Rab5a can alter the survival of pathogens that replicate intracellularly. Overexpression of constitutively active Rab5a reduced the survival of *Listeria monocytogenes* (in CHO cells) and of *Leishmania donovani* (Duclos et al., 2000; Prada-Delgado et al., 2005). The treatment with Rab5a antisense oligonucleotides, or the expression of dominant-negative Rab5a enhanced survival of *Listeria monocytogenes* (Alvarez-Dominguez et al., 1996; Prada-Delgado et al., 2005), while dominant-negative Rab5a decreased the survival of *Mycobacterium* avium (Kelley and Schorey, 2004). Evidence for an active targeting of Rab5a by these pathogens comes from the finding that *Listeria monocytogenes*, *Mycobacterium bovis* BCG and *Salmonella typhimurium* cause the retention of Rab5a on phagosomal membranes in order to delay phagosome maturation (Prada-Delgado et al., 2005; Mukherjee et al., 2000; Via et al., 1997).

However, a comparative study in neutrophil phagocytes showed that in contrast to mycobacterial phagosomes, Rab5a only becomes transiently recruited to phagosomes harbouring *Staphylococcus aureus* (Perskvist et al., 2002). The results from the work presented here demonstrated that neither the expression of constitutively active or dominant-negative Rab5a mutants, nor a Rab5a knock-down had a significant effect on the killing and degradation of phagocytosed *E. coli*. Taken together, these findings indicate that while some intracellular pathogens target processes linked to Rab5, the destruction of extracellular bacteria does not critically depend on Rab5a function.

4.1.3 Perturbation of Rab5a function stimulates bacterial phagocytosis

The expression of dominant-negative Rab5a had a very strong stimulatory effect on the phagocytosis of *E. coli*, and the knock-down of Rab5a also increased bacterial uptake. One interpretation of these results is that functional Rab5a or the activation of a downstream effector pathway exerts an inhibitory effect on phagocytosis, which is repressed by the mutant

protein or by a decrease in its expression level. Intriguingly, the overexpression of constitutively active Rab5a also enhanced phagocytosis of *E. coli*, and a similar stimulatory effect has previously been shown for the uptake of latex beads (Duclos et al., 2000).

It would be difficult to understand how a dominant-negative and constitutively active mutant of the same protein can produce a similar phenotype, when effects on only the immediate downstream effector pathways are taken into account. It should be considered, however, that the mutant proteins are locked in different conformations (the GDP-bound Rab5DN versus the GTP-bound Rab5CA). These conformations particularly affect the sequestration of guanine-exchange factors (GEFs) and GTPase-activating proteins (GAP), which within the Ras superfamily are often promiscous with respect to their target GTPases (Feig, 1999). An accumulation of Rab5DN-GEF or Rab5CA-GAP complexes could therefore impinge on the activity of other small GTPases that are involved in phagocytosis. Along this line, it could be surmised that a GAP sequestered by the overexpression of Rab5CA can also bind to another small GTPase which, when remaining in its active (GTP-bound) state because its GAP is not available, exerts a stimulatory effect on phagocytosis.

What seems clear from these results is that macrophage phagocytosis is sensitive to the absolute expression level of Rab5, and the balance between its active and inactive state.

4.1.4 Quantitative link between bacterial phagocytosis and macrophage apoptosis

The elevated levels of bacterial phagocytosis in the macrophage lines expressing mutant Rab5a proteins were associated with a substantial increase in apoptosis. Lower doses of *E. coli* elicited also lower levels of cell death, and a very similar quantitative correlation was observed in wt macrophages. Interestingly, normal macrophages reached a maximum with respect to their phagocytic capacity and apoptosis induction that could not be augmented further by offering higher amounts of bacteria. The overexpression of Rab5a mutants, or Rab5a knock-down, seemed able to breach this limit and enforce higher rates of uptake.

Although it cannot be fully excluded that the expression of the tested Rab5a mutants has a direct effect on apoptosis induction, the normal response to other apoptotic stimuli such as UV irradiation or staurosporine treatment suggested that the increase in macrophage apoptosis was linked to the stimulation of phagocytosis. A non-specific death-promoting effect of the formation of giant phagosomes in these cells is also very unlikely, as

macrophages stimulated with LPS form giant vacuoles to a similar extent and did not undergo apoptosis. These LPS-induced vacuoles may derive from a promotion of endosome fusion due to signalling from Toll-like receptors. The upregulation of mutant Rab5a (or EGFP) protein expression that was observed upon LPS stimulation (not shown) could also play a role, as in cells expressing very high levels of constitutively active Rab5, a constitutive vacuole enlargement due to enhanced endosome-endosome fusion has been reported (Stenmark 1994).

The quantitative relationship between the internal bacterial load and apoptosis demonstrated in this work has also been observed in macrophages that phagocytose *Streptococcus pneumoniae* (Ali et al., 2003). In the latter study, higher levels of uptake were produced by using opsonised bacteria, but no evidence was found for the involvement of apoptosis-promoting signalling emanating from complement or Fcγ receptors. Neither does signalling from Toll-like receptors play a substantial role in cell death induction, as macrophages derived from mice deficient in the signalling adaptor protein Myd88 are not protected from apoptosis after uptake of *E. coli* (Hacker et al., 2002). By contrast, cell death appears slightly enhanced in these cells, and one study reported that the suppression of signalling via NFκB (which is induced by TLR activation and largely dependent on Myd88 (Akira et al., 2006)) actually increases phagocytosis-induced macrophage apoptosis (Groesdonk et al., 2006). It thus appears that intracellular events directly associated with the breakdown of intraphagosomal bacteria are responsible for the activation of death-inducing pathways.

It can be assumed that the incidence of these degradation events is increased in macrophages expressing mutant Rab5, because bacterial breakdown proceeded normally in these cells. Since treatment with bafilomycin A_1 inhibited degradation and reduced apoptosis, the most straightforward interpretation may be that phagocytosis-induced apoptosis is related to the accumulation of a very late, probably terminal phagosome maturation stage. Inhibition of phagosomal maturation by bafilomycin A_1 would thus prevent the progression to this late phagolysosomal stage and reduce apoptosis (Figure 32).

Several studies have implicated lysosomal proteases (which also become delivered to phagolysosomes) leaking into the cytosol upon organelle permeabilisation in the activation of apoptosis-inducing pathways (Stoka et al., 2007). Albee et al. reported that treatment with pepstatin A, an inhibitor of aspartic proteases including the cathepsin family, decreases macrophage death upon phagocytosis of *E. coli*, concomitant with a reduction in cathepsin D and caspase-3 activation (Albee et al., 2007). Although this also fits in with the model of

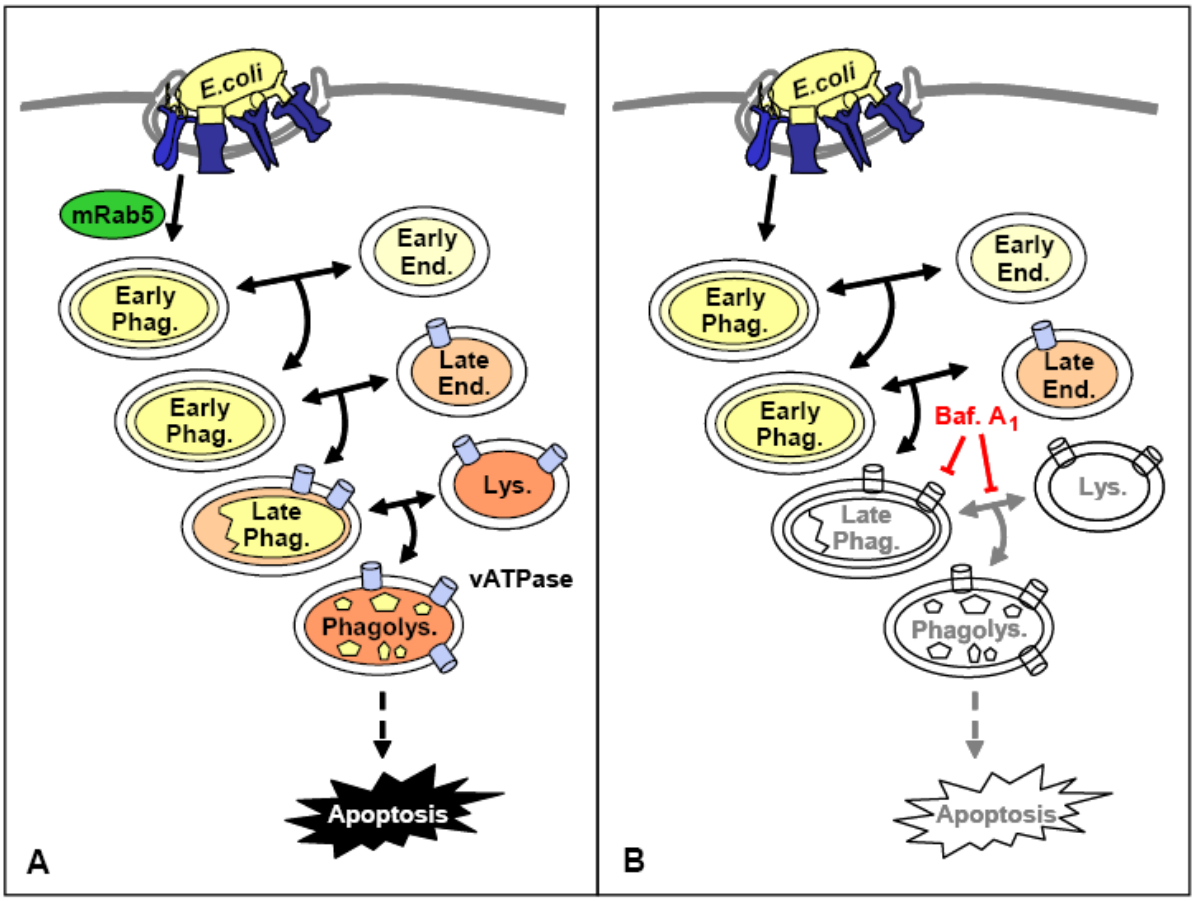


Fig. 32 Influence of mutant Rab5a proteins and bafilomycin A_1 on macrophage apoptosis

After phagocytosis, nascent phagosomes engage in a series of sequential fusion events with early, late endosomes, and lysosomes. Phagosome maturation to the phagolysosomal stage through the acquisition of hydrolases and acidifying vATPases causes bacterial killing and breakdown. **A**. The overexpression of constitutively active or dominant-negative Rab5a enhances phagocytosis while not affecting bacterial killing and breakdown. The accumulation of phagosomes of a late maturation stage or associated downstream events enhance macrophage apoptosis. **B**. Treatment with the vATPase inhibitor bafilomycin A_1 inhibits maturation to the phagolysosomal stage and reduces apoptosis.

degradation-dependent macrophage apoptosis presented here, the authors linked the protection from cell death at least partially to the lack of cathepsin-induced direct activation of caspase-3, implying (phago)lysosomal permeabilisation and liberation of proteases into the cytosol in the first place. Such a release has been reported in neutrophil phagocytes, where cathepsin-D has been observed to leak out from granules during constitutive apoptosis, and suggested to cause processing of caspase-8 (Conus et al., 2008). A potential analoguous

liberation of cathepsins or other proteases from phagolysosomes into the cytosol has not been analysed in this work, yet it would have to involve an alternative pathway because the cleavage of caspase-8 is not observed during bacterial phagocytosis (Hacker et al., 2002).

4.1.5 Mitochondrial translocation of the BH3-only protein Bim during phagocytosis-induced cell death

Macrophage deficient in the pro-apoptotic protein Bim are protected to a large degree from apoptosis induced by the phagocytosis of *Escherichia coli*, demonstrating that the protein is involved in this death (Kirschnek et al., 2005). Fractionation experiments had strongly suggested that Bim protein upregulated by the exposure to bacteria translocates to mitochondria, and an analysis by confocal microscopy carried out in the present study confirmed that the protein indeed accumulates at this organelle upon apoptosis induction.

Intriguingly, the stimulation of macrophages with LPS alone, which does not induce apoptosis, also upregulated the expression of Bim and caused translocation of the protein to mitochondria. Therefore, these events on their own are not sufficient for apoptosis induction by Bim. A second signal is required for its activation, which must derive from events associated with phagocytosis and bacterial breakdown.

Some differences had been noticed regarding the mitochondrial translocation of individual macrophage Bim isoforms in previous fractionation experiments. Whereas (uninduced) BimL was present mainly in the mitochondrial fraction, BimEL was found exclusively in the high molecular weight/cytoskeletal fraction (Kirschnek et al., 2005). Upon stimulation with *E. coli* or LPS, BimL relocalised to and accumulated at mitochondria, whereas BimEL largely remained in the cytoskeletal fraction (Kirschnek et al., 2005). Confocal microscopy analysis revealed a similar behaviour of the two long Bim isoforms in a different cellular system, where the expression of single isoforms could be induced by tetracyclin in HeLa cells. In these cells, overexpressed BimL localised predominantly to mitochondria, while overexpressed BimEL appeared to remain partly bound to microtubules. Because mitochondrial translocation is a prerequisite for cell death induction (discussed below), these findings suggest that the apoptosis-inducing activity of Bim in macrophages appears to be mediated primarly by the BimL isoform.

What regions of Bim are required for mitochondrial translocation? One isoform of Bim that is – by all evidence available – not subject to any posttranslational modifications and upon overexpression very potently and rapidely induces apoptosis in HeLa cells, is BimS (Weber et al., 2007). BimS immediatly accumulates at mitochondria when overexpressed and inserts into the outer mitochondrial membrane (Weber et al., 2007). However, a mutants that lacks the C-terminal transmembrane domain fails to do so, and does not longer induce cell death. The attachment of the mitochondrial tail anchor sequence of yeast Tom5 to this mutant restores mitochondrial localisation and apoptosis. These findings indicate that the mitochondrial translocation of Bim is necessary for its apoptosis-inducing activity, that translocation is mediated by the C-terminal transmembrane domain (which is also present in BimL and BimEL), and suggests that the C-terminus has no function other than mitochondrial targeting.

Such a dependency of the pro-apoptotic activity on mitochondrial localisation has also been noticed for the BH3-only protein Bid. The death-inducing potency of its cleavage product tBid is further enhanced by N-myristoylation, which targets it to the outer mitochondrial membrane (Zha et al., 2000), and the tethering of tBid to membranes strongly increases the activation of Bax on liposomes and its ability to permeabilise the membrane (Oh et al., 2006). Of the remaining BH3-only proteins, only Puma possess a consensus sequence for targeting to the outer mitochondrial membrane in its C-terminal domain (Weber et al., 2007). The activity of the three most potent “activators” of the intrinsic apoptosis pathway – Bim, Bid and Puma – therefore appears to be regulated no only by their interaction with Bcl2-like or Bax-like proteins, but also by their ability to translocate to mitochondrial membranes.

In conclusion, the findings in this work concerning the apoptosis of macrophages have demonstrated that the phagocytosis-induced death of these phagocytes is connected to the uptake, and intracellular killing and degradation of *Escherichia coli* bacteria. This connection was quantitative, and required a relatively large number of bacteria to be internalised during a prolonged period of phagocytosis. But even high doses of extracellular bacteria did not induce the death of all macrophages, likely due to the limitation of their phagocytic capacity to a level that within a macrophage population may allow an appropriate balance between efficient killing and removal of pathogens through self-sacrifice, and survival. A breaching of this “barrier” by enforced overstimulation of phagocytosis (as induced by expression of mutant Rab5a proteins) may shift this balance to predominantly apoptosis. It would be interesting to

see whether in an *in vivo* infection model involving extracellular bacteria, such enforced phagocytosis would be detrimental to the immune response and the host.

The protection from apoptosis elicited by the inhibition of phagosome maturation linked events associated with the phagosomal degradation of bacteria to apoptosis induction. The exact nature of these events remains unknown, although the generation of reactive oxygen or nitrogen species could be excluded. An involvement of the BH3-only protein Bim in phagocytosis-associated macrophage apoptosis had been shown previously (Kirschnek et al., 2005), and data presented here strengthens the view that upregulation and mitochondrial translocation of Bim is required but not sufficient for cell death induction, implying post-translational modifications of the protein.

Yet Bim only accounts for about half of this death (Kirschnek et al., 2005). During this work, Hoxb8 macrophages overexpressing Bcl-2, or being deficient in Bim or Puma were also generated. The next step of this project should involve the analysis of these cell lines to verify the role and relative contribution of the two BH3-only proteins in macrophage death. The reconstitution of single Bim isoforms into Bim-/- cells would answer the question whether BimL is in majority responsible for apoptosis induction, and mutations in particularly the extra domain of BimEL may deliver insights into the mechanism by which this isoform is sequestered to microtubules. Furthermore, reconstitution experiments with versions of Bim or Puma bearing mutations in their BH3 domain, through which interactions with Bcl2-like and possibly also Bax-like proteins are mediated, will help to decipher the pattern and relevance of interactions taking place within the Bcl-2 family during phagocytosis-induced macrophage apoptosis.

4.2 Bcl-2 family proteins involved in neutrophil apoptosis

The death of neutrophils is tightly regulated, as their short lifespan must be prolonged during acute infection, while at the same time allowing a rapid switch to apoptosis once the pathogen is cleared to avoid excessive tissue damage. In this work, several members of the Bcl-2 family involved in the regulation of the mitochondrial pathway were identified to mediate spontaneous neutrophil apoptosis *in vitro*, among them the pro-apoptotic BH3-only proteins Bim and Puma, and possibly the anti-apoptotic protein A1.

4.2.1 Bim and Puma are the major inducers of spontaneous neutrophil apoptosis

Neutrophils overexpressing Bcl-2 were almost completely protected from ageing-associated apoptosis, indicating that the Bcl2-dependent part of the intrinsic pathway mediates the vast majority of this cell death. About half of this protection was also conferred by the loss of Bim, marking this BH3-only protein as the major inducer of spontaneous neutrophil apoptosis.

Interestingly, the additional absence of Puma enhanced protection further only during ageing in the absence, but not in the presence of stem cell factor (SCF), suggesting that signalling from the SCF receptor may contribute to the regulation of Puma activity. How the activity of this BH3-only protein is controlled is not known. During SCF withdrawal, a slight increase in the expression level of Puma was noticable, and also the appearance of a slightly higher molecular weight form, suggesting phosphorylation of the protein. The behaviour of Puma during neutrophil ageing in the presence of SCF was not analysed, but a lack of upregulation and/or possibly phosphorylation would suggest that the activity of Puma is regulated also on this level.

An upregulation and potential phosphorylation during SCF-withdrawal was also noticed for the two Bim isoforms BimL and BimEL, but whether they promote the pro-apoptotic activity of Bim and are required for apoptosis induction is unclear at present. In neutrophils stimulated with LPS, BimL and BimEL have also been observed to become upregulated, and similar higher molecular weight forms that suggest phosphorylation appeared, in particular with respect to BimEL (Bauer et al., 2007). On one hand this may indicate that the upregulated (and potentially phosphorylated) Bim was not active, on the other hand higher levels of active Bim may also have been neutralised by the observed co-upregulation of A1 and Mcl-1 expression (Bauer et al., 2007).

Present evidence suggests that at least the phosphorylation of the protein mediated by extracellular signal-regulated kinase (ERK) is not involved in regulating Bim activity. Several studies investigating altogether five serine phosphorylation sites on Bim (four of them unique to BimEL but one also present in BimL) have associated ERK-mediated phosphorylation of the protein rather with increased proteasomal degradation, than enhanced pro-apoptotic potency (Hacker et al., 2006; Hubner et al., 2008; Ley et al., 2003, 2005; Luciano et al., 2003). Although the levels of Bim protein (but not mRNA) have been reported to increase in dependence of a reduction of ERK activity in monocyte/macrophage-derived osteoclasts

deprived of macrophage colony-stimulating factor (Akiyama et al., 2003), pharmacological inhibition of the kinase does not affect spontaenous apoptosis of human or murine neutrophil (Aoshiba et al., 1999; Villunger et al., 2000).

The combined absence of Bim and Puma could still not confer the same level of protection from apoptosis as that offered by the overexpression of Bcl-2, suggesting that further BH3-only proteins become activated. Bmf has been found to accumulate in ageing neutrophils *in vitro* (Villunger et al., 2003b), but knock-out mice do not show significant abnormalities in their haematopoietic system (Labi et al., 2008). Neither is granulopoiesis affected in mice deficient in Noxa, Bad, Bik, Hrk, or Bid (Coultas et al., 2005; 2007; Kaufmann et al., 2007; Ranger et al., 2003; Villunger et al., 2003a; Yin et al., 1999). One explanation may be that a contribution of these BH3-only proteins on its own is too weak to become noticable. They may act in concert though, and the co-overexpression of Noxa and Bad, for example, has been shown to induce still significant apoptosis in cells deprived of tBid, Bim, and Puma (Kim et al., 2006). In addition, the loss of an anti-apoptotic protein may contribute to apoptosis induction, and in all neutrophil lines within the Hoxb8 panel, such a loss – that of A1 – was indeed observable.

4.2.2 Anti-apoptotic A1 but not Mcl-1 is lost during neutrophil ageing

A1 has a clear pro-survival function in neutrophils, because cytokines or bacterial products that promote granulocyte survival also cause an upregulation of its expression (Baran et al., 1996; Bauer et al., 2007; Colotta et al., 1992; Cox et al., 1992; Chuang et al., 1998; Francois et al., 2005), and this apoptosis inhibition is reduced in A1-deficient neutrophils (Hamaski et al., 1998). The observed kinetics of the disappearance of A1 upon stem cell factor deprivation in Hoxb8 neutrophils, i.e. before the onset of significant apoptosis, together with the fact that apoptosis is accelerated in neutrophils lacking A1 (Hamasaki et al., 1998) suggests that this loss is of physiological relevance.

It is generally agreed that anti-apoptotic proteins confer protection by acting as a "sink" for activated BH3-only proteins. Bcl-xl and Mcl-1 in addition exert protection – at least from "sensitiser" BH3-only proteins – by sequestering pro-apoptotic Bak (Willis et al., 2005). Whether A1 can also directly inhibit the activation of Bak is still a matter of debate. One study has reported a weak binding of human A1 to detergent-activated Bak (Simmons et al., 2008). An interaction between mouse A1 and Bak, however, was not detectable (Willis et

al., 2005). This discrepancy may also be related to differences in the C-terminal sequence between the human and murine form. The C-terminus of human A1 appears to at least partially direct the protein to membranes, as the protein is associated with the mitochondrial outer membrane to some extent (Duriez et al., 2000; Simmons et al., 2008; Werner et al., 2002). The analogous region of murine A1, by contrast, appears not have this ability. Proteins fused to the C-terminal domain of Bcl-xl, for example, can be redirected to mitochondria (Kaufmann et al., 2003), but not proteins fused to the C-terminus of A1 (Herold et al., 2006; Kaufmann et al., 2003). Also, the death-inducing overexpression of Bak cannot be inhibited by murine A1 (but by Bcl-xl) (Holmgreen et al., 1999).

These findings question whether a sequestration of Bak by A1 does contribute to the promotion of neutrophil survival, and rather favour a protective effect by way of acting as a "sink" for BH3-only proteins. But does the loss of A1 set free BH3-only proteins, or does the upregulation of a BH3-only protein induce the degradation of A1, as has been reported in some cellular settings for Mcl-1 bound by Noxa (Czabotar et al., 2007; Lee et al., 2008; Willis et al., 2005)? This question is particularly relevant in the Bim/Puma-/- neutrophils, where residual cell death may critically depend on the A1-Noxa axis. Because an anti-apoptotic effect of A1 on the basis of Bak sequestration seems unlikely, a loss of A1 would only promote apoptosis induction if this loss was upstream of Noxa, and not downstream, i.e. a liberated Noxa would contribute to the induction of apoptosis by sequestering Mcl-1 away from Bak. Such a displacement has been observed in cells that upregulate Noxa in response to UV irradiation, and also in cells infected with adenovirus E1A where apoptosis induction involves the displacement of Mcl-1 from Bak by the viral protein E1A (Cuconati et al., 2003; Willis et al., 2005). The amount of Noxa freed in this way may be considerable, as the only other Bcl2-like protein binding to Noxa is Mcl-1 (Chen et al., 2005), and a significant part of Mcl-1 will be tied up by its interaction with Bak.

In support of such a model, an upregulation of Noxa mRNA has not been observed during ageing-associated apoptosis of murine bone-marrow derived neutrophils (Villunger et al., 2003b). Furthermore, blocking transcription by treatment with actinomycin D causes a rapid loss of A1 mRNA (but not Bcl-xl mRNA), indicating that transcripts are very short-lived (Moulding et al., 2001). As A1 is subject to a rapid constitutive turnover by proteasomal degradation (Herold et al., 2006; Kucharczak et al., 2005), transcriptional shutdown would rapidely decrease protein levels. Although no data are yet available that would confirm the loss of transcription upon stem cell factor withdrawal, it has been shown that A1 mRNA is

upregulated by cytokines or TLR ligands that enhance neutrophil survival (Chuang et al., 1998; Colotta et al., 1992; Cox et al., 1992; Kobayashi et al., 2003; Moulding et al., 2001).

Another issue that should be considered in this context concerns the binding spectra of A1 and Bcl-2. Because Bcl-2 does not bind Noxa (Chen et al., 2005), the question arises whether overexpression of the former would be able to compensate for the loss of A1. Most likely this would still be the case, as Noxa on its own appears unable to induce neutrophil death, and all of its potential collaborator BH3-onlies can be bound by Bcl-2 (Chen et al., 2005).

With respect to the identity of the other BH3-only protein inducing apoptosis in concert with Noxa in Bim-Puma double-deficient cells, Bad seems to be a promising candidate. Although its upregulation has not been observed upon ageing of bone-marrow derived neutrophils (Villunger et al., 2003b), increased protein levels may actually not be required to activate the protein. Bad is kept inactive by constitutive phosphorylation in response to growth receptor signalling (Zha et al., 1996), and phosphorylation of the protein has been reported to be increased by TLR agonists that prolong the lifespan of neutrophils (Francois et al., 2005). Accordingly, growth factor withdrawal may cause hypophosphorylation and activation of Bad.

Apoptosis induction upon ageing was not accompagnied by a disappearance of Mcl-1 in any Hoxb8 neutrophil line. This was surprising, as loss of this anti-apoptotic protein has been reported in cultured human peripheral blood neutrophils (Moulding et al., 1998; 2001). The discrepancy may be due to differences between human and murine neutrophils, or particularities of the Hoxb8 system with respect to the expression of Mcl-1. Apoptotic wt and Bim-/- Hoxb8 neutrophil cultures were marked by the appearance of two lower molecular weight bands of Mcl-1 of ~25 and 15 kD. A splice variant of Mcl-1 of a similar size as the larger band, Mcl-1S (26 kD), has been reported to be expressed in several cell types of the human haematopoietic lineage, including blood neutrophils (Bingle et al., 2000). It lacks the second exon and therefore the BH1 and BH2 domains, and has a frame shift-induced alternate C-terminus. Upon overexpression in human lung epithelial cells (A549 cell line) or Chinese Hamster Ovarian (CHO) cells, Mcl-1S promotes cell death (Bingle et al., 2000).

However, because the 25 kD band did not appear in ageing Bim/Puma-/- and Bcl-2 neutrophils that are largely resistant to apoptosis induction, a putative generation of Mcl-1S would require an apoptosis-induced upregulation of alternative splicing activity towards Mcl-1 mRNA. Yet when transcript levels of both Mcl-1 and Mcl-1S were compared in a human

myeloid leukaemia line upon apoptosis induction via staurosporine, etoposide, or serum deprivation, their ratio did not change (Bae et al., 2000). Also, the lack of disappearance of the 25 kD form (as opposed to full-length Mcl-1) in consequence of a strongly increased protein stability can be ruled out, as both Mcl-1 and Mcl-1S display similar instabilities when expressed in CHO cells (Bae et al., 2000).

An alternative explanation is that the lower molecular weight forms of Mcl-1 represent breakdown products of the protein. This was supported by the observation that their appearance could be blocked by the application of the caspase inhibitor zVAD. Indeed, *in vitro* translated murine Mcl-1 that is incubated with extracts from apoptotic cells has been shown to be cleaved into two fragments comprising 26 and 14 kD, respectively (Clohessy et al., 2004). Treatment with zVAD but not with cathepsin and proteasome inhibitors abolishes cleavage, and the incubation of human Mcl-1 with recombinant caspase-3 produces fragments of identical sizes (Clohessy et al., 2004; Michels et al., 2004). All these findings indicate that caspase activation in already apoptotic neutrophils leads to partial Mcl-1 degradation.

Caspase-mediated cleavage has also been reported for Bcl-2 and Bcl-xl in certain conditions, creating fragments that were pro-apoptotic upon overexpression (Cheng et al., 1997; Clem et al., 1998; Kirsch 1999). Ectopic expression of the corresponding Mcl-1 fragment induces apoptosis in MEF cells (Michels et al., 2004), but not in murine myeloid progenitor cells (Clohessy et al., 2004). The differences may relate to varying expression levels. Even if the Mcl-1 fragment produced in Hoxb8 neutrophils was pro-apoptotic, it would only be able to reinforce apoptosis, but no act as an initiator.

Increased expression of Bim or Puma has been reported to stabilise Mcl-1 levels (Czabotar et al., 2007; Mei et al., 2005; Wuilleme-Toumi et al., 2007), possibly by masking lysine residues, or by displacing the Mule E3-ligase complex known to ubiquitinate Mcl-1 (Zhong et al., 2005). Although such a stabilisation might theoretically explain the observed lack of Mcl-1 loss in the present work, it does not take place in this system since Mcl-1 levels do not decline in Bim-Puma double-knock out cells. It thus appears that Mcl-1 does not play a direct role in ageing-associated Hoxb8 neutrophil apoptosis. Hypothesising that a disappearance of Mcl-1 would result from an upregulation of Noxa as has been shown before (Czabotar et al., 2007; Lee et al., 2008; Willis et al., 2005), stable Mcl-1 levels may further indicate that Noxa levels do not increase during neutrophil apoptosis, and that Noxa activation requires the loss of A1.

4.2.3 Bcl2-independent neutrophil apoptosis

Hoxb8 neutrophils overexpressing Bcl-2 still underwent spontaneous apoptosis to a small but significant extent. Although this death might also be a consequence of the loss of A1, the model concerning the mode of action of A1 described above suggests that is does not.

Spontaneous neutrophil apoptosis has also been associated with the activation of caspase-8 (Conus et al., 2008; Maianski et al., 2004; Scheel-Toellner et al., 2004). Although the generation of cleaved Bid, which can be neutralised by Bcl-2, has also been observed in some of these studies (Maianski et al., 2004; Scheel-Toellner et al., 2004), direct processing of the effector caspase-3 by caspase-8 is not inhibitable by anti-apoptotic proteins. One study linked the activation of caspase-8 to the leakage of cathepsin-D from acidic granules, with caspase processing taking place probably in microenvironments of lower pH around the permeabilised granules, in which cathepsin activity would be at least partially conserved (Conus et al., 2008). Another report proposed an activation of caspase-8 due to spontaneous clustering of Fas receptors in ceramide-rich lipid rafts that is driven by an upregulation of ceramide production (Scheel-Toellner et al., 2004).

The investigators also showed that the levels of reduced glutathione, a powerful antioxidant, fell early during neutrophil culture, suggesting enhanced consumption by a rise in reactive oxygen species (ROS), or alternativey by virtue of gluthatione downregulation, allowing increased ROS generation. The application of ROS scavengers or of inhibitors of the membraneous NADPH oxidase mediating ROS production, reduced cathepsin-D leakage, and Fas receptor clustering, respectively, as well as cathepsin-8 activation, and was associated with a reduction of apoptosis to some extent. Also, neutrophils from patients with chronic granulomatous disease that is caused by a genetic deficiency in membrane-associated NADPH oxidases, show delayed constitutive apoptosis (Conus et al., 2008; Kasahara et al., 1997; Scheel-Toellner et al., 2004). Although it remains molecularly unexplained why the redox state changes during the life-span of neutrophils, these observations suggest that an ensuing caspase-8 activation may be responsible for the Bcl-2 independent death in Hoxb8 neutrophils.

Taken together, the obtained results demonstrate that constitutive neutrophil apoptosis is driven by the activation of Bim and Puma, and also suggest a contribution of the loss of A1. A minor part of this death is not inhibitable by Bcl-2, and may be caused by caspase-8

activation. A model of the proposed molecular interactions taking place during apoptosis induction is presented in Figure 33.

Several experiments would lend themselves to verify the involvement of A1 in ageing-associated neutrophil apoptosis in the absence of stem cell factor, and to test its proposed mode of action. First, the knock-down of A1 in wt neutrophils should enhance apoptosis. Conversely, an overexpression of A1 in Bim/Puma-deficient neutrophils should enhance protection, and if this protein is the only factor constituting the protection "gap" to Bcl-2 overexpression, then neutrophils overexpressing A1 should be protected to a similar extent as *bcl2*-transgenic neutrophils. Whether the loss of A1 is indeed induced by transcriptional downregulation could be verified in a quantitative RT-PCR of A1 transcripts during stem cell factor withdrawal. A possible alternative, the promotion of the proteasomal degradation of A1 by Noxa, would be excluded by the finding that A1 is still lost in Bim/Puma-/- neutrophils where Noxa has been elimiated by RNA interference knock-down. The proposed liberation of Noxa from its sequestration to A1 could be verified by immunoprecipitation, in this case the levels of A1 bound to Noxa should decrease upon factor deprivation.

Further downstream, according to the presented model, in Bim/Puma-/- neutrophils, freed Noxa would cause the activation of a Bax-like protein in concert with another activated BH3-only "sensitiser" protein. Bad may assume this function, and because it is only active in a hypophosphorylated state, it would be worth checking whether stem cell factor withdrawal in neutrophils induces Bad dephosphorylation. In the absence of activator BH3-only proteins such as Bim and Puma, Noxa and its cooperator most likely induce apoptosis through the activation of Bak via displacement of Mcl-1 and Bcl-xl. Accordingly, a knock-down of Bak should render neutrophils deficient in Bim and Puma as insensitive to apoptosis induction as neutrophils overexpressing Bcl-2.

In addition, the Bim/Puma-deficient neutrophil line offers an excellent opportunity for reconstitution experiments with Bim or Puma. In this way, it could be analysed whether apoptosis is induced by specific isoforms of the proteins, and mutagenesis could identify relevant domains and residues.

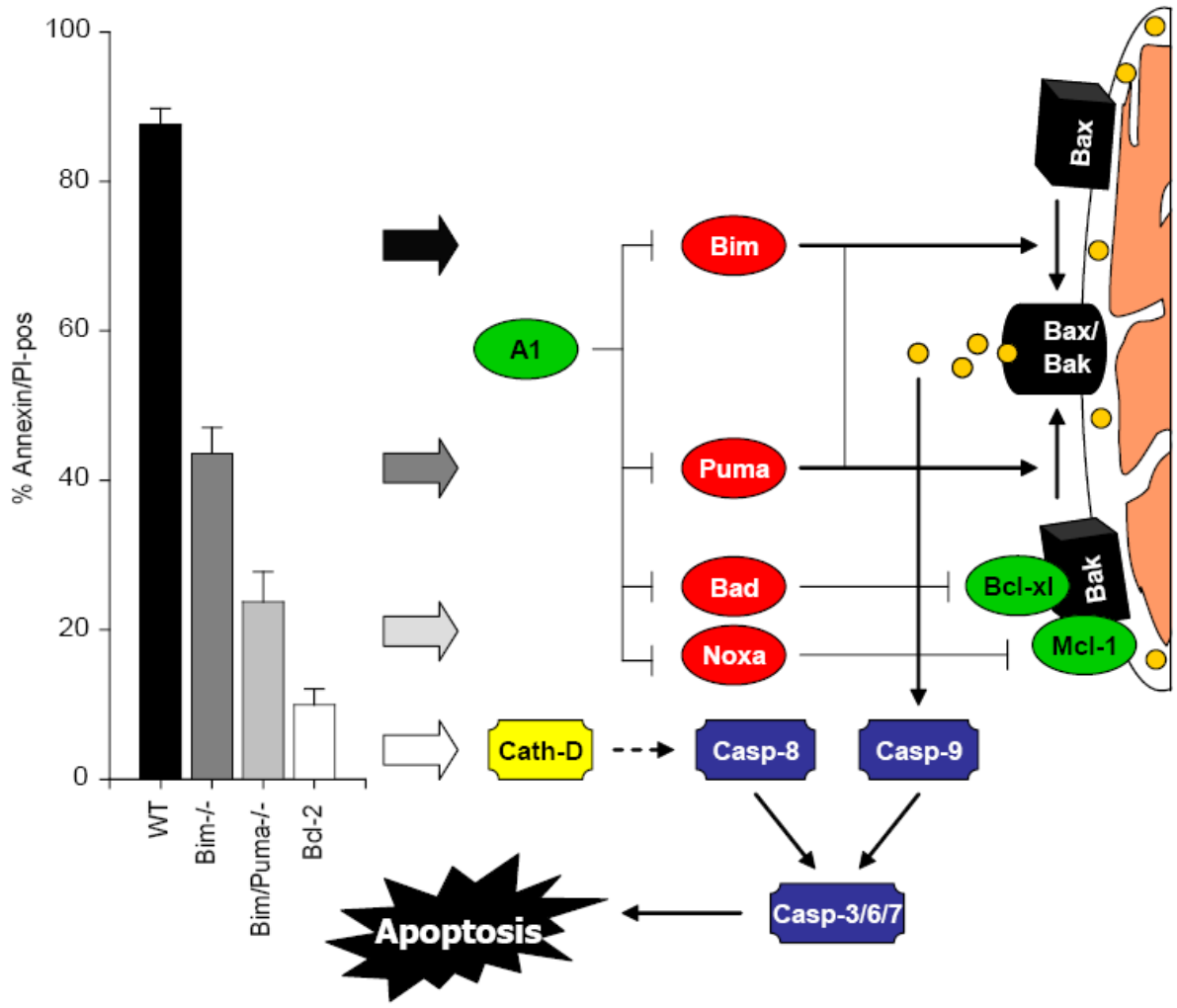


Fig. 33 Proposed model of protein interactions during induction of neutrophil apoptosis

The bar diagram on the left shows the extent of protection from stem cell factor withdrawal-induced neutrophil apoptosis conveyed by loss of the pro-apoptotic BH3-only proteins Bim, or Bim and Puma, and by overexpression of the anti-apoptotic protein Bcl-2 (apoptosis measured by Annexin/PI-positivity after 24 hours, reproduced from Figure 23). Bim and Puma are most likely able directly to activate Bax and/or Bak, whether they themselves require post-translational modifications to become active is not known at present. The involvement of Bad and Noxa is speculative but may explain why there is still significant death in neutrophils lacking both Bim and Puma. According to the proposed model, both proteins cause the activation of Bak via displacement of Bcl-xl and Mcl-1 in these cells. Bad may be activated by inactivation of a kinase (causing hypophosphorylation), and Noxa may become active by the downregulation of A1 expression (causing liberation from Noxa-A1 complexes). In wt cells, the loss of A1 may also cause enhanced levels of free Bim and Puma. A small part of neutrophil death cannot be inhibited by the overexpression of Bcl-2, for which the activation of caspase-8 via the protease cathepsin-D may be responsible. Cathepsin-D has been suggested to leak out of granules as neutrophils age.

4.3 The role of apoptosis in the shutdown of neutrophil immune functions

Neutrophils that die at sites of infection in the termination phase of the immune response are subsequently removed by macrophages. By all evidence available, this death does not differ from the one observed during constitutive neutrophil apoptosis. Granulocytes in inflamed tissues eventually undergo apoptosis *in situ* as judged by morphological appearance (Cox et al., 1995; Grigg et al., 1991; Savill et al., 1989, 1991, 1992). Cultured inflammatory neutrophils undergo apoptosis even slightly faster than resting or circulating neutrophils, and Bim-deficiency partially protects from this death (Savill et al., 1989; Villunger et al., 2000; 2003b).

Phagocytosis of apoptotic neutrophils promotes deactivation of the phagocytes and the release of anti-inflammatory cytokines, thereby promoting the resolution of inflammation. In this work it was shown that in contrast to apoptotic wt cells, neutrophils overexpressing Bcl-2 that fail to undergo apoptosis are not removed by macrophages. Importantly, these "undead" neutrophils did not become senescent, but retained the ability to phagocytose bacteria, produce an oxidative burst and secrete IL-1β under conditions of prolonged SCF-withdrawal. This suggested that, at least *in vitro*, there is no mechanism in place other than apoptosis that shuts down the immune functions of neutrophils, and that neutrophils kept alive and active by prevention of apoptosis may cause hyperinflammation and damage to host cells in infected tissues.

In order to corroborate these findings in an *in vivo* setting, the collaborating group of U. Koedel (Ludwig-Maximilian-Universität München) carried out a study on meningitis induced experimentally by infection with *Streptococcus pneumonia* (*S. pneumonia*), in mice overexpressing Bcl-2 in the haematopoietic compartment. Bacterial meningitis was chosen as experimental model because the initially uncontained bacterial replication in the cerebrospinal fluid (CSF) causes a very strong inflammatory innate immune response that is driven mainly by neutrophils infiltrating across the blood-brain barrier (Koedel et al., 2002a). These neutrophils are thought to be responsible to a significant degree for the secondary brain damage often associated with meningitis (Tuomanen et al., 1989; Weber et al., 1997). Accordingly, despite the antibiotic treatment of patients, mortality from *S. pneumonia*-induced meningitis is high (20-35%), and one-third of survivors are suffering from sequelae of the brain damage inflicted (Bohr et al., 1983; de Gans et al., 2002; van de Beek et al.,

2002). In the experimental model used by U. Koedel and co-workers, *S. pneumoniae* are injected into the cisterna magna. Following treatment with antibiotics after 24 hours, the inflammation begins to resolve and apoptosis of neutrophils can be readily observed *in situ*, peaking 48 hours after infection (Klein et al., 2006).

4.3.1 Bcl-2 induced protection from apoptosis induction conserves neutrophil effector functions and inhibits neutrophil clearance *in vitro* and *in vivo*

Apoptotic neutrophils are disposed of by macrophages, and clearance critically depends on the extent of apoptosis of the target cells (Savill et al., 1989). An earlier study had surprisingly found that inflammatory neutrophils from *bcl2*-transgenic mice (Bcl-2 expression was restricted to the myeloid lineage) failed to undergo spontaneous apoptosis *in vitro* but were still taken up to significant degrees by inflammatory macrophages, suggesting that a process of neutrophil ageing exists that produces "eat-me" signals independently of apoptosis (Lagasse et al., 1994). In the system used in this work, neutrophils overexpressing Bcl-2 were equally protected from apoptosis, but even after prolonged periods of growth factor withdrawal were not taken up by macrophages.

These differences are unlikely to relate to the fact that in the former study, the interaction between macrophages and neutrophils were investigated using cells isolated from an inflammatory environment *in vivo*. A study by Savill et al. has shown that apoptotic neutrophils are taken up with similar kinetics by macrophages derived from inflamed sites, or by blood monocyte-derived macrophages (Savill et al., 1989). In support of a defective neutrophil clearance on the basis of blocked apoptosis, 72 hours after infection with *S. pneumonia*, the leukocyte count in the CSF of *bcl2*-transgenic mice had dropped only by 35%, compared to 85% in wt mice. Initital leukocyte recruitment was similar, and since infiltration ceased after antibiotic treatment, only extended survival in association with impaired removal can account for the higher cell count in the transgenic animals.

The phagocytosis of apoptotic neutrophils usually triggers the production of anti-inflammatory transforming growth factor (TGF)-β by macrophages (Fadok et al., 1998). Therefore, the fact that the level of TGF-β in the brain of *bcl2*-transgenic mice was not elevated ~10-fold from 24 – 48 hours post-infection as in wt animals but almost stayed at base levels, can very likely be attributed to the absence of neutrophil clearance in the mutant animals. TGF-β has an anti-inflammatory effect by suppressing the secretion of pro-

inflammatory cytokines and by inhibiting neutrophil infiltration (Byrne et al., 2002; Fadok et al., 1998; Kim et al., 2004; Malipiero et al., 2006), and its exogenous administration has been shown to dampen early meningeal inflammation (Pfister et al., 1992). On the opposide side of the cytokine profile, *bcl2*-transgenic mice showed much higher residual levels of the pro-inflammatory cytokines G-CSF and IL-1β in the brain. Neither cytokine appears to be involved in neutrophil recruitment across the blood-brain barrier (Metcalf et al., 1996; Saukkonen et al., 1990; Zwijnenburg et al., 2003), but G-CSF enhances granulocyte survival (Colotta et al., 1992), and IL-1β promotes the production of other pro-inflammatory cytokines such as TNF-α and IL-6 (Zwijnenburg et al., 2003).

In models of endotoxin-induced acute inflammatory lung injury, infiltrating neutrophils represent a major souce of IL-1β in the lung (Parsey et al., 1998), and *in vitro* experiments conducted during this work showed that secretion of this cytokine from Hoxb8 neutrophils could be stimulated by exposure to *S. pneumoniae*. Neutrophils overexpressing Bcl-2 and deprived of stem cell factor for 72 hours retained the ability to secrete IL-1β, suggesting that the augmentation of pro-inflammatory cytokines observed during meningitis *in vivo* is not only due to a lack of TGF-β production, but also to continued neutrophil activity. In addition, Bcl2-overexpressing neutrophils remained proficient at executing respiratory bursts and phagocytosis of bacteria. Also these effector functions are likely to contribute to damage of host tissue, as the pharmacological induction of granule release during meningitis exacerbates brain damage (Tauber et al., 1988).

4.3.2 Aggravation of meningitis in Bcl-2 mice and alleviation by pharmacological killing of neutrophils

The continued presence and activity of neutrophils in *bcl2*-transgenic mice was associated with an strong increase in tissue damage characterised by a much higher number and severity of local bleedings in the cortex of the brain 72 hours after infection. The course of the disease was also strongly aggravated, as indicated by a clinical score twice as high as in wt animals, and the fact that 3 out of 12 transgenic mice died during the experiments, while all wt mice survived and eventually recovered.

All these results strongly suggest that the hyperinflammation, and more severe tissue damage and disease observed in *bcl2*-transgenic animals is caused by the sustained presence and action of neutrophils in the brain. Accordingly, in wt mice the course of the disease could

be ameliorated by treatment with roscovitine, a pharmacological agent that has been shown to promote the resolution of inflammation in other models of neutrophil-dependent inflammation by inducing granulocyte death (Rossi et al., 2006). Roscovitine is an inhibitor of cyclin-dependent kinase 1, and its death-promoting effect can be inhibited *in vivo* and *in vitro* by caspase inhibitors, demonstrating that it induces apoptosis (Rossi et al., 2006). When the drug was tested on Hoxb8 neutrophils, a dose-dependent apoptosis induction in wt cells could be confirmed. However, apoptosis induction was blocked in Bcl2-overexpressing neutrophils, indicating that roscovitine primarily engages the mitochondrial pathway.

In conclusion, this work has demonstrated that the inhibition of neutrophil apoptosis preserves neutrophil effector functions. This strongly suggests that the promotion and sustainment of inflammation observed during experimental meningitis is not only caused by a lack of anti-inflammatory phagocytotic clearance of neutrophils, but also through their continued execution of immune functions. It is plausible that apoptosis represents the only safeguard against neutrophil-mediated hyperinflammation, a safeguard that is likely to be circumvented also in acute inflammatory conditions and disorders such as sepsis, the systemic inflammatory response syndrome, or inflammatory bowel disease in consequence of an excessive production of neutrophil survival-promoting cytokines (Ertel et al., 1998; Ina et al., 1999; Jimenez et al., 1997).

The pharmacological induction of neutrophil apoptosis can promote the resolution of inflammation, as shown in this study and in others (Rossi et al., 2006; Sawatzky et al., 2006). Drugs such as roscovitine that induce neutrophil apoptosis in inflamed tissue are therefore promosing candidates for anti-inflammatory therapy, and treatments that prolong the lifespan of neutrophils may be worth considering in situations where neutrophil function is compromised. The factors that these agents could target may also include certain members of the Bcl-2 family. An activation of Bim or Puma would probably affect too many cell types and have undesirable side effect. The present work also suggested that the loss of A1 contributes to apoptosis induction in neutrophils. If further studies prove it to be indeed relevant, the targeting of A1 during inflammation may be very promising. Its anti-apoptotic function seems to be most important in haematopoietic cell lineages, and in mice deficient in A1 there was no evidence of illness up to 12 months of age (apart from an increased loss of hair) (Hamasaki et al., 1998). Furthermore, A1-deficiency has been reported to result in attenuation of inflammation during acute infection (Orlofsky et al., 2002), and because the

turnover of A1 mRNA and protein is very high, therapeutical downregulation could be achieved very fast, and also be terminated very fast at the end of therapy. *In vivo*, such an A1-centered approach could be tested for example in a model of experimental meningitis using a mouse where loss of A1 would be initiated in neutrophils after the immune response has been mounted. Such a conditional knock-down could for example be obtained by generating a mouse with a transgene encoding shRNA against *a1* transcripts, whose expression can be induced by addition of tetracyclin, and that bears a promoter only active in neutrophils. If successful, the next steps would involve the identification of pathways regulating A1 expression, and finding or developing specific inhibitors of these pathways.

4.4 Benefits and disadvantages of phagocyte apoptosis to the host immune response

Phagocyte apoptosis forms part of most innate immune responses to infection, but depending on the time of its onset and also the specific type of phagocyte involved, it can impact differentially on its course. Limited neutrophil apoptosis early during infection can have beneficial side effects in certain situations, such as an increased killing of mycobacteria in infected macrophages through the acquisition of microbicidal granule contents derived from phagocytosed apoptotic neutrophils (Tan et al., 2006). More extensive apoptosis, however, would in most cases be detrimental to the host. As a safeguard, neutrophil lifespan is prolonged by pro-inflammatory cytokines. Furthermore, the clearance of apoptotic neutrophils is delayed until infiltrating monocytes have differentiated into macrophages (Newman et al., 1982), and apoptotic cells that are not removed revert to secondary necrosis, which promotes inflammation (Stern et al., 1996). Once the infection is cleared, neutrophil apoptosis is crucial for the resolution of inflammation, as demonstrated in the present work. Apart from aiding in the termination of the immune response, the uptake of apoptotic cells also promotes the repair of tissue damage, through the induction of vascular endothelial growh factor and hepatocyte growth factor secretion from macrophages (Golpon et al., 2004; Morimoto et al., 2001).

The apoptosis of macrophages during infection may have different consequences. These phagocytes are long-lived (in the range of weeks to months), and have a crucial role in the initiation (by sensing infection), termination (by clearance of apoptotic neutrophils), and

aftermath (removal of damaged tissue) of the immune response. There is also evidence that during and at the end of immune responses, inflammatory macrophages leave the inflamed site via lymphatic vessels, and emigrate to draining lymph nodes (Bellingan et al., 1996; Lan et al., 1993), where they can present antigen to T cells. It can be expected that in most circumstances, an abolishment of these functions by macrophage death will be disadvantageous to the host.

Nevertheless, macrophage apoptosis can be beneficial in response to certain pathogens, for example by limiting the spread of mycobacteria that preferentially infect these phagocytes (Fratazzi et al., 1997). Previous studies have demonstrated that the phagocytosis of extracellular low-virulence *Escherichia coli* can also induce apoptosis, and the present work has linked it to the uptake and digestion of a large number of bacteria. Macrophage death in these circumstances may have several benefits. First, the prolonged presence of large quantities of cytotoxic material inside the cell may entail a considerable risk for cellular damage, and apoptosis thus eliminates a potentially dysfunctional or tumorigenic cell. Second, the neutralisation of cytotoxic bacterial components by one macrophage and their delivery in form of apoptotic bodies to bystander macrophages may enable overall safer and faster clearance of the pathogen. And third, the uptake of apoptotic macrophages (and also neutrophils) by dendritic cells (Rubartelli et al., 1997) can have immunomodulatory functions.

Dendritic cells, the most potent antigen-presenting cells, are able to present peptides derived from ingested apoptotic cells to thymocytes, and elicit $CD4^+$ or $CD8^+$ T cell-mediated immune responses (Albert et al., 1998; Bellone et al., 1997; Inaba et al., 1998). For example, dendritic cells that phagocytose dying macrophages infected with *Salmonella* can present bacteria-derived peptides on MHC class I and II (Yrlid et al., 2000). The so-called cross-presentation of phagocytosed antigen on MHC I involves leakage of antigen into the cytosol, where it is processed by the proteasome and loaded onto MHC class I molecules via the classical pathway (Ackerman and Cresswell, 2004). Efficient antigen presentation is, however, dependent on the maturation status of the dendritic cell, and the nature of the ingested material (Albert, 2004). While phagocytosis of necrotic cells and exposed intracellular material usually stimulates maturation, the uptake of apoptotic cells in most cases does not (Basu et al., 2000; Gallucci et al., 1999; Rovere et al., 1998; Sauter et al., 2000), and can under certain circumstances even suppress LPS-driven maturation of dendritic cell (Stuart et al., 2002; Urban et al., 2001).

The apoptosis of phagocytes is therefore not only an important element in the modulation of the innate immune response, but can also impact on the development of an adaptive immune response.

5 Summary

The apoptosis of phagocytes affects the course and the termination of responses of the innate immune system during infection. This work examined the molecular events underlying cell death induction in macrophages during bacterial phagocytosis, the involvement of Bcl-2 family members in the induction of neutrophil apoptosis, and the relevance of apoptosis for the termination of neutrophil immune functions at the end of the immune response.

Previous studies had shown that the uptake of low-virulence *Escherichia coli* bacteria can induce the apoptosis of macrophages, but it had remained unclear how phagocytosis was connected to this death. In the work presented here, a link between the uptake, killing and degradation of *Escherichia coli* bacteria and the induction of apoptosis in macrophages could be demonstrated. This link was quantitative, as higher doses of bacteria led to increased levels of macrophage apoptosis. Both phagocytic capacity and cellular death reached a maximum at a certain dose of bacteria but could be elevated further through the overexpression of mutant versions of the small GTPase Rab5a. Both constitutively active and dominant-negative Rab5a, as well as a knock-down of the endogenous protein, enhanced bacterial uptake and macrophage death to a considerable degree, suggesting that phagocytosis is sensitive to the balance between active and inactive Rab5a, and the absolute levels of the protein.

As the intracellular killing and breakdown of *Escherichia coli* bacteria was not affected by the alterations in Rab5a activity or protein level, these findings suggested that the increased apoptosis was linked to a higher incidence of degradation events or an accumulation of phagosomes of a late maturation stage in the mutant macrophages. Accordingly, the treatment of macrophages with bafilomycin A_1, a phagosome acidification inhibitor, reduced killing and degradation of phagocytosed bacteria and significantly decreased macrophage apoptosis. The exact nature of the molecular signalling produced in response to such an accumulation of late-stage phagosomes remains unknown but the involvement of reactive nitrogen or oxygen species could be excluded. Macrophage apoptosis can therefore be initiated as a direct result of the uptake and digestion of pyogenic extracellular bacteria. *In vivo*, such apoptosis may serve as a means safely to dispose of phagocytes that have accumulated a large amount of bacterial toxins that could potentially damage the host cell, and a subsequent uptake of apoptotic macrophages by dendritic cells may have immunomodulatory functions.

In the termination phase of the immune response, the apoptosis of short-lived neutrophils and their removal by macrophages is a critical factor. In the control of neutrophil death, two pro-apoptotic Bcl-2 family protein were identified to play a role, namely the BH3-only proteins Bim and Puma. The deficiency in these proteins conferred considerable protection against stem cell factor deprivation-induced apoptosis. Furthermore, in all neutrophil lines the anti-apoptotic protein A1 was lost upon factor withdrawal, suggesting an involvement in apoptosis induction.

Several studies have associated a prolongation of neutrophil lifespan or defects in neutrophil clearance with inflammatory disorders and autoimmune disease. It was not known, however, whether neutrophils are harmful merely through their sustained presence, or also because of a continued execution of immune functions. This work demonstrated that aged neutrophils that were kept alive through the overexpression of Bcl-2 did not become senescent, but retained their capacity to phagocytose *Streptococcus pneumonia*, and produce a microbicidal respiratory burst and secrete pro-inflammatory IL-1β in response to bacteria. Aged *bcl2*-transgenic neutrophils were also not removed by macrophages.

This indicated that there exists no way other than apoptosis to turn off and clear aged neutrophils. Accordingly, in a model of experimental meningitis caused by infection with *Streptococcus pneumonia*, mice overexpressing Bcl-2 in the haematopoietic compartment displayed higher levels of leukocytes in the inflamed brain, and an increase in pro-inflammatory combined with a decrease in anti-inflammatory cytokines. In consequence, these mice suffered from massive cerebral bleedings and the course of the disease was strongly aggravated. Furthermore, while all wt mice survived and eventually recovered from the infection, a portion of the *bcl2*-transgenic animals died during the experiments. These findings stress the importance of neutrophil apoptosis in the termination phase of the innate immune response, not only with regard to the clearance of apoptotic cells but also considering the shutdown of neutrophil effector functions. Drugs that can reduce the lifespan of neutrophils are therefore promising candidates for anti-inflammatory therapy.

This study has thus shown under which conditions phagocyte death takes place in the macrophage effector arm of the innate immune system during infection with pyogenic bacteria, and demonstrated that apoptosis within the neutrophil effector arm is crucial for the termination of the immune response and the preclusion of hyperinflammation and host tissue damage.

6 Index of References

Acehan, D., X. Jiang, et al. (2002). "Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation." Mol Cell **9**(2): 423-32.

Ackerman, A. L. and P. Cresswell (2004). "Cellular mechanisms governing cross-presentation of exogenous antigens." Nat Immunol **5**(7): 678-84.

Adams, J. M. and S. Cory (2002). "Apoptosomes: engines for caspase activation." Curr Opin Cell Biol **14**(6): 715-20.

Akira, S., S. Uematsu, et al. (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.

Akiyama, T., P. Bouillet, et al. (2003). "Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim." Embo J **22**(24): 6653-64.

Albee, L., B. Shi, et al. (2007). "Aspartic protease and caspase 3/7 activation are central for macrophage apoptosis following infection with Escherichia coli." J Leukoc Biol **81**(1): 229-37.

Albert, M. L. (2004). "Death-defying immunity: do apoptotic cells influence antigen processing and presentation?" Nat Rev Immunol **4**(3): 223-31.

Albert, M. L., M. Jegathesan, et al. (2001). "Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells." Nat Immunol **2**(11): 1010-7.

Albert, M. L., B. Sauter, et al. (1998). "Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs." Nature **392**(6671): 86-9.

Albina, J. E., S. Cui, et al. (1993). "Nitric oxide-mediated apoptosis in murine peritoneal macrophages." J Immunol **150**(11): 5080-5.

Ali, B. R., C. Wasmeier, et al. (2004). "Multiple regions contribute to membrane targeting of Rab GTPases." J Cell Sci **117**(Pt 26): 6401-12.

Ali, F., M. E. Lee, et al. (2003). "Streptococcus pneumoniae-associated human macrophage apoptosis after bacterial internalization via complement and Fcgamma receptors correlates with intracellular bacterial load." J Infect Dis **188**(8): 1119-31.

Alpuche Aranda, C. M., J. A. Swanson, et al. (1992). "Salmonella typhimurium activates virulence gene transcription within acidified macrophage phagosomes." Proc Natl Acad Sci U S A **89**(21): 10079-83.

Alvarez-Dominguez, C., A. M. Barbieri, et al. (1996). "Phagocytosed live Listeria monocytogenes influences Rab5-regulated in vitro phagosome-endosome fusion." J Biol Chem **271**(23): 13834-43.

Ambrosini, G., C. Adida, et al. (1997). "A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma." Nat Med **3**(8): 917-21.

Anant, J. S., L. Desnoyers, et al. (1998). "Mechanism of Rab geranylgeranylation: formation of the catalytic ternary complex." Biochemistry **37**(36): 12559-68.

Antonsson, B., S. Montessuit, et al. (2000). "Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria." Biochem J **345 Pt 2**: 271-8.

Antonsson, B., S. Montessuit, et al. (2001). "Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells." J Biol Chem **276**(15): 11615-23.

Aoshiba, K., S. Yasui, et al. (1999). "Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils." J Immunol **162**(3): 1692-700.

Aouacheria, A., F. Brunet, et al. (2005). "Phylogenomics of life-or-death switches in multicellular animals: Bcl-2, BH3-Only, and BNip families of apoptotic regulators." Mol Biol Evol **22**(12): 2395-416.

Ashkenazi, A. and V. M. Dixit (1998). "Death receptors: signaling and modulation." Science **281**(5381): 1305-8.

Bae, J., C. P. Leo, et al. (2000). "MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain." J Biol Chem **275**(33): 25255-61.

Baran, J., K. Guzik, et al. (1996). "Apoptosis of monocytes and prolonged survival of granulocytes as a result of phagocytosis of bacteria." Infect Immun **64**(10): 4242-8.

Basanez, G., A. Nechushtan, et al. (1999). "Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations." Proc Natl Acad Sci U S A **96**(10): 5492-7.

Basanez, G., J. C. Sharpe, et al. (2002). "Bax-type apoptotic proteins porate pure lipid bilayers through a mechanism sensitive to intrinsic monolayer curvature." J Biol Chem **277**(51): 49360-5.

Bastiani, M., M. C. Vidotto, et al. (2005). "An avian pathogenic Escherichia coli isolate induces caspase 3/7 activation in J774 macrophages." FEMS Microbiol Lett **253**(1): 133-40.

Basu, S., R. J. Binder, et al. (2000). "Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway." Int Immunol **12**(11): 1539-46.

Bauer, A., S. Kirschnek, et al. (2007). "Inhibition of apoptosis can be accompanied by increased Bim levels in T lymphocytes and neutrophil granulocytes." Cell Death Differ **14**(9): 1714-6.

Bauer, A., A. Villunger, et al. (2006). "The NF-kappaB regulator Bcl-3 and the BH3-only proteins Bim and Puma control the death of activated T cells." Proc Natl Acad Sci U S A **103**(29): 10979-84.

Bellingan, G. J., H. Caldwell, et al. (1996). "In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes." J Immunol **157**(6): 2577-85.

Bellone, M., G. Iezzi, et al. (1997). "Processing of engulfed apoptotic bodies yields T cell epitopes." J Immunol **159**(11): 5391-9.

Bidani, A. and T. A. Heming (1995). "Effects of bafilomycin A1 on functional capabilities of LPS-activated alveolar macrophages." J Leukoc Biol **57**(2): 275-81.

Bidani, A., B. S. Reisner, et al. (2000). "Bactericidal activity of alveolar macrophages is suppressed by V-ATPase inhibition." Lung **178**(2): 91-104.

Bingle, C. D., R. W. Craig, et al. (2000). "Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death." J Biol Chem **275**(29): 22136-46.

Boatright, K. M., M. Renatus, et al. (2003). "A unified model for apical caspase activation." Mol Cell **11**(2): 529-41.

Bohr, V., B. Hansen, et al. (1983). "Sequelae from bacterial meningitis and their relation to the clinical condition during acute illness, based on 667 questionnaire returns. Part II of a three part series." J Infect **7**(2): 102-10.

Boldin, M. P., E. E. Varfolomeev, et al. (1995). "A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain." J Biol Chem **270**(14): 7795-8.

Borregaard, N. and J. B. Cowland (1997). "Granules of the human neutrophilic polymorphonuclear leukocyte." Blood **89**(10): 3503-21.

Bouillet, P., S. Cory, et al. (2001). "Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim." Dev Cell **1**(5): 645-53.

Bouillet, P., D. Metcalf, et al. (1999). "Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity." Science **286**(5445): 1735-8.

Bouillet, P., J. F. Purton, et al. (2002). "BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes." Nature **415**(6874): 922-6.

Bowman, E. J., A. Siebers, et al. (1988). "Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells." Proc Natl Acad Sci U S A **85**(21): 7972-6.

Boyd, J. M., G. J. Gallo, et al. (1995). "Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins." Oncogene **11**(9): 1921-8.

Brown, P. D. and S. A. Lerner (1998). "Community-acquired pneumonia." Lancet **352**(9136): 1295-302.

Bucci, C., R. G. Parton, et al. (1992). "The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway." Cell **70**(5): 715-28.

Bucci, C., P. Thomsen, et al. (2000). "Rab7: a key to lysosome biogenesis." Mol Biol Cell **11**(2): 467-80.

Bursch, W., H. S. Taper, et al. (1985). "Quantitative histological and histochemical studies on the occurrence and stages of controlled cell death (apoptosis) during regression of rat liver hyperplasia." Virchows Arch B Cell Pathol Incl Mol Pathol **50**(2): 153-66.

Byrne, A. and D. J. Reen (2002). "Lipopolysaccharide induces rapid production of IL-10 by monocytes in the presence of apoptotic neutrophils." J Immunol **168**(4): 1968-77.

Carlos, T. M. and J. M. Harlan (1994). "Leukocyte-endothelial adhesion molecules." Blood **84**(7): 2068-101.

Carroll, M. C. and M. B. Fischer (1997). "Complement and the immune response." Curr Opin Immunol **9**(1): 64-9.

Cassatella, M. A. (1995). "The production of cytokines by polymorphonuclear neutrophils." Immunol Today **16**(1): 21-6.

Cassatella, M. A. (1999). "Neutrophil-derived proteins: selling cytokines by the pound." Adv Immunol **73**: 369-509.

Cerretti, D. P., C. J. Kozlosky, et al. (1992). "Molecular cloning of the interleukin-1 beta converting enzyme." Science **256**(5053): 97-100.

Chai, T. J. and J. Foulds (1977). "Purification of protein A, an outer membrane component missing in Escherichia coli K-12 ompA mutants." Biochim Biophys Acta **493**(1): 210-5.

Chautan, M., G. Chazal, et al. (1999). "Interdigital cell death can occur through a necrotic and caspase-independent pathway." Curr Biol **9**(17): 967-70.

Chen, S. M., J. S. Dumler, et al. (1994). "Identification of a granulocytotropic Ehrlichia species as the etiologic agent of human disease." J Clin Microbiol **32**(3): 589-95.

Cheng, E. H., D. G. Kirsch, et al. (1997). "Conversion of Bcl-2 to a Bax-like death effector by caspases." Science **278**(5345): 1966-8.

Cheng, E. H., M. C. Wei, et al. (2001). "BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis." Mol Cell **8**(3): 705-11.

Chinnaiyan, A. M., K. O'Rourke, et al. (1995). "FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis." Cell **81**(4): 505-12.

Chitnis, D., C. Dickerson, et al. (1996). "Inhibition of apoptosis in polymorphonuclear neutrophils from burn patients." J Leukoc Biol **59**(6): 835-9.

Chittenden, T., C. Flemington, et al. (1995). "A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions." Embo J **14**(22): 5589-96.

Chou, J. J., H. Li, et al. (1999). "Solution structure of BID, an intracellular amplifier of apoptotic signaling." Cell **96**(5): 615-24.

Chuang, P. I., E. Yee, et al. (1998). "A1 is a constitutive and inducible Bcl-2 homologue in mature human neutrophils." Biochem Biophys Res Commun **249**(2): 361-5.

Claus, V., A. Jahraus, et al. (1998). "Lysosomal enzyme trafficking between phagosomes, endosomes, and lysosomes in J774 macrophages. Enrichment of cathepsin H in early endosomes." J Biol Chem **273**(16): 9842-51.

Clem, R. J., E. H. Cheng, et al. (1998). "Modulation of cell death by Bcl-XL through caspase interaction." Proc Natl Acad Sci U S A **95**(2): 554-9.

Clem, R. J. and L. K. Miller (1993). "Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus." J Virol **67**(7): 3730-8.

Clohessy, J. G., J. Zhuang, et al. (2004). "Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells." Br J Haematol **125**(5): 655-65.

Colotta, F., F. Re, et al. (1992). "Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products." Blood **80**(8): 2012-20.

Conus, S., R. Perozzo, et al. (2008). "Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation." J Exp Med **205**(3): 685-98.

Cory, S., D. C. Huang, et al. (2003). "The Bcl-2 family: roles in cell survival and oncogenesis." Oncogene **22**(53): 8590-607.

Coultas, L., P. Bouillet, et al. (2005). "Concomitant loss of proapoptotic BH3-only Bcl-2 antagonists Bik and Bim arrests spermatogenesis." Embo J **24**(22): 3963-73.

Coultas, L., S. Terzano, et al. (2007). "Hrk/DP5 contributes to the apoptosis of select neuronal populations but is dispensable for haematopoietic cell apoptosis." J Cell Sci **120**(Pt 12): 2044-52.

Cox, G., J. Crossley, et al. (1995). "Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo." Am J Respir Cell Mol Biol **12**(2): 232-7.

Cox, G., J. Gauldie, et al. (1992). "Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro." Am J Respir Cell Mol Biol **7**(5): 507-13.

Cox, J. A., A. Y. Jeng, et al. (1987). "Comparison of subcellular activation of the human neutrophil NADPH-oxidase by arachidonic acid, sodium dodecyl sulfate (SDS), and phorbol myristate acetate (PMA)." J Immunol **138**(6): 1884-8.

Coxon, A., T. Tang, et al. (1999). "Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo. A role for granulocyte/macrophage colony-stimulating factor." J Exp Med **190**(7): 923-34.

Cross, A. R. and A. W. Segal (2004). "The NADPH oxidase of professional phagocytes--prototype of the NOX electron transport chain systems." Biochim Biophys Acta **1657**(1): 1-22.

Cuconati, A., C. Mukherjee, et al. (2003). "DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells." Genes Dev **17**(23): 2922-32.

Cunha, F. Q., J. Assreuy, et al. (1993). "Phagocytosis and induction of nitric oxide synthase in murine macrophages." Immunology **79**(3): 408-11.

Czabotar, P. E., E. F. Lee, et al. (2007). "Structural insights into the degradation of Mcl-1 induced by BH3 domains." Proc Natl Acad Sci U S A **104**(15): 6217-22.

Dancey, J. T., K. A. Deubelbeiss, et al. (1976). "Neutrophil kinetics in man." J Clin Invest **58**(3): 705-15.

Day, C. L., L. Chen, et al. (2005). "Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands." J Biol Chem **280**(6): 4738-44.

de Gans, J. and D. van de Beek (2002). "Dexamethasone in adults with bacterial meningitis." N Engl J Med **347**(20): 1549-56.

Degli Esposti, M. (2002). "Sequence and functional similarities between pro-apoptotic Bid and plant lipid transfer proteins." Biochim Biophys Acta **1553**(3): 331-40.

Degli Esposti, M. and C. Dive (2003). "Mitochondrial membrane permeabilisation by Bax/Bak." Biochem Biophys Res Commun **304**(3): 455-61.

Delamarre, L., M. Pack, et al. (2005). "Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate." Science **307**(5715): 1630-4.

DeLeo, F. R. (2004). "Modulation of phagocyte apoptosis by bacterial pathogens." Apoptosis **9**(4): 399-413.

Denault, J. B. and G. S. Salvesen (2002). "Caspases: keys in the ignition of cell death." Chem Rev **102**(12): 4489-500.

Desagher, S., A. Osen-Sand, et al. (1999). "Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis." J Cell Biol **144**(5): 891-901.

Desjardins, M., L. A. Huber, et al. (1994). "Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus." J Cell Biol **124**(5): 677-88.

Desjardins, M., N. N. Nzala, et al. (1997). "Maturation of phagosomes is accompanied by changes in their fusion properties and size-selective acquisition of solute materials from endosomes." J Cell Sci **110 (Pt 18)**: 2303-14.

Deveraux, Q. L. and J. C. Reed (1999). "IAP family proteins--suppressors of apoptosis." Genes Dev **13**(3): 239-52.

Dijkers, P. F., R. H. Medema, et al. (2000). "Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1." Curr Biol **10**(19): 1201-4.

Dirac-Svejstrup, A. B., T. Sumizawa, et al. (1997). "Identification of a GDI displacement factor that releases endosomal Rab GTPases from Rab-GDI." Embo J **16**(3): 465-72.

Dockrell, D. H., M. Lee, et al. (2001). "Immune-mediated phagocytosis and killing of Streptococcus pneumoniae are associated with direct and bystander macrophage apoptosis." J Infect Dis **184**(6): 713-22.

Duclos, S., R. Diez, et al. (2000). "Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages." J Cell Sci **113 Pt 19**: 3531-41.

Duriez, P. J., F. Wong, et al. (2000). "A1 functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor." J Biol Chem **275**(24): 18099-107.

Dzhagalov, I., A. St John, et al. (2007). "The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages." Blood **109**(4): 1620-6.

Edwards, S. W. (1994). Biochemistry and Physiology of the Neutrophil. New York, Cambridge University Press.

Ekoff, M., T. Kaufmann, et al. (2007). "The BH3-only protein Puma plays an essential role in cytokine deprivation induced apoptosis of mast cells." Blood **110**(9): 3209-17.

Ellis, R. E., J. Y. Yuan, et al. (1991). "Mechanisms and functions of cell death." Annu Rev Cell Biol **7**: 663-98.

Enders, A., P. Bouillet, et al. (2003). "Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells." J Exp Med **198**(7): 1119-26.

Ertel, W., M. Keel, et al. (1998). "Circulating mediators in serum of injured patients with septic complications inhibit neutrophil apoptosis through up-regulation of protein-tyrosine phosphorylation." J Trauma **44**(5): 767-75; discussion 775-6.

Eskes, R., S. Desagher, et al. (2000). "Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane." Mol Cell Biol **20**(3): 929-35.

Fadok, V. A., D. L. Bratton, et al. (2001). "Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases." J Immunol **166**(11): 6847-54.

Fadok, V. A., D. L. Bratton, et al. (1998). "Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF." J Clin Invest **101**(4): 890-8.

Fecho, K. and P. L. Cohen (1998). "Fas ligand (gld)- and Fas (lpr)-deficient mice do not show alterations in the extravasation or apoptosis of inflammatory neutrophils." J Leukoc Biol **64**(3): 373-83.

Feig, L. A. (1999). "Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases." Nat Cell Biol **1**(2): E25-7.

Fesik, S. W. (2000). "Insights into programmed cell death through structural biology." Cell **103**(2): 273-82.

Fischer, S. F., G. T. Belz, et al. (2008). "BH3-only protein Puma contributes to death of antigen-specific T cells during shutdown of an immune response to acute viral infection." Proc Natl Acad Sci U S A **105**(8): 3035-40.

Fischer, S. F., P. Bouillet, et al. (2007). "Proapoptotic BH3-only protein Bim is essential for developmentally programmed death of germinal center-derived memory B cells and antibody-forming cells." Blood **110**(12): 3978-84.

Fischer, U., R. U. Janicke, et al. (2003). "Many cuts to ruin: a comprehensive update of caspase substrates." Cell Death Differ **10**(1): 76-100.

Franchi, L., C. McDonald, et al. (2006). "Nucleotide-binding oligomerization domain-like receptors: intracellular pattern recognition molecules for pathogen detection and host defense." J Immunol **177**(6): 3507-13.

Francois, S., J. El Benna, et al. (2005). "Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF-kappaB signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated Bad." J Immunol **174**(6): 3633-42.

Frankenberg, T., S. Kirschnek, et al. (2008). "Phagocytosis-induced apoptosis of macrophages is linked to uptake, killing and degradation of bacteria." Eur J Immunol **38**(1): 204-15.

Fratazzi, C., R. D. Arbeit, et al. (1997). "Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages." J Immunol **158**(9): 4320-7.

Funato, K., W. Beron, et al. (1997). "Reconstitution of phagosome-lysosome fusion in streptolysin O-permeabilized cells." J Biol Chem **272**(26): 16147-51.

Gaillard, J. L., P. Berche, et al. (1987). "In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2." Infect Immun **55**(11): 2822-9.

Gallucci, S., M. Lolkema, et al. (1999). "Natural adjuvants: endogenous activators of dendritic cells." Nat Med **5**(11): 1249-55.

Ganz, T. and R. I. Lehrer (1998). "Antimicrobial peptides of vertebrates." Curr Opin Immunol **10**(1): 41-4.

Godson, C., S. Mitchell, et al. (2000). "Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages." J Immunol **164**(4): 1663-7.

Golpon, H. A., V. A. Fadok, et al. (2004). "Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth." Faseb J **18**(14): 1716-8.

Gomez-Bougie, P., R. Bataille, et al. (2005). "Endogenous association of Bim BH3-only protein with Mcl-1, Bcl-xL and Bcl-2 on mitochondria in human B cells." Eur J Immunol **35**(3): 971-6.

Goping, I. S., A. Gross, et al. (1998). "Regulated targeting of BAX to mitochondria." J Cell Biol **143**(1): 207-15.

Griffiths, G. J., L. Dubrez, et al. (1999). "Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis." J Cell Biol **144**(5): 903-14.

Grigg, J. M., J. S. Savill, et al. (1991). "Neutrophil apoptosis and clearance from neonatal lungs." Lancet **338**(8769): 720-2.

Groesdonk, H. V., S. Schlottmann, et al. (2006). "Escherichia coli prevents phagocytosis-induced death of macrophages via classical NF-kappaB signaling, a link to T-cell activation." Infect Immun **74**(10): 5989-6000.

Gross, A., J. Jockel, et al. (1998). "Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis." Embo J **17**(14): 3878-85.

Guermonprez, P., J. Valladeau, et al. (2002). "Antigen presentation and T cell stimulation by dendritic cells." Annu Rev Immunol **20**: 621-67.

Guzman, C. A., E. Domann, et al. (1996). "Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of Listeria monocytogenes." Mol Microbiol **20**(1): 119-26.

Gyrd-Hansen, M., T. Farkas, et al. (2006). "Apoptosome-independent activation of the lysosomal cell death pathway by caspase-9." Mol Cell Biol **26**(21): 7880-91.

Hacker, G., K. Suttner, et al. (2006). "TLR-dependent Bim phosphorylation in macrophages is mediated by ERK and is connected to proteasomal degradation of the protein." Int Immunol **18**(12): 1749-57.

Hacker, H., C. Furmann, et al. (2002). "Caspase-9/-3 activation and apoptosis are induced in mouse macrophages upon ingestion and digestion of Escherichia coli bacteria." J Immunol **169**(6): 3172-9.

Hamasaki, A., F. Sendo, et al. (1998). "Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene." J Exp Med **188**(11): 1985-92.

Han, J., C. Flemington, et al. (2001). "Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals." Proc Natl Acad Sci U S A **98**(20): 11318-23.

Harada, H., B. Quearry, et al. (2004). "Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity." Proc Natl Acad Sci U S A **101**(43): 15313-7.

Hart, P. D. and M. R. Young (1991). "Ammonium chloride, an inhibitor of phagosome-lysosome fusion in macrophages, concurrently induces phagosome-endosome fusion, and opens a novel pathway: studies of a pathogenic mycobacterium and a nonpathogenic yeast." J Exp Med **174**(4): 881-9.

Hatakeyama, S., A. Hamasaki, et al. (1998). "Multiple gene duplication and expression of mouse bcl-2-related genes, A1." Int Immunol **10**(5): 631-7.

Hau, T. (1990). "Bacteria, toxins, and the peritoneum." World J Surg **14**(2): 167-75.

Hauser, A. R. and J. N. Engel (1999). "Pseudomonas aeruginosa induces type-III-secretion-mediated apoptosis of macrophages and epithelial cells." Infect Immun **67**(10): 5530-7.

Hegde, R., S. M. Srinivasula, et al. (1998). "Blk, a BH3-containing mouse protein that interacts with Bcl-2 and Bcl-xL, is a potent death agonist." J Biol Chem **273**(14): 7783-6.

Hengartner, M. O. and H. R. Horvitz (1994). "C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2." Cell **76**(4): 665-76.

Henning, U., H. D. Royer, et al. (1979). "Cloning of the structural gene (ompA) for an integral outer membrane protein of Escherichia coli K-12." Proc Natl Acad Sci U S A **76**(9): 4360-4.

Herold, M. J., J. Zeitz, et al. (2006). "The stability and anti-apoptotic function of A1 are controlled by its C terminus." J Biol Chem **281**(19): 13663-71.

Herrmann, M., R. E. Voll, et al. (1998). "Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus." Arthritis Rheum **41**(7): 1241-50.

Hersh, D., D. M. Monack, et al. (1999). "The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1." Proc Natl Acad Sci U S A **96**(5): 2396-401.

Heyworth, C. M., A. D. Whetton, et al. (1992). "Stem cell factor directly stimulates the development of enriched granulocyte-macrophage colony-forming cells and promotes the effects of other colony-stimulating factors." Blood **80**(9): 2230-6.

Hilbi, H., J. E. Moss, et al. (1998). "Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB." J Biol Chem **273**(49): 32895-900.

Hinds, M. G., M. Lackmann, et al. (2003). "The structure of Bcl-w reveals a role for the C-terminal residues in modulating biological activity." Embo J **22**(7): 1497-507.

Hinds, M. G., C. Smits, et al. (2007). "Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets." Cell Death Differ **14**(1): 128-36.

Hodge, S., G. Hodge, et al. (2003). "Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells." Immunol Cell Biol **81**(4): 289-96.

Hoebe, K., E. Janssen, et al. (2004). "The interface between innate and adaptive immunity." Nat Immunol **5**(10): 971-4.

Holmgreen, S. P., D. C. Huang, et al. (1999). "Survival activity of Bcl-2 homologs Bcl-w and A1 only partially correlates with their ability to bind pro-apoptotic family members." Cell Death Differ **6**(6): 525-32.

Horwitz, M. A. and F. R. Maxfield (1984). "Legionella pneumophila inhibits acidification of its phagosome in human monocytes." J Cell Biol **99**(6): 1936-43.

Hsu, S. Y. and A. J. Hsueh (1998). "A splicing variant of the Bcl-2 member Bok with a truncated BH3 domain induces apoptosis but does not dimerize with antiapoptotic Bcl-2 proteins in vitro." J Biol Chem **273**(46): 30139-46.

Hsu, S. Y., P. Lin, et al. (1998). "BOD (Bcl-2-related ovarian death gene) is an ovarian BH3 domain-containing proapoptotic Bcl-2 protein capable of dimerization with diverse antiapoptotic Bcl-2 members." Mol Endocrinol **12**(9): 1432-40.

Hsu, Y. T., K. G. Wolter, et al. (1997). "Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis." Proc Natl Acad Sci U S A **94**(8): 3668-72.

Hsu, Y. T. and R. J. Youle (1997). "Nonionic detergents induce dimerization among members of the Bcl-2 family." J Biol Chem **272**(21): 13829-34.

Hsu, Y. T. and R. J. Youle (1998). "Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations." J Biol Chem **273**(17): 10777-83.

Huang, D. C., S. Cory, et al. (1997). "Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death." Oncogene **14**(4): 405-14.

Hubner, A., T. Barrett, et al. (2008). "Multisite phosphorylation regulates Bim stability and apoptotic activity." Mol Cell **30**(4): 415-25.

Huynh, M. L., V. A. Fadok, et al. (2002). "Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation." J Clin Invest **109**(1): 41-50.

Imaizumi, K., M. Tsuda, et al. (1997). "Molecular cloning of a novel polypeptide, DP5, induced during programmed neuronal death." J Biol Chem **272**(30): 18842-8.

Ina, K., K. Kusugami, et al. (1999). "Increased mucosal production of granulocyte colony-stimulating factor is related to a delay in neutrophil apoptosis in Inflammatory Bowel disease." J Gastroenterol Hepatol **14**(1): 46-53.

Inaba, K., S. Turley, et al. (1998). "Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells." J Exp Med **188**(11): 2163-73.

Inohara, N., D. Ekhterae, et al. (1998). "Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-XL." J Biol Chem **273**(15): 8705-10.

Irmler, M., M. Thome, et al. (1997). "Inhibition of death receptor signals by cellular FLIP." Nature **388**(6638): 190-5.

Jahraus, A., T. E. Tjelle, et al. (1998). "In vitro fusion of phagosomes with different endocytic organelles from J774 macrophages." J Biol Chem **273**(46): 30379-90.

Jeffers, J. R., E. Parganas, et al. (2003). "Puma is an essential mediator of p53-dependent and -independent apoptotic pathways." Cancer Cell **4**(4): 321-8.

Jiang, X. and X. Wang (2000). "Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1." J Biol Chem **275**(40): 31199-203.

Jimenez, M. F., R. W. Watson, et al. (1997). "Dysregulated expression of neutrophil apoptosis in the systemic inflammatory response syndrome." Arch Surg **132**(12): 1263-9; discussion 1269-70.

Johansson, A., A. J. Jesaitis, et al. (1995). "Different subcellular localization of cytochrome b and the dormant NADPH-oxidase in neutrophils and macrophages: effect on the production of reactive oxygen species during phagocytosis." Cell Immunol **161**(1): 61-71.

Jourdan, M., J. De Vos, et al. (2000). "Regulation of Bcl-2-family proteins in myeloma cells by three myeloma survival factors: interleukin-6, interferon-alpha and insulin-like growth factor 1." Cell Death Differ **7**(12): 1244-52.

Kagan, B. L., A. Finkelstein, et al. (1981). "Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes." Proc Natl Acad Sci U S A **78**(8): 4950-4.

Karsan, A., E. Yee, et al. (1997). "Fibroblast growth factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms." Am J Pathol **151**(6): 1775-84.

Kasahara, Y., K. Iwai, et al. (1997). "Involvement of reactive oxygen intermediates in spontaneous and CD95 (Fas/APO-1)-mediated apoptosis of neutrophils." Blood **89**(5): 1748-53.

Kaufmann, T., S. Schlipf, et al. (2003). "Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane." J Cell Biol **160**(1): 53-64.

Kaufmann, T., L. Tai, et al. (2007). "The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest." Cell **129**(2): 423-33.

Kawai, T., O. Adachi, et al. (1999). "Unresponsiveness of MyD88-deficient mice to endotoxin." Immunity **11**(1): 115-22.

Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-57.

Kim, H., M. Rafiuddin-Shah, et al. (2006). "Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies." Nat Cell Biol **8**(12): 1348-58.

Kim, S., K. B. Elkon, et al. (2004). "Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells." Immunity **21**(5): 643-53.

Kirsch, D. G., A. Doseff, et al. (1999). "Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c." J Biol Chem **274**(30): 21155-61.

Kirschnek, S., S. Ying, et al. (2005). "Phagocytosis-induced apoptosis in macrophages is mediated by up-regulation and activation of the Bcl-2 homology domain 3-only protein Bim." J Immunol **174**(2): 671-9.

Kischkel, F. C., S. Hellbardt, et al. (1995). "Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor." Embo J **14**(22): 5579-88.

Klein, M., R. Paul, et al. (2006). "Protein expression pattern in experimental pneumococcal meningitis." Microbes Infect **8**(4): 974-83.

Knoepfler, P. S., D. B. Sykes, et al. (2001). "HoxB8 requires its Pbx-interaction motif to block differentiation of primary myeloid progenitors and of most cell line models of myeloid differentiation." Oncogene **20**(39): 5440-8.

Kobayashi, S. D., K. R. Braughton, et al. (2003). "Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils." Proc Natl Acad Sci U S A **100**(19): 10948-53.

Koedel, U., W. M. Scheld, et al. (2002). "Pathogenesis and pathophysiology of pneumococcal meningitis." Lancet Infect Dis **2**(12): 721-36.

Koedel, U., F. Winkler, et al. (2002). "Role of Caspase-1 in experimental pneumococcal meningitis: Evidence from pharmacologic Caspase inhibition and Caspase-1-deficient mice." Ann Neurol **51**(3): 319-29.

Krueger, A., I. Schmitz, et al. (2001). "Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex." J Biol Chem **276**(23): 20633-40.

Krysko, D. V., K. D'Herde, et al. (2006). "Clearance of apoptotic and necrotic cells and its immunological consequences." Apoptosis **11**(10): 1709-26.

Kucharczak, J. F., M. J. Simmons, et al. (2005). "Constitutive proteasome-mediated turnover of Bfl-1/A1 and its processing in response to TNF receptor activation in FL5.12 pro-B cells convert it into a prodeath factor." Cell Death Differ **12**(9): 1225-39.

Kuida, K., J. A. Lippke, et al. (1995). "Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme." Science **267**(5206): 2000-3.

Kuwana, T., L. Bouchier-Hayes, et al. (2005). "BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly." Mol Cell **17**(4): 525-35.

Kuwana, T., M. R. Mackey, et al. (2002). "Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane." Cell **111**(3): 331-42.

Labi, V., M. Erlacher, et al. (2008). "Loss of the BH3-only protein Bmf impairs B cell homeostasis and accelerates gamma irradiation-induced thymic lymphoma development." J Exp Med **205**(3): 641-55.

Lacronique, V., A. Mignon, et al. (1996). "Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice." Nat Med **2**(1): 80-6.

Lagasse, E. and I. L. Weissman (1994). "bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages." J Exp Med **179**(3): 1047-52.

Lakin, N. D. and S. P. Jackson (1999). "Regulation of p53 in response to DNA damage." Oncogene **18**(53): 7644-55.

Lan, H. Y., D. J. Nikolic-Paterson, et al. (1993). "Trafficking of inflammatory macrophages from the kidney to draining lymph nodes during experimental glomerulonephritis." Clin Exp Immunol **92**(2): 336-41.

Lee, A., M. K. Whyte, et al. (1993). "Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators." J Leukoc Biol **54**(4): 283-8.

Lee, E. F., P. E. Czabotar, et al. (2008). "A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis can proceed without Mcl-1 degradation." J Cell Biol **180**(2): 341-55.

Letai, A., M. C. Bassik, et al. (2002). "Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics." Cancer Cell **2**(3): 183-92.

Leuenroth, S. J., P. S. Grutkoski, et al. (2000). "The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis." J Leukoc Biol **68**(1): 158-66.

Levy, B. D., C. B. Clish, et al. (2001). "Lipid mediator class switching during acute inflammation: signals in resolution." Nat Immunol **2**(7): 612-9.

Ley, R., K. Balmanno, et al. (2003). "Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim." J Biol Chem **278**(21): 18811-6.

Ley, R., K. Hadfield, et al. (2005). "Identification of a DEF-type docking domain for extracellular signal-regulated kinases 1/2 that directs phosphorylation and turnover of the BH3-only protein BimEL." J Biol Chem **280**(18): 17657-63.

Li, H., H. Zhu, et al. (1998). "Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis." Cell **94**(4): 491-501.

Li, P., D. Nijhawan, et al. (1997). "Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade." Cell **91**(4): 479-89.

Li, P., H. Allen, et al. (1995). "Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock." Cell **80**(3): 401-11.

Lindsten, T., A. J. Ross, et al. (2000). "The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues." Mol Cell **6**(6): 1389-99.

Lithgow, T., R. van Driel, et al. (1994). "The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane." Cell Growth Differ **5**(4): 411-7.

Liu, K. and G. Li (1998). "Catalytic domain of the p120 Ras GAP binds to RAb5 and stimulates its GTPase activity." J Biol Chem **273**(17): 10087-90.

Liu, X., S. Dai, et al. (2003). "The structure of a Bcl-xL/Bim fragment complex: implications for Bim function." Immunity **19**(3): 341-52.

Lorber, B. and R. M. Swenson (1975). "The bacteriology of intra-abdominal infections." Surg Clin North Am **55**(6): 1349-54.

Luciano, F., A. Jacquel, et al. (2003). "Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function." Oncogene **22**(43): 6785-93.

Lukacs, G. L., O. D. Rotstein, et al. (1990). "Phagosomal acidification is mediated by a vacuolar-type H(+)-ATPase in murine macrophages." J Biol Chem **265**(34): 21099-107.

Lundqvist-Gustafsson, H. and T. Bengtsson (1999). "Activation of the granule pool of the NADPH oxidase accelerates apoptosis in human neutrophils." J Leukoc Biol **65**(2): 196-204.

Luo, X., I. Budihardjo, et al. (1998). "Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors." Cell **94**(4): 481-90.

Lutter, M., M. Fang, et al. (2000). "Cardiolipin provides specificity for targeting of tBid to mitochondria." Nat Cell Biol **2**(10): 754-61.

Maianski, N. A., F. P. Mul, et al. (2002). "Granulocyte colony-stimulating factor inhibits the mitochondria-dependent activation of caspase-3 in neutrophils." Blood **99**(2): 672-9.

Maianski, N. A., D. Roos, et al. (2003). "Tumor necrosis factor alpha induces a caspase-independent death pathway in human neutrophils." Blood **101**(5): 1987-95.

Makin, G. W., B. M. Corfe, et al. (2001). "Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate." Embo J **20**(22): 6306-15.

Malipiero, U., U. Koedel, et al. (2006). "TGFbeta receptor II gene deletion in leucocytes prevents cerebral vasculitis in bacterial meningitis." Brain **129**(Pt 9): 2404-15.

Marani, M., T. Tenev, et al. (2002). "Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis." Mol Cell Biol **22**(11): 3577-89.

Marriott, H. M., C. D. Bingle, et al. (2005). "Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance." J Clin Invest **115**(2): 359-68.

Marsden, V. S. and A. Strasser (2003). "Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more." Annu Rev Immunol **21**: 71-105.

Martin, S. J., C. P. Reutelingsperger, et al. (1995). "Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl." J Exp Med **182**(5): 1545-56.

Martinou, J. C., M. Dubois-Dauphin, et al. (1994). "Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia." Neuron **13**(4): 1017-30.

Matsuda, T., H. Saito, et al. (1999). "Ratio of bacteria to polymorphonuclear neutrophils (PMNs) determines PMN fate." Shock **12**(5): 365-72.

McCartney-Francis, N., W. Jin, et al. (2004). "Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-beta 1 signaling pathway." J Immunol **172**(6): 3814-21.

McNiece, I. K., K. E. Langley, et al. (1991). "Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages." Exp Hematol **19**(3): 226-31.

Meagher, L. C., J. S. Savill, et al. (1992). "Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B2." J Leukoc Biol **52**(3): 269-73.

Medzhitov, R., P. Preston-Hurlburt, et al. (1998). "MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways." Mol Cell **2**(2): 253-8.

Mei, Y., W. Du, et al. (2005). "Puma(*)Mcl-1 interaction is not sufficient to prevent rapid degradation of Mcl-1." Oncogene **24**(48): 7224-37.

Metcalf, D. and N. A. Nicola (1991). "Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro: effects of combination with colony-stimulating factors." Proc Natl Acad Sci U S A **88**(14): 6239-43.

Metcalf, D., L. Robb, et al. (1996). "Role of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in the development of an acute neutrophil inflammatory response in mice." Blood **88**(10): 3755-64.

Michels, J., J. W. O'Neill, et al. (2004). "Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage." Oncogene **23**(28): 4818-27.

Mikhailov, V., M. Mikhailova, et al. (2003). "Association of Bax and Bak homo-oligomers in mitochondria. Bax requirement for Bak reorganization and cytochrome c release." J Biol Chem **278**(7): 5367-76.

Mikhailov, V., M. Mikhailova, et al. (2001). "Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane." J Biol Chem **276**(21): 18361-74.

Moldoveanu, T., Q. Liu, et al. (2006). "The X-ray structure of a BAK homodimer reveals an inhibitory zinc binding site." Mol Cell **24**(5): 677-88.

Monack, D. M., C. S. Detweiler, et al. (2001). "Salmonella pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1." Cell Microbiol **3**(12): 825-37.

Monack, D. M., B. Raupach, et al. (1996). "Salmonella typhimurium invasion induces apoptosis in infected macrophages." Proc Natl Acad Sci U S A **93**(18): 9833-8.

Morimoto, K., H. Amano, et al. (2001). "Alveolar macrophages that phagocytose apoptotic neutrophils produce hepatocyte growth factor during bacterial pneumonia in mice." Am J Respir Cell Mol Biol **24**(5): 608-15.

Motoyama, N., F. Wang, et al. (1995). "Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice." Science **267**(5203): 1506-10.

Moulding, D. A., C. Akgul, et al. (2001). "BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis." J Leukoc Biol **70**(5): 783-92.

Moulding, D. A., J. A. Quayle, et al. (1998). "Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival." Blood **92**(7): 2495-502.

Muchmore, S. W., M. Sattler, et al. (1996). "X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death." Nature **381**(6580): 335-41.

Munro, S. (2004). "Organelle identity and the organization of membrane traffic." Nat Cell Biol **6**(6): 469-72.

Muzio, M., A. M. Chinnaiyan, et al. (1996). "FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex." Cell **85**(6): 817-27.

Muzio, M., G. S. Salvesen, et al. (1997). "FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens." J Biol Chem **272**(5): 2952-6.

Muzio, M., B. R. Stockwell, et al. (1998). "An induced proximity model for caspase-8 activation." J Biol Chem **273**(5): 2926-30.

Naik, E., E. M. Michalak, et al. (2007). "Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly via the BH3-only protein Noxa." J Cell Biol **176**(4): 415-24.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." Mol Cell **7**(3): 683-94.

Navarre, W. W. and A. Zychlinsky (2000). "Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies." Cell Microbiol **2**(4): 265-73.

Nechushtan, A., C. L. Smith, et al. (1999). "Conformation of the Bax C-terminus regulates subcellular location and cell death." Embo J **18**(9): 2330-41.

Nechushtan, A., C. L. Smith, et al. (2001). "Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis." J Cell Biol **153**(6): 1265-76.

Newman, S. L., J. E. Henson, et al. (1982). "Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages." J Exp Med **156**(2): 430-42.

Nijhawan, D., M. Fang, et al. (2003). "Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation." Genes Dev **17**(12): 1475-86.

O'Connor, L., A. Strasser, et al. (1998). "Bim: a novel member of the Bcl-2 family that promotes apoptosis." Embo J **17**(2): 384-95.

O'Mahony, D. S., U. Pham, et al. (2008). "Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages." Int J Med Sci **5**(1): 1-8.

O'Reilly, L. A., L. Cullen, et al. (2000). "The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells." Am J Pathol **157**(2): 449-61.

O'Reilly, L. A., C. Print, et al. (2001). "Tissue expression and subcellular localization of the pro-survival molecule Bcl-w." Cell Death Differ **8**(5): 486-94.

O'Sullivan, M. P., S. O'Leary, et al. (2007). "A caspase-independent pathway mediates macrophage cell death in response to Mycobacterium tuberculosis infection." Infect Immun **75**(4): 1984-93.

Oda, E., R. Ohki, et al. (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science **288**(5468): 1053-8.

Oda, K., Y. Nishimura, et al. (1991). "Bafilomycin A1 inhibits the targeting of lysosomal acid hydrolases in cultured hepatocytes." Biochem Biophys Res Commun **178**(1): 369-77.

Oh, K. J., S. Barbuto, et al. (2006). "A membrane-targeted BID BCL-2 homology 3 peptide is sufficient for high potency activation of BAX in vitro." J Biol Chem **281**(48): 36999-7008.

Oh, Y. K. and J. A. Swanson (1996). "Different fates of phagocytosed particles after delivery into macrophage lysosomes." J Cell Biol **132**(4): 585-93.

Oltersdorf, T., S. W. Elmore, et al. (2005). "An inhibitor of Bcl-2 family proteins induces regression of solid tumours." Nature **435**(7042): 677-81.

Opferman, J. T., A. Letai, et al. (2003). "Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1." Nature **426**(6967): 671-6.

Orlofsky, A., L. M. Weiss, et al. (2002). "Deficiency in the anti-apoptotic protein A1-a results in a diminished acute inflammatory response." J Immunol **168**(4): 1840-6.

Paddison, P. J., A. A. Caudy, et al. (2002). "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." Genes Dev **16**(8): 948-58.

Parker, L. C., M. K. Whyte, et al. (2005). "The expression and roles of Toll-like receptors in the biology of the human neutrophil." J Leukoc Biol **77**(6): 886-92.

Parsey, M. V., D. Kaneko, et al. (1999). "Neutrophil apoptosis in the lung after hemorrhage or endotoxemia: apoptosis and migration are independent of IL-1beta." Clin Immunol **91**(2): 219-25.

Parsey, M. V., R. M. Tuder, et al. (1998). "Neutrophils are major contributors to intraparenchymal lung IL-1 beta expression after hemorrhage and endotoxemia." J Immunol **160**(2): 1007-13.

Pavlov, E. V., M. Priault, et al. (2001). "A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast." J Cell Biol **155**(5): 725-31.

Pellegrini, M., G. Belz, et al. (2003). "Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim." Proc Natl Acad Sci U S A **100**(24): 14175-80.

Perskvist, N., K. Roberg, et al. (2002). "Rab5a GTPase regulates fusion between pathogen-containing phagosomes and cytoplasmic organelles in human neutrophils." J Cell Sci **115**(Pt 6): 1321-30.

Petros, A. M., D. G. Nettesheim, et al. (2000). "Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies." Protein Sci **9**(12): 2528-34.

Pfeffer, S. and D. Aivazian (2004). "Targeting Rab GTPases to distinct membrane compartments." Nat Rev Mol Cell Biol **5**(11): 886-96.

Pfister, H. W., K. Frei, et al. (1992). "Transforming growth factor beta 2 inhibits cerebrovascular changes and brain edema formation in the tumor necrosis factor alpha-independent early phase of experimental pneumococcal meningitis." J Exp Med **176**(1): 265-8.

Pitt, A., L. S. Mayorga, et al. (1992). "Alterations in the protein composition of maturing phagosomes." J Clin Invest **90**(5): 1978-83.

Print, C. G., K. L. Loveland, et al. (1998). "Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant." Proc Natl Acad Sci U S A **95**(21): 12424-31.

Putcha, G. V., K. L. Moulder, et al. (2001). "Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis." Neuron **29**(3): 615-28.

Puthalakath, H., D. C. Huang, et al. (1999). "The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex." Mol Cell **3**(3): 287-96.

Puthalakath, H., A. Villunger, et al. (2001). "Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis." Science **293**(5536): 1829-32.

Qin, H., S. M. Srinivasula, et al. (1999). "Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor 1." Nature **399**(6736): 549-57.

Ranger, A. M., J. Zha, et al. (2003). "Bad-deficient mice develop diffuse large B cell lymphoma." Proc Natl Acad Sci U S A **100**(16): 9324-9.

Raulet, D. H. (2004). "Interplay of natural killer cells and their receptors with the adaptive immune response." Nat Immunol **5**(10): 996-1002.

Reeves, E. P., H. Lu, et al. (2002). "Killing activity of neutrophils is mediated through activation of proteases by K+ flux." Nature **416**(6878): 291-7.

Reinhardt, R. L., S. J. Kang, et al. (2006). "T helper cell effector fates--who, how and where?" Curr Opin Immunol **18**(3): 271-7.

Ren, Y., J. Tang, et al. (2003). "Increased apoptotic neutrophils and macrophages and impaired macrophage phagocytic clearance of apoptotic neutrophils in systemic lupus erythematosus." Arthritis Rheum **48**(10): 2888-97.

Richter-Dahlfors, A., A. M. Buchan, et al. (1997). "Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo." J Exp Med **186**(4): 569-80.

Rinkenberger, J. L., S. Horning, et al. (2000). "Mcl-1 deficiency results in peri-implantation embryonic lethality." Genes Dev **14**(1): 23-7.

Roberts, R. L., M. A. Barbieri, et al. (2000). "Dynamics of rab5 activation in endocytosis and phagocytosis." J Leukoc Biol **68**(5): 627-32.

Rodrigues, V. S., M. C. Vidotto, et al. (1999). "Apoptosis of murine peritoneal macrophages induced by an avian pathogenic strain of Escherichia coli." FEMS Microbiol Lett **179**(1): 73-8.

Rodriguez, I., K. Matsuura, et al. (1996). "A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection." J Exp Med **183**(3): 1031-6.

Rosenfeld, J. L., R. H. Moore, et al. (2001). "Lysosome proteins are redistributed during expression of a GTP-hydrolysis-defective rab5a." J Cell Sci **114**(Pt 24): 4499-508.

Rossi, A. G., D. A. Sawatzky, et al. (2006). "Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis." Nat Med **12**(9): 1056-64.

Rot, A. and U. H. von Andrian (2004). "Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells." Annu Rev Immunol **22**: 891-928.

Rovere, P., C. Vallinoto, et al. (1998). "Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function." J Immunol **161**(9): 4467-71.

Rubartelli, A., A. Poggi, et al. (1997). "The selective engulfment of apoptotic bodies by dendritic cells is mediated by the alpha(v)beta3 integrin and requires intracellular and extracellular calcium." Eur J Immunol **27**(8): 1893-900.

Russell, J. H. and T. J. Ley (2002). "Lymphocyte-mediated cytotoxicity." Annu Rev Immunol **20**: 323-70.

Saikumar, P., Z. Dong, et al. (1998). "Role of hypoxia-induced Bax translocation and cytochrome c release in reoxygenation injury." Oncogene **17**(26): 3401-15.

Sakahira, H., M. Enari, et al. (1999). "Functional differences of two forms of the inhibitor of caspase-activated DNase, ICAD-L, and ICAD-S." J Biol Chem **274**(22): 15740-4.

Salvesen, G. S. and V. M. Dixit (1999). "Caspase activation: the induced-proximity model." Proc Natl Acad Sci U S A **96**(20): 10964-7.

Sattler, M., H. Liang, et al. (1997). "Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis." Science **275**(5302): 983-6.

Saukkonen, K., S. Sande, et al. (1990). "The role of cytokines in the generation of inflammation and tissue damage in experimental gram-positive meningitis." J Exp Med **171**(2): 439-48.

Sauter, B., M. L. Albert, et al. (2000). "Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells." J Exp Med **191**(3): 423-34.

Savill, J. (1992). "Macrophage recognition of senescent neutrophils." Clin Sci (Lond) **83**(6): 649-55.

Savill, J. (1997). "Apoptosis in resolution of inflammation." J Leukoc Biol **61**(4): 375-80.

Savill, J. (1998). "Apoptosis. Phagocytic docking without shocking." Nature **392**(6675): 442-3.

Savill, J., J. Smith, et al. (1992). "Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis." Kidney Int **42**(4): 924-36.

Savill, J. S., A. H. Wyllie, et al. (1989). "Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages." J Clin Invest **83**(3): 865-75.

Savina, A., C. Jancic, et al. (2006). "NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells." Cell **126**(1): 205-18.

Sawatzky, D. A., D. A. Willoughby, et al. (2006). "The involvement of the apoptosis-modulating proteins ERK 1/2, Bcl-xL and Bax in the resolution of acute inflammation in vivo." Am J Pathol **168**(1): 33-41.

Scaffidi, C., S. Fulda, et al. (1998). "Two CD95 (APO-1/Fas) signaling pathways." Embo J **17**(6): 1675-87.

Scherr, M. and M. Eder (2007). "Gene silencing by small regulatory RNAs in mammalian cells." Cell Cycle **6**(4): 444-9.

Serhan, C. N. (2007). "Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways." Annu Rev Immunol **25**: 101-37.

Serhan, C. N., S. Hong, et al. (2002). "Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals." J Exp Med **196**(8): 1025-37.

Sharpe, J. C., D. Arnoult, et al. (2004). "Control of mitochondrial permeability by Bcl-2 family members." Biochim Biophys Acta **1644**(2-3): 107-13.

Shibue, T., K. Takeda, et al. (2003). "Integral role of Noxa in p53-mediated apoptotic response." Genes Dev **17**(18): 2233-8.

Shinjyo, T., R. Kuribara, et al. (2001). "Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors." Mol Cell Biol **21**(3): 854-64.

Sibley, L. D., E. Weidner, et al. (1985). "Phagosome acidification blocked by intracellular Toxoplasma gondii." Nature **315**(6018): 416-9.

Siegel, D. P. (1984). "Inverted micellar structures in bilayer membranes. Formation rates and half-lives." Biophys J **45**(2): 399-420.

Simmons, M. J., G. Fan, et al. (2008). "Bfl-1/A1 functions, similar to Mcl-1, as a selective tBid and Bak antagonist." Oncogene **27**(10): 1421-8.

Simonsen, A., R. Lippe, et al. (1998). "EEA1 links PI(3)K function to Rab5 regulation of endosome fusion." Nature **394**(6692): 494-8.

Srinivasula, S. M., M. Ahmad, et al. (1998). "Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization." Mol Cell **1**(7): 949-57.

Srinivasula, S. M., M. Ahmad, et al. (1996). "Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases." Proc Natl Acad Sci U S A **93**(25): 14486-91.

Stark, M. A., Y. Huo, et al. (2005). "Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17." Immunity **22**(3): 285-94.

Steel, D. M. and A. S. Whitehead (1994). "The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein." Immunol Today **15**(2): 81-8.

Steinman, R. M., S. Turley, et al. (2000). "The induction of tolerance by dendritic cells that have captured apoptotic cells." J Exp Med **191**(3): 411-6.

Stenmark, H. and V. M. Olkkonen (2001). "The Rab GTPase family." Genome Biol **2**(5): REVIEWS3007.

Stenmark, H., R. G. Parton, et al. (1994). "Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis." Embo J **13**(6): 1287-96.

Stennicke, H. R., Q. L. Deveraux, et al. (1999). "Caspase-9 can be activated without proteolytic processing." J Biol Chem **274**(13): 8359-62.

Stern, M., J. Savill, et al. (1996). "Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha v beta 3/CD36/thrombospondin recognition mechanism and lack of phlogistic response." Am J Pathol **149**(3): 911-21.

Stoka, V., V. Turk, et al. (2007). "Lysosomal cysteine cathepsins: signaling pathways in apoptosis." Biol Chem **388**(6): 555-60.

Strasser, A. (2005). "The role of BH3-only proteins in the immune system." Nat Rev Immunol **5**(3): 189-200.

Strasser, A., A. W. Harris, et al. (1991a). "bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship." Cell **67**(5): 889-99.

Strasser, A., S. Whittingham, et al. (1991b). "Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease." Proc Natl Acad Sci U S A **88**(19): 8661-5.

Stuart, L. M., M. Lucas, et al. (2002). "Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation." J Immunol **168**(4): 1627-35.

Sturgill-Koszycki, S., P. H. Schlesinger, et al. (1994). "Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase." Science **263**(5147): 678-81.

Sulston, J. E. and H. R. Horvitz (1977). "Post-embryonic cell lineages of the nematode, Caenorhabditis elegans." Dev Biol **56**(1): 110-56.

Suzuki, M., R. J. Youle, et al. (2000). "Structure of Bax: coregulation of dimer formation and intracellular localization." Cell **103**(4): 645-54.

Takano, T., C. B. Clish, et al. (1998). "Neutrophil-mediated changes in vascular permeability are inhibited by topical application of aspirin-triggered 15-epi-lipoxin A4 and novel lipoxin B4 stable analogues." J Clin Invest **101**(4): 819-26.

Takeda, K. and S. Akira (2005). "Toll-like receptors in innate immunity." Int Immunol **17**(1): 1-14.

Tan, B. H., C. Meinken, et al. (2006). "Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens." J Immunol **177**(3): 1864-71.

Tang, D. and V. J. Kidd (1998). "Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis." J Biol Chem **273**(44): 28549-52.

Tauber, M. G., U. Borschberg, et al. (1988). "Influence of granulocytes on brain edema, intracranial pressure, and cerebrospinal fluid concentrations of lactate and protein in experimental meningitis." J Infect Dis **157**(3): 456-64.

Tedder, T. F., D. A. Steeber, et al. (1995). "L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites." J Exp Med **181**(6): 2259-64.

Terradillos, O., S. Montessuit, et al. (2002). "Direct addition of BimL to mitochondria does not lead to cytochrome c release." FEBS Lett **522**(1-3): 29-34.

Terrones, O., B. Antonsson, et al. (2004). "Lipidic pore formation by the concerted action of proapoptotic BAX and tBID." J Biol Chem **279**(29): 30081-91.

Thome, M., P. Schneider, et al. (1997). "Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors." Nature **386**(6624): 517-21.

Thornberry, N. A. and Y. Lazebnik (1998). "Caspases: enemies within." Science **281**(5381): 1312-6.

Thorsteinsdottir, U., A. Mamo, et al. (2002). "Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion." Blood **99**(1): 121-9.

Tsujimoto, Y., J. Cossman, et al. (1985). "Involvement of the bcl-2 gene in human follicular lymphoma." Science **228**(4706): 1440-3.

Tuomanen, E. I., K. Saukkonen, et al. (1989). "Reduction of inflammation, tissue damage, and mortality in bacterial meningitis in rabbits treated with monoclonal antibodies against adhesion-promoting receptors of leukocytes." J Exp Med **170**(3): 959-69.

Uematsu, S. and S. Akira (2006). "Toll-like receptors and innate immunity." J Mol Med **84**(9): 712-25.

Ullrich, H. J., W. L. Beatty, et al. (1999). "Direct delivery of procathepsin D to phagosomes: implications for phagosome biogenesis and parasitism by Mycobacterium." Eur J Cell Biol **78**(10): 739-48.

Ullrich, O., H. Horiuchi, et al. (1994). "Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange." Nature **368**(6467): 157-60.

Ullrich, O., H. Stenmark, et al. (1993). "Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins." J Biol Chem **268**(24): 18143-50.

Underhill, D. M. and A. Ozinsky (2002). "Phagocytosis of microbes: complexity in action." Annu Rev Immunol **20**: 825-52.

Ungermann, C. and D. Langosch (2005). "Functions of SNAREs in intracellular membrane fusion and lipid bilayer mixing." J Cell Sci **118**(Pt 17): 3819-28.

Urban, B. C., N. Willcox, et al. (2001). "A role for CD36 in the regulation of dendritic cell function." Proc Natl Acad Sci U S A **98**(15): 8750-5.

van de Beek, D., B. Schmand, et al. (2002). "Cognitive impairment in adults with good recovery after bacterial meningitis." J Infect Dis **186**(7): 1047-52.

Van de Craen, M., W. Declercq, et al. (1999). "The proteolytic procaspase activation network: an in vitro analysis." Cell Death Differ **6**(11): 1117-24.

van Zandbergen, G., J. Gieffers, et al. (2004). "Chlamydia pneumoniae multiply in neutrophil granulocytes and delay their spontaneous apoptosis." J Immunol **172**(3): 1768-76.

Vandivier, R. W., V. A. Fadok, et al. (2002). "Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis." J Clin Invest **109**(5): 661-70.

Veis, D. J., C. M. Sorenson, et al. (1993). "Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair." Cell **75**(2): 229-40.

Vercammen, D., R. Beyaert, et al. (1998). "Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor." J Exp Med **187**(9): 1477-85.

Verhoven, B., R. A. Schlegel, et al. (1995). "Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes." J Exp Med **182**(5): 1597-601.

Verma, S., L. J. Zhao, et al. (2001). "Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis." J Biol Chem **276**(7): 4671-6.

Vieira, O. V., R. J. Botelho, et al. (2002). "Phagosome maturation: aging gracefully." Biochem J **366**(Pt 3): 689-704.

Villunger, A., E. M. Michalak, et al. (2003a). "p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa." Science **302**(5647): 1036-8.

Villunger, A., L. A. O'Reilly, et al. (2000). "Fas ligand, Bcl-2, granulocyte colony-stimulating factor, and p38 mitogen-activated protein kinase: Regulators of distinct cell death and survival pathways in granulocytes." J Exp Med **192**(5): 647-58.

Villunger, A., C. Scott, et al. (2003b). "Essential role for the BH3-only protein Bim but redundant roles for Bax, Bcl-2, and Bcl-w in the control of granulocyte survival." Blood **101**(6): 2393-400.

Walensky, L. D., K. Pitter, et al. (2006). "A stapled BID BH3 helix directly binds and activates BAX." Mol Cell **24**(2): 199-210.

Wang, H. G., N. Pathan, et al. (1999). "Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD." Science **284**(5412): 339-43.

Wang, K., X. M. Yin, et al. (1996). "BID: a novel BH3 domain-only death agonist." Genes Dev **10**(22): 2859-69.

Watson, R. W., H. P. Redmond, et al. (1996). "Neutrophils undergo apoptosis following ingestion of Escherichia coli." J Immunol **156**(10): 3986-92.

Weber, A., S. A. Paschen, et al. (2007). "BimS-induced apoptosis requires mitochondrial localization but not interaction with anti-apoptotic Bcl-2 proteins." J Cell Biol **177**(4): 625-36.

Weber, J. R., K. Angstwurm, et al. (1997). "Heparin inhibits leukocyte rolling in pial vessels and attenuates inflammatory changes in a rat model of experimental bacterial meningitis." J Cereb Blood Flow Metab **17**(11): 1221-9.

Wei, M. C., T. Lindsten, et al. (2000). "tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c." Genes Dev **14**(16): 2060-71.

Wei, M. C., W. X. Zong, et al. (2001). "Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death." Science **292**(5517): 727-30.

Weinmann, P., P. Gaehtgens, et al. (1999). "Bcl-Xl- and Bax-alpha-mediated regulation of apoptosis of human neutrophils via caspase-3." Blood **93**(9): 3106-15.

Werner, A. B., E. de Vries, et al. (2002). "Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax." J Biol Chem **277**(25): 22781-8.

Werner, G., H. Hagenmaier, et al. (1984). "Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity." J Antibiot (Tokyo) **37**(2): 110-7.

White, E. (2006). "Mechanisms of apoptosis regulation by viral oncogenes in infection and tumorigenesis." Cell Death Differ **13**(8): 1371-7.

Whyte, M. K., L. C. Meagher, et al. (1993). "Impairment of function in aging neutrophils is associated with apoptosis." J Immunol **150**(11): 5124-34.

Williams, T. J. and M. J. Peck (1977). "Role of prostaglandin-mediated vasodilatation in inflammation." Nature **270**(5637): 530-2.

Willis, S. N., L. Chen, et al. (2005). "Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins." Genes Dev **19**(11): 1294-305.

Wolfs, J. L., P. Comfurius, et al. (2005). "Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction." Cell Mol Life Sci **62**(13): 1514-25.

Wolter, K. G., Y. T. Hsu, et al. (1997). "Movement of Bax from the cytosol to mitochondria during apoptosis." J Cell Biol **139**(5): 1281-92.

Wuilleme-Toumi, S., V. Trichet, et al. (2007). "Reciprocal protection of Mcl-1 and Bim from ubiquitin-proteasome degradation." Biochem Biophys Res Commun **361**(4): 865-9.

Wyllie, A. H. (1980). "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation." Nature **284**(5756): 555-6.

Xiang, Z., A. A. Ahmed, et al. (2001). "Essential role of the prosurvival bcl-2 homologue A1 in mast cell survival after allergic activation." J Exp Med **194**(11): 1561-69.

Yagisawa, M., A. Yuo, et al. (1996). "Superoxide release and NADPH oxidase components in mature human phagocytes: correlation between functional capacity and amount of functional proteins." Biochem Biophys Res Commun **228**(2): 510-6.

Yamaguchi, H. and H. G. Wang (2002). "Bcl-XL protects BimEL-induced Bax conformational change and cytochrome C release independent of interacting with Bax or BimEL." J Biol Chem **277**(44): 41604-12.

Yamamoto, A., S. Taniuchi, et al. (2002). "Role of reactive oxygen species in neutrophil apoptosis following ingestion of heat-killed Staphylococcus aureus." Clin Exp Immunol **129**(3): 479-84.

Yang, E., J. Zha, et al. (1995). "Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death." Cell **80**(2): 285-91.

Yin, X. M., K. Wang, et al. (1999). "Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis." Nature **400**(6747): 886-91.

You, H., M. Pellegrini, et al. (2006). "FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal." J Exp Med **203**(7): 1657-63.

Yrlid, U. and M. J. Wick (2000). "Salmonella-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells." J Exp Med **191**(4): 613-24.

Yu, J., Z. Wang, et al. (2003). "PUMA mediates the apoptotic response to p53 in colorectal cancer cells." Proc Natl Acad Sci U S A **100**(4): 1931-6.

Yu, J., L. Zhang, et al. (2001). "PUMA induces the rapid apoptosis of colorectal cancer cells." Mol Cell **7**(3): 673-82.

Zerial, M. and H. McBride (2001). "Rab proteins as membrane organizers." Nat Rev Mol Cell Biol **2**(2): 107-17.

Zha, J., H. Harada, et al. (1996). "Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L)." Cell **87**(4): 619-28.

Zha, J., S. Weiler, et al. (2000). "Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis." Science **290**(5497): 1761-5.

Zhang, B., J. Hirahashi, et al. (2003). "Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation." J Biol Chem **278**(31): 28443-54.

Zhong, Q., W. Gao, et al. (2005). "Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis." Cell **121**(7): 1085-95.

Zhu, Y., B. J. Swanson, et al. (2004). "Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells." Proc Natl Acad Sci U S A **101**(20): 7681-6.

Zimmermann, R. and W. Wickner (1983). "Energetics and intermediates of the assembly of Protein OmpA into the outer membrane of Escherichia coli." J Biol Chem **258**(6): 3920-5.

Zong, W. X., T. Lindsten, et al. (2001). "BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak." Genes Dev **15**(12): 1481-6.

Zou, H., Y. Li, et al. (1999). "An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9." J Biol Chem **274**(17): 11549-56.

Zwijnenburg, P. J., T. van der Poll, et al. (2003). "IL-1 receptor type 1 gene-deficient mice demonstrate an impaired host defense against pneumococcal meningitis." J Immunol **170**(9): 4724-30.

Zychlinsky, A., C. Fitting, et al. (1994). "Interleukin 1 is released by murine macrophages during apoptosis induced by Shigella flexneri." J Clin Invest **94**(3): 1328-32.

Zychlinsky, A., M. C. Prevost, et al. (1992). "Shigella flexneri induces apoptosis in infected macrophages." Nature **358**(6382): 167-9.

Zychlinsky, A., K. Thirumalai, et al. (1996). "In vivo apoptosis in Shigella flexneri infections." Infect Immun **64**(12): 5357-65.

Zysk, G., L. Bejo, et al. (2000). "Induction of necrosis and apoptosis of neutrophil granulocytes by Streptococcus pneumoniae." Clin Exp Immunol **122**(1): 61-6.

Acknowledgements

I want to thank Prof. Dr. Georg Häcker for entrusting me with this very interesting and productive project, and for his continuous support and advice, encouragement and overall excellent supervision.

I am very grateful to Prof. Dr. Michael Groll from the Faculty of Chemistry for assuming the official supervision of my thesis.

I wish to thank Dr. Susanne Kirschnek for the initial technical supervision, and collaboration within the phagocyte project. I am also grateful to Dr. Stefan Linder for helpful advice on confocal microscopy.

A special thanks goes to my current and former collegues that were responsible for the very warm, friendly and supportive working environment, with whom I could discuss ideas and doubts, share problems, and who also initiated and participated in a lot of social activities outside the lab. I also want to thank Charles Bradshaw for endless hours of discussing about science, PhDing and other matters, and for (incidentally) being a source of Rab5 knowledge and antibodies.

My biggest thanks goes to my parents, who were always there for advice, motivation and encouragement, and have continuously supported me throughout my studies and this PhD.