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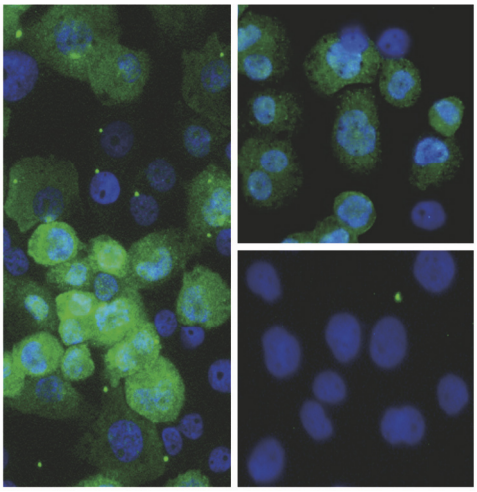
Prevention of Inflammatory Cellular Responses by Ethanol and Hemin – Interplay Between Inflammasomes and Processes Inhibiting Inflammation



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Prevention of inflammatory cellular responses by ethanol and hemin – interplay between inflammasomes and processes inhibiting inflammation

Katariina Nurmi

Academic Dissertation

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"Science has explained nothing; the more we know the more fantastic the world becomes and the profounder the surrounding darkness."

– Aldous Huxley

To my dear family

Contents

List of Original Publications

This thesis is based on the following publications, which are referred to by their Roman numerals in the text.

I **Nurmi K**, Methuen T, Mäki T, Lindstedt KA, Kovanen PT, Sandler C, Eklund KK. (2009) Ethanol induces apoptosis in human mast cells. Life Sciences 85: 678-684.

II **Nurmi K**, Virkanen J, Rajamäki K, Niemi K, Kovanen PT, Eklund KK. (2013) Ethanol inhibits activation of NLRP3 and AIM2 inflammasomes in human macrophages – a novel anti-inflammatory action of alcohol. PLoS One 8: e78537.

III **Nurmi K**, Kareinen I, Virkanen J, Rajamäki K, Vaali K, Levonen A-L, Fyhrquist N, Matikainen S, Kovanen PT, Eklund KK. Hemin and cobalt protoporphyrin inhibit the activation of NLRP3 inflammasome by enhancing autophagy – a novel mechanism of inflammasome regulation. ***Submitted.***

The original publications are reproduced with the permission of the copyright holders. In addition, some previously unpublished data are presented.

Katariina Nurmi´s contribution to the articles

I Participated in the conception and design of individual experiments, was the sole conductor of the apoptosis and cell viability measurements, and conducted some (50%) of the mast cell proliferation measurements ([H^3] thymidine incorporation). Analyzed data, interpreted the results of experiments and participated in writing the manuscript.

II Participated in the conception and design of the research, performed experiments, and participated in ICP-MS measurements. Analyzed data, interpreted results, and participated in writing and editing the manuscript.

III Participated in the conception and design of the research and performed experiments. Performed some (50%) of the *in vivo* mouse peritonitis experiments and participated in the ICP-MS measurements. Analyzed data, interpreted results, and participated in writing and editing the manuscript.

Abbreviations

3-MA	3-methyladenine
AIM2	absent in melanoma 2
ALR	absent in melanoma 2 [AIM2]-like receptor
AMPK	adenosine monophosphate-activated protein kinase
APAF1	apoptotic protease-activating factor-1
ARDS	acute respiratory distress syndrome
ASC	apoptosis-associated speck-like protein containing a caspase-recruitment domain
Atg	autophagy-related protein
ATG16L1	autophagy-related protein 16-like 1
ATP	adenosine triphosphate
Bach1	BTB and CNC homolog 1
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2- antagonist/killer-1
Barkor	Beclin 1-associated autophagy-related key regulator
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma
Bcl-w	Bcl-2-like-2
Bcl-xL	Bcl-2-like protein
BH3	Bcl-2 homology-3 domain
Bid	BH3-interacting domain death antagonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-like-11
BIR	baculoviral inhibitory repeat domain
Bnip3L	Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3-like
Bok	Bcl-2-related ovarian killer
CAPS	cryopyrin-associated periodic syndromes
CARD	caspase activation and recruitment domain
CD	cluster of differentiation
c-FLIP	cellular FLICE inhibitory protein
CHD	coronary heart disease
cIAP	cellular inhibitors of apoptosis protein
CINCA	chronic infantile neurologic cutaneous articular syndrome
CO	carbon monoxide
COP	CARD-only protein
CPA3	carboxypeptidase A3
CRP	C-reactive protein
DAMP	danger-associated molecular patterns
DAPk	death-associated protein kinase
DD	death domain
DED	death effector domain
DIABLO	direct IAP-binding protein with low pI
DISC	death-inducing signaling complex
DPI	diphenylene iodium
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
FasR	Fas receptor
FCAS	familial cold autoinflammatory syndrome
FcεRI	high-affinity Immunoglobulin (Ig)E receptor
FIP200	focal adhesion kinase family-interacting protein of 200 kDa
GM-CSF	granulocyte macrophage colony-stimulating factor
GPSM3	G protein signaling modulator-3
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
HMGB	high-mobility group box 1
HO	heme oxygenase
Hrk	hara-kiri
Hsc70	heat shock 70 kDa protein 8
HSP	heat shock protein
HtrA2	high-temperature requirement A2
IAP	inhibitor of apoptosis protein
IBM	IAP-binding motif
ICE	IL-1β converting enzyme
IFI16	interferon inducing protein 16
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IL-1RA	IL-1 receptor antagonist
IRAK	IL-1 receptor activated kinase
Jak-STAT	Janus kinase and signal transducer and activator of transcription
Jnk	c-Jun N-terminal kinase
Keap1	kelch-like ECH-associated protein 1
LAMP2A	lysosomal-associated membrane protein 2
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LRRFIP2	Fli-I-interacting protein 2
MALT	mucosa-associated lymphoid tissue lymphoma translocation protein
MAPK	mitogen-activated kinase
Mcl-1	myeloid cell leukemia sequence-1
MCP	monocyte chemotactic protein
M-CSF	macrophage colony-stimulating factor
MC_T	tryptase-positive mast cell type
MC_{TC}	chymase tryptase-positive mast cell type
miRNA	microRNA
MOMP	mitochondrial outer membrane permeabilization

MSU	monosodium urate
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
Mule	Mcl-1 ubiquitin ligase E3
MWS	Muckle-Wells syndrome
MyD88	myeloid differentiation factor 88
NAC	N-acetyl cysteine
NACHT	central nucleotide-binding and oligomerization domain
NAD	NACHT-associated domain
NADPH	nicotinamide adenine dinucleotide phosphate
NAIP	NLR family apoptosis-inhibitory protein
NBR1	neighbor of BRCA1 gene 1
NFκB	nuclear factor κ-light chain-enhancer of activated B cells
NK-cell	natural killer cell
NLR	nucleotide-binding domain and leucine-richrepeat-containing receptor
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	nucleotide-binding domain and leucine -rich repeat-containing family, pyrin domain-containing 3
NO	nitric oxide
NOMID	neonatal onset multi-system inflammatory disorder
Noxa	phorbol-12-myristate-13-acetate-induced protein 1
Nrf2	NF-E2-related factor-2
P2X	P2X purinergic receptor
PAMP	pathogen-associated molecular patterns
PI3K	phoshatidylinositol-3 kinase
PI3P	phosphatidylinositol 3-phosphate
PMA	phorbol 12-myristate 13-acetate
PML	promyelotic leukemia protein
POP	pyrin-only protein
PPA2	pyrophosphatase 2
PRR	pattern recognition receptor
PS	phosphatidyl serine
Puma	Bcl-2-binding component-3
PYD	pyrin
PYHIN	pyrin domain and hematopoietic expression, interferon-inducible nature, and nuclear localization domain
RA	rheumatoid arthritis
RANTES	regulated on activation normal T-cell expressed and secreted
RIP1	receptor-associated protein 1
RIPK1	receptor-associated protein kinase 1
ROS	reactive oxygen species
Rubicon	RUN domain- and cysteine-rich domain-containing, Beclin 1-interacting
S1P	sphingosine-1-phosphate
SAA	serum amyloid A
SCF	stem cell factor
SGT1	suppressor of G2 allele of skp1
Smac	second mitochondrial activator of caspases
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SQSTM1	sequestosome 1
STAT	signal transducers and activators of transcription
SYK	spleen tyrosine kinase
TEPM	thioglycollate elicited peritoneal macrophage
TGFβ	transforming growth factor-β
T_H	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TRADD	TNF receptor-associated-protein with death domain
TRAF2	TNF receptor-associated protein 2
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
TRIF	TIR-domain-containing adaptor – inducing interferon-β
TRIM	tripartite-motif protein
TRX	thioredoxin
TUNEL	Terminal transferase mediated nick end labelling
TXNIP	thioredoxin-interacting protein
ULK	unc-51-like kinase
UTP	uridine triphosphate
UVRAG	UV irradiation resistance-associated gene
VEGF	vascular endothelial growth factor
VMP1	vacuole membrane protein 1
VPS	vacuolar sorting protein
WIPI	WD-repeat protein interacting with phosphoinositide
XIAP	X-linked inhibitor of apoptosis

Abstract

The innate immune system responds to infection or injury by initiating nonspecific inflammation, which functions to limit the spread of harmful microbes or the damage caused by tissue injury. The cells of the innate immune system are the first to encounter danger signals, and they mediate the rapid local immune response. Inflammatory reactions are normally beneficial for the host, and inflammation is usually resolved when the threat has been removed. However, in chronic inflammatory diseases, the danger signals either are not cleared or continue to be formed. Pathogen-derived molecules and danger signals induce the activation of pattern recognition receptors (PRRs) in the innate immune cells. Several families of PRRs exist, and their interplay is needed for the induction of efficient immune defense reactions.

Nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) are intracellular receptors that respond to a plethora of danger- and pathogen-associated molecular patterns. Their activation induces the assembly and activation of cytosolic multiprotein complexes called inflammasomes. Inflammasomes act as a primary checkpoint for the activation and secretion of the strong proinflammatory cytokines interleukin (IL)-1β and IL-18. Similar to other cellular functions, innate immune responses are operated via a complicated interplay between signalosomes. To become activated inflammasomes require the coordinated activation of Toll-like receptors (TLRs) and NLRs, which induce the activation and assembly of inflammasome complexes. The consequent secretion of inflammasome-derived cytokines is, in turn, modulated by autophagy. Inflammasome activation and autophagy also interact with cellular death pathways. Cellular death acts to limit the spread of intracellular pathogens by denying a protective niche to these pathogens, thereby inhibiting their replication and predisposing them for detection by the immune system.

The aim of this study was to investigate the roles of ethanol and hemin in the modulation of innate immune cell functions, as well as the mechanisms underlying the reported protective effects of ethanol and hemin against chronic inflammatory diseases. Alcohol is the most commonly and widely used drug in the world. The consequences of alcohol consumption depend on both the pattern of consumption and the amounts consumed. Alcohol abuse predisposes to more frequent and severe infections, whereas the light to moderate consumption of alcoholic beverages has been associated with a reduced incidence of chronic inflammatory conditions, such as cardiovascular diseases and rheumatoid arthritis. These seemingly different responses may both derive from attenuated reactions of innate immunity. In the present thesis study, ethanol was shown to reduce the viability and proliferation of mast cells. This reduced viability resulted from the immunologically silent apoptotic death of mast cells. In macrophages, ethanol reduced the pyroptotic cell death induced by inflammasome activation and instead directed cell death toward apoptosis.

Excessive inflammasome activation is a prominent feature of several chronic inflammatory diseases. The mechanisms that restrain inflammasome activation were studied in greater detail in cultured macrophages. Ethanol dose-dependently inhibited inflammasome activation and the secretion of IL-1β in human macrophages. It was further shown that the inhibitory effect of ethanol was mediated by a reduction in lysosomal disruption and the release of active cathepsin B, which thus contributed to diminished inflammasome assembly.

The majority of mammalian cells are constantly renewed. Enormous numbers of senescent red blood cells are phagocytosed daily by macrophages. In certain pathologies, such as malarial infection, massive hemolysis occurs that exceeds the capacity of the scavenging and degradation systems of hemoglobin. As a consequence, free heme and hemin are released into the circulation. Free heme and hemin are cytotoxic and proinflammatory compounds. However, heme and hemin are also potent inducers of the heme oxygenase-1 (HO-1) enzyme, which possesses anti-inflammatory and cytoprotective effects. In the present thesis study, hemin and its synthetic derivative cobalt protoporphyrin (CoPP) blocked inflammasome activation and assembly. Decreased secretion of IL-1β was also observed *in vivo* in a nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 3 (NLRP3)-dependent peritonitis model in mice. The inhibitory effects of hemin and CoPP were partially dependent on the induction of HO-1 transcription by NF-E2-related factor-2 (Nrf2) and the enzymatic activity of HO-1. The inhibitory effects of hemin and CoPP were mediated by increased degradation of inflammasome components, which was due to elevated autophagy.

Overall, the results of this study demonstrate that ethanol and hemin markedly prevent inflammatory cellular responses in macrophages and mast cells. This inhibition may contribute to the cardioprotective effect of ethanol and the anti-inflammatory effects of hemin. An enhanced molecular understanding of the mechanisms by which ethanol and hemin inhibit inflammation may help reveal new therapeutic options in the treatment of chronic inflammatory diseases.

I Introduction

The immune system has evolved to detect potential threats and to provide protection against microbial infections and other harmful agents. The mammalian defense system consists of innate and adaptive components that function via close interactions with each other. The innate immune system relies on both cellular and humoral mechanisms to eradicate threats. Hence, the key function of the immune system is to initiate the immune response by the direct recognition of molecular patterns that signal danger or intrusion by pathogens (Matzinger 2002, Medzhitov & Janeway 2002). The cells of the innate immune system serve as a first line of defense, as exemplified by the ability of activated macrophages and mast cells to secrete powerful proinflammatory cytokines and mediators that act as strong attractants for other immune cells, thereby initiating local and systemic inflammation (Beutler 2004, da Silva *et al*. 2014).

Several pattern recognition receptors (PRRs) account for the recognition of danger signals. Among them, nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) are intracellular receptors that induce the assembly of large proinflammatory signalosomes, called inflammasomes, in response to a wide variety of pathogen-derived and endogenous danger signals (Latz *et al*. 2013). Activated inflammasomes induce the secretion of the strong proinflammatory mediators interleukin (IL)-1β and IL-18. Despite the tight control of inflammasome activation, the excessive activation of the nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 3 (NLRP3) inflammasome has been associated with the pathogenesis of severe autoinflammatory diseases, and of certain common chronic inflammatory diseases, such as atherosclerosis, gout, and type 2 diabetes (Kastner *et al*. 2010, Duewell *et al*. 2010, Rajamaki *et al*. 2010, Martinon *et al*. 2006, Grant & Dixit 2013). Therefore, the mechanisms of inhibition, resolution, and downmodulation are a crucial area of inflammasome research, yet, one that has received less attention.

Proteins involved in intracellular signaling have a tendency to form networks that permit the temporal and spatial regulation of signals, as well as the local enrichment of signal-mediating components to enable proximity-driven signal transduction (Wu 2013). The major signalosomes of the cell engage in constant interactions to combine extracellular and intracellular signals with the appropriate outcomes. Recent evidence has revealed the existence of major interplay and multifaceted modulatory functions among autophagy, apoptosis and inflammasome activation, yet we have just begun to unravel these connections.

Alcohol, heme, and hemin are potent immunomodulatory agents. Alcohol induces a biphasic modulation of the immune response. The effects of alcohol depend critically on the dose and duration of alcohol intake. Chronic abuse of alcohol increases the incidence of severe infections and certain tumors (Goral *et al*. 2008), whereas the light to moderate consumption of alcohol reduces the risk of chronic inflammatory diseases such as coronary heart disease

(Rimm *et al*. 1999) and rheumatoid arthritis (RA) (Jin *et al*. 2014). Moreover, endogenous heme and hemin contribute to both the enhancement and resolution of inflammation. Free heme and hemin induce inflammation and cytotoxic effects. The anti-inflammatory and cytoprotective effects of heme and hemin have been associated with the induction of heme oxygenase-1 (HO-1) and with the degradation products of heme and hemin (Kumar & Bandyopadhyay 2005, Abraham & Kappas 2008, Gozzelino *et al*. 2010).

The aim of this study was to explore the effects of ethanol and hemin on two cellular key players of innate immune defense, mast cells and macrophages, as well as the consequences of such effects on the pathogenesis of chronic inflammatory diseases. Moreover, we studied the signaling pathways that mediate the modulation of mast cell and macrophage functions, and we discovered links in the multilayered crosstalk among apoptosis, inflammasome and autophagy.

II Review of the Literature

1. Innate immune cells

Innate immune cells are the first cells to encounter infectious agents. Initial bacterial recognition and the rapid initiation of the immune response in vertebrates rely mainly on innate immune cells, which are derived from hematopoietic stem cells in the bone marrow. With the exception of natural killer (NK) cells, which originate from the lymphoid lineage, mast cells, macrophages, dendritic cells, and granulocytes i.e., neutrophils, eosinophils, and basophils are derived from a common myeloid progenitor (Kitamura *et al*. 1993, Hoebeke *et al*. 2007, Galli *et al*. 2011). Many of the functions of immune cells are confined to certain cell types, but they also share common features. For example, neutrophil numbers are greatly expanded during infection, and although neutrophils are the workhorses of bacterial phagocytosis, macrophages and dendritic cells also phagocytose pathogens. In addition to phagocytic bacterial clearance, innate immune cells secrete a plethora of mediators that orchestrate the immune response via the further recruitment of cells of the innate and adaptive immune defense systems. Furthermore, macrophages and dendritic cells present antigens to adaptive immune cells, which is crucial for the initiation of adaptive immune responses (Beutler 2004). Mast cells and macrophages, which are relevant to this study, are discussed in greater detail below.

1.1 Mast cells

1.1.1 Mast cell heterogeneity and distribution

The most distinctive morphological features of mast cells are their secretory cytoplasmic granules and monolobular nucleus (Kritikou *et al*. 2015). Mast cells were first described by Paul Erlich, who also named them (Beaven 2009). The term “mast cells”, or “Mastzellen”, is derived from the German word “mästung”, meaning “to fatten or feed something”. The name refers to the metachromatically stained granules of mast cells, which Erlich thought to represent internalized material that had been fed to mast cells by other cells (Beaven 2009). Mast cells migrate from the bone marrow as immature precursors to differentiate and proliferate in mucosal and connective tissues (Kitamura *et al*. 1993). In tissues, mast cell differentiation is principally regulated by stem cell factor (SCF), the ligand of the c-Kit receptor, which is secreted by fibroblasts and endothelial cells (Galli *et al*. 1993, Broudy *et al*. 1994). In addition to SCF, local concentrations of IL-3 and T helper (T_H) type

2-associated cytokines, such as IL-4 and IL-9, and a wide array of other factors contribute to mast cell maturation (Galli *et al*. 1993, Galli *et al*. 2005). The wealth of factors that guide mast cell differentiation leads to the formation of a heterogeneous mast cell population, with variations in phenotype, granule content, receptor expression, and cytokine production (Friend *et al*. 1996, Galli *et al*. 2005).

Mast cells are categorized into subtypes according to their granule protease content. The two main mast cell subtypes are the tryptase-positive (MC_T) and chymase tryptase-positive (MC_{TC}) types, which contain predominantly tryptase or tryptase, chymase, cathepsin G, and carboxypeptidase A3 (CPA3), respectively (Irani *et al*. 1986, Schechter *et al*. 1990, Dougherty *et al*. 2010). However, there are exceptions in the protease content: some tryptase-positive mast cells have also been shown to contain CPA3 in their granules (Dougherty *et al*. 2010). Mast cells are relatively long-lived cells; resident mast cells may survive from months up to years in tissues (Kiernan 1979, Dichlberger *et al*. 2015). Due to the plasticity of mast cells, the tissue microenvironment has a profound impact on mast cell phenotypes, including the granule protease content of mature mast cells. The sole mast cell progenitor has the capacity to differentiate into any subtype (Maaninka *et al*. 2013). In addition, the phenotype is reversible and can be altered during the mast cell's lifespan in response to changes in the microenvironment, such as inflammation (Friend *et al*. 1996). In humans, mast cells are located predominantly at the interface of the host and the external environment. The MC_{TC} subtype is mostly found in connective tissues, such as the skin and the submucosa of the bowel, whereas MC_T predominates in the bowel mucosa, lung subepithelium and alveoli (Irani *et al*. 1986). Their strategic location allows mast cells to react quickly to intruding environmental pathogens, danger signals, and toxins (Galli *et al*. 2011). Upon stimulation, the number of mast cells can be increased by the enhanced recruitment and maturation of progenitors and by prolonged survival (Galli & Tsai 2010). In addition, the proliferation of resident mast cells has been reported (Galli & Tsai 2010).

1.1.2 Mast cell activation and mediators

Mast cells bear numerous cell surface receptors and are activated by various stimuli. The best studied mast cell receptor is the high-affinity immunoglobulin (Ig) E receptor (FcεRI), which is activated in allergic reactions. Antigen binding to IgE induces the cross-linkage of IgE. The subsequent aggregation of IgE-bound FcεRI receptors induces the phosphorylation of the cytoplasmic regions of the receptors, thereby leading to a signaling cascade via spleen tyrosine kinase (Syk) or other protein tyrosine kinases (Kawakami & Galli 2002). The activation of these protein tyrosine kinases induces the phosphorylation of several signaling proteins, resulting in the release of granule contents to the extracellular space and the *de novo* synthesis of mediators (Kawakami & Galli 2002). Bacterial infections induce mast cell activation through PRRs. Mast cells express many Toll-like receptors (TLRs) and are reported to induce the secretion of cytokines in response to the activation of TLR2 and TLR4 (Supajatura *et al*. 2002). Further, Supajatura *et al*. demonstrated that mast cell-deficient mice reconstituted with TLR4-/- mast cells showed significantly increased mortality compared to

mice reconstituted with TLR4+/+ mast cells in response to cecal ligation and puncture-induced sepsis, highlighting the vital importance of mast cell function during bacterial infection.

Mast cells are endowed with the ability to release and generate a variety of newly synthesized and presynthesized mediators with proinflammatory, anti-inflammatory, and immunosuppressive activities (da Silva *et al*. 2014). Upon stimulation, mast cells can release preformed mediators stored in their granules, including 1) the mast cell proteases chymase, tryptase, cathepsin B, and CP3A; 2) the biogenic amines histamine and serotonin; 3) the proteoglycans heparin and chondroitin sulfates; 4) lysosomal enzymes and other enzymes, such as β-hexosaminidase; and 5) numerous cytokines, chemokines, growth factors and peptides, such as monocyte chemotactic protein (MCP), regulated on activation normal T-cell expressed and secreted (RANTES), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), SCF, and preformed tumor necrosis factor (TNF) α (da Silva *et al*. 2014). Activation also induces the synthesis of lipid mediators, including leukotrienes, prostaglandins, and platelet-activating factor, from membrane phospholipids. *De novo*-generated cytokines and chemokines include several interleukins, such as IL-1α, IL-1β, IL-6, IL-18, and TNFα (da Silva *et al*. 2014). Additionally, mast cells secrete newly synthesized growth factors, such as granulocyte macrophage colony-stimulating factor (GM-CSF), nitric oxide (NO), and complement factors (da Silva *et al*. 2014).

Mast cells contribute to a variety of physiological processes, such as wound healing, angiogenesis and the recruitment of immune cells such as neutrophils during inflammation (Weller *et al*. 2011). Mast cells can also participate in bacterial clearance by phagocytosis and the subsequent degradation of bacteria in acidified vacuoles, and they can kill bacteria via the secretion of NO and antimicrobial peptides (Malaviya *et al*. 1994, Rao & Brown 2008). However, increased numbers of mast cells, alterations in their distribution and phenotypes, and adverse mast cell activation are observed in numerous inflammatory conditions. Perhaps the most well-known conditions to which mast cells contribute are allergic reactions and asthma (Bischoff 2007, Dougherty *et al*. 2010). Furthermore, increased mast cell numbers and their dysregulated activation have been reported in metabolic diseases, such as diabetes and atherosclerosis. In atherosclerosis, mast cells contribute to plaque development by various mechanisms. Among others, mast cells secrete heparin and chymase, which increase foam cell formation by inducing the fusion of low-density lipoprotein (LDL) particles on the surface of exocytosed mast cell granules, after which the lipid particle-containing granules are phagocytosed by macrophages (Kokkonen & Kovanen 1989, Kovanen 1993). Similarly, the assembly of activated mast cells in plaques has been shown to contribute to plaque progression and instability, e.g., by increasing angiogenesis in the plaque and by eventually promoting plaque rupture via a histamine-induced coronary spasm (Kaartinen *et al*. 1994, Lappalainen *et al*. 2004, Laine *et al*. 1999). Further evidence of the role of mast cells in metabolic diseases was provided by Liu *et al*. in a study demonstrating that the number of mast cells in the adipose tissue of obese humans was increased. In addition, mast cell

deficiency was shown to improve glucose metabolism and to inhibit inflammation of the adipose tissue in a mouse model (Liu *et al*. 2009). Dysfunctions of mast cells have also been implicated in the pathology of cancer and autoimmune diseases (Rao & Brown 2008).

1.2 Macrophages

1.2.1 Macrophage heterogeneity and distribution

Macrophages are mononuclear cells that are identified by their receptor expression and characteristic staining. Elie Metchnikoff is generally regarded as the father of the concept of phagocytosis and the discoverer of phagocytic cells (Cavaillon 2011). A heterogeneous population of monocytes exits the bone marrow and differentiates into macrophages in the tissues; thus, these circulating monocytes form a reservoir for the replenishment of tissue macrophages in some tissues and during inflammation or pathologies (Italiani & Boraschi 2014). However, the local proliferation maintains the population of tissue-resident macrophages under normal conditions in most tissues (Haldar & Murphy 2014). Macrophages are long-lived cells. For example, resident murine lung macrophages can live up to 8 months, and lifespans ranging from several weeks to months have been reported for resident liver macrophages, depending on the species and experimental circumstances (Murphy *et al*. 2008, Naito *et al*. 1997). Macrophages are found virtually everywhere in the body. Some tissue-specific macrophages with characteristic phenotypes and specialized functions and locations have been given specific names; these include Kupffer cells in the liver and gastrointestinal tract, Langerhans cells in the epidermis, osteoclasts in the bone, microglial cells in the central nervous system, alveolar macrophages in the lung, and histiocytes in the connective tissue (Haldar & Murphy 2014).

Macrophages respond avidly to signals from the surrounding microenvironment, such as signals generated during inflammation or infection, and in response to secreted mediators. These signals induce rapid and reversible changes in macrophage responses by modifying their gene expression (Murray & Wynn 2011a, Zhou *et al*. 2014). Based on their gene expression profiles, macrophages are classified as subpopulations: M1, M2, and regulatory macrophages. Thus, in the macrophage phenotypic polarization, M1 and M2 represent the opposite ends of a continuum, rather than confined phenotypes applicable to all macrophages (Mosser & Edwards 2008, Murray & Wynn 2011a, Zhou *et al*. 2014). Proinflammatory M1 polarization is induced by the T_H1 cytokine interferon-γ (IFNγ), TNFα, and TLR activation. M1 macrophages are prototypical immune effector cells, and their actions are vital for host defense. Upon activation, M1-polarized macrophages secrete high levels of proinflammatory cytokines and exhibit increased pathogen- and tumor cell-killing capacity (Edwards *et al*. 2006, Mosser & Edwards 2008, Italiani & Boraschi 2014). The classical T_H2 cytokines IL-4 and IL-13 induce the generation of M2-polarized macrophages, which suppress inflammation and promote wound healing by secreting collagen and polyamine precursors, among other mediators (Edwards *et al*. 2006, Mosser & Edwards 2008, Italiani & Boraschi 2014). The third macrophage subpopulation, regulatory macrophages, resembles M2 macrophages, and their polarization is induced by IgG immune complexes in

the presence of TLR agonists (Edwards *et al*. 2006, Mosser & Edwards 2008). Other factors that induce regulatory macrophage polarization include apoptotic cells, prostaglandins, and IL-10 (Edwards *et al*. 2006, Mosser & Edwards 2008). Regulatory macrophages produce the anti-inflammatory mediators IL-10 and TGF-β1, and they induce T_H2 and regulatory T cell responses, which further suppress immune reactions (Edwards *et al*. 2006, Mosser & Edwards 2008, Galli *et al*. 2011).

1.2.2 Macrophage Activation

Macrophages are efficient phagocytes, which, in addition to bacterial clearance, secrete mediators that recruit more immunity effector cells to the site of inflammation. Furthermore, macrophages promote immune responses by presenting antigens to cells of adaptive immune defence. Macrophages are endowed with a variety of PRRs that sense bacterial ligands and endogenous danger signals. The activation of macrophage PRRs enhances phagocytosis, bacterial killing, and the secretion of strong proinflammatory mediators (PRR activation is explained in greater detail in Chapter 4.1) (Galli *et al*. 2011). Proinflammatory mediator release at the site of inflammation recruits circulating monocytes to the tissue and guides them to differentiate into proinflammatory type M1 macrophages, which further enhances pathogen clearance or the progression of inflammation (Murray & Wynn 2011b). In addition to their roles in immune defense, macrophages perform important functions that contribute to homeostasis in tissues and the body. Macrophages express several different scavenger receptors, which contribute to the removal of senescent red blood cells (explained in greater detail in Chapter 2.2.1) and apoptotic and necrotic cells, in addition to performing other roles (Mosser & Edwards 2008).

Macrophages have a marked impact on immune reactions and systemic metabolism, and they contribute to a variety of different processes, including angiogenesis, cancer, and reproduction (Murray & Wynn 2011b, Zhou *et al*. 2014). The pathogenic functions of macrophages are associated with several chronic inflammatory diseases and metabolic pathologies, such as atherosclerosis (Murray & Wynn 2011b). One of the defining signs of atherosclerosis is the accumulation of macrophages in the intima of the arterial wall. Macrophages contribute critically to the progression of atherosclerosis at all stages by the secretion of proinflammatory cytokines and foam cell formation (Duewell *et al*. 2010, Rajamaki *et al*. 2010, van Tits *et al*. 2011, Fenyo & Gafencu 2013). Macrophage colony-stimulating factor (M-CSF) and GM-CSF are among the most important mediators of macrophage differentiation in plaques; however, various other stimuli in plaques or, possibly, a variety of monocyte populations, from which macrophages derive, contribute to the formation of heterogeneous macrophage populations (Fenyo & Gafencu 2013, Chinetti-Gbaguidi *et al*. 2015). One example of macrophage plasticity was demonstrated by van Tits *et al*., who reported that cultured M2 macrophages were more prone to the uptake of minimally oxidized LDL and foam cell formation. Subsequent to oxidized LDL uptake, the gene expression and cytokine secretion of M2 macrophages shifted toward the M1 phenotype (van Tits *et al*. 2011). Macrophage polarization may also alter the progression of type 2 diabetes. Macrophage infiltration to the white adipose tissue results in local

inflammation (Xu *et al*. 2003). On the other hand, the polarization of macrophages toward the M2 phenotype has been suggested to protect against obesity, insulin resistance and glucose intolerance (Odegaard *et al*. 2007). In addition, macrophages are among the most important effector cells in arthritis (Gierut *et al*. 2010). In synovial tissue, the normally quiescent macrophages become activated, and the subsequent secretion of proinflammatory mediators, such as IL-1 and TNFα, further accelerates inflammation and the consequent joint destruction (Kennedy *et al*. 2011). However, in addition to macrophages, other cells in inflamed joints are capable of secreting proinflammatory cytokines (Kennedy *et al*. 2011).

2. Ethanol and heme as immunomodulatory compounds

Both ethanol and heme/hemin are potent immunomodulatory compounds. The consequences of ethanol consumption are highly dependent on the amounts consumed and the pattern of use. Acute and chronic alcohol consumption induce biphasic immunologic responses. Acute ethanol exposure attenuates inflammatory responses; conversely, chronic alcohol consumption augments inflammation. Moreover, the pro-oxidant and cytotoxic effects of free heme/hemin are widely acknowledged. The deleterious effects of heme/hemin are potently reversed by the induction of HO-1 (Abraham & Kappas 2008). The essential role of HO-1 in cellular homeostasis is emphasized by its high evolutionary conservation from bacteria to higher plants and animals (Wilks 2002).

2.1 Ethanol

Alcoholic beverages have been ingested since the Neolithic Stone Age, and alcohol has been the most commonly abused drug throughout history (Szabo & Mandrekar 2009). Alcohol consumption has been reported to induce beneficial effects in addition to several serious health hazards. The heavy, regular, consumption of alcohol increases the risk of injury, violence, suicide, poisoning, cirrhosis, cardiovascular ailments (e.g., cardiomyopathy), high blood pressure, hemorrhagic stroke, acute respiratory distress syndrome (ARDS), and several types of cancer (Thun *et al*. 1997, Goral *et al*. 2008). In addition, alcohol abuse increases susceptibility to bacterial and viral infections (Molina *et al*. 2010). Moreover, alcoholics experience infections that are more severe and exhibit impaired resolution (Molina *et al*. 2010). However, the effects of alcohol consumption are highly dependent on the pattern and amount of alcohol consumption, as well as on the affected organs. Thus, moderate alcohol consumption, i.e., daily intake up to 30 g of ethanol, has been associated with a lower risk of coronary heart disease (CHD) (Rimm *et al*. 1999). Although moderate alcohol consumption is socially accepted and can be part of a healthy lifestyle, it is widely acknowledged that alcohol consumption should not be encouraged (Rimm *et al*. 1999, O'Keefe *et al*. 2007).

2.1.1 Effects of ethanol on immune cell activation

Alcohol has direct effects on immune cell functions. The varying effects of acute and prolonged ethanol exposure on lipopolysaccharide (LPS)-induced signaling were demonstrated by Manderkar *et al*. In their study, acute alcohol exposure increased the levels of IL-1 receptor-activated kinase-M (IRAK-M) in monocytes (Mandrekar *et al*. 2009). IRAK-M is a negative regulator of the LPS-induced signaling cascade, and its activation leads to the reduced activation of nuclear factor κ-light chain-enhancer of activated B cells (NFκB) and to hyporesponsiveness of peripheral blood monocytes to LPS (Mandrekar *et al*. 2009). In contrast, prolonged exposure of monocytes to ethanol reduced IRAK-M, thereby increasing the activation of NFκB and subsequent secretion of TNFα (Mandrekar *et al*. 2009). Acute ethanol exposure has also been reported to reduce the LPS-induced secretion of TNFα and IL-8 by inhibiting p38 mitogen activated kinase (p38 MAPK), another signaling protein downstream of TLR activation (Arbabi *et al*. 1999). A number of studies have reported a general trend toward reduced inflammatory responses, which are reflected by diminished TLR signaling and proinflammatory mediator release, as well as increased levels of IL-10, in response to acute ethanol exposure (Boe *et al*. 2001, Pruett *et al*. 2004, Pruett & Pruett 2006). The reduced secretion of proinflammatory mediators induced by acute ethanol exposure suppresses neutrophil chemotaxis and thus can diminish pathogen clearance and immune responses (Arbabi *et al*. 1999, Boe *et al*. 2001). In addition, alcohol impairs the function of adaptive immunity by downregulating $CD4^+$ T cell responses and by decreasing the absolute numbers of T cells and B cells (Molina *et al*. 2010).

The biphasic effect of ethanol may be further explained by the effects of ethanol on acute-phase proteins. TLR activation-induced elevation of serum amyloid A (SAA) and IL-6 leves were decreased shortly after the administration of both higher and lower doses of ethanol in mice. However, the higher concentration of ethanol alone activated the acute-phase response at later time points, during which ethanol was already metabolized (Pruett & Pruett 2006). The pattern of acute-phase protein expression may reflect the immune suppression that occurs in the presence of low ethanol concentrations and, consequently, the proinflammatory effects reported at high dosages (Pruett & Pruett 2006). Pruett *et al*. hypothesized that the increase in acute-phase proteins might be due to the ethanol-induced leakiness of the gastrointestinal tract, which allows LPS to enter the circulation. Indeed, highly elevated levels of LPS are detected in the blood of alcoholics and in animal models (Hines & Wheeler 2004, Rao *et al*. 2004). In addition, ethanol and its metabolite acetaldehyde have been shown to increase intestinal permeability by disrupting epithelial tight junctions (Rao *et al*. 2004). Furthermore, chronic alcoholics show elevated levels of proinflammatory cytokines in their blood, and increased TNFα secretion from Kupffer cells is considered a sign that predicts the beginning of liver injury (Hines & Wheeler 2004, Nagy 2004, Goral *et al*. 2008). Ethanol-induced liver injury is further aggravated by increased oxygen radical production by Kupffer cells and the enhanced expression of classical and alternative pathway complement components, particularly C3 (Hines & Wheeler 2004, Bykov *et al*. 2006, Bykov *et al*. 2007). The synthesis of C3 may indirectly be due to the increased LPS levels in the blood (Bykov *et al*. 2007).

Although complement activation normally drives the clearance of damaged cellular material and the subsequent resolution of inflammation, ethanol-induced increases in the expression of early and classical pathway complement components contribute to tissue damage (Bykov *et al*. 2006, Bykov *et al*. 2007). The effects of acute and chronic ethanol exposure on LPS-induced signaling and inflammatory markers are summarized in Table 1.

Moderate or acute alcohol	High or chronic alcohol
IRAK-M activation ↑	IRAK-M activation ↓
IRAK1 ↓	IRAK1 ↑
IKK activity ↓	IKK activity ↑
NFκB activation ↓	NFκB activation ↑
ERK1/2 activation ↓	ERK1/2 activation ↑
p38 MAPK ↓	p38 MAPK ↑
Proinflammatory mediator secretion ↓	Proinflammatory mediator secretion ↑
IL-10 and TGF-β secretion ↑	IL-10 ↑
SAA ↓	SAA ↓ at early time points; SAA ↑ at late time points
CRP ↓	CRP ↑
Leukocyte numbers: no significant change	Leukocyte numbers: no significant change

Table 1. The biphasic effects of acute and chronic ethanol exposure on LPS-induced signaling pathways, inflammatory mediator secretion, and systemic markers of inflammation. Changes in the markers are expressed as comparisons to cells or animals not treated or fed with ethanol or to abstainers. CRP, C-reactive protein; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; IL, interleukin; IRAK, IL-1 receptor activated kinase; MAPK, mitogen activated kinase; NFκB, nuclear factor κ-light chain-enhancer of activated B cells; SAA, serum amyloid A; TGF-β, transforming growth factor-β. Data were derived from Kishore *et al*. 2001, Imhof *et al*. 2004, Oliveira *et al*. 2010, and references mentioned in the text.

Increased membrane fluidity is one plausible mechanism of the decreased TLR signaling induced by acute alcohol exposure. This may disturb the recruitment of TLRs to lipid rafts and the formation of membrane-bound protein complexes (Goral *et al*. 2008). However, it has been shown that TLR signaling is affected differently in response to the separate activation of TLR2 and TLR4 and to their simultaneous activation in the presence of ethanol (Oak *et al*. 2006). This finding is in contrast to the model of lipid raft disturbance that should apply to all TLRs without exception (Goral *et al*. 2008).

Ethanol has been shown to affect the numbers of circulating immune defense cells. In a model of binge drinking, blood samples drawn from human volunteers after alcohol ingestion revealed an early, transient proinflammatory state (20 min after alcohol ingestion), during which the numbers of circulating leucocytes, monocytes and NK cells were increased, as was the level of TNFα but not of IL-1β (Afshar *et al*. 2015). Furthermore, during this period blood cells responded to LPS stimulation with enhanced TNFα secretion. The early proinflammatory state was followed by an anti-inflammatory response (2 h and 5 h after alcohol ingestion), during which the numbers of immune cells were reduced and

proinflammatory cytokine secretion was inhibited and shifted toward an anti-inflammatory response via the enhanced secretion of IL-10 (Afshar *et al*. 2015). Instead, chronic alcohol ingestion increases the numbers of blood monocytes; however, due to a reduction in their capability to phagocytose and induce intracellular microbial killing by, for example, the generation of reactive oxygen species (ROS), pathogen clearance is impaired (Molina *et al*. 2010). Undoubtedly, several aspects contribute to ethanol-induced suppression and stimulation of immune responses. The more severe infections experienced by alcoholics may also derive, in part, from diminished phagocytotic activity of immune cells: both acute ethanol exposure and (even more uniformly) chronic alcohol exposure have been shown to reduce phagocytosis (Goral *et al*. 2008).

2.1.2 Alcohol consumption in relation to coronary heart disease

Lifestyle and dietary habits have a strong impact on cardiovascular health. Population meta-analysis studies have consistently demonstrated positive correlations between moderate alcohol consumption and reduced risk of CHD and myocardial infarction compared to abstainers (i.e., non-drinkers) (O'Keefe *et al*. 2007, Costanzo *et al*. 2010). In stark contrast to moderate alcohol consumption, heavy drinking and binge drinking strongly increase the risk of CHD and mortality from cardiovascular events (Costanzo *et al*. 2010). The protective effect of moderate alcohol consumption may derive from alterations in a number of biochemical factors, including reductions in systemic markers of inflammation, such as C-reactive protein (CRP) and proinflammatory mediators; decreased platelet aggregation, blood clotting and fibrinolysis; increased vasorelaxation; elevated levels of natriuretic peptide; and increased insulin sensitivity (Imhof & Koenig 2003, O'Keefe *et al*. 2007, Costanzo *et al*. 2010). Furthermore, other substances in alcoholic beverages, such as antioxidants in wines, have also been suggested to contribute to the beneficial effects of alcoholic drinks. However, a systematic review of studies analyzing the effects of consumption of specific drinks on the risk of CHD revealed that all types of alcoholic drinks lowered the risk of CHD (Rimm *et al*. 1996). Therefore, alcohol was concluded to be the common denominator in the beneficial effects (Rimm *et al*. 1996).

Alcohol-induced increases in high-density lipoprotein (HDL) cholesterol and in the main apolipoprotein of HDL, apolipoprotein A I, along with lower fibrinogen concentrations, were considered the most likely mechanisms of protection against CHD in a meta-analysis of population studies (Rimm *et al*. 1999). The ability of moderate alcohol consumption to increase blood HDL levels has been suggested to be dependent on genetic predisposition. A polymorphism that slows the oxidizing activity of the alcohol dehydrogenase enzyme was shown to cause increased HDL levels and reduced risk of myocardial infarctions, implying that ethanol itself, not its metabolites or other substances in alcoholic beverages, accounts for these beneficial effects (Hines *et al*. 2001). However, data reported by Hines *et al*. suggested that the increase in HDL was not the only contributing factor in the cardioprotective effects of ethanol. A meta-analysis of randomized trials examining the effects of lipid modifying interventions revealed that a simple increase in the amount of circulating HDL did not reduce the risk of CHD events or deaths (Briel *et al*. 2009). Thus, there are reasons to be

suspicious of the hypothesis that the HDL-raising effects of moderate alcohol consumption explain its cardioprotective effects. Population studies have weaknesses, particularly in terms of the analysis of alcohol consumption, the heterogeneity of the study groups, and the insufficient analysis of multiple factors that affect cardiovascular health. Therefore, the observed association between elevated HDL levels and improvements in cardiovascular health may not reflect a causal relationship.

2.2 Heme

Heme is a protoporphyrin IX molecule, in which an iron atom is linked at the center of four pyrrole rings. Heme is a ubiquitous protein in aerobic cells, and it is required to serve as a prosthetic group in proteins involved in a variety of fundamental biological processes, including the transport and storage of gases in hemoglobin and myoglobin, mitochondrial respiration, signal transduction, detoxification reactions, steroid biosynthesis, and antioxidant defense (Ryter & Tyrrell 2000). Several variants of heme exist in nature. The most common form in mammals is type b heme, which is present in hemoglobin and myoglobin (Larsen *et al*. 2012). Type a is found in cytochrome c oxidase, and type c is in cytochrome c (Larsen *et al*. 2012). The iron in heme can be reversibly oxidized to different oxidative states (Ryter & Tyrrell 2000). The most common states in hemoproteins being Fe^{2+}, which is present in heme (ferroprotoporphyrin), which has a neutral charge; and Fe^{3+}, which is present in hemin (ferriprotoporphyrin), which has a positive charge that binds anions (Kumar & Bandyopadhyay 2005). The molecular structures of heme and hemin are illustrated in Figure 1.

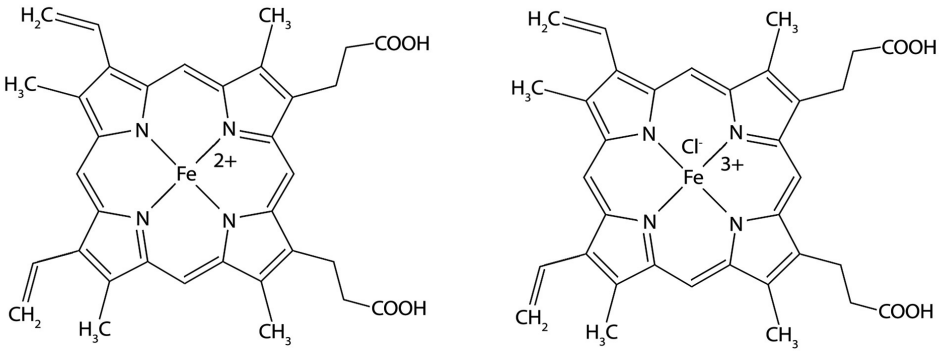

Figure 1. Structures of heme and hemin. Heme (ferroprotoporphyrin) is on the left and hemin (ferriprotoporphyrin) is on the right.

2.2.1 Heme is bound to hemoproteins

Hepatic Kupffer cells and splenic macrophages are largely responsible for the phagocytosis of senescent red blood cells. They recycle approximately 2 x 10^{11} red blood cells daily, which equates to nearly 3 kg of iron and hemoglobin in a year (Mosser & Edwards 2008, Kovtunovych *et al*. 2010). Subsequently, the iron released by the degradation of heme is returned to the circulation for reuse (Mosser & Edwards 2008). The enormous amount of

heme processed in macrophages requires HO-1. Deficiency in HO-1 encoding gene *Hmox1* in mice resulted in the death of macrophages and disrupted the removal of senescent red blood cells from the circulation (Kovtunovych *et al*. 2010). Upon hemolysis, hemoglobin is released to the circulation, and free hemoglobin can be subsequently oxidized, resulting in the release of heme/hemin (Larsen *et al*. 2012). Because free heme/hemin is toxic and lipophilic, its release is avoided by the binding of hemoglobin to haptoglobin (Kumar & Bandyopadhyay 2005, Larsen *et al*. 2012). The formed hemoglobin:haptoglobin complex is subsequently cleared by macrophages and hepatocytes (Kumar & Bandyopadhyay 2005, Larsen *et al*. 2012). In malaria and certain other pathological conditions, such as sickle cell anemia and ischemia reperfusion, severe hemolysis results in the massive release of hemoglobin from red blood cells. During such pathological processes, haptoglobin can no longer bind all the hemoglobin, resulting in the release of heme/hemin (Larsen *et al*. 2012). In plasma, free heme/hemin is scavenged by an acute-phase protein hemopexin or albumin, α1-microglobulin, and lipoproteins, but these scavenging mechanisms can also be saturated upon high release of hemoglobin (Balla *et al*. 2005, Kumar & Bandyopadhyay 2005, Larsen *et al*. 2012)

2.2.2 Heme-induced cytotoxicity and ROS formation

The cytotoxic effects of free heme/hemin are mediated by its pro-oxidant properties, and its capability to produce ROS is generally attributed to its iron atom (Balla *et al*. 1991, Kumar & Bandyopadhyay 2005). The intercalation of hemin into cellular lipid bilayers increases cell permeability by disrupting the bilayer composition, which may predispose the cell to lysis (Schmitt *et al*. 1993). Damage induced by iron-derived ROS is implicated in several vascular disorders, including atherosclerosis, microangiopathic hemolytic anemia, vasculitis and reperfusion injury, due to the direct cytotoxic activities of iron-containing heme/hemin and the oxidation of lipoproteins (Balla *et al*. 1991, Balla *et al*. 2005). Heme and iron chelate highly sensitize endothelial cells to injury induced by exogenous and endogenous oxidants (Balla *et al*. 2005). In addition, LDL oxidized by hemin is cytotoxic to endothelial cells (Balla *et al*. 1991). Furthermore, in macrophages, heme induces cell death with features of necrosis by two independent mechanisms involving the generation of ROS and by inducing the production of TNFα (Fortes *et al*. 2012). TNFα further activates TNF receptor 1 (TNFR) 1, which subsequently induces cell death (Fortes *et al*. 2012). The above mentioned mechanisms may mediate the deleterious effects of massive heme release. Heme may also provoke ROS generation via nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Thus, in intestinal epithelial cells, heme was shown to induce the activation of NADPH oxidases, which led to production of ROS (Barcellos-de-Souza *et al*. 2013). It is controversial whether heme/hemin can act as a Fenton reagent, i.e., support the formation of free radicals from hydrogen peroxide. However, heme is capable of catalyzing a process that turns rather slowly reactive organic hydroperoxides into highly reactive lipid peroxyl, alkyl, and alkoxyl radicals, which can induce lipid peroxidation (Ryter & Tyrrell 2000).

2.2.3 Proinflammatory effects of heme

Heme possesses proinflammatory abilities, which are, like its cytotoxic effects, considered to be mainly due its ability to generate ROS (Dutra *et al*. 2014). The capability of free heme to provoke an immune defense response was demonstrated by hemin injection, which led to the local migration and accumulation of neutrophils (Graca-Souza *et al*. 2002). Furthermore, heme was shown to induce the secretion of leukotriene B_4 from macrophages, which again acted as a chemoattractant for neutrophils, thereby promoting their recruitment (Monteiro *et al*. 2011). Hemin was also shown to trigger an oxidative burst and actin polymerization, which indicate the activation of neutrophils (Graca-Souza *et al*. 2002). Heme/hemin can contribute to inflammation in the vascular wall by increasing oxidative stress, vascular permeability, adhesion molecule expression and leukocyte infiltration (Wagener *et al*. 2003, Kumar & Bandyopadhyay 2005). Furthermore, heme has been reported to act as a TLR4 ligand to trigger the secretion of TNFα, a process that requires the iron in heme, because protoporphyrin IX, i.e., the direct precursor of heme that does not contain an iron atom, was shown to inhibit the effect of heme (Figueiredo *et al*. 2007). However, heme-induced TNFα secretion did not proceed through the signaling pathway used in LPS-induced activation, which may indicate that heme uses a different site for binding to TLR4 than LPS (Figueiredo *et al*. 2007).

The data presented by Li *et al*. and Dutra *et al*. concerning heme as an activator of the NLRP3 inflammasome are contradictory (Li *et al*. 2014b, Dutra *et al*. 2014). Li *et al*. demonstrated that heme and protoporphyrin IX induced the activation of the NLRP3 inflammasome and the secretion of mature IL-1β in the absence of serum (Li *et al*. 2014b). On the contrary, Dutra *et al*. showed that the activation of the inflammasome, which was induced by the synergistic effects of heme and pathogen-associated molecular patterns (PAMPs), required the presence of serum (Dutra *et al*. 2014). Furthermore, Dutra *et al*. observed that several other protoporphyrins devoid of an iron atom did not induce the secretion of IL-1β. The heme-induced activation of the NLRP3 inflammasome was dependent on ROS generated by the mitochondria and NADPH oxidase, potassium efflux from the cell, and Syk phosphorylation, but the activation of the NLRP3 inflammasome was independent of adenosine triphosphate (ATP) release and its receptor, purinergic receptor $P2X_7$ (Dutra *et al*. 2014). The results concerning the P2X receptor were again discrepant with the previous study by Li *et al*., in which the P2X receptors $P2X_7$ and $P2X_4$ were required for IL-1β secretion (Li *et al*. 2014b). In addition to these contradictory results, yet more discrepancies were found by studies showing that hemin in fact inhibits NLRP3 inflammasome activation. Kim *et al*. demonstrated that hemin reduced the expression of proIL-1β and inhibited the secretion of mature IL-1β induced by NLRP3 inflammasome activation. The ROS-dependent dissociation of thioredoxin-interacting protein (TXNIP) from thioredoxin (TRX) has been shown to induce NLRP3 inflammasome activation, and Kim *et al*. further demonstrated that hemin blocked the dissociation of TXNIP from TRX (Zhou *et al*. 2010, Kim & Lee 2013). In accordance with the study by Kim *et al*., hemin was shown to protect epithelial cells from LPS-induced damage by reducing the LPS-induced expression of NLRP3 inflammasome components and the assembly of the NLRP3 inflammasome, as well as the

subsequent secretion of IL-1β (Li *et al*. 2014a). Furthermore, hemin blocked cecal ligation and puncture-induced pulmonary damage, neutrophil infiltration, edema, and the secretion of IL-1β and IL-18 by reducing the expression of NLRP3 inflammasome components, the assembly of the NLRP3 inflammasome, and the production of malondialdehyde and ROS (Luo *et al*. 2014). All three latter studies suggested that the inhibitory effect was mediated by the induction of HO-1. The results of the above mentioned studies (Kim & Lee 2013, Dutra *et al*. 2014, Li *et al*. 2014a, Li *et al*. 2014b, Luo *et al*. 2014) are summarized in Table 2.

Heme-induced NLRP3 inflammasome activation	Hemin-induced NLRP3 inflammasome inhibition
Heme induces the secretion of IL-1β (Dutra *et al.* 2014, Li *et al.* 2014b).	Hemin blocks the secretion of IL-1β (Kim & Lee 2013, Li *et al.* 2014a, Luo *et al.* 2014).
Heme does not reduce the expression of pro-IL-1β protein (Dutra *et al.* 2014).	Hemin reduces the expression of proIL-1β, NLRP3, ASC, and caspase-1 induced by inflammatory stimuli (Kim & Lee 2013, Li *et al.* 2014a, Luo *et al.* 2014). Hemin blocks the nuclear translocation of NFκB (Li *et al.* 2014a).
The dependence of inflammasome activation on serum is controversial (Dutra *et al.* 2014, Li *et al.* 2014b).	
Inflammasome activation is dependent on mitochondrial and NADPH-generated ROS (Dutra et al. 2014).	Hemin blocks the dissociation of TXNIP from TRX (Kim & Lee 2013).
Inflammasome activation is dependent on potassium efflux (Dutra *et al.* 2014).	Hemin inhibits NLRP3 inflammasome assembly (Kim & Lee 2013, Li *et al.* 2014a, Luo *et al.* 2014).
Inflammasome activation is dependent on Syk phosphorylation (Dutra *et al.* 2014).	Hemin blocks the production of malondialdehyde (Luo *et al.* 2014).
The dependence of inflammasome activation on ATP and the P2X receptor is controversial (Dutra *et al.* 2014, Li *et al.* 2014b).	Inhibitory effects are suggested to be mediated by the induction of HO-1 (Kim & Lee 2013, Li *et al.* 2014a, Luo *et al.* 2014).

Table 2. Effects of heme and hemin on NLRP3 inflammasome activation. Data are derived from references mentioned in the table.

2.2.4 Heme oxygenase-1

Heme/hemin is degraded by the heme oxygenase (HO) enzyme, which catabolizes the rate-limiting step in the oxidative degradation of heme/hemin to carbon monoxide (CO), catalytic iron, and biliverdin, which is rapidly further converted to bilirubin (Tenhunen *et al*. 1968, Tenhunen *et al*. 1969). The induction of HO is the other role of heme/hemin in inflammation. Small amounts of heme induce cytoprotective and anti-inflammatory effects through the activation of HO, whereas high concentrations act in a proinflammatory and cytotoxic manner (Wagener *et al*. 2003). HO in humans exists in two isoforms. The constitutive form, HO-2, is expressed mainly in the brain and testis. The ubiquitous inducible form, HO-1, exhibits low basal expression but is readily induced in response to various stress signals, such as cytokines, heavy metals, its substrate heme/hemin, and other synthetic metalloporphyrins (Abraham & Kappas 2008, Paine *et al*. 2010). HO-1 is principally regulated at the expression level (Abraham & Kappas 2008, Paine *et al*. 2010), and similar to many other antioxidant genes, the *HMOX1* possesses several antioxidant response elements in its enhancer regions, which act as binding sites for transcription factors (Paine *et al*. 2010). The transcription of HO-1 is regulated by several factors, including the transcriptional repressor BTB and

CNC homolog 1 (Bach1), which binds the stress-responsive elements in the promoter of *HMOX1* in the absence of activating stimuli, as well as by the transcription factors NF-E2-related factor-2 (Nrf2), NFκB, and activating protein-1 (Gozzelino *et al*. 2010, Paine *et al*. 2010). Nrf2 is the most prominent HO-1 activating transcription factor in response to oxidative cellular stress (Alam *et al*. 1999). Recent evidence has demonstrated that Nrf2 is the principal transcription factor that mediates HO-1 activation under injurious conditions, as the disruption of cellular homeostasis activates both HO-1 and autophagy. An unexpected link between these two pathways was revealed when Nrf2 and sequestosome 1 (SQSTM1, also called p62), a protein that acts as a receptor for selective autophagy, were shown to mutually enhance the expression of each other (Jain *et al*. 2010, Itakura & Mizushima 2011).

HO-1 exerts potent immunomodulatory actions that promote cell survival, as well as antioxidant and anti-inflammatory effects. The importance of HO-1 is exemplified by the severe diseases observed in the absence of HO-1 in two human patients and in mouse knockout models. The symptoms or findings observed in HO-1 deficiency include anemia, elevated levels of heme and iron deposits, signs of chronic inflammation, high blood lipid concentrations, and endothelial and vascular injury (Poss & Tonegawa 1997a, Yachie *et al*. 1999, Wagener *et al*. 2003, Kovtunovych *et al*. 2010). The cytoprotective and anti-inflammatory effects of HO-1 have been well demonstrated. HO-1-deficient mouse models showed exaggerated immune responses and mortality in response to inflammatory stimuli, such as LPS (Poss & Tonegawa 1997a, Poss & Tonegawa 1997b, Brouard *et al*. 2002, Kapturczak *et al*. 2004, Gozzelino *et al*. 2010). One important mechanism that down-modulates inflammation is the inhibition of the secretion of proinflammatory mediators. The induction of HO-1 expression by hemoglobin reversed LPS-induced TNFα secretion *in vivo* and increased survival from toxemia (Otterbein *et al*. 1997). In addition, the overexpression of HO-1 or its induction with hemin blocked the secretion of TNFα via the induction of autophagy (Waltz *et al*. 2011). Furthermore, the activation of TLR4 alone initiated the expression of HO-1, suggesting the existence of a negative feedback loop (Immenschuh *et al*. 1999, Waltz *et al*. 2011). In addition to TLR signaling, the activation of other signaling pathways, including p38 MAPK, phoshatidylinositol-3 kinase (PI3K)/Akt, and the Janus kinase and signal transducer and activator of transcription (Jak-STAT) pathway also induce the expression of HO-1 (Paine *et al*. 2010). The beneficial effects of HO-1 are not only mediated by its counteractions against the effects of free heme/hemin but have also been attributed to the degradation products: CO, biliverdin, and bilirubin. The observation that the administration of CO and bilirubin mimics the anti-inflammatory and antiapoptotic effects of HO-1 suggests that the protective effects of HO-1 activation are mediated by degradation products (Kapitulnik 2004, Gozzelino *et al*. 2010, Constantin *et al*. 2012). However, interestingly, Kim *et al*. showed that CO induced the expression of HO-1 and the pharmacological activation of HO-1 induced CO, both of which inhibited endoplasmic reticulum stress-induced apoptosis (Kim *et al*. 2007, Gozzelino *et al*. 2010).

Many of the above mentioned effects of HO-1 confer protection against chronic inflammatory diseases, such as atherosclerosis and RA. The beneficial effects of HO-1 in the context of

chronic inflammatory diseases include reduced inflammation, reduced oxidative damage, degradation of proinflammatory and cytotoxic free heme/hemin, and enhanced cell survival. Pronounced expression of HO-1 in macrophages and in the endothelium of atherosclerotic lesions has been reported (Wang *et al*. 1998, Ishikawa *et al*. 2001a). Accordingly, oxidized LDL has been shown to induce HO-1 expression in cultured macrophages, implicating that oxidized LDL may promote HO-1 expression in plaques (Wang *et al*. 1998). Furthermore, the formation and progression of atherosclerotic lesions, as well as monocyte chemotaxis, were reduced by pharmacological induction or the overexpression of HO-1 in hypercholesterolemic animals, and consistent with that finding, lesion development was exacerbated upon pharmacological inhibition of HO-1 (Ishikawa *et al*. 2001a, Ishikawa *et al*. 2001b, Juan *et al*. 2001). Similarly, in animal models of RA, markers of inflammation such as joint swelling, cartilage degradation, inflammatory tissue proliferation, and the secretion of proinflammatory mediators were reduced upon the induction of HO-1 with cobalt protoporphyrin (CoPP) and cilostazol, the latter of which additionally increased the apoptosis of inflammatory synovial tissue cells (Benallaoua *et al*. 2007, Park *et al*. 2010).

3. Apoptosis

Apoptosis, a type of programmed cell death, is crucial for tissue homeostasis and normal development. Many developmental processes and immune defense reactions produce cells that must be removed without provoking unnecessary immune reactions, such as the removal of self-reactive T cells during their maturation in the thymus or the disposal of neutrophils after inflammation has been resolved (Savill 1997, Saikumar *et al*. 1999). Apoptosis has been suggested to be involved in the resolution of inflammation; accordingly, immune reactions have been shown to induce apoptosis, e.g., via TLR2 activation (Aliprantis *et al*. 1999, Saikumar *et al*. 1999, Martin *et al*. 2012). Aberrant apoptosis has been associated with a range of diseases varying from developmental disorders and neurodegeneration to cancer and dysregulated immune defense (Fuchs & Steller 2011).

3.1 Morphological and biochemical features of apoptosis

Unlike other cell death mechanisms, most notably necrosis/necroptosis and pyroptosis, apoptotic cell death is immunologically silent (Kaczmarek *et al*. 2013, Sangiuliano *et al*. 2014). During necrosis, proinflammatory mediators present in various cellular compartments of the dying cell are released, which provokes inflammatory reactions in neighboring cells (Sangiuliano *et al*. 2014). In contrast, apoptosis is characterized by the following morphology: (1) cells and nuclei shrink, with the ensuing condensation of the nucleus and chromatin; (2) cells become rounded and retract from neighboring cells; and (3) the plasma membrane remains intact and forms blebs that may be "pinched off" as small

vesicles called “apoptotic bodies” (Saraste & Pulkki 2000). The morphological alterations that occur during apoptotic cell death are illustrated in Figure 2. Biochemically, apoptosis is defined by the fragmentation of DNA by DNases into typical 180- to 200-base-pair pieces (Saraste & Pulkki 2000). The cleavage of cellular proteins, organelles, and nuclear lamina as well as the morphological changes are largely mediated by caspases (Saraste & Pulkki 2000, Taylor *et al*. 2008). The reasons for the cleavage of proteins and the condensation and fragmentation of DNA have yet to be elucidated. However, it has been proposed that these events facilitate the clearance of apoptotic cells (the clearance of apoptotic cells is explained in greater detail in Chapter 3.3) and prevent inflammatory reactions (Taylor *et al*. 2008, Martin *et al*. 2012).

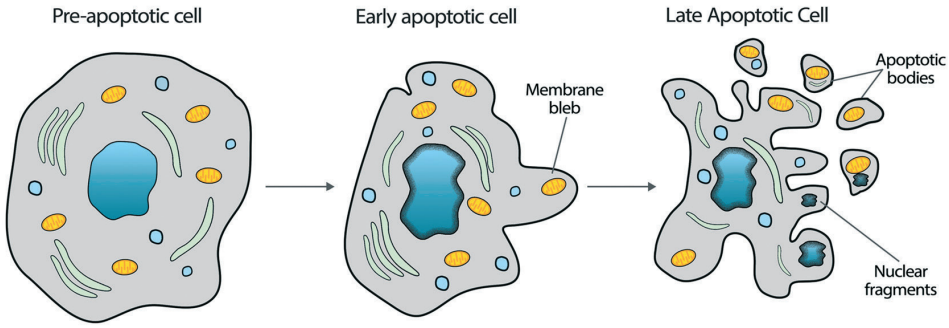

Figure 2. Morphology of apoptosis. Similar morphological features of apoptosis are observed across different cell types and species. Early in the apoptotic process the cell shrinks and the nucleus and chromatin condensate. The condensation of chromatin, i.e., pyknosis, is a hallmark of apoptosis. Plasma membrane blebbing (the formation of plasma membrane protrusions) is followed by the fragmentation of the nucleus. The separation of the blebs from the cell forms apoptotic bodies containing cellular organelles and nuclear fragments. Data are derived from Ziegler & Groscurth 2004, Elmore 2007, and references mentioned in the text.

3.2 Apoptotic cell death

Apoptotic cell death can be triggered by intrinsic or extrinsic routes. Intrinsic and extrinsic apoptosis differ by the signaling events that precede the activation of executor caspases, which mediate the morphological and biochemical changes typical of apoptosis. The apoptosome is a large intracellular protein complex formed during intrinsic apoptosis. The apoptosome and the corresponding protein complexes involved in extrinsic apoptosis are required for the activation of caspase-proteases. Caspase-activating complexes serve as scaffolds that induce the activating cleavage of procaspases by concentrating the procaspases to enable proximity-induced autocatalysis or to allosterically regulate caspase activation (Rodriguez & Lazebnik 1999, Adams & Cory 2002). Two evolutionarily conserved protein families are the key mediators of apoptotic events: the B cell lymphoma (Bcl-2) family proteins, which regulate mitochondrial integrity; and caspases, which execute the cleavage phase (Thornberry & Lazebnik 1998, Youle & Strasser 2008). Caspases are cysteine aspartic acid-specific proteases that cleave substrate proteins after an aspartic acid residue. Caspases are translated as inactive proenzymes that usually require proteolytic cleavage to become active (Taylor *et al*. 2008). The caspase family proteins are classified as initiator caspases

(caspase-2, caspase-8, caspase-9, and caspase-10) and executioner caspases (caspase-3, caspase-6, and caspase-7), which are activated by initiator caspases (Taylor *et al*. 2008, Duprez *et al*. 2009). Other caspase family proteins function in innate immune responses (caspase-1, caspase-4, caspase-5, and caspase-12) (Taylor *et al*. 2008). Procaspases are composed of a variable-length N-terminal prodomain, a large subunit (p20) and a small C-terminal unit (p10). Initiator caspases possess a long prodomain, which contains protein-protein interaction-mediating regions, including death effector domains (DED) and caspase activation and recruitment domains (CARD) (Duprez *et al*. 2009). The DED and CARD domains are used to recruit caspases to activation complexes (Duprez *et al*. 2009). Caspase activation during cell death is characteristic of apoptosis, and if the function of caspases (caspase-8 in particular) is inhibited, the cell switches to necroptosis in response to apoptotic stimuli (He *et al*. 2009, Kaczmarek *et al*. 2013).

3.2.1 Extrinsic apoptosis

Extrinsic apoptosis is initiated by extracellular ligands that induce the activation of the TNFR family death receptors, including Fas receptor (FasR), TNFR1, and TRAIL-R. The pathways of extrinsic apoptosis induced by the activation of FasR, TRAIL-R and TNFR1 are illustrated in Figure 3. Subsequent to the binding of the Fas ligand, the oligomerization of the FasR induces a signaling cascade that results in the formation of the death-inducing signaling complex (DISC) (Kischkel *et al*. 1995, Peter & Krammer 2003). In DISC the FADD adapter protein binds the cytoplasmic part of the receptor through homotypic DD-DD (death domain) interactions, which is followed by the binding of procaspase-8 to FADD (Kischkel *et al*. 1995, Peter & Krammer 2003). The recruitment of procaspase-8 to the complex induces its activation (Peter & Krammer 2003). The DISC formed in response to FasR and TRAIL-R signaling remains at the plasma membrane (Wilson *et al*. 2009).

The activation of TNFR1 induces the formation of two distinct signaling complexes through the partial dissociation of the first complex from the plasma membrane and by the binding of new subunits in the cytoplasm. The activation of TNFR1 instantly recruits a scaffold protein, TRADD, to its cytoplasmic tail via homotypic interactions between their DDs, which leads to the recruitment of signaling proteins, i.e., TRAF2, RIPK1, and the cellular inhibitors of apoptosis proteins (cIAPs) (Muppidi *et al*. 2004). The formed complex is called complex I, and its formation activates the transcription factor NFκB (Muppidi *et al*. 2004). Complex II is formed later (> 2 h) upon the dissociation and internalization of RIPK1, TRAF2, and TRADD, as well as the recruitment of FADD and caspase-8/-10 (Muppidi *et al*. 2004). The formation of complex II is modified by the balance between stress and survival signals, e.g., NFκB and c-Jun N-terminal kinase (Jnk) signaling. In complex IIA, RIPK1 is ubiquinated (Wilson *et al*. 2009). This complex is sensitive to the rate of protein synthesis, and it is regulated by the NFκB-dependent expression of c-FLIP and by the Jnk-dependent degradation of c-FLIP (Wang *et al*. 2008, Wilson *et al*. 2009). The activation of complex IIB is induced by the deubiquination and dissociation of RIPK1, and it is inhibited by cIAPs that ubiquinate RIPK1 (Wang *et al*. 2008, Wilson *et al*. 2009). Both complexes IIA and IIB provide a platform for the activation of procaspase-8. Regardless of the pathway of extrinsic

apoptosis, the activation of caspase-8 initiates the cleavage of effector caspases (Wang *et al*. 2008, Wilson *et al*. 2009). Extrinsic apoptosis may require amplification through the intrinsic pathway; e.g., in B cells caspase-8 cleaves Bid to induce intrinsic apoptosis (Wilson *et al*. 2009).

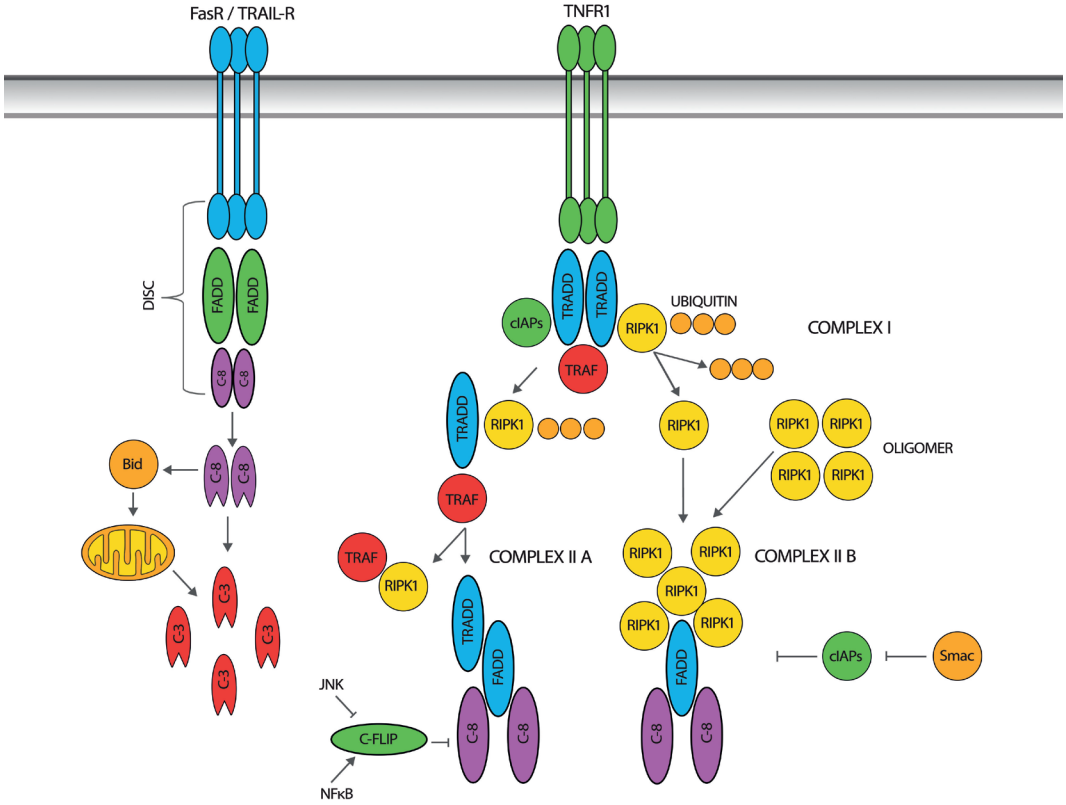

Figure 3. Signaling pathways of extrinsic apoptosis. Some steps are omitted for clarity. The activation of FasR or TRAIL-R induces the assembly of the DISC complex (shown on the left). The activation of the TNFR induces the immediate formation of complex I, and later dissociation events induce the formation of cytosolic complexes IIA and IIB (shown on the right). The activation of caspase-8 leads to the activation of the effector caspases caspase-3, caspase-6, and caspase-7 (not all the activated caspases are shown in the figure), which induce the cleavage of cellular components. Bid, BH3-interacting domain death antagonist; C-3, caspase-3; C-8, caspase-8; cIAP, cellular inhibitors of apoptosis protein; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FasR, Fas receptor; c-FLIP, cellular FLICE inhibitory protein; JNK, c-Jun N-terminal kinase; NFκB, nuclear factor κ-light chain-enhancer of activated B cells; RIPK1, receptor-associated protein kinase 1; Smac, second mitochondrial activator of caspases; TNF, tumor necrosis factor; TRADD, TNF receptor-associated-protein with death domain; TRAF, TNF receptor-associated protein 2; TRAIL, TNF-related apoptosis-inducing ligand. Data are derived from references mentioned in the text.

3.2.2 Intrinsic apoptosis

Intrinsic apoptosis can be initiated by cellular stress induced by diverse stimuli, such as toxic agents and DNA damage. Intrinsic apoptosis beginns by the permeabilization of the outer mitochondrial membrane, and it is regulated by the members of the Bcl-2 protein family (Taylor *et al*. 2008). The activation pathways of intrinsic apoptosis are presented in Figure 4. The Bcl-2 family contains both proapoptotic and antiapoptotic proteins. The proapoptotic Bcl-2-family membranes include Bax, Bak, and Bok (Bcl-2-related ovarian killer) (Youle

& Strasser 2008). Upon the induction of apoptosis, the proapoptotic Bcl-2 family members permeabilize the mitochondrial outer membrane, thereby causing the release of cytochrome c from the space between the outer and inner mitochondrial membranes into the cytosol (Youle & Strasser 2008). The process that leads to the release of cytochrome c is called mitochondrial outer membrane permeabilization (MOMP). Cytochrome c is released through pores formed by Bax and Bak (Youle & Strasser 2008, Subburaj *et al.* 2015). The mechanisms of pore formation and the physical nature of the pore have remained elusive for long. However, Subburaj *et al*. recently suggested that activated Bax is inserted on mitochondrial outer membrane in the monomeric state. Subsequent to its insertion, Bax rapidly forms dimers that further oligomerize into variable size pore structures (Subburaj *et al.* 2015). In the cytosol, cytochrome c associates with APAF1 and induces its oligomerization; then, the oligomerized APAF1 forms a complex with caspase-9, resulting in the formation of a large complex, called the apoptosome. (Rodriguez & Lazebnik 1999, Zou *et al*. 1999). The apoptosome is thought to be the actual active form of caspase-9. Active caspase-9 initiates a cascade that cleaves the downstream executioner caspases (Rodriguez & Lazebnik 1999). The functions of the pro-death proteins are opposed by the antiapoptotic Bcl-2 proteins, such as Bcl-2, Bcl-xL (Bcl-2-like protein), Bcl-w (Bcl-2-like-2), Bcl-2L10, Bcl-2A1, and Mcl-1 (myeloid cell leukemia sequence-1) (Youle & Strasser 2008). Additionally, the proapoptotic proteins include a group of BH3-only proteins, such as Bim (Bcl-2-like-11), Bid, Bad (Bcl-2 antagonist of cell death), Puma (Bcl-2-binding component-3), Noxa (phorbol-12-myristate-13-acetate-induced protein 1), Bik (Bcl-2-interacting killer), Hrk (harakiri), and Mule (*M*cl-1 *u*biquitin *l*igase *E*3) (Shamas-Din *et al*. 2011). These proteins are proposed to either inhibit the antiapoptotic proteins or to enhance the oligomerization and insertion of the proapoptotic proteins into the mitochondrial membrane by interacting with or directly binding to them (Willis *et al*. 2007, Shamas-Din *et al*. 2011).

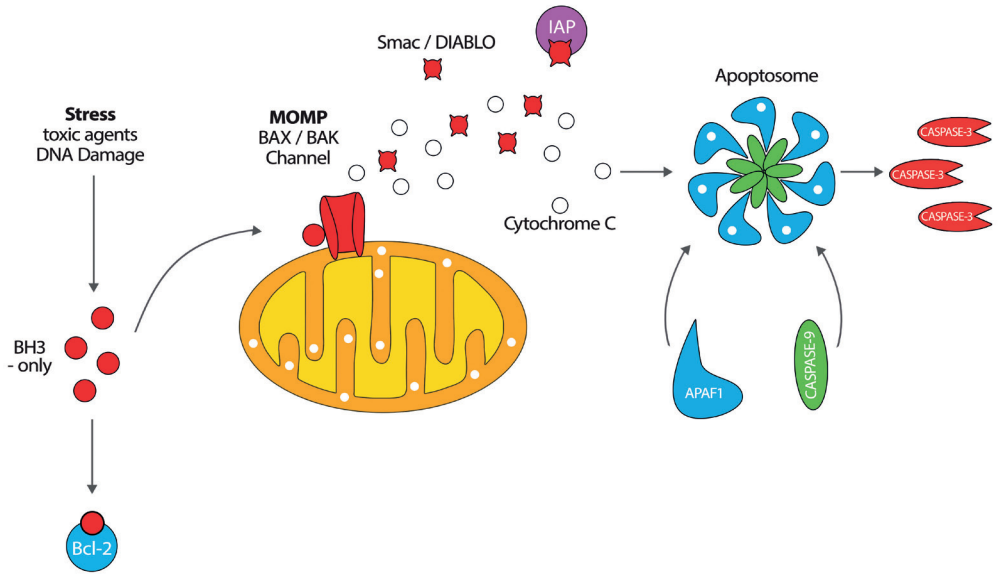

Figure 4. Signaling pathways of intrinsic apoptosis. Some steps are omitted for clarity. Intrinsic apoptosis is induced by cellular stress, which initiates MOMP. Bax and Bak form pores through which cytochrome c and IAP-regulatory proteins, such as Smac/DIABLO, are released from the intermembrane space of mitochondria. BH3-

only proteins inhibit antiapoptotic proteins by binding them. BH3-only proteins also interact with proapoptotic proteins, thereby enhancing their functions. Cytochrome c induces the oligomerization of APAF1, which results in the formation of the apoptosome. Smac/DIABLO allows the activation of caspases by binding IAPs. The activation of caspase-9 induces the activation of the effector caspases caspase-3, caspase-6, and caspase-7 (not all activated caspases are shown in the figure), which mediate the destruction of cellular components. APAF1, apoptotic protease-activating factor-1; Bak, Bcl-2- antagonist/killer-1; Bax, Bcl-2-associated X protein; Bcl-2, B cell lymphoma; IAPs, inhibitor of apoptosis proteins; MOMP, mitochondrial outer membrane permeabilization; Smac/DIABLO, second mitochondrial activator of caspases/direct IAP-binding protein with low pI. Data are derived from references mentioned in the text.

3.2.3 Inhibitors of apoptosis

The death of a cell is an irreversible process; therefore, protein families that prevent apoptosis exist. Among the inhibitors of apoptosis proteins (IAPs), the mammalian IAP, X-linked inhibitor of apoptosis (XIAP), is one of the most potent and most widely studied caspase inhibitors (Salvesen & Duckett 2002). IAPs bind directly to activated caspases through their baculovirus-inhibitory repeat domain (BIR) (Silke & Vucic 2014). IAPs seem to be regulated by at least three different levels: transcriptionally/post-transcriptionally, via degradation in proteasomes, and by regulatory proteins (Salvesen & Duckett 2002). When cells are committed to die, IAPs are inactivated by interactions with regulatory proteins, such as Smac/DIABLO, which are localized to the mitochondrial inter-membrane space, and are released simultaneously with cytochrome c (Verhagen *et al*. 2000). Smac/DIABLO and another IAP regulatory protein, high-temperature requirement A2 (HtrA2, also known as Omi) bind IAPs directly through their IAP-binding motifs (Suzuki *et al*. 2001, Salvesen & Duckett 2002). Two negative regulators of IAPs that lack IAP-binding motifs have been recognized. They are also thought to interact with IAPs, although the mechanism of binding must differ from that exerted by other IAP-regulatory proteins (Salvesen & Duckett 2002).

3.3 Clearance of apoptotic cells

Apoptotic cell death does not provoke inflammation because during apoptosis no proinflammatory mediators are released. Instead, apoptotic cells are rapidly and efficiently removed by phagocytosis (Ravichandran & Lorenz 2007, Ravichandran 2011). The quick removal of apoptotic cells is ensured by secreted chemotactic signals that recruit phagocytes to the target. Among these signals is the lipid lysophosphatidylcholine, which is cleaved from membranous phosphatidylcholine by the caspase-3 activated calcium-independent phospholipase A2 (Lauber *et al*. 2003). Lauber *et al*. could not demonstrate that sphingosine-1-phosphate (S1P) functions as a chemotactic agent; however, Gude *et al*. showed that S1P is a potent chemotactic molecule for monocytic line cells and for primary monocytes and macrophages (Gude *et al*. 2008). Additionally, ATP/UTP, when found in the extracellular space, signals cell death. Therefore, it also acts as a danger signal for neighboring cells (Elliott *et al*. 2009).The subcutaneous injection of nucleotides into mice induced chemotaxis of monocytes and macrophages, and the deletion of the nucleotide recognition receptor was shown to inhibit the clearance of apoptotic cells (Elliott *et al*. 2009). In addition, a soluble

fragment of fractalkine protein is a well-characterized phagocyte attractant, and the absence of its receptor was shown to impair macrophage recruitment to apoptotic B cells at germinal centers of lymphoid follicles *in vivo* (Truman *et al*. 2008). Apoptotic cells also secrete signals that lure phagocytes to engulf them. Phosphatidyl serine (PS) is a phospholipid that is normally not exposed to phagocytes because in living cells, PS is located only on the inner leaflet of the plasma membrane (Fadok *et al*. 1992). However, Fadok *et al*. demonstrated that apoptotic cells flip PS on the cell surface, which can function as an "eat me" signal. To avoid unwanted neutrophil migration and inflammatory reactions, cells are also capable of secreting lactoferrin, which is a well-recognized anti-inflammatory glycoprotein (Bournazou *et al*. 2009).

4. Signalosomes of innate immunity

Innate immunity signaling is not merely an action of independent receptors. Instead proteins recruited and activated by receptor activation have a tendency to form protease cascades and signaling networks that permit the temporal and spatial regulation of the signal, as well as to induce local enrichment of signal-mediating components to enable proximity-driven signal transduction (Wu 2013). In the signaling networks, positive and negative feedback loops may amplify or reduce the signal, respectively, ensuring faster signal transduction and the efficient reduction in noise. Because of the obvious benefits to the host, innate immunity signaling cascades are highly conserved in evolution (Gay & Gangloff 2007, Wu 2013). Many receptors of innate immunity form regulatory networks in which the activation of one receptor is a prerequisite for the activation of another receptor. For example, the stimulation of TLRs increases the expression of some NLRs (Becker & O'Neill 2007). Furthermore, the oligomerization of inflammasome proteins into an inflammasome complex allows the rapid proximity-induced activation of caspases and the amplification of the signal (Lu *et al*. 2014). The formation of higher-order oligomeric structures further explains why receptor complexes can be observed as visible punctate morphologies in cells (Wu 2013).

4.1 Pattern recognition receptors

The recognition of danger is a fundamental step in the initiation of immune responses and the protection of the host. Distinguishing between harmless and dangerous is by no means simple and contains the risk of errors. The first hypothesis regarding immune system recognition mechanisms suggested that the immune system discriminates between self (entities defined during early development) and nonself (e.g., pathogenic entities). However, when it became apparent that the immune system does not reject all nonself (e.g., the pregnant woman does

not reject the fetus) and that adaptive immune responses could not be activated without adjuvants, Janeway proposed that innate immune cells were responsible for recognizing a large array of molecular patterns that distinguish noninfectious self from infectious nonself (Janeway 2013). In addition to pathogen-derived exogenous danger signals, an increasing number of endogenous signals capable of eliciting immune reactions were acknowledged, which led to the development of the danger model (Matzinger 2002). The danger model suggests that the immune system alerts to danger signals during infection, inflammation or tissue injury; thus, virtually any endogenous molecule is regarded as a danger signal when it is encountered in an unusual location (Matzinger 2002). Molecules sensed as danger signals are now classified according to their origin: PAMPs, which originate from pathogens, or danger-associated molecular patterns (DAMPs), which originate, for example, from altered or misplaced selfmolecules (Medzhitov & Janeway 2000, Beutler 2004, Seong & Matzinger 2004).

Exogenous and endogenous danger signals are sensed by a limited number of conserved germ line-encoded receptors, PRRs, which recognize conserved pathogenic molecules and endogenous signals derived from dying or stressed cells (Matzinger 2002). PRRs target molecules that are broadly shared by pathogens and are not readily altered by mutations due to their indispensability for the organism (Janeway 2013, Medzhitov & Janeway 1997). PRRs are primarily expressed by cells capable of presenting antigens, such as macrophages, dendritic cells and B cells (Medzhitov & Janeway 1997). PRRs can be classified into at least five families: (1) TLRs, (2) NLRs, (3) Absent in Melanoma 2 (AIM2)-like receptors (ALRs), (4) Retinoic acid inducible gene I-like receptors, and (5) C-type lectin receptors (Akira *et al*. 2006, Takeuchi & Akira 2010, Kumar *et al*. 2013). TLRs are classical receptors of innate immunity that perform most microbial detection in mammals (Beutler 2004). The bacterial and viral ligands sensed by TLRs are diverse, including proteins, nucleic acids, lipids and lipopeptides (Beutler 2004, Akira *et al*. 2006, Gay & Gangloff 2007, Beutler 2009). Some TLRs recognize different PAMPs of the same pathogen; in addition, there are redundancies in ligand recognition between different PRRs (Kawai & Akira 2011).

4.1.1 Toll-like receptors

All TLR paralogs are type I transmembrane receptors that belong to the IL-1/TLR receptor superfamily, along with IL-1 and IL-18 receptors (Dunne & O'Neill 2003, Akira *et al*. 2006). Different TLRs are located on intracellular membranes or on the cell surface. TLR3 and 7-9 recognize intracellular organisms and are located on the membranes of intracellular vesicles, whereas TLR1, 2, and 4-6 are located on the cell surface, where they sense ligands from the extracellular space (Kawai & Akira 2011). The leucine-rich repeat (LRR) of TLRs senses ligands, and upon ligand recognition, TLRs form either hetero- or homomeric complexes with each other (Kawai & Akira 2011). Their activation culminates in the innate immune cell responses, which are induced by the activation of NFκB and the subsequent transcription of proinflammatory cytokines (Doyle & O'Neill 2006). Probably the best-studied molecule recognized by PRRs is the bacterial endotoxin LPS. LPS was first characterized by Pfeiffer in 1892, and later positional cloning studies revealed that LPS is recognized by TLR4

(Poltorak *et al*. 1998, Beutler 2004). LPS is an outer membrane component of the cell wall of Gram-negative bacteria, and in large quantities it induces septic shock and lethality due to the systemic release of proinflammatory cytokines, such as TNFα, IL-1 and IL-6 (Beutler 2004). The signal transduction that occurs after the activation of the endotoxin receptor TLR4 proceeds via two different routes: the myeloid differentiation factor 88 (MyD88)-dependent pathway, which leads to the early-phase activation of NFκB and MAPK, or the TIR-domain-containing adaptor-inducing interferon-β (TRIF)-dependent pathway, which leads to the late-phase activation of NFκB and MAPK, both of which are required for the induction of proinflammatory cytokine transcription (Kawai & Akira 2011).

Different PRRs cooperate in the activation of the NLRP3 inflammasome; the activation of TLR or cytokine receptors is a prerequisite for the activation of the NLRP3 receptor. Intracellular PRRs capable of inflammasome formation, the NLRs and ALRs, will be discussed in greater detail below.

4.2 Inflammasomes

The inflammasome acts as a checkpoint that regulates the secretion of strong proinflammatory cytokines, which elicit cellular protection and bacterial clearance (Latz *et al*. 2013). Inflammasomes are intracellular multiprotein complexes formed upon the activation of their receptor proteins (Latz *et al*. 2013). The inflammasome complex acts as a platform for the activation of (pro)inflammatory caspase-1 (Lu *et al*. 2014). The activated caspase-1 further mediates the proteolytic maturation of the proforms of the inflammatory cytokines IL-1β and IL-18 (Dinarello 1998). The activation of the inflammasome may result in cell death via the activation of apoptotic or pyroptotic pathways, depending on the strength of the inflammatory stimulus (Fernandes-Alnemri *et al*. 2007, Sagulenko *et al*. 2013). Cell death may help to attenuate the invasion of pathogens by denying their protective niche in the cell. To date, many different inflammasome-forming receptors have been recognized, and they are divided into two superfamilies according to their sensor protein: NLRs, which recognize a plethora of pathogen- or stress- and danger-associated signals (Davis *et al*. 2011), and ALRs, which specifically recognize double-stranded DNA (Rathinam *et al*. 2012).

4.2.1 Interleukin-1β (IL-1β)

IL-1β is a strong proinflammatory cytokine that affects virtually all cell types. The most prominent symptom caused by excessive IL-1β secretion is fever (Dinarello 1998, Dinarello 2009). IL-1β induces inflammatory and immune responses by enhancing neutrophil chemotaxis and the generation of proinflammatory cytokines (Apte & Voronov 2008). IL-1β also promotes cell differentiation, angiogenesis and hemopoiesis (Apte & Voronov 2008, Dinarello 2009). IL-1β is a member of the IL-1 protein family, which is further divided into three subfamilies: the IL-1, IL-18, and IL-36 subfamilies (Garlanda *et al*. 2013). The IL-1 subfamily encompasses IL-1β, IL-1α, IL-33 and the IL-1 receptor antagonist (IL-1RA) (Garlanda *et al*. 2013). All the members of the IL-1 subfamily are encoded by different

genes and have different patterns of expression and secretion (Rock *et al*. 2010). However, they all bind to the receptors that belong to IL-1 receptor family: IL-1β, IL-1α, and IL1RA bind to IL-1 receptor 1, and IL-33 binds to the IL-1-related protein ST2L (Schmitz *et al*. 2005, Palomo *et al*. 2015). Consequently IL-1β, IL-1α and IL-33 activate the cell, whereas IL-1RA acts as a competitive antagonist that physically inhibits the binding of IL-1β and IL-1α (Rock *et al*. 2010, Garlanda *et al*. 2013). In contrast to IL-1β, IL-1α and IL-33 are synthesized in a form that is ready to activate their respective receptors, and their cleaved forms also activate the receptors (Cohen *et al*. 2010, Kim *et al*. 2013, Palomo *et al*. 2015). IL-1α and IL-33 are released from damaged or dying cells and signal that cellular damage has occurred (Garlanda *et al*. 2013, Palomo *et al*. 2015). The released forms of IL-1α and IL-33, as well as secreted mature IL-1β are proinflammatory. Thus, in sterile inflammation, IL-1α has been suggested to initiate neutrophil recruitment, and the secretion of IL-1β has been implicated in the later recruitment of macrophages, which propagates inflammation (Rider *et al*. 2011).

IL-1β is secreted mainly by mononuclear cells, whereas nonphagocytic cells generally secrete only small amounts of IL-1β (Apte & Voronov 2008). The secretion of IL-1β is subjected to tight regulation at the levels of transcription, translation, maturation and secretion (Dinarello 2009). The transcription of IL-1β is induced by a wide variety of microbial products through the activation of the TLRs; it is also induced by nonmicrobial products, such as cytokines, including IL-1β itself, which activate cytokine receptors (Bauernfeind *et al*. 2009, Marucha *et al*. 1990, Gross *et al*. 2011). Unlike proIL-1α, the proform of IL-1β is not active (Dinarello 1996). IL-1β is activated by the cleavage of its N-terminal propiece between amino acids Asp^{116} and Ala^{117}; this cleavage is catalyzed by the cysteine protease caspase-1 (previously known as IL-1β converting enzyme, ICE) (Thornberry *et al*. 1992, Higgins *et al*. 1994). In the extracellular space, the released proIL-1β can also be cleaved into its mature form by other proteases, such as serine proteinase 3 (Fantuzzi *et al*. 1997, Joosten *et al*. 2009).

The IL-1β protein lacks a signal sequence, which would direct it to be secreted via the conventional endoplasmic reticulum and Golgi secretory route (Auron *et al*. 1984). Different mechanisms have been suggested for the secretion of IL-1β. Based on the targeting of IL-1β in the endosomal/lysosomal compartment, which allows the colocalization of proIL-1β with procaspase-1, Andrei *et al*. suggested that the targeting of IL-1β to this compartment may lead to its degradation in the lysosome. However, instead of the lysosomal degradation of IL-1β, the ATP-induced activation of the $P2X_7$ receptor and potassium efflux was found to lead to inflammasome activation and the processing of proIL-1β. Subsequently, mature IL-1β was shown to be secreted by lysosome exocytosis in a process dependent on an increase in the intracellular calcium concentration, the activation of phospholipase C, and calcium-independent and -dependent phospholipase A_2 (Andrei *et al*. 1999, Andrei *et al*. 2004). The activation of the $P2X_7$ receptor has also been suggested to induce the formation of plasma membrane-derived vesicles. In this model, the plasma membrane forms blebs in response to $P2X_7$ receptor activation, and biologically active IL-1β is secreted by this microvesicle shedding in a process that is dependent of the extracellular calcium concentration (MacKenzie

et al. 2001). Another $P2X_7$-dependent secretion model suggests that IL-1β is secreted in exosomes contained in multivesicular bodies, along with active caspase-1, cathepsin B, and lysosomal proteins (Qu *et al*. 2007b). However, unlike microvesicle shedding, this model is independent of the extracellular calcium concentration (Qu *et al*. 2007b). Furthermore, IL-1β has been suggested to be released to the extracellular space from autophagosomes (the autophagy-based unconventional secretion of IL-1β is explained in greater detail in Chapter 6.1) (Dupont *et al*. 2011). The release of IL-1β by some activators has also been associated with cell death. Inflammasome activation induces either lytic pyroptotic cell death, which releases the cell contents, or apoptosis (Sagulenko *et al*. 2013). The direct secretion of IL-1β across the plasma membrane has been proposed to precede cell death by an unknown mechanism (Verhoef *et al*. 2005). However, the secretion of IL-1β does not require cell death *per se*, as shown by the inhibition of lytic cell death with glycine, which reduced but did not block the secretion of IL-1β (Verhoef *et al*. 2005).

4.2.2 Structure of nucleotide-binding domain leucine-rich repeat-containing receptors

To date, as many as 22 genes that encode NLR proteins have been reported in humans (Man & Kanneganti 2015). NLR proteins share a typical domain organization characterized by three main structural domains: a C-terminal ligand-binding domain consisting of several LRRs; followed by a conserved central nucleotide-binding and oligomerization domain (NACHT, also referred to as NOD), which is common to all NLRs; and an N-terminal effector domain, which binds downstream signaling molecules (Istomin & Godzik 2009) (An example of an NLR protein, NLRP3, is shown in Figure 6 on page 41). Additionally, several NLRs contain a NACHT-associated domain (NAD). NLR proteins differ by their signal-transducing N-terminal effector domains. Based on their effector domains and phylogenetic evidence, inflammasome-forming NLR proteins can be classified into the following subgroups: NLRA, which contains an acidic transactivation domain; NLRB, which contains a BIR domain; NLRC, which contains a CARD domain; NLRP, which contains a death fold pyrin (PYD) domain; and NLRX, which contains an N-terminal domain unrelated to any other NLR (Martinon & Tschopp 2005, Proell *et al*. 2008, Ting *et al*. 2008). In addition to ligand sensing, LRR has been proposed to inhibit the oligomerization of the inflammasome by interacting with NACHT domains in the absence of an activating stimulus (Martinon & Tschopp 2005).

Upon activation certain NLR proteins, such as NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4 (NLR family CARD domain-containing protein 4), and NLRC4/NAIP (NLR family, apoptosis inhibitory protein) form cytosolic scaffolds termed inflammasomes, which enable the activation of the proform of caspase-1 (Martinon *et al*. 2009, Kofoed & Vance 2011, Khare *et al*. 2012, Rathinam *et al*. 2012, Latz *et al*. 2013). NLRPs that lack the CARD domain require an adaptor protein to bridge the NLR sensor proteins and caspase-1.The adaptor protein used in NLRP3 inflammasomes is a bipartite protein called apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), which is composed of an N-terminal pyrin domain (PYD) and a C-terminal CARD domain (Mariathasan *et al*. 2006, Mariathasan & Monack 2007). In addition to NLRs, other

intracellular sensors form inflammasomes. The receptor proteins of Absent in melanoma 2 (AIM2) and interferon-inducing protein 16 (IFI16) inflammasomes belong to the pyrin domain and hematopoietic expression, interferon-inducible nature, and nuclear localization domain-containing (PYHIN) receptor family (Hornung *et al*. 2009, Kerur *et al*. 2011). The AIM2 inflammasome consists of one or two DNA-binding HIN-200 domains at the C-terminus and an N-terminal PYD (Davis *et al*. 2011, Rathinam *et al*. 2012). Similar to NLRPs, AIM2 lacks a CARD domain; therefore, the assembly and activation of the AIM2 inflammasome requires ASC (Rathinam *et al*. 2012). The function of the prototypical ASC-harboring inflammasomes NLRP3 and AIM2 will be described in greater detail below.

4.2.3 Activation of the NLRP3 inflammasome

Inflammasomes recognize a range of environment- and host-derived DAMPs and microbial components through direct binding and/or through indirect mechanisms. Most inflammasome-forming receptors recognize a limited number of conserved microbial ligands. However, the activation of the NLRP3 inflammasome is triggered by several heterogeneous DAMPs. The activation of the NLRP3 inflammasome is bipartite: the first signal, also called priming, initiates the expression of the NLRP3 and proIL-1β proteins, and the second signal induces the assembly and activation of the NLRP3 inflammasome.

In unstimulated macrophages and monocytes the expression levels of the inflammasome sensor protein NLRP3 and of proIL-1β are generally low; therefore, a priming signal is required to induce their expression (Bauernfeind *et al*. 2009, Marucha *et al*. 1990). The priming signal is usually a proinflammatory compound from a microbial or endogenous source, such as LPS, but the acute-phase protein SAA or cytokines also activate TLRs or cytokine receptors to induce the activation of NFκB (Marucha *et al*. 1990, Gay & Gangloff 2007, Bauernfeind *et al*. 2009, O'Connor *et al*. 2003, Niemi *et al*. 2011). The inflammasome components NLRP3 and ASC are further subjected to post-translational regulation. To be able to induce the assembly and activation of the inflammasome complex, the NLRP3 receptor protein must be deubiquitinylated, which can be mediated by the endogenic deubiquitinase BRCC3 (Juliana *et al*. 2012, Py *et al*. 2013, Lopez-Castejon *et al*. 2013). The expression of BRCC3 may be stimulated by the MAPK pathway, indicating that TLR activation might deubiquinate NLRP3 and thereby favor the activation of the NLRP3 inflammasome (Py *et al*. 2013). Furthermore, the NO-mediated S-nitrosylation of NLRP3 prevents the assembly of the NLRP3 inflammasome (Hernandez-Cuellar *et al*. 2012). In addition, the activity of caspase-1 can be regulated by its oxidation, which has been shown to result in the glutathionylation of caspase-1, which inhibits its activity (Meissner *et al*. 2008).

Several kinases contribute to the inflammasome assembly and processing of IL-1β (Shio *et al*. 2009, Chuang *et al*. 2011, Hara *et al*. 2013, Martin *et al*. 2014). The oligomerization of the adaptor protein ASC is required for the formation of the active NLRP3 and AIM2 inflammasome complexes (inflammasome complex formation is explained in greater detail below). ASC is subjected to post-translational regulation by kinases, such as Syk, Jnk, and IKKα kinase (Hara *et al*. 2013, Martin *et al*. 2014). The phosphorylation of ASC by Syk and Jnk is required for the oligomerization of ASC and the activation of caspase-1 (Hara *et al*.

2013). However, Syk and Jnk seem to induce the localization of ASC to perinuclear area rather than inducing the oligomerization of ASC (Hara *et al*. 2013). In resting cells, ASC is found in the cytoplasm, mitochondria and nucleus (Bryan *et al*. 2009, Zhou *et al*. 2011, Misawa *et al*. 2013), and the redistribution of ASC to the perinuclear space is required for inflammasome complex formation and activation (Bryan *et al*. 2009). Martin *et al*. demonstrated that the nuclear localization of ASC is regulated by the kinase IKKα. Thus, in the absence of an activating stimulus, IKKα retains ASC in the nucleus (Martin *et al*. 2014). The negative regulation of inflammasome complex formation and activation by IKKα is illustrated in Figure 5. Inflammasome priming inhibits IKKα, which is followed by the relocalization of IKKα and ASC to the perinuclear area (Martin *et al*. 2014). The second signal that activates the NLRP3 inflammasome induces the recruitment of phosphatase PP2A, which allows the dissociation of IKKα from ASC, resulting in the assembly of the inflammasome (Martin *et al*. 2014). In addition to the conventional model of inflammasome activation induced by two separate priming and activation signals, it was recently demonstrated that a rapid NLRP3 inflammasome activation pathway is induced by the synergistic sequential activation of TLRs and NLRP3, which induces acute NLRP3 inflammasome activation via the kinase activity of IRAK-1 downstream of TLR activation (Lin *et al*. 2014).

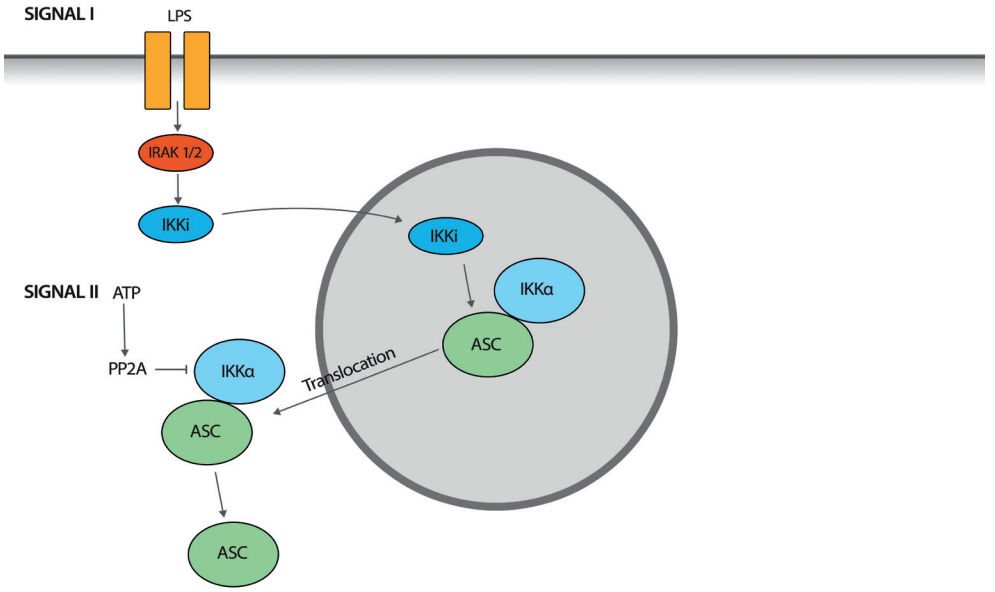

Figure 5. Negative regulation of inflammasome complex formation by IKKα. IKKα regulates the formation of ASC-dependent inflammasome complexes by controlling the subcellular location of ASC. In the absence of a priming signal, IKKα retains ASC in the nucleus. LPS induces the translocation of IKKi to the nucleus and allows the signaling events leading to the translocation of the ASC-IKKα complex to the perinuclear area. An NLRP3 inflammasome-activating signal (e.g., ATP) inhibits IKKα kinase activity by recruiting PP2A, which participates in the dissociation of ASC from IKKα. The liberated ASC is able to participate in NLRP3 inflammasome complex formation in the perinuclear area. ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; ATP, adenosine triphosphate; IKK, IκB kinase; IRAK, IL-1 receptor-activated kinase; LPS, lipopolysaccharide; PP2A, protein phosphatase 2. Data are derived from Martin *et al.* 2014.

In the absence of an activating stimulus, the NLRP3 receptor has been suggested to be in an autorepressed state, and a sufficient stimulus is needed to overcome the autorepression to

further induce the oligomerization of NLRP3 (Martinon & Tschopp 2005). The activation of the NLRP3 receptor induces the recruitment of inflammasome components and the assembly of the multiprotein complex. The assembly of the inflammasome is mediated by homotypic interactions between the receptor complex and two members of the death domain superfamily, PYD and CARD (Ferrao & Wu 2012). Upon inflammasome assembly, the PYD domain of NLRP3 interacts with the PYD of ASC, which induces the polymerization of ASC. ASC forms hollow filaments, thereby creating a platform for the nucleation of caspase-1 filaments, and flexibly linked CARD domains allow the polymerization of caspase-1, leading to the formation of filaments that continue the structure of ASC filaments (Lu *et al*. 2014). The oligomerization of caspase-1 induces its proximity-induced dimerization, which results in its autoproteolytic processing and the activation of the p10 and p20 subunits (Lu *et al*. 2014). According to a recent study by Lu *et al*., relatively few sensor molecules of the AIM2 and NLRP3 inflammasomes are needed for the assembly of the inflammasome complex. An active inflammasome complex is a large structure (approximately 2 µm), and upon inflammasome assembly the oligomerization of ASC is readily visualized as structures called ASC specks (Lu *et al*. 2014, Bauernfeind *et al*. 2009, Hornung *et al*. 2009). In another model proposed by Man *et al*., *Salmonella* infection induced the formation of an inflammasome complex in which caspase-1 and caspase-8 are located in the center and are surrounded by ASC and both NLRP3 and NLRC4 (Man *et al*. 2014). However, it has also been questioned whether ASC speck formation is always related to inflammasome activation and whether inflammasome activation can also be induced without speck formation (Elliott & Sutterwala 2015). The structures of the NLRP3 inflammasome in the unoligomerized and oligomerized states, the latter representing the active inflammasome complex, are illustrated in Figure 6.

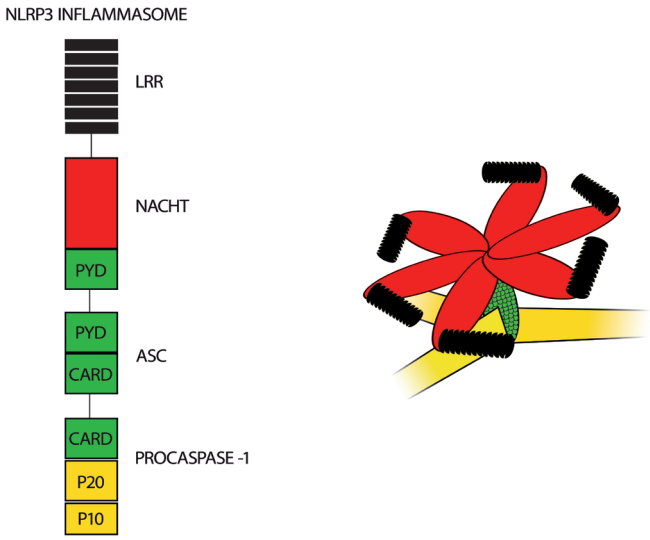

Figure 6. Structure of the NLRP3 inflammasome. The domains of the NLRP3 inflammasome are depicted on the left, and the active multiprotein inflammasome complex, according to the model of Lu *et al*., is shown on the right (Lu *et al*. 2014). The LRRs of the NLRP3 inflammasome sense the activating stimulus, and the central NACHT domain mediates the oligomerization of the complex. This oligomerization induces the proximity-induced cleavage of caspase-1, which results in the formation of heterotetrameric complexes consisting of two p10 and two p20 subunits. The complex of p10 and p20 is the activated form of caspase-1. ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; CARD, caspase recruitment domain; LRR, leucine-rich repeat; NACHT, nucleotide-binding and oligomerization domain; PYD, pyrin domain. Data are derived from references mentioned in the text.

Many of the DAMPs that activate the NLRP3 inflammasome are misplaced cellular components, which are exposed to receptors that normally do not encounter them or are generated in response to inflammation, infection, cellular damage, metabolic dysregulation or environmental stress. A great variety of different exogenous and endogenous activators have been described, including bacterial nucleic acids (Sha *et al*. 2014) and bacterial toxins, such as nigericin (Mariathasan *et al*. 2006); DNA and RNA viruses (Lupfer & Kanneganti 2013); fungi and protozoa, e.g., the fungal pathogen *Candida albicans* and the hemozoin formed during malarial infection (Franchi *et al*. 2012, Shio *et al*. 2009); ATP (Mariathasan *et al*. 2006); and particles such as silica and asbestos (Dostert *et al*. 2008), cholesterol crystals, and uric acid crystals (Duewell *et al*. 2010, Rajamaki *et al*. 2010, Martinon *et al*. 2006). Although the precise mechanism of NLRP3 inflammasome activation has yet to be elucidated, it is generally accepted that instead of the direct ligation of an agonist, disturbances in cellular homeostasis mediate the activation of the NLRP3 inflammasome.

The most common mechanisms implicated in the transduction of the activating signal to the NLRP3 inflammasome are potassium efflux, the disruption of lysosomal integrity, and the production of ROS (Davis *et al*. 2011, Man & Kanneganti 2015). It seems that more than one activating signal is often induced simultaneously. The three main mechanisms suggested to mediate the activation of the NLRP3 inflammasome are summarized below and are illustrated in Figure 7. Subphysiological amounts of intracellular potassium induced by NLRP3 activators, such as ATP and nigericin, have been shown to activate the NLRP3 inflammasome (Mariathasan *et al*. 2006, Petrilli *et al*. 2007). Reciprocally, high extracellular potassium concentrations, which prevent the lowering of intracellular potassium by inhibiting potassium efflux, inhibit the activating effect of various NLRP3 activators (Petrilli *et al*. 2007). Extracellular ATP induces potassium efflux by the activation of the $P2X_7$ receptor, which opens pannexin-1 hemichannels that form pores on the cell membrane, thereby leading to potassium efflux (Pelegrin & Surprenant 2006). However, the role of pannexin in potassium efflux seems controversial: the receptor $P2X_7$ is a cation channel itself, and a deficiency in pannexin-1 was shown to not impair the activation of the NLRP3 inflammasome (Qu *et al*. 2011).

Lysosomal destabilization has been implicated as the activating mechanism, especially in response to crystals (Hornung *et al*. 2008, Duewell *et al*. 2010, Rajamaki *et al*. 2010). The phagocytosis of crystals and particles induces lysosomal swelling, which results in the leakage of lysosomal contents, along with lysosomal proteases, to the cytoplasm (Hornung *et al*. 2008). Additionally, the noncrystalline substance nigericin induces the release of cathepsin B from seemingly intact lysosomes (Hentze *et al*. 2003). Cathepsin B has been suggested to induce the activation of the NLRP3 inflammasome because the pharmacological inhibition or deficiency in cathepsins B and/or L blocks the activation of the NLRP3 inflammasome by nigericin and various particulate matters (Hentze *et al*. 2003, Hornung *et al*. 2008, Duewell *et al*. 2010, Rajamaki *et al*. 2010). In addition, purified lysosomal cathepsin B has been shown to cleave caspase-1 (Vancompernolle *et al*. 1998). The release of cathepsin B has also been suggested to be required for necrosis-like cell death during NLRP3 inflammasome

activation induced by nigericin, *S. flexneri* or disease-associated mutated NLRP3 protein (Hentze *et al*. 2003, Willingham *et al*. 2007). However, the mechanisms that mediate NLRP3 activation in response to cathepsin B are not yet fully understood.

Cellular redox imbalance has been strongly implicated in the activation of the NLRP3 inflammasome. Antioxidants, such as diphenylene iodium (DPI) and N-acetyl cysteine (NAC), and the deletion of the NADPH subunit p22*phox* inhibit NLRP3 activation in response to several activators, which first led to an assumption that NLRP3 was activated in response to ROS generated by NADPH oxidases (Dostert *et al*. 2008, Rubartelli *et al*. 2011). However, this theory was called into question by analyses of monocytes from patients with chronic granulomatous disease, who have defective NADPH-oxidase activity and consequently are unable to produce ROS via the NADPH system. It was shown that the activation of the NLRP3 inflammasome and the secretion of IL-1β in the monocytes of these patients were similar to, or even higher than, cells with normal NADPH activity (van de Veerdonk *et al*. 2010, Meissner *et al*. 2010). Therefore, rather than NADPH oxidases, oxygen radicals derived from dysfunctional or senescent mitochondria and oxidized mitochondrial DNA are now considered the source of ROS that induces the activation of the NLRP3 inflammasome (Zhou *et al*. 2011, Shimada *et al*. 2012). One mechanism of ROS-induced inflammasome activation is the oxidation of TRX, which enables the dissociation of TXNIP from TRX and its subsequent binding to NLRP3, which induces the activation of the NLRP3 inflammasome (Zhou *et al*. 2010).

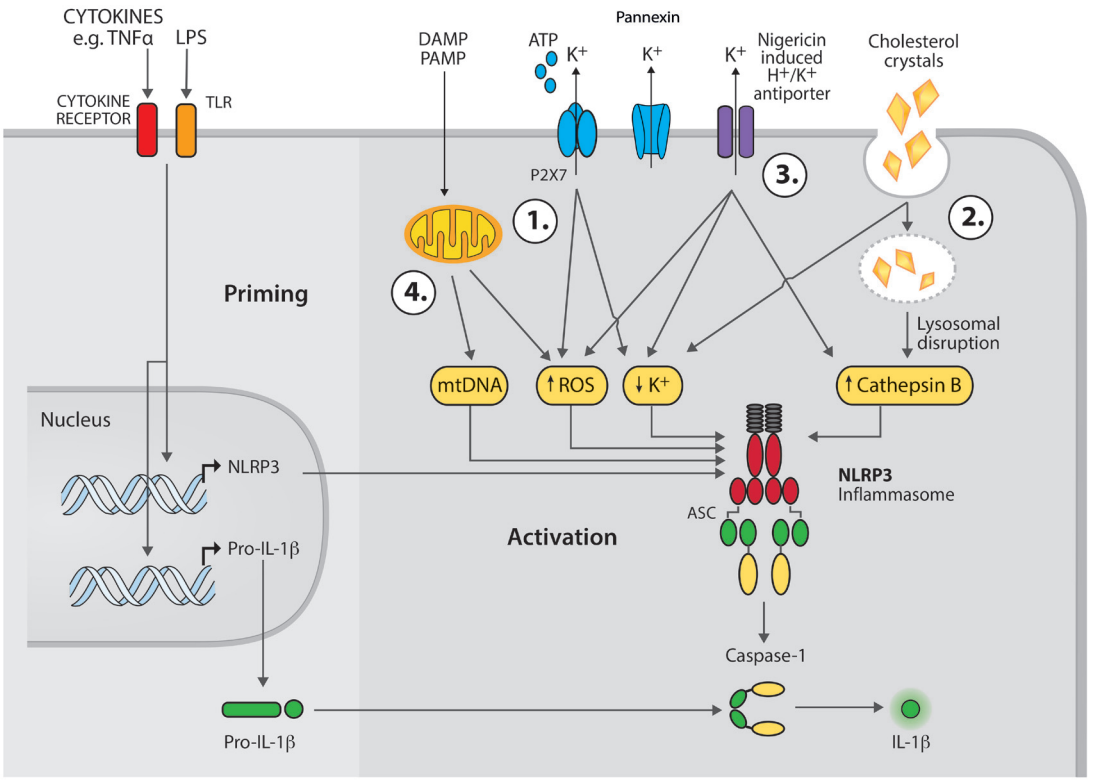

Figure 7. Proposed models of NLRP3 inflammasome activation. Priming is presented on the left, and the most frequently suggested intermediate signaling pathways leading to the activation of the NLRP3 inflammasome are presented on the right. The priming of the NLRP3 inflammasome is induced by LPS or cytokines that activate TLRs or cytokine receptors, respectively. Priming induces the expression of NLRP3 and proIL-1β proteins. (1) Extracellular ATP activates the $P2X_7$ receptor, which is an ATP-gated ion channel. The stimulation of $P2X_7$ is proposed to open the hemichannel pannexin-1 on the membrane. The activation of $P2X_7$ induces potassium efflux, which results in a decrease in the intracellular potassium concentration. The reduced potassium concentration induces the activation of the NLRP3 inflammasome. (2) Phagocytosed crystals and particles induce lysosomal swelling and disruption, which results in the leakage of lysosomal proteases, including cathepsin B. Active cathepsin B induces the activation of the NLRP3 inflammasome. (3) One NLRP3 inflammasome activator can induce several activating events. Nigericin induces the release of cathepsin B from nonruptured lysosomes (nonruptured lysosomes are not shown for clarity), ROS formation, and potassium efflux. (4) Several DAMPs and PAMPs induce mitochondrial dysfunction either directly or via potassium efflux or lysosomal destabilization. Mitochondrial dysfunction increases the production of mitochondrial ROS and the release of oxidized mtDNA. Both mitochondrial ROS and mtDNA induce the activation of the NLRP3 inflammasome. The autocleavage and activation of caspase-1 is induced in the NLRP3 inflammasome complex. Active caspase-1 cleaves proIL-1β to its mature form, which is secreted from the cell. ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; ATP, adenosine triphosphate; DAMP, danger-associated molecular patterns; IL-1β, interleukin 1β; LPS, lipopolysaccharide; mtDNA, mitochondrial DNA; NLRP3, nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain containing 3; $P2X_7$, P2X purinergic receptor 7; PAMP, pathogen-associated molecular patterns; ROS, reactive oxygen species; TLR, Toll-like receptor; TNF, tumor necrosis factor. Data are derived from references mentioned in the text; figure adapted from (Nurmi *et al.* 2013).

4.2.4 Activation of the AIM2 inflammasome

AIM2 inflammasome activation is induced by double-stranded DNA from bacterial, viral or synthetic sources, irrespective of the DNA sequence or GC content (Hornung *et al*. 2009, Rathinam *et al*. 2010, Jin *et al*. 2012, Rathinam *et al*. 2012). However, the length of the sequence may restrict the activation; double-stranded DNA of less than 80 base pairs ceases to activate the AIM2 inflammasome (Rathinam *et al*. 2012). In general, the assembly and activation of the AIM2 inflammasome do not require priming (Bauernfeind *et al*. 2011). However, an early priming event induced by IFNβ and IFNγ has also been described for AIM2 (Burckstummer *et al*. 2009, DeYoung *et al*. 1997, Schattgen & Fitzgerald 2011). Furthermore, IFN-mediated priming was shown to be required for inflammasome activation in response to *Francisella* infection (Henry *et al*. 2007). Upon activation, AIM2 forms a star-shaped ternary complex with ASC in the center of the star (Lu *et al*. 2014). Cytosolic double-stranded DNA is a direct ligand for the sensor protein HIN-200 of the AIM2 inflammasome (Jin *et al*. 2012). Positively charged HIN-200 binds the sugar-phosphate backbone of both strands of double-stranded DNA, which displaces the PYD domain of the AIM2 inflammasome, thereby allowing PYD to bind ASC (Jin *et al*. 2012). Jin *et al*. have also suggested that in the absence of an activating stimulus the PYD and HIN domains of the AIM2 inflammasome are complexed in a manner that inhibits its accidental activation. An intriguing question is, although all bacteria and DNA viruses contain DNA, why is the AIM2 inflammasome activated only by certain pathogens. At least a partial answer to this question was offered by the recent discovery of virulence factors expressed by some bacteria. *Francisella tularensis* ssp. *novicida* was demonstrated to encode the short palindromic repeat Clustered, regularly interspaced, short palindromic repeats-CRISPR-associated (CRISPER-Cas) system, which is assumed to protect the integrity of the bacterial envelope, thereby reducing the release of DNA from bacteria (Sampson *et al*. 2014). The reduced release of bacterial DNA downgrades the activation of the AIM2 inflammasome and subsequent cell death, which would otherwise reduce the survival of *F. novicida* (Sampson *et al*. 2014).

4.2.5 Inhibition of the NLRP3 inflammasome

Considering the severity of autoinflammatory diseases, such as Muckle-Wells syndrome, that are caused by the inappropriate activation of the NLRP3 inflammasome (Rigante 2012) and the consequences of prevalent chronic inflammatory diseases, such as atherosclerosis, gout, and type 2 diabetes, it is obvious that stringent control of NLRP3 inflammasome activation is needed (Strowig *et al*. 2012). Therefore, inhibition, resolution and down-modulation mechanisms are crucial, although a less well known area of inflammasome research. In this chapter, the focus is on the mechanisms that reduce NLRP3 inflammasome activation that are relevant to this study. Viral and bacterial inhibitors are not included because they are not relevant to this thesis study. The negative regulators of the NLRP3 inflammasome that are described below are summarized in Table 3.

A number of endogenic protein interactors with the inflammasome proteins ASC and caspase-1 have been described, and most of them share considerable sequence similarity

with the PYD domain of ASC or the CARD domain of caspase-1 (Stehlik & Dorfleutner 2007). ASC and caspase-1 inhibitors evolved through gene duplications in the course of evolution, and the timing of these duplication events corresponds to the different presence of these genes among species (Kersse *et al*. 2007, Stehlik & Dorfleutner 2007, da Cunha *et al*. 2008). Pyrin-only proteins (POPs), including pyrin, POP1, and POP2 (the last two are present only in the human genome), interact with the PYD domain of ASC, thereby blocking the recruitment of ASC to the inflammasome complex (Stehlik & Dorfleutner 2007). CARD-containing proteins (CARD-only proteins, COPs), such as Iceberg, INCA, COP1, caspase-12, and the short variant of Nod2 (all of which are found in the human genome, whereas only caspase-12 is present in the murine genome), are hypothesized to function in a manner similar to POPs. COPs interfere with the recruitment and/or processing of caspase-1 by interacting with its CARD domain (Stehlik & Dorfleutner 2007). Similarly, leucine-rich repeat Fli-I-interacting protein 2 (LRRFIP2) has been shown to inhibit caspase-1 activation by binding to NLRP3 (Jin *et al*. 2013). Jin *et al*. demonstrated that LRRFIP2 binds the NLRP3 protein through its N-terminus and simultaneously interacts with the caspase-1 pseudosubstrate Flightless-I. The recruitment of Flightless-I inhibited caspase-1 activation. In accordance with that finding, the silencing of Flightless-I reversed the inhibitory effect of LRRFIP2 (Jin *et al*. 2013).

Promyelocytic leukemia protein (PML) acts as a scaffold protein in structures called PML nuclear bodies (Dowling *et al*. 2014). PML nuclear bodies are markers of cellular stress that can recruit diverse regulatory proteins that mediate their functions in several cellular processes (including senescence, apoptosis, tumor suppression, metabolic control, and anti-viral defense) (Sahin *et al*. 2014). However, PML is capable of interacting with ASC, and as a consequence of ASC-PML interaction ASC is retained in the nucleus, thereby leading to reduced inflammasome activation (Dowling *et al*. 2014). Additionally, a hematopoietic-restricted protein, G protein signaling modulator-3 (GPSM3), has been shown to interact with NLRP3. The association of GPSM3 with the C-terminus of NLRP3 inhibited the secretion of IL-1β, possibly by reducing interactions between LRR and activating ligands (Giguere *et al*. 2014). The described interaction between GPSM3 and NLRP3 was increased by heat shock protein HSPA8 (Giguere *et al*. 2014). Indeed, the interaction of NLRP3 with a complex of HSP90 and ubiquitin ligase-associated protein, suppressor of G2 allele of *skp1* (SGT1), has been shown to prevent spontaneous activation of NLRP3 while maintaining NLRP3 in a signaling-competent state for the activatory stimulus, which induces the dissociation of SGT1 and possibly also HSP90 from NLRP3 (Mayor *et al*. 2007). Furthermore, heat shock did prevent caspase-1 activation in response to the activation of NLRP1 with anthrax lethal toxin and the activation of NLRP3 with nigericin (Levin *et al*. 2008). In addition, caspase-1 activity was also inhibited by whole-cell lysate from heat shocked cells (Levin *et al*. 2008).

The cells of the adaptive immune system can also dampen the activation of innate immune responses by cell-cell contacts and cytokines. The inhibitory actions of adaptive immunity may help to resolve the activation of innate immunity after the adaptive immune responses have been activated (Chen & Sun 2013). The anti-inflammatory effects of type I IFNs may

be mediated, at least in part, by their inhibitory effects on inflammasome activation. Type I IFNs were shown to suppress the activity of the NLRP1 and NLRP3 inflammasomes as well as the expression of proIL-1β via signal transducers and activators of transcription (STAT)1- and STAT3-dependent signaling, respectively (Guarda *et al*. 2011). Guarda *et al*. found that the main mechanism of the reduced expression of proIL-1β was the secretion of IL-10, which was induced by the synergistic effects of LPS and type I IFNs (Guarda *et al*. 2011). In accordance with reduced inflammasome activation and the secretion of IL-1β *in vitro*, the induction of type I IFN production reduced monocyte recruitment and the secretion of IL-1β *in vivo* in response to intraperitoneal alum injection or challenge with *C. albicans* (Guarda *et al*. 2011). The induction of IL-10 is specific to type I IFNs, and Guarda *et al*. reported that IFNγ lacks the inhibitory effect of type I IFNs under the same experimental circumstances. In contrast, Mayer-Barber *et al*. reported that IFNγ dampened the expression of IL-1β, although the suppression of cytokine signaling was observed in monocyte-macrophages but not in dendritic cells (Mayer-Barber *et al*. 2011). Cell-cell contact-mediated blockage of caspase-1 activation and the subsequent secretion of IL-1β in mouse macrophages and dendritic cells by effector and memory $CD4^+$T cells has been reported to require the expression of T cell TNF superfamily ligands (Guarda *et al*. 2009). Furthermore, cell-cell contact between monocytes and IFNβ-primed active human memory T cells was shown to reduce $P2X_7$-mediated inflammasome activation in monocytes by downgrading the expression of the $P2X_7$ receptor (Beynon *et al*. 2012). The reduced expression of the $P2X_7$ receptor was partially dependent on the secretion of soluble Fas ligand (Beynon *et al*. 2012). The results reported by Beynon *et al*. suggested a potential feedback loop triggered by IFNβ, which could augment the cessation of immune responses after infection.

Missense mutations in Tripartite-motif protein (TRIM) family proteins have been linked to autoinflammatory diseases. Pyrin, also called TRIM20, is mutated in familial Mediterranean fever, and the overexpression of another member of the TRIM family, TRIM30, has been shown to inhibit NLRP3 inflammasome activation in response to ATP (Hu *et al*. 2010). In accordance with this, a deficiency in TRIM30 increased the secretion of IL-1β in response to several well-known NLRP3 inflammasome activators. The inhibitory effect of TRIM30 was mediated by reduced ROS production (Hu *et al*. 2010). NO exhibits pleiotropic effects on immune responses. The anti-inflammatory effects of NO have been suggested to partially derive from its suppressive effect on inflammasome activation (Mao *et al*. 2013). NO was shown to reduce the IL-1β secretion induced by the activation of the NLRP3 inflammasome. To a lesser extent NO also reduced AIM2- and NLRC4-induced IL-1β secretion. In keeping with that finding, a deficiency in endogenous inducible NO synthase enhanced the secretion of IL-1β *in vivo* (Mao *et al*. 2013). The deletion of inducible NO synthase was shown to increase mitochondrial ROS production and mitochondrial damage, implying that NO-mediated inflammasome inhibition results from reduced ROS production and subsequently diminished inflammasome activation (Mao *et al*. 2013). Additionally, certain drugs have been shown to reduce inflammasome activation. Sulfonylurea-containing compounds, including glyburide, cytokine release-inhibitory drugs, and the small-molecule drug MCC950, have been shown to inhibit the secretion of IL-1β (Lamkanfi *et al*. 2009, Coll &

O'Neill 2011, Coll *et al*. 2015). All the above mentioned drugs have been shown to inhibit the activation of caspase-1 by reducing the assembly of the ASC-dependent inflammasomes (Coll & O'Neill 2011, Coll *et al*. 2015). A variety of endogenous microRNAs (miRNAs), small RNA pieces (typically approximately 23 nucleotides) that do not encode proteins, are implicated in the posttranscriptional control of protein mRNA (Bartel 2009). Various single-nucleotide polymorphisms associated with the pathogenesis of several common disorders have the potential to interfere with the function of miRNAs that may modulate inflammasome pathway components (Glinsky 2008). However, only one miRNA, miR-223, has been shown to inhibit NLRP3 inflammasome activity so far. miR-223 targets NLRP3 transcripts, and it was found to reduce NLRP3 transcript levels (Bauernfeind *et al*. 2012). Therefore, Bauernfeind *et al*. concluded that miR-223 sets a threshold for NLRP3 inflammasome activation by reducing the availability of short-lived NLRP3 transcripts.

Autophagy and certain autophagic and apoptotic signaling proteins have been shown to interact with and inhibit inflammasome activation via multiple mechanisms, which are discussed in greater detail in Chapters 6.1 and 6.3 Additionally, the inhibitory effects of HO-1 on the activation of the NLRP3 inflammasome are discussed in Chapter 2.2.3.

Regulator	Mechanism	Reference
Deubiquitinases	Ubiquination of NLRP3 inhibits the assembly of the inflammasome	(Juliana *et al.* 2012, Lopez-Castejon *et al.* 2013, Py *et al.* 2013)
S-nitrosylation	S-nitrosylation of NLRP3 inhibits the assembly of the inflammasome	(Hernandez-Cuellar *et al.* 2012)
Superoxidase dismutase 1	Oxidation of caspase-1 inhibits caspase-1 activity	(Meissner *et al.* 2008)
Syk and Jnk	Phosphorylation of ASC allows its localization to the perinuclear area	(Hara *et al.* 2013)
IKKα	Retains ASC in the nucleus preventing spontaneous inflammasome activation	(Martin *et al.* 2014)
Pyrin-only proteins (POPs)	Interactions with ASC prevent inflammasome assembly	(Stehlik & Dorfleutner 2007)
CARD-only proteins (COPs)	Interactions with caspase-1 prevent the processing of caspase-1	(Stehlik & Dorfleutner 2007)
LRRFIP2	Interactions with NLRP3 recruit Flightless-I, which prevents caspase-1 processing	(Jin *et al.* 2013)
PML	Interactions with ASC retain ASC in the nucleus	(Dowling *et al.* 2014)
GPSM3	Interactions with LRR inhibit the secretion of IL-1β	(Giguere *et al.* 2014)
HSP90-SGT complex	Interactions with NLRP3 prevent spontaneous inflammasome activation	(Mayor *et al.* 2007)
Heat shock	Induces a cellular factor/modification that inhibits caspase-1 activity	(Levin *et al.* 2008)
Type I interferons	Inhibits NLRP3 activation and the expression of proIL-1β	(Guarda *et al.* 2011)
Type II interferon IFNγ	Reduces IL-1β expression	(Mayer-Barber *et al.* 2011)

Effector and memory CD^+ T cells	Cell-cell contact inhibits caspase-1 activation	(Guarda *et al.* 2009)
INFβ-primed memory T cells	Reduces the expression of $P2X_7$	(Beynon *et al.* 2012)
TRIM30	Decreases ROS production	(Hu *et al.* 2010)
NO	Decreases ROS production	(Mao *et al.* 2013)
Sulfonylurea-containing compounds	Prevents inflammasome assembly	(Lamkanfi *et al.* 2009, Coll & O'Neill 2011, Coll *et al.* 2015)
miR-223	Reduces the level of NLRP3 transcripts	(Bauernfeind et al. 2012)
Autophagy	Degrades inflammasome components, and reduces mitochondrial ROS production and the release of mitochondrial DNA	(Harris *et al.* 2011, Zhou *et al.* 2011, Shi *et al.* 2012, Shimada *et al.* 2012)
HO-1	Reduces inflammasome assembly and ROS production	(Kim & Lee 2013, Li *et al.* 2014a, Luo *et al.* 2014)

Table 3. Summary of mechanisms that inhibit the NLRP3 inflammasome. Data are derived from the references listed in the table.

4.3 NLRP3 inflammasome in pathophysiological conditions

As a part of the innate immune system, the role of inflammasome activation is to maintain homeostasis, yet deregulated function of the inflammasome is detrimental to the host. The diseases affected by excessive or aberrant inflammasome function and IL-1β secretion range from autoimmune diseases and rare autoinflammatory diseases to much more common multifactorial conditions, such as atherosclerosis, gout, type 2 diabetes, and Crohn´s disease. Most autoinflammatory diseases and several autoimmune diseases are caused by known mutations in pyrin, NLRP1, NLRP3, NLRC4 or AIM2 inflammasomes. Autoinflammatory and autoimmune diseases are generally thought as a spectrum of diseases in which autoinflammatory diseases form one end and autoimmune diseases the other (Lamkanfi *et al*. 2011). Numerous autoinflammatory diseases with varying etiologies and symptoms have been described; nevertheless, the common denominator of these diseases is the dysregulated generation of IL-1β in the absence of apparent infection or antigen-specific cellular and humoral responses (Rigante 2012, Saavedra *et al*. 2015). In autoimmune diseases, on the other hand, the adaptive immune system recognizes self molecules and attacks them with antibodies and antigen-specific T cells (Lamkanfi *et al*. 2011). Thus, understanding the molecular mechanisms of these diseases and inflammasome functions will offer means to develop more efficient therapies. NLRP3 inflammasome-related autoimmune and autoinflammatory diseases as well as the NLRP3-derived inflammatory components of atherosclerosis and gout that are relevant for the study are discussed below.

4.3.1 NLRP3-associated autoinflammatory and autoimmune diseases

Mutations in the *NLRP3* gene that encodes the NLRP3 protein, i.e., cryopyrin are associated with autoinflammatory diseases. Cryopyrin-associated periodic syndromes (CAPS) encompass familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile neurologic cutaneous articular syndrome (CINCA), also

called neonatal onset multi-system inflammatory disorder (NOMID) (Rigante 2012). All three syndromes are associated with autosomal dominant gain-of-function mutations in the *NLRP3* gene, which lead to the continuous activation of the NLRP3 inflammasome and the secretion of IL-1β (Goldbach-Mansky 2011, Rigante 2012). Systemic inflammation and the release of IL-1β cause episodes of fever, which are typical of all CAPS (Goldbach-Mansky 2011, Rigante 2012). In addition, systemic inflammation mediates the organ damage associated with MWS and CINCA (Goldbach-Mansky 2011). CAPS can be seen as a continuum defined by their severity, with FCAS being the mildest and CINCA being the most severe (Lamkanfi *et al*. 2011, Rigante 2012). FCAS is characterized by rashes, fever, and arthralgia occurring in an episodic manner, usually 1-2 hours after cold exposure (Hoffman *et al*. 2001). Some of the mutations linked to FCAS have also been associated with MWS, and, although more severe, MWS symptoms resemble the symptoms of FCAS (Maksimovic *et al*. 2008). MWS manifests as episodic fever with abdominal pain, myalgia, arthralgia, arthritis, urticaria, conjunctivitis, and later-occurring deafness, as well as possible AA amyloidosis (Cuisset *et al*. 1999, Maksimovic *et al*. 2008). The symptoms of CINCA occur early, often at birth, and are the most severe of all CAPS. The clinical phenotype of CINCA includes fever; rash; inflammations of the eye, inner ear, and central nervous system; possible amyloidosis; and cognitive and physical disabilities (Goldbach-Mansky 2011). Three different strategies to block IL-1β in CAPS have been used in clinical trials: a recombinant human IL-1R antagonist (Anakinra®), which is approved for the treatment of RA for example in the United States (US); a recombinant IL-1R-Ig fusion protein (Rilonacept®), which is accepted for the treatment of CAPS for example in the US; and a humanized anti-IL-1β antibody (Canakinumab®), which is accepted for treatment of CAPS for example in the US (Goldbach-Mansky 2011). All the above mentioned medications were found beneficial in the treatment of CAPS in clinical trials (Goldbach-Mansky 2011).

Several autoimmune diseases, including RA, inflammatory bowel disease, celiac disease, type 1 diabetes, and systemic lupus erythematosus, are associated with polymorphisms in genes that encode NLRP3 or NLRP1 inflammasome-associated proteins (Saavedra *et al*. 2015). Although autoimmune diseases are defined by the inappropriate recognition of self-antigens by the adaptive immune system, the components of innate immune defense, PRRs, macrophages, and mast cells play a key role in the pathogenesis of RA (Lee *et al*. 2002, Gierut *et al*. 2010). RA is the most common type of inflammatory arthritis. In RA, the chronic inflammation of synovial tissue leads to the infiltration of $CD4^+$ T cells, B cells, mast cells, and macrophages; increased concentrations of proinflammatory cytokines; and the aggressive growth of synovial tissue, which fills the joint, destroying the articular structure (Firestein 2003, Gierut *et al*. 2010). In RA, the expression of *NLRP3* is enhanced in the synovium (Rosengren *et al*. 2005). Certain polymorphisms of NLRP3 inflammasome components have been shown to correlate with increased susceptibility to RA (Mathews *et al*. 2014, Yang *et al*. 2014). Intriguingly, in genetically modified mice (*A20/TNFAIP3* deleted in myeloid cells) that spontaneously develop arthritis, the deletion of IL-1R, NLRP3 and caspase-1, but not of TNFR1, attenuated the joint inflammation (Vande Walle *et al*. 2014). TNFα inhibitors have been shown to be more efficient than the IL-1R antagonist

Anakinra® in the treatment of RA (Mertens & Singh 2009). Unfortunately, neither treatment helps all patients. In the case of Anakinra®, this may be because Anakinra® is a competitive IL-1R antagonist, rather than a direct inhibitor of IL-1β (Firestein 2003, Mertens & Singh 2009).

4.3.2 Multifactorial diseases

The above mentioned autoinflammatory diseases are induced by mutations that affect the functions of the NLRP3 inflammasome. However, the dysregulated activation of the NLRP3 inflammasome is implicated in the pathogenesis of several chronic diseases, including atherosclerosis, gout, type 2 diabetes, and Alzheimer´s disease (Ozaki *et al*. 2015). In many cases, the altered metabolism related to the pathogenesis of these diseases induces the accumulation of DAMPs, which activate the inflammasome (Martinon *et al*. 2006, Duewell *et al*. 2010, Rajamaki *et al*. 2010, Grant & Dixit 2013). In atherosclerosis, the deposition of circulating LDL particles into the arterial wall, together with the recruitment and proliferation of immune cells leads to chronic inflammation, and the progressive growth of plaques, thus contributing to the narrowing of the vessel lumen (Cochain & Zernecke 2015). Inflammation is considered the principal driving force in the pathogenesis of atherosclerosis. In the arterial wall, LDL particles are oxidized and consequently taken up by macrophages (Moore & Tabas 2011, Weber & Noels 2011). Oxidized LDL particles have been shown to prime and activate the NLRP3 inflammasome via cathepsin B and ROS generation (Jiang *et al*. 2012). In support of that finding, Sheedy *et al*. demonstrated that scavenger receptor cluster of differentiation (CD) 36 mediated the uptake of oxidized LDL particles and primed the NLRP3 inflammasome in cooperation with TLR4 and TLR6. In macrophages, oxidized LDL particles were further crystallized to cholesterol crystals in lysosomes, which led to lysosomal destabilization and the subsequent activation of the NLRP3 inflammasome (Sheedy *et al*. 2013). In accordance with that finding, the deletion of CD36 was shown to inhibit the accumulation of cholesterol in plaques, and to suppress IL-1β secretion *in vivo* (Sheedy *et al*. 2013).

Additionally, extracellularly added cholesterol crystals have been shown to induce lysosomal destabilization and subsequent NLRP3 inflammasome activation in both mouse and human macrophages in culture (Duewell *et al*. 2010, Rajamaki *et al*. 2010). The influence of the NLRP3 inflammasome on atherogenesis was demonstrated *in vivo* in an atherosclerosis-prone mouse model in which the deletion of the NLRP3 inflammasome in bone marrow-derived cells significantly reduced the lesion size and the inflammasome-dependent secretion of IL-18 (Duewell *et al*. 2010). The significance of IL-1β in the pathogenesis of atherosclerosis was further emphasized by findings demonstrating that the deletion of *Il1b* in hypercholesterolemic (ApoE-deficient) mice reduced atherosclerotic lesions in the aorta (Kirii *et al*. 2003). The deficiency in IL-1β led to diminished expression of adhesion molecules (Kirii *et al*. 2003). Another metabolite shown to activate NLRP3 is monosodium urate (MSU) crystals, which are formed in gout as a consequence of high blood urate levels (So 2008). In patients with gout, deposits of MSU induce inflammatory episodes, during which neutrophils are recruited and inflammatory mediators are secreted locally in the joint

(So 2008). Indeed, MSU crystals have been shown to induce the activation of the NLRP3 inflammasome in cultured macrophages and in an *in vivo* mouse model of MSU-induced peritonitis (Martinon *et al*. 2006). Martinon *et al*. also demonstrated that, in mice deficient in IL-1R or the inflammasome components ASC and caspase-1, neutrophil recruitment was significantly reduced. In accordance with the results of Martinon *et al*., IL-1R and its downstream signaling molecule MyD88 were required for the secretion of IL-1β and other proinflammatory cytokines in another model of MSU peritonitis, although in this model, only nonhematopoietic cells responded to deletion of IL-1R or MyD88 with reduced neutrophil recruitment (Chen *et al*. 2006). However, although MSU deposits remain in the joints constantly, gout attacks occur only occasionally. Recent results reported by Joosten *et al*. may offer a partial explanation for this discrepancy; they demonstrated that MSU *per se*, without prior priming, did not elicit the secretion of IL-1β. However, the combination of MSU and free fatty acids induced the potent secretion of IL-1β *in vitro*, and intraarticular joint injections of MSU and free fatty acids induced arthritis *in vivo* (Joosten *et al*. 2010). Curiously, the *in vivo* joint inflammation was independent of NLRP3 but dependent on ASC and caspase-1 (Joosten *et al*. 2010). The role of IL-1β as a key mediator of gout was further emphasized by the results of clinical trials of Canakinumab®, Rilonacept®, and Anakinra®, all of which have been shown to efficiently reduce gout flare-ups (Schlesinger *et al*. 2011, Schumacher *et al*. 2012, Ottaviani *et al*. 2013).

5. Autophagy

To preserve their functionality, living cells must constantly remove aberrant and excess proteins, cell organelles, and protein aggregates. Senescent cytoplasmic constituents are recycled by two major degradation systems: lysosomes and proteasomes. Lysosomes degrade both extracellular and intracellular material. Extracellular material is delivered to lysosomes by the endocytic pathway, whereas cytosolic material is transported to lysosomes via autophagy. Autophagy (Greek for “self-eating”) is an evolutionarily conserved process of eukaryotic cells that maintains homeostasis by controlling the quality and number of cell organelles (Levine *et al*. 2011, Noda & Inagaki 2015). Previously, autophagy was thought to be nonselective, but increasing evidence suggests that in addition to the nonselective autophagy induced by stress, basal autophagy selectively targets damaged and obsolete organelles as well as protein aggregates for degradation in lysosomes (Lamb *et al*. 2013, Sica *et al*. 2015). A low basal level of autophagosomal activity is constantly present in cells. However, autophagy is rapidly induced by environmental stress, such as nutrient or growth factor deprivation and hypoxia (Behrends *et al*. 2010, Lamb *et al*. 2013). During starvation, nonessential cellular components are recycled in autophagosomes to amino acids, lipids and carbohydrates to sustain cell viability and homeostasis (He & Klionsky 2009).

5.1 Mechanisms of autophagy

In mammalian cells, three different forms of autophagy can be distinguished: macroautophagy, microautophagy, and chaperone-mediated autophagy (illustrated in Figure 8) (Mizushima *et al*. 2011). Despite differences in cargo sequestration, all autophagic pathways transport their cargo to lysosomes for degradation (Wirawan *et al*. 2012, Damme *et al*. 2015). The mechanisms and functions of macroautophagy are discussed below.

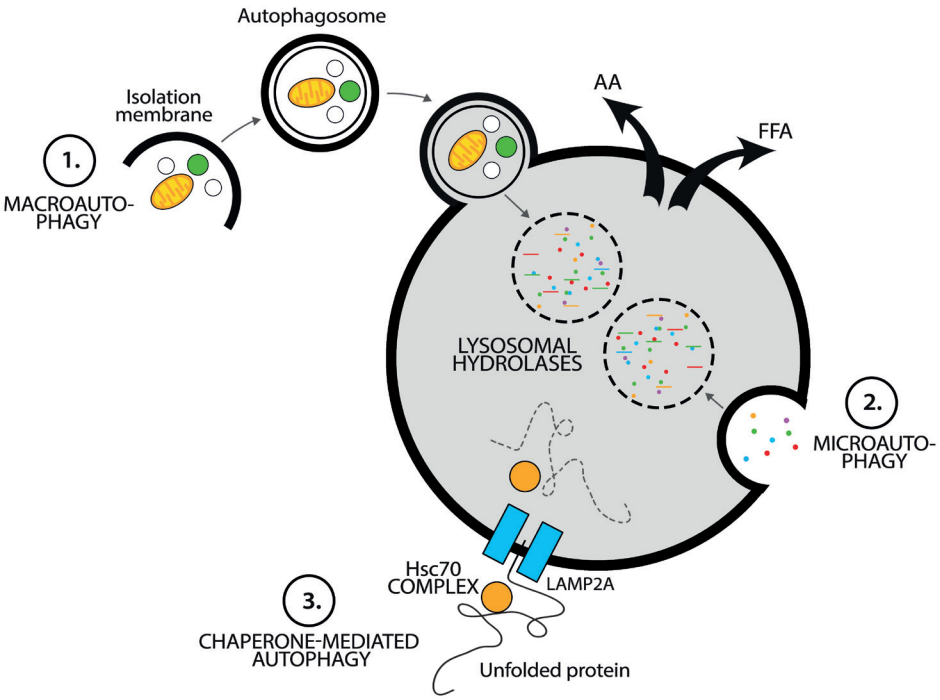

Figure 8. Principal types of autophagy. All autophagic routes target the cargo to the lysosome (the lysosome is shown in the figure). 1) Macroautophagy sequesters cytoplasm and cell organelles to specialized double-membraned vesicles called autophagosomes, which transport the cargo to lysosomes. Both nonselective and selective macroautophagy exist. During selective macroautophagy, cell organelles, such as mitochondria, are targeted to autophagosomes by proteins acting as autophagy receptors. 2) During microautophagy, the lysosomal membrane invaginates and a portion of the cytoplasm is engulfed. 3) Chaperone-mediated autophagy directly targets proteins that possess a KFERQ sequence on their surface to the lysosome. Proteins are bound by Hsc70 and its co-chaperones. The cargo proteins are translocated across the lysosomal membrane by a translocation complex formed by the multimerization of the lysosomal membrane protein LAMP2A. The autophagosomal cargo is degraded in lysosomes. Autophagy has great degradative capacity and leads to the degradation of entire organelles, large protein aggregates, and lipids. The degradation products, such as amino acids, nucleic acids, and fatty acids, are transported from the lysosome to the cytosol and used for anabolic processes, such as the synthesis of macromolecules or for energy production. AA, amino acid; FFA, free fatty acid; Hsc70, heat shock 70 kDa protein 8; LAMP2A, A isoform of lysosomal-associated membrane protein 2. Data are derived from references in the text.

5.1.1 Autophagic process

Macroautophagy (referred to hereafter as autophagy) is the best-characterized form of autophagy, and it is thought to represent the major type of autophagy (Mizushima *et al*. 2011). During autophagy, a portion of the cytoplasm and cellular components, ranging from soluble molecules to entire organelles, is encapsulated in a double-membraned vesicle called the autophagosome (Lamb *et al*. 2013). A mature autophagosome can subsequently fuse with an endosome, and ultimately with a lysosome, in which the cargo and the inner membrane of the autophagosome are degraded by lysosomal hydrolases (Yang & Klionsky 2010, Hamacher-Brady 2012). In mammalian cells, autophagosomes can form in multiple locations. The source of the lipids used to form autophagosomal membranes is unclear; the endoplasmic reticulum, endosomes, Golgi complex, mitochondria, plasma membrane and nuclear membrane have all been suggested to contribute to the autophagosome formation (Chen & Klionsky 2011, Mizushima *et al*. 2011). Whether some or all of the above mentioned sources participate in membrane delivery likely depends on the site and conditions of autophagosome formation, as well as on the targeted cargo (Chen & Klionsky 2011, Mizushima *et al*. 2011).

5.1.2 Autophagosome formation

The formation of the autophagosome begins with the nucleation of an isolation membrane, also called the phagophore, a double-lipid bilayer that eventually encloses the cytosolic cargo. The cargo-loaded mature autophagosomes subsequently fuse with lysosomes, thereby forming structures called autolysosomes. The autophagic process, beginning with the formation of the autophagosome and ending in the degradation of the autolysosomal contents, is outlined in Figure 9. Each stage of the autophagic process is regulated by an ordered interaction of conserved autophagy-related proteins (Atg), and a failure of signaling or the disturbance of these consecutive steps at any point can interrupt the flow (for clarity, autophagy proteins are hereafter referred to using the human ortholog nomenclature) (Parzych & Klionsky 2014). The core signaling complexes required for autophagosome formation are divided into four key complexes based on their functional properties. First, the farthest upstream step that controls the induction of autophagosome formation is the recruitment of the ULK1 complex to the isolation membrane assembly site (Itakura & Mizushima 2010, Yang & Klionsky 2010, Parzych & Klionsky 2014). Second, the nucleation of the isolation membrane is mediated by the active PI3K complex (Itakura & Mizushima 2010, Yang & Klionsky 2010, Parzych & Klionsky 2014). Third, WIPI proteins and two transmembrane proteins, ATG9 and VMP1, are recruited to the forming isolation membrane (Itakura & Mizushima 2010, Yang & Klionsky 2010, Parzych & Klionsky 2014). Fourth, two ubiquitin-like protein conjugation systems, the ATG12 and LC3 systems, are recruited to the isolation membrane to control the elongation and closure of the autophagosome (Itakura & Mizushima 2010, Yang & Klionsky 2010, Parzych & Klionsky 2014).

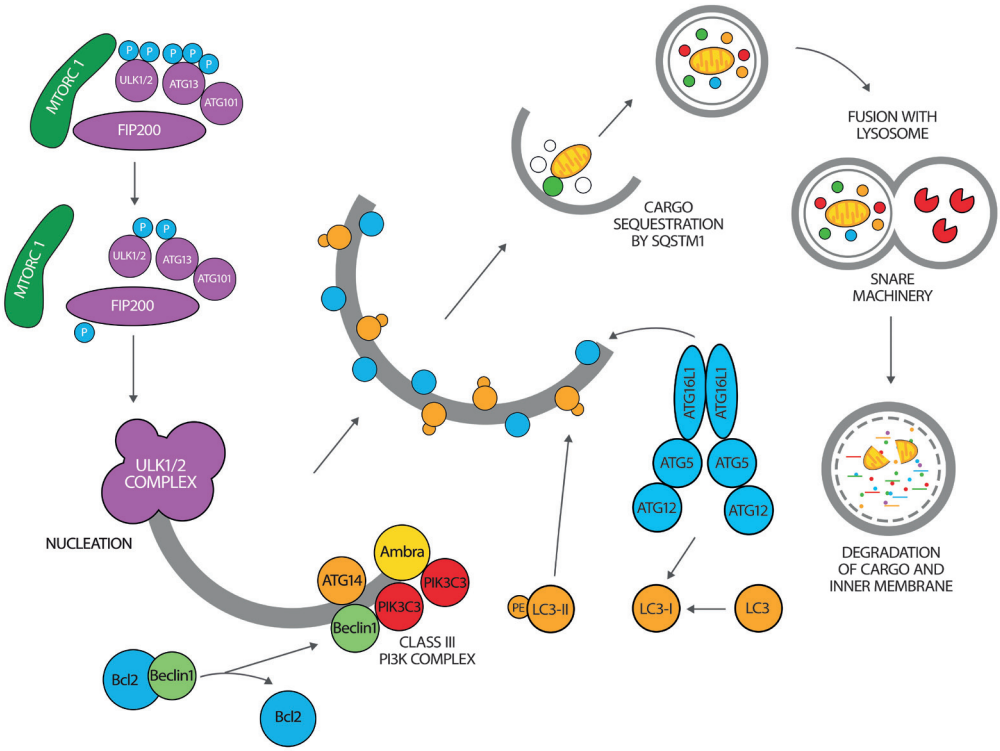

Figure 9. Autophagic process. Some steps are omitted for clarity. The sequential arrival of the protein complexes to the autophagosome assembly site results in the formation of the isolation membrane. ULK1, ATG13, ATG101, and FIP200 are mainly cytosolic proteins, which form a complex irrespective of nutrient status. In the nourished state, the ULK1 complex is inhibited by MTORC1. Starvation induces the dissociation of MTORC1, which activates the ULK1 complex. The next complex recruited to the forming isolation membrane is the PI3K complex, which consists of PIK3C3, PIK3R4, ATG14L (also called Barkor), Beclin1, and the Beclin1-interacting protein AMBRA1. Through the actions of PIK3C3, this complex produces the lipid PI3P. In mammalian cells, ATG9, WIPI1, WIPI2, and DFCP1, along with VMP1 (not shown in the figure) are also required for the formation of the isolation membrane. The elongation and complete closure of the isolation membrane are controlled by two ubiquitin-like conjugation systems. The first complex is the ATG12-ATG5 conjugate, which interacts with the dimeric ATG16L1 to form the ATG16L1 complex. The ATG16L1 complex associates with the isolation membrane to promote its expansion, and Atg16L1 later dissociates from the completed autophagosome. For the second elongation complex, the precursor of LC3 protein is first cleaved by ATG4 to LC3-I; LC3-I is then conjugated to the lipid PE. The conjugation of PE may be aided by the ATG16L1 complex. The conjugated form of LC3, called LC3-II, is a structural component of both the inner and outer membranes of the autophagosome. During the elongation stage the isolation membrane grows in size, and the ubiquitinated cargo is sequestered by cargo receptor proteins, such as SQSTM1, to the forming autophagosome (SQSTM1 is depicted as a green ball). The cargo loaded isolation membrane seals itself to form the completed autophagosome. For the final stage of maturation, the autophagosome is transported along the microtubules toward a lysosome to form the final degradative compartment. The fusion of the autophagosome with the lysosome is aided by autophagy proteins and SNARE machinery proteins. This fusion allows lysosomal acid hydrolases to degrade the sequestered material and the inner limiting membrane of the autophagosome. The resulting macromolecules are transported back to the cytosol, and the LC3-II proteins at the outer limiting membrane are recycled by ATG4 back to LC3-I and PE. AMBRA1, Activating molecule in Beclin-1-related autophagy-1; ATG16L1; autophagy-related 16-like 1; DFCP1, double FYVE-containing protein 1; FIP200, focal adhesion kinase family-interacting protein of 200 kDa; LC3, microtubule-associated protein 1 light-chain 3; MTORC1, mammalian target of rapamycin complex; PE, phosphatidylethanolamine; PI3K, phoshatidylinositol-3 kinase PI3P, phosphatidylinositol 3-phosphate; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SQSTM1, sequestosome 1; ULK1, UNC-51-like kinase; VMP1, vacuole membrane protein 1; WIPI, WD-repeat protein interacting with phosphoinositide. Data are derived from references mentioned in the text.

The majority of the autophagy-inducing signaling pathways that sense cellular stress converge at the mammalian target of rapamycin (mTOR). mTOR forms a complex, called MTORC1, that under growth-promoting conditions associates with ULK1 (or with ULK2, another mammalian Atg1 ortholog that may have a partially redundant role with ULK1), thereby phosphorylating the ULK1 and ATG13 components of the ULK1 complex (Hosokawa *et al*. 2009, Parzych & Klionsky 2014). At the ULK1 complex, MTORC1 acts as a negative regulator, inhibiting the formation of the autophagosome under nutrient-rich conditions and promoting cell growth by upregulating protein translation and ribosome biogenesis (Wullschleger *et al*. 2006). Under catabolic conditions, MTORC1 is inactivated and it dissociates from the ULK1 complex, which is then dephosphorylated (Parzych & Klionsky 2014). Subsequently ULK1 complex translocates to the site of autophagosome formation and initiates the formation of isolation membrane (Parzych & Klionsky 2014). Additionally, several other energy level- and stress-sensing kinases can promote autophagy and/or activate the ULK1 complex, including the major energy-sensing kinase adenosine monophosphate-activated protein kinase (AMPK), protein kinase C, and the MAPKs: extracellular signal-regulated kinase (ERK), p38, and Jnk (Sridharan *et al*. 2011).

The autophagic process is not only controlled at the level of initiation. Autophagosome nucleation and formation are also regulated by several inhibitory and activating proteins that attach to the core complexes or influence their assembly. The Beclin 1-interacting proteins, such as Bcl-2, inhibit autophagosome nucleation by regulating the availability of Beclin 1. The dissociation of Beclin 1 from Bcl-2 is required for the activation of the PI3K complex that locally produces PI3P, which is needed for the initiation of autophagosome formation (Burman & Ktistakis 2010, Mizushima *et al*. 2011, Parzych & Klionsky 2014). The activity of the PI3K complex is regulated by the association of several subunits, such as Beclin 1-associated autophagy-related key regulator (Barkor or ATG14L), Activating molecule in Beclin-1-related autophagy-1 (AMBRA1), UV irradiation resistance-associated gene (UVRAG), Endophilin B1 (also called Bif-1), and RUN domain and cysteine-rich domain-containing, Beclin 1-interacting (Rubicon) subunits, which either promote or inhibit autophagosome formation (Yang & Klionsky 2010, Parzych & Klionsky 2014). UVRAG has also been shown to regulate autophagosome maturation into autolysosomes (Liang *et al*. 2008, Kim *et al*. 2015).

Autophagy is a bulk process that enables cell survival under harsh conditions. In addition, autophagy can specifically select cargo by the actions of proteins that may be called autophagy receptors. The selective autophagic degradation of organelles, such as mitochondria and peroxisomes, and the targeting of ubiquitinated protein aggregates or bacteria to the autophagosomes are examples of this phenomenon (Kundu *et al*. 2008, Zhang & Ney 2009, Mortensen *et al*. 2010, Huybrechts *et al*. 2009, Kim *et al*. 2008). Mammalian autophagy receptors, such as SQSTM1 (also called p62), the neighbor of *BRCA1* gene 1 (NBR1), and Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3-like (Bnip3L), act as adaptors that connect the cargo, which is often marked by ubiquitination, to LC3-II (Bjorkoy *et al*.

2005, Mizushima *et al*. 2011). The selective sequestration of ubiquinated proteins or protein aggregates and large organelles can be mediated by SQSTM1 (Bjorkoy *et al*. 2005). The direct binding of SQSTM1 both to the hydrophobic amino acids of ubiquitin and to LC3 is needed to target the ubiquinated substrate to autophagosomes (Pankiv *et al*. 2007, Kim *et al*. 2008).

5.2 Autophagy in physiology and pathophysiology

Autophagy plays key roles in multiple specific cellular processes. Basal constitutive autophagy is required to prevent the accumulation of polyubiquitinated protein aggregates. This process is critical for the maintenance of cellular homeostasis, particularly in postmitotic cells such as neurons and muscle cells. Therefore, it is not surprising that malfunctions in autophagy predispose cells to pathophysiological conditions. The dysregulation of autophagy has been described in neurodegenerative diseases and cancer, as well as in disturbances of embryogenesis and developmental processes (Wu *et al*. 2013).

5.2.1 Autophagy maintains homeostasis

The degradation of protein aggregates and senescent mitochondria is vital for preserving essential cellular functions and preventing the accumulation of ROS (Cuervo *et al*. 2005). Most misfolded proteins are ubiquitinated and eliminated via the proteasomes. However, autophagy is required for the removal of large protein aggregates, i.e., inclusion bodies, which form during cellular stresses such as oxidative stress, infection and toxication (Zatloukal *et al*. 2002, Canadien *et al*. 2005, Kaniuk *et al*. 2007). The polyubiquitin-binding protein SQSTM1, a component of ubiquitinated cellular inclusion bodies, is needed for the targeting of ubiquinated aggregates to autophagosomes (Bjorkoy *et al*. 2005, Pankiv *et al*. 2007). The loss of basal autophagy results in the massive accumulation of SQSTM1 and protein aggregates, which disrupts cellular homeostasis (Komatsu *et al*. 2005, Hara *et al*. 2006, Pankiv *et al*. 2007, Saitoh *et al*. 2008). Additionally, exposure to oxidative stress increases the intracellular level of SQSTM1 (Ishii *et al*. 1997). Recently, reciprocal interactions were demonstrated between SQSTM1 and the transcription factor Nrf2, which is an important transcriptional regulator for several cytoprotective genes and antioxidant enzymes (Jain *et al*. 2010). Both SQSTM1 and Nrf2 are needed to preserve cellular functions during stress. In response to oxidative stress, Nrf2 induced the expression of SQSTM1, which induced the constitutive activation of Nrf2, thereby forming a signaling cascade that positively regulated the expression of both proteins (Jain *et al*. 2010). Abnormal accumulation of inclusion bodies and SQSTM1 is associated with many neurodegenerative and other diseases, such as Parkinson´s disease, Alzheimer´s disease and Huntington´s disease, inclusion body myopathies, an experimental model of type 2 diabetes in rats, and alcoholic and non-alcoholic liver disorders (Kuusisto *et al*. 2001, Kuusisto *et al*. 2002, Rub *et al*. 2014, Komatsu *et al*. 2005, Kaniuk *et al*. 2007, Zatloukal *et al*. 2002). In these disorders, the capacity of cellular rescue systems, such as increased chaperone expression and proteasomal degradation, may be overwhelmed by the chronic stress caused by the degenerative disease or alcohol (Zatloukal *et al*. 2002).

5.2.2 Role of autophagy in inflammation

Autophagy is versatile process capable of controlling and modulating both the innate and adaptive immunity responses. Autophagy may have first evolved as a mechanism for the clearance of intracellular microbes; therefore, it aims to ensure the survival of cells and to restrict the spread of pathogens (Deretic 2011). The induction of autophagy facilitates the recognition and elimination of intruding pathogens by increasing the efficiency of the immune response. Indeed, autophagy has been reported to enhance antigen presentation and to contribute to the T cell selection process in the thymus (Schmid & Munz 2005). Autophagy contributes directly to the degradation and abrogation of a variety of pathogens, such as viruses or bacteria, including *Shigella*, *Mycobacteria*, and *Salmonella*, that reside in the cytoplasm or phagosomes (Gutierrez *et al*. 2004, Deretic 2009). In addition, autophagy is involved in the elimination of normally extracellular pathogens that invade the host cell interior, such as *Streptococcus pyogenes* (Nakagawa *et al*. 2004). As a counterattack in the course of evolution, pathogens have developed multiple mechanisms to escape autophagic clearance, which initially led to the misconception that autophagy promotes the survival of pathogens (Schmid & Munz 2007, Deretic 2009). Autophagy also plays a role in the central function of innate immunity, i.e., the recognition of pathogens. Autophagy is induced by the activation of the PRRs, including the TLRs and NLRs. Furthermore, autophagy can modulate innate immune signaling, including NLR activation. The engagement of the TLRs in response to stimuli such as LPS, single stranded RNA, and zymosan enhances autophagosome formation (Xu *et al*. 2007, Delgado *et al*. 2008). TLR4-induced autophagy proceeds via receptor-interacting protein 1 (RIP1), p38 MAPK, and TRIF, which mediate the slower response downstream of TLR4 activation (Xu *et al*. 2007). Instead, MyD88 was reported to not contribute to the TLR4-induced activation of autophagy (Xu *et al*. 2007). On the contrary, the activation of autophagy in response to the activation of TLR7 depended on MyD88 (Delgado *et al*. 2008). However, NFκB, which is also stimulated during TLR activation and is needed for the induction of transcription of many proinflammatory cytokines, was shown to act as a negative regulator of autophagy under certain circumstances (Djavaheri-Mergny *et al*. 2006).

6. Crosstalk between signalosomes

Multiple shared components with dual activities, i.e., components of one process that also directly regulate another process, are known to function in signaling pathways for autophagy, inflammasomes and apoptosis. Interactions among autophagy, inflammasomes and apoptosis are likely because all of the above mentioned pathways respond to cellular stress. Thus, the investigation of the reciprocal interactions between signalosomes is of outmost importance for our understanding of intracellular signaling cascades and the possible outcomes of inhibiting or enhancing any one of these pathways. In many syndromes and diseases, it is more than likely

that the function of more than one pathway is involved. In addition, several currently existing drugs or those in the development phase inhibit or aim to modulate autophagy, inflammasomes or apoptosis. For instance, several cancer treatments induce apoptosis by the activation of caspases (Thorburn 2008). Crosstalk between apoptosis and autophagy or inflammasomes may fundamentally alter the outcomes of these treatments (Thorburn 2008). In the next section, crosstalk between autophagy and inflammasomes, between autophagy and apoptosis and between inflammasomes and apoptosis is discussed. The main links between these pathways are presented in Figure 10 on page 64.

6.1 Crosstalk between autophagy and NLRs

Complicated interactions between autophagy and the functions of the NLRP3 and AIM2 inflammasomes have been reported in several studies, whereas the activation of the NLRC4 inflammasome has been shown to directly inhibit autophagosome formation via the association of NLRC4 with Beclin 1 (Dupont *et al*. 2011, Harris *et al*. 2011, Jounai *et al*. 2011, Nakahira *et al*. 2011, Zhou *et al*. 2011, Shi *et al*. 2012). The loss of autophagy protein ATG16L1 has been shown to enhance the secretion of IL-1β and IL-18 in response to LPS (Saitoh *et al*. 2008). Thus, autophagy is mostly described as a negative regulator of NLRP3 and AIM2 inflammasome activation (Harris *et al*. 2011, Nakahira *et al*. 2011, Zhou *et al*. 2011, Shi *et al*. 2012). However, the secretion of IL-1β has also been reported to depend on the activation of autophagy (Dupont *et al*. 2011). Autophagy can inhibit the activation of the NLRP3 inflammasome by reducing the availability of NLRP3 inflammasome agonists, such as intracellular ROS. A basal autophagic process called mitophagy (Mortensen *et al*. 2010), targets damaged mitochondria for autophagic degradation, thereby reducing the generation of mitochondrial ROS and the leakage of mtDNA, which are potent activators of the NLRP3 inflammasome (Zhou *et al*. 2011, Nakahira *et al*. 2011, Shimada *et al*. 2012).

Both the TLR-induced priming of the NLRP3 inflammasome and activation of the NLRP3 and AIM2 inflammasomes induce autophagy, and further enhancement of autophagy has been shown to target the components of the activated NLRP3 and AIM2 inflammasomes to autophagosomes for subsequent degradation (Harris *et al*. 2011, Shi *et al*. 2012). Upon activation of the inflammasome, the oligomerized ASC within the inflammasome complex is ubiquitinated, which leads to the recruitment of ASC and possibly the entire inflammasome complex to autophagosome by SQSTM1 (Shi *et al*. 2012). Subsequent to their targeting to autophagosomes the inflammasome components are degraded (Shi *et al*. 2012). Harris *et al*. reported the sequestration of only the proform and mature form of IL-1β, but not of caspase-1, into autophagosomes (Harris *et al*. 2011). Three independent groups have reported that IL-1β co-localizes with autophagosomes, although one of the studies reported a very different outcome for the stimulation of autophagy in combination with inflammasome activation. Harris *et al*. and Shi *et al*. reported that stimulating autophagy via

rapamycin or starvation inhibits inflammasome activation, thereby reducing the secretion of mature IL-1β. In contrast, results by Dupont *et al*. demonstrated that the secretion of mature IL-1β was enhanced in response to starvation-induced autophagy (Dupont *et al*. 2011). These contradictory results regarding the secretion of IL-1β upon the activation of autophagy were unified in a model proposed by Deretic *et al*. According to this model, the induction of autophagy is required for the secretion of IL-1β early in the process. However, the degradation of inflammasome components in autophagosomes becomes dominant at later stages, and the secretion of IL-1β is consequently reduced (Deretic *et al*. 2012).

Thus, the results of Dupont *et al*. also present a plausible mechanism for the unconventional secretion of leaderless proteins. In addition to IL-1β, high-mobility group box 1 (HMGB1), a major DAMP released from cells during necrosis, was reported to be secreted via this autophagy-based route. Dupont *et al*. suggested that the autophagosome creates a compartment that brings cathepsin B and the activated inflammasome complex close together, instead of being a mere carrier. The compartmentalization of the activated inflammasome complex together with cathepsin B would allow cathepsin B to cleave proIL-1β and/or possibly enhance inflammasome activation (Dupont *et al*. 2011, Hornung *et al*. 2008). Because cytokines are vital mediators of innate and adaptive immune responses, it is no surprise that cytokines are also reported to modulate autophagy. TNFα and the classical T_H1 cytokine IFNγ have been reported to enhance autophagy, whereas the T_H2 cytokines IL-4 and IL-13 were shown to inhibit the autophagic control of mycobacteria (Harris & Keane 2010, Gutierrez *et al*. 2004, Harris *et al*. 2007). Additionally, IL-1β promotes autophagy, which suggests that IL-1β limits its own processing by controlling inflammasome activity (Harris 2013).

6.2 Crosstalk between autophagy and apoptosis

The association between autophagy and cell death pathways has been known for years. Autophagic structures are commonly observed in dying cells (Kroemer & Levine 2008, Shen & Codogno 2011). Autophagy is activated in stressed cells as a survival mechanism; consequently, autophagic structures are often present in dying cells. Therefore, it was originally suggested that autophagy functions as an independent programmed cell death mechanism. However, other than the Human Immunodeficiency Virus (HIV) envelope glycoprotein-induced cell death of $CD4^+$ T cells that requires the induction of autophagy, but for which the induction of apoptosis is dispensable, there is little evidence that autophagy causes cell death independently of apoptosis or other pathways of programmed cell death in mammals (Espert *et al*. 2006, Shen & Codogno 2011). Although the concept of "autophagic cell death" may exist only for experimentally manipulated cells (Pattingre *et al*. 2005, Thorburn 2008), it has been shown that autophagy and apoptosis share convergence points at which signals derived from one pathway modulate the other. However, autophagy and apoptosis can also function in concert. For example, in apoptotic cells autophagy maintains sufficient energy levels for the generation of signals that promote phagocytosis, thereby assuring the clearance

of apoptotic cells (Qu *et al*. 2007a). Resolving the tangled interactions between autophagy and apoptosis is complicated because both are induced by similar stimuli and both modify each other's functions. The majority of the interactions known to date seem to favor the function of one process over the other.

Under normal growth conditions, Beclin 1, a critical protein in the autophagy initiation complex, is bound to antiapoptotic Bcl-2 family members, such as Bcl-2, which is a prototypical antiapoptotic protein. In fact, Beclin 1 has been shown to be a BH3-only protein, and similar to other BH3-only proteins, Beclin 1 is capable of physically interacting with the BH3 receptor domains of Bcl-2 and with the BH3 receptor domains of other antiapoptotic proteins, such as Bcl-xL and Mcl-1 (Feng *et al*. 2007, Maiuri *et al*. 2007). The interaction between Bcl-2 and Beclin 1 inhibits the activation of autophagy. Therefore, the phosphorylation-induced dissociation of Bcl-2 from Beclin 1 is required for the induction of autophagy. This phosphorylation could be mediated by either JNK1, which phosphorylates Bcl-2, or by death-associated protein kinase (DAPk) 1, which mediates the phosphorylation of Beclin 1 (Wei *et al*. 2008, Zalckvar *et al*. 2009a, Zalckvar *et al*. 2009b). In addition to phosphorylation, Bcl-2 can be displaced from Beclin 1 by HMGB1, which also acts to hamper apoptosis (Tang *et al*. 2010). The translocation of HMGB1 from the nucleus to the cytoplasm does not require the type of severe insult that would lead to necrosis; instead, ROS and other autophagy-inducing stimuli are sufficient (Tang *et al*. 2010). In addition, the binding of the proapoptotic BH3 proteins to Bcl-2 releases Beclin 1 (Maiuri *et al*. 2007). However, despite the increased level of dissociated Beclin 1, the overexpression of the proapoptotic Bax decreased autophagy by inducing the caspase-mediated cleavage of Beclin 1 (Luo & Rubinsztein 2010).

Although Bcl-2 inhibits autophagy by interacting with Beclin 1, the effect of this interaction is not necessarily reciprocal. Several authors have shown that the antiapoptotic effect of Bcl-2 is not affected by its interaction with Beclin 1, which might be due to the weaker physical interactions between Beclin 1 and Bcl-2 or possible counteracting regulatory sequences in Beclin 1 (Feng *et al*. 2007, Ciechomska *et al*. 2009, Wirawan *et al*. 2010). On the contrary, Cho *et al*. reported that modifications of Beclin 1 expression affected apoptosis. The deletion of Beclin 1 using siRNA was shown to lower the threshold for the induction of apoptosis, and consistent with that finding, the overexpression of Beclin 1 reduced apoptosis induced by a recombinant TRAIL (Cho *et al*. 2009). In addition, most of the Atg proteins can be cleaved by caspases and calpains 1 and 2, and the cleaved forms of Beclin 1 and ATG5, another autophagy protein, have thus far been shown to increase apoptosis (Yousefi *et al*. 2006, Norman *et al*. 2010, Wirawan *et al*. 2010). The cleaved forms of ATG5 and Beclin 1 were shown to localize to mitochondria and to induce the release of cytochrome c by binding to Bcl-xL (Yousefi *et al*. 2006, Luo & Rubinsztein 2007, Wirawan *et al*. 2010). ATG5-induced cell death has also been shown to proceed through interactions with FADD. In a study by Pyo *et al*., IFNγ induced autophagic vacuole formation in cells, and the cells subsequently underwent caspase-dependent death via the interaction of ATG5 with FADD (Pyo *et al*. 2005). However, the activation of apoptosis by the cleaved form of ATG5 was

considered to be independent of autophagy (Yousefi *et al*. 2006, Luo & Rubinsztein 2007). Adding complexity to the relationship between autophagy and FADD-dependent cell death results indicate that the inhibition of caspase-8, the apical caspase of extrinsic apoptosis, causes exaggerated autophagy, which results in cell death (Yu *et al*. 2004, Bell *et al*. 2008). Therefore, FADD and caspase-8 were suggested to protect T cells from necroptotic cell death caused by excessive autophagy (Bell *et al*. 2008). Thus, apoptosis has been suggested to also restrict the autophagic response by the caspase-mediated cleavage of autophagic proteins, which often results in the inhibition of autophagy (Cho *et al*. 2009, Norman *et al*. 2010, Wirawan *et al*. 2010). Because mitochondria are key players in the initiation of intrinsic apoptosis and autophagy is a major pathway for mitochondrial turnover, it is conceivable that autophagy raises the threshold for the induction of apoptosis by degrading compromised mitochondria, which could release cytochrome c (Thorburn 2008). However, the mechanisms and consequences of such a functional connection remain to be elucidated.

6.3 Crosstalk between inflammasome activation and apoptosis

The ability of caspase-8 to cleave the proforms of IL-1β and IL-18 and to induce apoptotic cell death suggests a role for caspase-8 as a switch between the inflammatory response and cell death. Accumulating evidence has revealed connections between caspase-8 and both canonical and noncanonical inflammasomes. Noncanonical inflammasomes induce the caspase-8-dependent processing of IL-1β in response to the activation of certain receptors, dectin-1 and FasR, as well as endoplasmic reticulum stress. The endoplasmic reticulum stress-induced secretion of IL-1β was dependent on the priming of the cells via the activation of TLR4, which induced both the transcription of IL-1β and TRIF-dependent signaling, which is needed for caspase-8 activation and subsequent cytokine secretion (Shenderov *et al*. 2014). The cross linkage of the FasR or the formation of a FADD-RIPK1 complex mediated the activation of caspase-8, which was followed by the maturation and secretion of inflammasome-dependent cytokines and cell death or, as occurs in response to the Yersinia protein YopJ, by the subsequent activation of caspase-1 and cell death (Bossaller *et al*. 2012, Philip *et al*. 2014). Furthermore, the activation of dectin-1 by extracellular fungal and mycobacterial antigens induced the assembly of a CARD9-Bcl-10-MALT1 complex (MALT, mucosa-associated lymphoid tissue lymphoma translocation protein), which was required for the transcription of proIL-1β. The further association and activation of the noncanonical MALT1-caspase-8-ASC complex to the above mentioned CARD9-Bcl-10-MALT1 complex mediated the caspase-8-dependent maturation of IL-1β (Gringhuis *et al*. 2012). In addition, Grinhuis *et al*. demonstrated that, unlike the extracellular fungal antigens, the internalized fungal antigens of some *Candida albicans* strains were able to induce canonical NLRP3-dependent IL-1β processing. These results were supported by other studies reporting an absolute requirement for the NLRP3 inflammasome in the secretion of mature IL-1β induced by the fungal cell wall component β-glucan and strains of *C. albicans* (Ganesan *et al*. 2014, Kankkunen *et al*. 2010). Internalized fungal antigens simultaneously activated caspase-1

and caspase-8, both of which contributed to the secretion of IL-1β in response to β-glucans and heat-killed, but not live, *C. albicans* (Ganesan *et al*. 2014). However, the induction of cell death did not require NLRP3 or ASC (Ganesan *et al*. 2014).

The activation of caspase-8 has been strongly associated with canonical inflammasome activation. Recent findings have demonstrated that caspase-8 is capable of enhancing the transcription of proIL-1β and NLRP3, as well as the secretion of mature IL-1β via FADD (Gurung *et al*. 2014). Furthermore, the activation of the AIM2 and NLRP3 inflammasomes, particularly the association of NLRP3 with ASC, induced the processing of caspase-8 independently of caspase-1 (Sagulenko *et al*. 2013, Gurung *et al*. 2014). Additionally, co-operation between caspase-8 and caspase-1 has been suggested. Activated caspase-8 was shown to enhance the posttranslational maturation of IL-1β via the caspase-8-induced activation of caspase-1, which would explain the observed reduction in the secretion of IL-1β and IL-18 in the absence of caspase-8 (Gurung *et al*. 2014). Similarly, *Salmonella* (*enterica* serovar Typhimurium) infection, which induces the activation of the NLRC4 inflammasome, required caspase-8 for the transcription of proIL-1β (Man *et al*. 2013). The activation of the NLRC4 inflammasome was shown to recruit both caspase-1 and caspase-8 to the ASC structure of the NLRC4 inflammasome (Man *et al*. 2013).

Several authors have shown that caspase-8 associates with ASC through the pyrin domain of ASC (Masumoto *et al*. 2003, Pierini *et al*. 2012, Sagulenko *et al*. 2013). The existence of crosstalk between caspase-8 and inflammasomes was further verified by results demonstrating an interaction between caspase-8 and ASC of the AIM2 inflammasome. In the absence of caspase-1, AIM2 inflammasome activation was shown to result in the processing of caspase-8 and subsequent apoptotic cell death (Pierini *et al*. 2012, Sagulenko *et al*. 2013). Sagulenko *et al*. proposed that the continuum between caspase-8-driven apoptosis and caspase-1-induced pyroptosis depends on the strength of the activating signal: weaker inflammasome stimulation induces apoptosis, whereas the initiation of pyroptosis predominates in the presence of a strong stimulus (Sagulenko *et al*. 2013). On the contrary, an inhibitory role for caspase-8 in the activation of the NLRP3 inflammasome has also been reported. Thus, in a study by Kang *et al*., caspase-8 deficiency promoted the secretion of mature IL-1β and IL-18 and increased mortality in response to LPS alone *in vivo* and *in vitro* in dendritic cells (Kang *et al*. 2013). The discrepant results in response to NLRP3 inflammasome activation may, however, be due to the use of a dendritic cell-targeted conditional deletion in the study of Kang *et al*. instead of the *Rip3 casp8* double knockout used in the study by Gurung *et al*., as well as the use of different inflammasome-activating stimuli in the two studies.

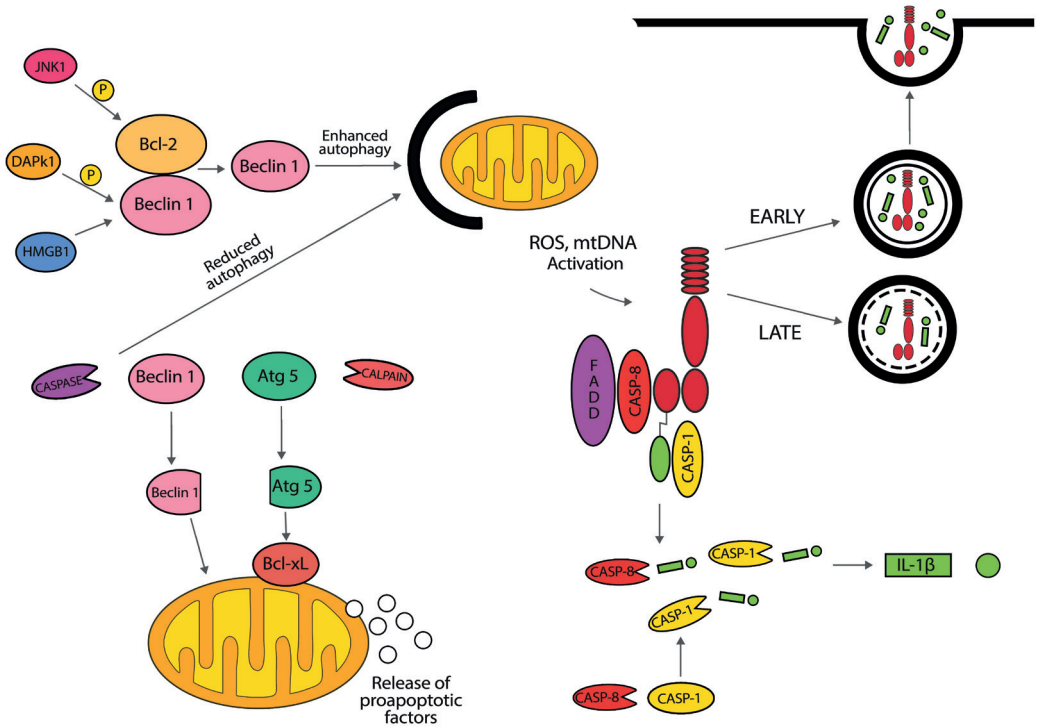

Figure 10. Convergence points between apoptosis, inflammasome activation and autophagy. The main links between apoptosis, inflammasomes and autophagy discussed in Chapters 6.1, 6.2, and 6.3 are illustrated here. Some details are omitted for clarity. Atg5 protein and a complex of Bcl-2 and Beclin 1 act as switches between autophagy and cell death. The antiapoptotic Bcl-2 binds Beclin 1, and the dissociation of Beclin 1 can be mediated by the phosphorylation of Bcl-2 by JNK1 or the phosphorylation of Beclin 1 by DAPk1. Additionally, HMGB1 binds Beclin 1 upon the translocation of HMGB1 to the cytosol, thereby disrupting the interaction between Bcl-2 and Beclin 1. Most autophagic proteins can be cleaved by calpains and caspases, which often results in reduced autophagic activity. The caspase-induced cleavage of Beclin 1 inhibits autophagy but enhances apoptosis by releasing proapoptotic factors from mitochondria. Additionally, the calpain-mediated cleavage of Atg5 results in the formation of a truncated form of Atg5, which associates with Bcl-xL on mitochondria and induces the release of cytochrome c. Autophagy modulates inflammasome activation on several levels. Autophagy suppresses NLRP3 inflammasome activation by removing damaged mitochondria, which are sources of ROS and mtDNA. Autophagy modulates inflammasome activation differentially at early and late time points during autophagy activation. Initially, autophagy-based unconventional secretion enables the secretion of IL-1β (Early); later, inflammasome components are sequestered and degraded in autophagosomes when the induction of autophagy is continued (Late). Diverse interconnections exist between inflammatory signaling and apoptosis. Caspase-8 can be activated during canonical and noncanonical inflammasome activation. Fungal antigens activate caspase-8, which can cleave proIL-1β and promote inflammasome-independent cell death. FADD may function as a platform for the activation of procaspase-8 in the NLRP3 inflammasome. Furthermore, caspase-8 and FADD act as upstream regulators of the NLRP3 inflammasome by promoting the expression of NLRP3 inflammasome components and the activation of the NLRP3 inflammasome. Caspase-8 can also enhance IL-1β processing by activating caspase-1. However, caspase-8 has also been demonstrated to inhibit the secretion of inflammasome-dependent cytokines (not shown in the figure). Atg, autophagy-related protein; Bcl-xL, Bcl-2-like protein; CASP, caspase; DAPk1, death-associated protein kinase 1; FADD, Fas-associated death domain; HMGB1, high-mobility group box 1; JNK1, c-Jun N-terminal kinase; mtDNA, mitochondrial DNA; NLRP3, nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 3; ROS, reactive oxygen species. Data are derived from references mentioned in the text.

III Aims of the Study

The aims of this study were to elucidate the regulation of the innate immune system and to identify novel means of inhibiting innate immune responses that could be applied to the treatment of inflammatory diseases in the future. Another aim was to explore the molecular mechanisms of the immune-suppressive effects of ethanol, particularly whether the effects of ethanol are mediated by its actions on cells of the innate immune system. A final aim was to study the mechanisms that regulate the activation of the NLRP3 inflammasome to discover novel means of inhibiting the exacerbated inflammasome activation observed in the pathogenesis of several chronic inflammatory diseases.

The specific aims of the study were the following:

1. To explore whether the immunosuppressive effects of ethanol exposure are mediated by the downregulation of mast cell functions.

2. To study the mechanisms that modulate the release of proinflammatory cytokines in human macrophages in response to acute ethanol exposure (treatment of the cells with ethanol for 1 h or 3 h prior to and during the activation) and, in particular, to study the effect of ethanol on the function of the NLRP3 inflammasome.

3. To explore the molecular mechanisms that regulate the function of the NLRP3 inflammasome and, in particular, to study whether the endogenous molecule hemin modulates the activation of the NLRP3 inflammasome in macrophages.

IV Materials and Methods

1. Methods used in publications I-III

Table 4 summarizes the main methods used in the present study; more detailed materials and methods are presented in the original publications. The references provided in Table 4 refer to more detailed descriptions of protocols published elsewhere or to manufacturer-provided protocols.

Table 4. Methods used in the present study.

***In vitro* stimulation and inhibition**		**Reference/ Manufacturer**
Isolation, maturation and stimulation of human primary macrophages	II, III	(Nakanishi *et al.* 2009)
Isolation, maturation and stimulation of mouse bone marrow-derived mast cells of wild-type Balb/C mice	I	(Eklund *et al.* 1994)
Isolation, maturation and stimulation of mouse bone marrow-derived, and thioglycollate-elicited peritoneal macrophages of wild-type C57BL and Nrf2- or HO-1- deficient mice	III	(Tzima *et al.* 2009)
Isolation, maturation and stimulation of human cord blood-derived mast cells	I	(Saito *et al.* 1995, Lappalainen *et al.* 2007)
Culture and stimulation of human cell line mast cells (HMC-1)	I	(Butterfield *et al.* 1988)
Culture and stimulation of human cell line macrophages (THP-1)	II,III	(Tsuchiya *et al.* 1980)
Lipofectamin-induced transfection of double-stranded DNA	II, III	Invitrogen
***In vivo* stimulation and inhibition**		
Sterile MSU-induced peritonitis of wild-type C57BL mice	III	
Detection		
[^{3}H] Thymidine incorporation	I	
Terminal transferase mediated nick end labelling (TUNEL) assay	I	ApopTag
Reverse transcriptase polymerase chain reaction	I	
Quantitative real-time reverse transcriptase polymerase chain reaction	II, III	(Heid *et al.* 1996)
Western blotting	II, III	(Burnette 1981)
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	II, III	(Laemmli *et al.* 1970)
BCA assay	II, III	Pierce
Inductively Coupled Plasma-Mass Spectrometry	II, III	(ISO 17294-2:2005 Water quality)
Cathepsin B/L activity measurement	II, III	(Luheshi *et al.* 2012)
Cytotoxicity detection	II, III	Roche
Thin-layer chromatography	II	(Lahdesmaki *et al.* 2009)

Enzyme-linked Immunosorbent Assay (ELISA)		
Human cytokines IL-1β, IL-18, and TNFα	II, III	R&D Systems
Mouse cytokine IL-1β	III	R&D Systems
Caspase-3,-8, or -9 activity assay	I	R&D Systems
Cell death detection	I	Roche
Cell imaging		
Fluorescence imaging of cathepsin B and lysosomes	II	BIOMOL
Immunofluorescent microscopy of ASC	II, III	
Double immunofluorescenct microscopy of ASC and SQSTM1	III	
Generation of CORM-3		
Synthesis of CORM-3 from CORM II	III	(Clark *et al.* 2003)
Genotyping of HO-1-deficient mice		
Reverse transcriptase polymerase chain reaction	III	(Tzima *et al.* 2009)
Crystal preparation		
Preparation of cholesterol crystals	II	(Rajamaki *et al.* 2010)
Preparation of monosodium urate crystals	III	(Scanu *et al.* 2010)
Statistical analysis		
Student´s t-test	I, II, III	
Wilcoxon matched-pairs signed rank test	II	
Mann-Whitney´s U test	III	

V Results and Discussion

1. Ethanol induces apoptosis and reduces inflammatory responses

Alcohol abuse predisposes to infectious diseases, which are more frequent and more severe among alcoholics. In contrast, the moderate use of alcohol has been associated with a reduced risk of coronary heart disease (Rimm *et al*. 1999, Molina *et al*. 2010). The common denominator of increased susceptibility to infections and reduction in cardiovascular diseases could be a reduction in innate immune system function. Mast cells are key players in innate immune responses. They are located in tissues that are in close contact with the external environment, such as mucosal surfaces of the upper alimentary tract, in which they encounter pathogens but may also be exposed to high alcohol concentrations (Irani *et al*. 1986, Galli *et al*. 2011). In addition to the mucosal surfaces of the upper alimentary tract, fairly high (over 8‰) ethanol concentrations have been observed in portal vein blood. Furthermore, among alcoholics peripheral blood ethanol concentrations of 3‰ to 4‰ or even over 7‰ are not uncommon at the time of hospitalization (Slukvin & Jerrells 1995).

1.1 Ethanol reduces human and mouse mast cell viability and proliferation (Study I)

The effect of ethanol on mast cell viability was studied using a human mast cell line (HMC-1) and mouse bone marrow-derived mast cells. HMC-1 is a malignant human mast cell line that is phenotypically similar to human MC_T type mast cells, despite the lack of functional high-affinity FcεRI receptors (Butterfield *et al*. 1988, Nilsson *et al*. 1994). A dose-dependent reduction in the viability of both HMC-1 cells and mouse bone marrow-derived mast cells (mBMMCs) was observed after a 4 day culture in the presence of ethanol. There was a significant reduction in the viability of HMC-1 cells in the presence of 344 mM (20‰) ethanol (Study I, Figure 1A). The daily quantification of the viability of the HMC-1 cells revealed a gradual reduction in the number of viable cells in the presence of ethanol concentrations from 43 mM to 860 mM (2.5‰ to 50‰, respectively) (Study I, Figure 1B). In the presence of the highest ethanol concentration (1720 mM; 100‰) a total loss of viability during the first day of culture was observed. The reduction in viability was independent of growth factors because in HMC-1 cells, survival is mediated by the constitutively active c-kit, and mBMMCs are sustained by IL-3 (Nilsson *et al*. 1994). Consistent with our results, ethanol at concentrations of 60 mM and higher has been shown to reduce the viability of Hep G2 cells in a model of liver damage (Cameron *et al*. 1998).

The reduction in the number of viable mast cells was connected to a reduction in proliferation. The exposure of HMC-1 cells to ethanol at concentrations of 86 mM to 3440 mM (50‰ - 200‰) significantly reduced their proliferation (Study I, Figure 2). Because ethanol is readily metabolized to acetaldehyde, we cultured HMC-1 cells and mBMMCs in the presence of acetaldehyde. Acetaldehyde is a product of ethanol metabolism that exhibits carcinogenic and neurotoxic effects (Koivisto & Salaspuro 1998, Lee *et al*. 2005). Surprisingly, even at the very high concentrations of 500 µM and 1000 µM, which were similar to the concentrations used in the study by Koivisto *et al*., acetaldehyde had no effect on the viability or the proliferation of HMC-1 cells or mBMMCs (Study I, Figures 1C and 2). In contrast to our results, Koivisto *et al*. reported an increase in the proliferation of a human adenocarcinoma cell line cultured in the presence of acetaldehyde for 5 weeks (Koivisto & Salaspuro 1998). However, significantly reduced viability of an intestinal goblet cell line has also been reported at low (25 µM to 100 µM) concentrations of acetaldehyde (Elamin *et al*. 2014). In the latter study, the reduction in viability was attributed to increased ROS production (Elamin *et al*. 2014). The discrepant effects of acetaldehyde may be explained by different mechanisms of proliferation among different cell types. Taken together, the results indicate that ethanol, but not acetaldehyde, dose-dependently reduced mast cell viability and proliferation.

1.2 Ethanol induces apoptosis in mast cells and macrophages (Studies I and II)

To further elucidate the events leading to the significant loss of mast cell viability in the presence of ethanol, we studied the mechanisms of cell death. A significant induction of apoptosis was noted in HMC-1 cells in the presence of ethanol at concentrations of 43 mM (2.5‰) and higher (Study I, Figure 3). In mBMMCs, apoptosis was initially reduced at low ethanol concentrations. However, the numbers of apoptotic nuclei increased at concentrations of 344 mM (20‰) and higher. These findings suggest that mBMMCs may be more susceptible to the cytotoxic effects of ethanol because the attenuation of mBMMC viability was more pronounced at lower ethanol concentrations compared with HMC-1 cells (Study I, Figure 1A). To confirm the induction of apoptosis, the activity of caspase-3, which is the apical caspase of the apoptotic process, was measured. Again, ethanol dose-dependently increased caspase-3 activity in HMC-1 cells (Study I, Figure 5A). This finding was also supported by the modest inhibition of ethanol-induced apoptosis in the presence of a caspase-3 inhibitor (data not shown). The highest caspase-3 activities were measured in the presence of ethanol at concentrations of 344 mM (20‰) and 860 mM (50‰). Caspase-3 activity was attenuated at the highest 1720 mM (100‰) concentration, conditions under which the number of apoptotic nuclei was also decreased. Combined with the observations of dramatic reductions in viability, this result suggests that at high concentrations, the toxic effects of ethanol dominate and cells experience lytic cell death instead of apoptosis. The ability of ethanol to induce apoptosis was further studied in human mast cells (huMCs) derived from peripheral cord blood $CD34^+$ cells, which are the best cellular model of human mast cells available. In huMCs, 860 mM (50‰) ethanol clearly increased the fragmentation of genomic DNA as indicated by TUNEL staining (Study I, Figure 4). Intranucleosomal

fragmentation of DNA and formation of characteristic ladder pattern is considered a definitive proof of apoptosis (Saraste & Pulkki 2000).

To further elucidate the apoptotic mechanism, the activities of caspases-8 and -9, which are activated in the extrinsic and intrinsic apoptosis pathways, respectively, were measured. The enzymatic activities of both caspase-8 and caspase-9 were modestly induced in mast cells exposed to ethanol (Study I, Figure 5B). In the intrinsic apoptosis pathway, proapoptotic Bax promotes the induction of apoptosis and antiapoptotic Bcl-2 inhibits the actions of Bax (Youle & Strasser 2008). We further examined the expression of apoptotic proteins because relationships among these proteins have been suggested to modulate the initiation of apoptosis (Perez-Navarro *et al*. 2005). Ethanol promoted the expression of Bax mRNA but had no effect on the expression of Bcl-2 mRNA, which suggests the promotion of intrinsic apoptosis if the expression of Bax is equally increased on the protein level (Study I, Figure 6). However, because increase in caspase-9 activity was very modest, it could not be definitively concluded that ethanol promotes intrinsic apoptosis. Previous studies have shown that ethanol induces apoptosis in many cell types, such as thymocytes, T and B lymphocytes, primary hepatocytes and hepatocyte cell lines, and cerebellar granule cells (Slukvin & Jerrells 1995, Neuman *et al*. 1999, Heaton *et al*. 2011). In response to ethanol exposure, intrinsic apoptosis was induced in cerebellar granule cells, and ethanol-induced apoptosis could be inhibited by an antioxidant (Heaton *et al*. 2011). In hepatoma HepG2 cells, ethanol has been shown to induce both intrinsic and extrinsic apoptosis (Nakayama *et al*. 2001, Castaneda & Kinne 2001). It was suggested that the dominant apoptosis mechanism is dependent on the ethanol concentration, i.e., low concentrations of ethanol induce extrinsic apoptosis and higher concentrations induce intrinsic apoptosis (Castaneda & Kinne 2001). Regardless, it seems that ethanol is capable of inducing both intrinsic and extrinsic apoptosis in hepatoma cells.

In conclusion, in the present study ethanol, but not its metabolite acetaldehyde, induced the dose-dependent apoptosis of mast cells. Whether intrinsic or extrinsic apoptosis (or both simultaneously) is activated in mast cells in response to ethanol requires further study.

Inflammasome activation by most inflammasome activators results in cell death (Fernandes-Alnemri *et al*. 2007). Depending on the strength of the inflammasome activation, either pyroptosis or apoptosis ensues (Sagulenko *et al*. 2013). Caspase-8-mediated apoptosis accompanies weaker inflammasome stimulation, whereas strong inflammasome activation promotes pyroptosis via caspase-1 activation (Sagulenko *et al*. 2013). Interestingly, we observed that ethanol was able to inhibit the activation of the NLRP3 inflammasome and caspase-1 in macrophages. Because ethanol did not inhibit the activation of caspase-8, these findings suggest that apoptosis, rather than pyroptosis, is induced in macrophages when inflammasome activation is blocked by ethanol (Study II, Figure 6C,D).

2. Ethanol and hemin inhibit the secretion of proinflammatory cytokines (Studies I, II, III, and unpublished data)

IL-1β and TNFα are strong proinflammatory mediators that play key roles in the propagation of immune responses. Because of the potentially harmful and damaging effects of IL-1β, its secretion is subjected to strict regulation at multiple levels (Dinarello 2009). The secretion of mature IL-1β requires two consecutive steps. The first activating signal, called priming, is accomplished via the activation of TLRs or cytokine receptors, which initiate the transcription of IL-1β and NLRP3 (Marucha *et al*. 1990, O'Connor *et al*. 2003, Bauernfeind *et al*. 2009, Gross *et al*. 2011). A second activating signal is required to induce the assembly and the activation of the inflammasome. As a result of inflammasome activation, mature IL-1β and IL-18 are secreted.

2.1 Ethanol inhibits the secretion of IL-1β and IL-18 in macrophages

Ethanol was shown to inhibit the secretion of inflammasome-dependent cytokines IL-1β and IL-18 in primary human macrophages and in a THP-1 monocyte macrophage cell line (Study II, Figures 1A-F and 2C-E). Incubating the cells in the presence of ethanol induced a significant dose-dependent reduction in IL-1β secretion in response to all studied well-known NLRP3 inflammasome activators, i.e., cholesterol crystals, extracellular ATP, SAA, and nigericin, which is a potassium-proton antiporter (Perregaux & Gabel 1994). The inhibitory effect was likely caused by the modulation of inflammasome signaling pathways because ethanol did not induce cell death in nonactivated or activated macrophages, as measured by the release of lactate dehydrogenase (LDH) and trypan blue exclusion (Study II, Figure S3A-C). Furthermore, the inhibitory effect of ethanol was rapid; the simultaneous addition of ethanol with the NLRP3 inflammasome activator also significantly inhibited the secretion of IL-1β (Study II, Figure S2A). In addition, the removal of ethanol after an initial treatment for 3 h reduced, but could not fully reverse, the inhibitory effect (Study II, Figure S2B), which implies that either the ethanol-induced changes in signaling were (at least partially) reversed more slowly or that some cytotoxicity was induced, although the cytotoxicity measurements strongly argue against the latter option.

In macrophages, ethanol is readily metabolized to acetaldehyde, primarily by the ethanol-metabolizing cytochrome P450 but also to a lesser extent by alcohol dehydrogenase (Hutson & Wickramasinghe 1999). However, in the present study the inhibitory effect was shown to be specific to ethanol because exposing THP-1 cells to acetaldehyde at concentrations as high as 1 mM did not inhibit IL-1β secretion (Study II, Figure S4A). In accordance with that result, the inhibition of alcohol dehydrogenase using 4-methylpyrazole did not reverse the inhibitory effect of ethanol in human primary macrophages (Study II, Figure S4B). In

conclusion, these results demonstrate that ethanol, but not acetaldehyde, was responsible for the diminished secretion of IL-1β and IL-18 in response to inflammasome activation.

2.2 Hemin inhibits the secretion of IL-1β and IL-18 in macrophages

Hemin and cobalt protoporphyrin (CoPP), which is a synthetic derivative of heme, were also shown to inhibit the secretion of IL-1β in primary human macrophages, mouse macrophages and THP-1 cells in response to several NLRP3 inflammasome activators, i.e., ATP, SAA, nigericin, and MSU crystals (Study III, Figures 1A-F and 2A,B). Similarly, the inflammasome-dependent secretion of IL-18 was also reduced in THP-1 cells in the presence of CoPP or hemin (Study III, Figure 3C). The reduced secretion of IL-1β and IL-18 could not be attributed to increased cell death because neither CoPP nor hemin exhibited significant cytotoxicity (Study III, Suppl. Figure 1G,H). There was some variation in sensitivity between the different cell types in their response to CoPP and hemin. THP-1 cells were more sensitive than human primary macrophages: THP-1 cells responded to 1-5 μM concentrations of CoPP and to 2-5 μM concentrations of hemin, whereas 20-50 μM concentrations of CoPP and 30-50 μM concentrations of hemin were required for inhibitory effects in human primary macrophages. However, in mouse bone marrow-derived macrophages (mBMMCs), 0.05-0.5 μM concentrations of CoPP were sufficient for an inhibitory effect, whereas a hemin concentration of 50 μM was required. It is noteworthy that hemin and CoPP could inhibit NLRP3 inflammasome activation by several different activators although the mechanisms that mediate the activation are most likely different. However, neither hemin nor CoPP reduced the inflammasome activation induced by cholesterol crystals (Study III unpublished data, data not shown). The lack of inhibition in response to cholesterol crystal activation implies that the mechanism mediating the cholesterol crystal-induced activation differs from the activating mechanisms of ATP, SAA, nigericin and MSU crystals.

2.3 Ethanol and hemin inhibit the activation of the AIM2 inflammasome but not the secretion of TNFα in macrophages

To determine whether the inhibitory effects of ethanol, hemin and CoPP were specific to the NLRP3 inflammasome, their effects on the activation of another ASC-dependent inflammasome, i.e., the AIM2 inflammasome, were studied. The activation of the AIM2 inflammasome can be induced with double-stranded DNA derived from synthetic, viral or bacterial sources (Hornung *et al*. 2009, Rathinam *et al*. 2012). In the present study, AIM2 inflammasome activation was induced by the transfection of synthetic double-stranded DNA. The exposure of THP-1 cells to ethanol induced a dose-dependent inhibition of IL-1β secretion in response to stimulation with double-stranded DNA while having no significant effect on *AIM2* expression (Study II, Figure 6A,B). The incubation of THP-1 cells in the presence of CoPP completely blocked the AIM2-induced secretion of IL-1β. However, CoPP also significantly inhibited the expression of *AIM2* (Study III, Figure 2E,F).

Generally, priming is not considered necessary for the activation of the AIM2 inflammasome (Bauernfeind *et al*. 2011). Nonetheless it is conceivable that the reduced expression of AIM2 contributed to the inhibitory effect of CoPP. In conclusion, these results demonstrate that, in addition to NLRP3 inflammasome activation, the activation of the AIM2 inflammasome and the subsequent secretion of IL-1β were also inhibited by ethanol and CoPP. Therefore, it is conceivable that their inhibitory effects may be more general and apply to all ASC-dependent inflammasomes. However, the possible inhibition of other inflammasomes and the mechanism of AIM2 inflammasome inhibition remain to be elucidated.

Both IL-1β and IL-18 are synthesized as proforms, which require caspase-1-mediated cleavage for their maturation and secretion (Dinarello 1998). To demonstrate that ethanol, hemin and CoPP specifically inhibited the inflammasome rather than merely inhibiting cytokine secretion in general, their effects on the TLR-induced secretion of TNFα were studied. TLR activation was induced either by its most widely used ligand, LPS, or by SAA, which activates both TLR2 and TLR4 (Niemi *et al*. 2011). Ethanol did not inhibit the secretion of TNFα in response to SAA activation (Study II unpublished data, data not shown). Furthermore, neither CoPP nor hemin had significant effects on the secretion of TNFα (Study III unpublished data, data not shown). These results confirmed that ethanol, CoPP and hemin did not induce general defects in macrophage responsiveness or the secretion of proinflammatory cytokines.

2.4 Ethanol inhibits the secretion of TNFα in activated mast cells

Ethanol consumption modulates the immune defense system and the ability of immune cells to activate and secrete proinflammatory cytokines by various mechanisms (see Chapter 2.1.1 in Review of the literature). Previously, Toivari *et al*. demonstrated that the degranulation and secretion of TNFα and IL-8 were blocked in response to ethanol exposure in mast cells (Toivari *et al*. 2000). A steep dose-dependent inhibition of TNFα secretion was observed in HMC-1 cells first challenged with ethanol and then activated with a combination of phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (Figure 11). In the first study, we showed that ethanol induced apoptosis in mast cells. Therefore, it was conceivable that the reduced secretion of TNFα was at least partially dependent on the initiation of apoptosis. However, IgE-activation has been shown to render mast cells less responsive to apoptosis (Xiang *et al*. 2006). In the present experiment, considerable cell death was observed in activated mast cells with ethanol at concentrations of 344 mM (20‰) or higher. However, the secretion of TNFα was reduced already in the presence of 43 mM and 86 mM (2.5‰ and 5‰) ethanol. In RA, mast cells in the inflamed tissue are mostly activated. Theoretically, ethanol could reduce the inflammatory effects of mast cells in the synovium both by inducing apoptosis and by inhibiting the secretion of proinflammatory cytokines, which would explain the observed protective effect of moderate alcohol consumption against the development of RA (Jin *et al*. 2014).

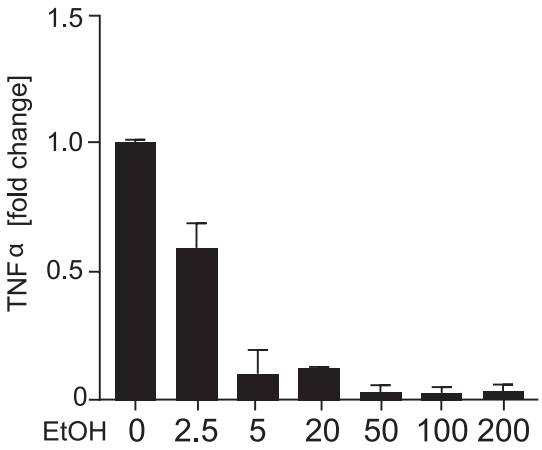

Figure 11. Ethanol dose-dependently inhibits the secretion of TNFα in activated mast cells. HMC-1 cells were incubated in the presence of ethanol at the indicated concentrations (‰) for 1 h and were then activated using PMA and the calcium ionophore A23187 for 20 h in the presence of ethanol. The secretion of TNFα was determined by ELISA and is expressed as fold changes compared to control cells. EtOH, ethanol; TNFα, tumor necrosis factor α. The data represent the means ±SEM of 2 independent experiments.

3. Ethanol and hemin do not disrupt NLRP3 inflammasome priming (Studies II and III)

Priming is an absolute requirement for the activation of the NLRP3 inflammasome because *IL1B* and *NLRP3* are not constitutively expressed. The expression of *IL1B* and *NLRP3* is commonly induced by the activation of TLRs or cytokine receptors, which signal at least partly via the activation of NFκB (Marucha *et al*. 1990, O'Connor *et al*. 2003, Bauernfeind *et al*. 2009, Franchi *et al*. 2009, Gross *et al*. 2011). In addition to priming, a separate second signal is needed for the activation of the inflammasome. However, some inflammasome activators are capable of inducing both priming and inflammasome-activating signals, in which case a separate priming step is not needed. Bacteria are able to induce both priming and the activation of the inflammasome because bacterial components activate TLRs and bacterial toxins activate the NLRP3 inflammasome (Harder *et al*. 2009). In addition, SAA activates TLR2 and TLR4, and it can also provide the second activating signal (Niemi *et al*. 2011).

Several studies have shown that ethanol inhibits TLR signaling (see Chapter 2.1.1 in Review of the literature). However, differences in these studies' experimental settings and duration of ethanol exposure complicate the interpretation of the results. In addition to the inhibitory effects of ethanol, it has been demonstrated that ethanol stimulates TLR-induced signaling and expression of the IL-1β. The addition of ethanol simultaneously with the combined activation of the TLR2 and TLR4 receptors increased the activation of NFκB, the phosphorylation of Jnk, and the nuclear binding of activator protein 1 (Oak *et al*. 2006). In addition, short-term ethanol intake has been shown to increase the expression

of *Il1b* and other cytokines *in vivo* via TLR4 signaling (Pascual *et al*. 2011). In the current study, human primary macrophages were first activated with LPS and then challenged with ethanol. Under these experimental conditions, ethanol did not reduce the expression of *IL1B*, and the expression of *NLRP3* was slightly augmented (Study II, Figure 2A,B). THP-1 cells constitutively express another IL-1 cytokine, IL-18, as a proform that must be cleaved by activated caspase-1 in the inflammasome (Lin *et al*. 2000). The ability of ethanol to inhibit the secretion of IL-18 in addition to the secretion of IL-1β indicates that the inhibitory effect of ethanol did not depend on priming and cytokine expression; instead, ethanol specifically inhibited the second inflammasome-activating signal (Study II, Figure 1E,F).

Similar to ethanol, CoPP and hemin did not reduce the expression of *IL1B* or *NLRP3* (Study III, Figure 3A,B). Instead, they significantly increased the expression of *IL1B*. This finding is in accordance with the results of Dutra *et al*., who demonstrated that heme did not reduce the expression of the proIL-1β protein in mouse macrophages (Dutra *et al*. 2014). In contrast to our results and the results of Dutra *et al*., hemin was shown to reduce the expression of inflammasome components *in vitro* in epithelial cells in response to LPS, and *in vivo* both in hepatic tissue in response to $_{D}$-Galactosamine and LPS and in lung tissue in response to cecal ligation and puncture (Li *et al*. 2014a, Kim & Lee 2013, Luo *et al*. 2014). In our studies, CoPP and hemin also inhibited the nigericin-induced secretion of IL-18 in THP-1 cells, implying that their inhibitory effects were independent of priming (Study III, Figure 3C). The discrepant effects of hemin on the priming step may have resulted from diverse experimental conditions. Taken together, our results suggest that the inhibition of IL-1β secretion by ethanol, hemin, and CoPP was not caused by the reduced expression of inflammasome components.

4. Effects of ethanol and hemin on signaling events that induce inflammasome activation (Studies II and III)

The NLRP3 inflammasome is activated by a wide variety of chemically and structurally diverse activators. Therefore, it has been suggested that all of these activators could not be direct ligands for the NLRP3 receptor (Latz *et al*. 2013). Many mechanisms have been suggested to trigger the activation of the NLRP3 inflammasome. The mechanisms most frequently associated with the activation of the inflammasome are 1) potassium efflux (Mariathasan *et al*. 2006, Petrilli *et al*. 2007), 2) mitochondrial ROS production (Zhou *et al*. 2011), and 3) lysosomal destabilization (Hornung *et al*. 2008).

4.1 Effects of ethanol and hemin on potassium efflux

The induction of potassium efflux is among the most frequently proposed mechanisms of NLRP3 inflammasome activation. Of the activators used in the present study, ATP, nigericin,

and cholesterol crystals have been demonstrated to induce potassium efflux (Mariathasan *et al*. 2006, Petrilli *et al*. 2007, Rajamaki *et al*. 2010). To explore the effects of ethanol and CoPP on potassium efflux, we directly measured the potassium concentrations of cell lysates using Inductively Coupled Plasma –Mass Spectrometry (ICP-MS). Neither ethanol nor CoPP alone induced changes in the intracellular potassium concentration in THP-1 cells, nor did they inhibit the potassium efflux induced by nigericin (Study II, Figure 4B, Study III, Figure 3D). Nigericin-induced potassium efflux has not been shown to induce the activation of $P2X_7$, which is thought to contribute to the potassium efflux induced by ATP and the subsequent inflammasome activation (Pelegrin & Surprenant 2006). Because ethanol, hemin, and CoPP inhibited both the ATP- and nigericin-induced secretion of IL-1β in human and mouse macrophages, it is conceivable that their inhibitory effect was not mediated by the inhibition of $P2X_7$. These data suggest that ethanol and CoPP target inflammasome-activating signaling pathways downstream of the $P2X_7$ receptor and potassium efflux.

4.2 Effects of ethanol on ROS

The production of ROS is another general mechanism that is hypothesized to mediate the activation of the NLRP3 inflammasome. Ethanol exposure increases the production of ROS primarily via its metabolism to acetaldehyde. All the pathways of ethanol metabolism produce oxygen radicals, such as the hydroxyethyl radicals generated via cytochrome P450 (Hutson & Wickramasinghe 1999, Das & Vasudevan 2007, Szuster-Ciesielska *et al*. 2009). Moreover, the mitochondrial dysfunction and damage caused by the ROS formed during ethanol metabolism has been associated with ethanol-induced cytotoxicity and apoptosis (Das & Vasudevan 2007). Interestingly, although increased mitochondrial ROS induce the activation of the NLRP3 inflammasome, the overproduction of ROS has been shown to oxidize and gluthathionylate caspase-1, which in turn inhibits caspase-1 activity (Meissner *et al*. 2008). To assess the role of ROS in the ethanol-induced inhibition of inflammasomes, we inhibited ROS production using NAC, a general ROS scavenger. NAC has been shown to not only inhibit NLRP3 inflammasome activation but also reduce LPS-induced priming, i.e., the expression of IL-1β (Bauernfeind *et al*. 2011). Consistent with our expectations, NAC potently inhibited ATP-induced inflammasome activation (Study II, Figure S6). However, NAC did not inhibit IL-1β secretion in THP-1 cells activated with SAA (Study II, Figure 4A), which led us to assume that NAC was able to scavenge ROS but could not inhibit SAA-induced inflammasome activation. The inhibitory effect of ethanol was not reversed or modulated in the presence of NAC in either ATP or SAA activated cells, which led to the conclusion that the possible induction of ROS by ethanol did not contribute to the inhibitory effect of ethanol in THP-1 cells. In the present study, the effects of hemin or CoPP on ROS production were not studied. In the future, it would be interesting to determine whether hemin and CoPP reduce ROS via the antioxidant effects of HO-1. It is also conceivable that hemin and CoPP induce the generation of mitochondrial ROS: Dutra *et al*. recently reported that heme increased mitochondrial ROS production (Dutra *et al*. 2014). Future studies should address whether the increased ROS production would in fact inhibit caspase-1 activity by

oxidizing and gluthathionylating caspase-1, thereby contributing to the inhibitory effect of hemin (Meissner *et al*. 2008).

4.3 Effects of ethanol and hemin on lysosomal disruption and the release of cathepsin B

The phagocytosis of particulate material, such as silica and cholesterol crystals, is required for the activation of the NLRP3 inflammasome (Hornung *et al*. 2008, Duewell *et al*. 2010, Rajamaki *et al*. 2010). The ingestion of particles elicits lysosomal swelling and disruption, which results in the leakage of cathepsin B to the cytoplasm and the subsequent activation of the inflammasome (Hornung *et al*. 2008, Duewell *et al*. 2010, Rajamaki *et al*. 2010). To assess the effects of ethanol on lysosomal integrity, cells were stained with acridine orange, which becomes concentrated in acidic compartments. The treatment of THP-1 cells with cholesterol crystals reduced the number of lysosomes and induced the formation of large, swollen lysosomes compared to the control cells (Study II, Figure 3A, and Figure 12). However, the loss and swelling of lysosomes was inhibited by incubating the cells in the presence of 171 mM (10‰) ethanol (Study II, Figure 3A, and Figure 12). Consistent with these results indicating that ethanol reduced lysosomal swelling, ethanol also diminished lysosomal rupture, as reflected by the decreased leakage of cathepsin B from lysosomes (Study II, Figure 3A, and Figure 12). The reduction in lysosomal disruption was not due to the diminished phagocytosis of cholesterol crystals, as indicated by cholesterol esterification, which was not altered by ethanol (Study II, Figure S5).

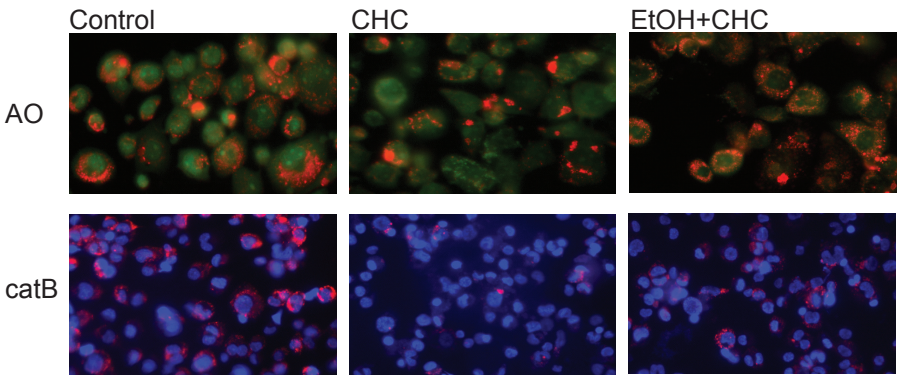

Figure 12. Ethanol inhibits lysosomal disruption and the release of cathepsin B. THP-1 cells were incubated in the presence of 171 mM ethanol (10‰) for 1 h prior to and during the cholesterol crystal-induced activation of the NLRP3 inflammasome. Live cells were stained with acridine orange (AO), and a change in color from green to red/orange was used to indicate the acidic pH of lysosomes. The fluorescently labeled cathepsin B substrate z-Arg-Arg-cresyl violet (catB; red) was used to monitor the cholesterol crystal-induced lysosomal swelling and the disruption and leakage of lysosomal contents. CHC, cholesterol crystals; EtOH, ethanol.

In addition to cholesterol crystals, a noncrystalline inflammasome activator, nigericin, has been shown to activate the NLRP3 inflammasome via the release of cathepsin B from

seemingly intact lysosomes (Hentze *et al*. 2003). We further studied the nigericin-induced release of cathepsin B to the cell culture medium by measuring the cleavage of Z-Phe-Arg-AMC, which is a substrate for both cathepsin B and cathepsin L, which closely resembles cathepsin B. Most studies reporting cathepsin B as a mediator of NLRP3 inflammasome activation have used a cathepsin B inhibitor that inhibits both cathepsin B and cathepsin L (Montaser *et al*. 2002). Therefore, the possibility that cathepsin L contributes to the activation of the NLRP3 inflammasome cannot be excluded. The cleavage of the cathepsin B/L substrate was found to be significantly reduced in cells incubated in the presence of 43 mM or 86 mM ethanol (2.5‰ and 5‰, respectively) (Study II, Figure 3B). Furthermore, CoPP, the other potent inflammasome inhibitor studied, was also shown to reduce the cleavage of the cathepsin B/L substrate very efficiently (Study III, Figure 3E,F). In conclusion, the results indicate that ethanol reduced the inflammasome activation induced by cholesterol crystals and nigericin at least partially by inhibiting the release of cathepsin B from lysosomes and that the inhibitory effect of CoPP may also be partially mediated by the diminished release of cathepsin B/L.

Cathepsin B has been shown to mediate NLRP3 inflammasome activation. However, the exact location of inflammasome activation or the possible cathepsin B-mediated cleavage and activation of caspase-1 (Vancompernolle *et al*. 1998) is not known. According to the current hypothesis, cathepsin B is released to the cytoplasm, where it activates the NLRP3 inflammasome (Vancompernolle *et al*. 1998, Henze & Wolfram 1988, Hornung *et al*. 2008). However, it was recently shown that autophagy contributes to the secretion of IL-1β (Dupont *et al*. 2011). Therefore, it has been suggested that inflammasomes are sequestered to autophagosomes, which offer a compartment for inflammasome activation (Dupont *et al*. 2011). The subsequent fusion of autophagosomes with lysosomes brings lysosomal proteases, such as cathepsin B in close contact with the NLRP3 inflammasome, which would allow cathepsin B to activate the inflammasome and/or cleave caspase-1 (Dupont *et al*. 2011, Andrei *et al*. 2004). The theory that inflammasomes are captured in autophagosomes and IL-1β is secreted from the autophagosomal compartment is further supported by data demonstrating the simultaneous release of inflammasome components and cathepsins upon inflammasome activation (Dupont *et al*. 2011, Andrei *et al*. 2004). In addition, the lysosomal release of cathepsins from mouse macrophages has also been reported to occur solely in response to the ATP-induced activation of $P2X_7$, in the absence of inflammasome priming or the processing of IL-1β, (Lopez-Castejon *et al*. 2010). This finding does not exclude the suggested role of autophagy in the secretion of cathepsins and inflammasome components, but it may reflect the ability of ATP to induce autophagy. Thus, the diminished secretion of active cathepsin B/L in the presence of ethanol and CoPP may not be primarily due to a reduction in inflammasome-activating signaling. Alternatively, the reduced release of active cathepsin B/L potentially implies a decrease in the secretion of IL-1β and inflammasome components from autophagosomes.

5. Ethanol and hemin inhibit inflammasome assembly and activation (Studies II and III)

Activating stimuli induce subsequent intermediate signaling that triggers the assembly of the NLRP3 inflammasome. ASC is an obligatory adaptor of the NLRP3 inflammasome, which is needed for the formation of the functional NLRP3 inflammasome complex. ASC mediates the assembly of the inflammasome via homotypic interactions. ASC itself is recruited to NLRP3 via PYD-PYD interactions, and CARD-CARD interactions mediate the recruitment of caspase-1 to ASC (Mariathasan *et al*. 2006, Mariathasan & Monack 2007). Upon activation, inflammasome components are localized to the perinuclear area, where the inflammasome complex is assembled (Zhou *et al*. 2011). In the perinuclear area, the formed inflammasome complex colocalizes with the endoplasmic reticulum and mitochondria (Zhou *et al*. 2011). The oligomerization of inflammasome components is a self-propagating process that typically results in the formation of one multiprotein complex per cell (Lu *et al*. 2014). The formed inflammasome complexes are large and can be easily visualized as specks by staining the inflammasome proteins (Masumoto *et al*. 1999, Bauernfeind *et al*. 2009).

5.1 Ethanol inhibits the assembly of the NLRP3 inflammasome

We took advantage of the ability of ASC to form visible specks to determine whether ethanol-induced inhibition was dependent on reduced inflammasome assembly. In THP-1 cells, nigericin elicited robust ASC speck formation, which was visualized by staining ASC with a monoclonal antibody. The incubation of the THP-1 cells in the presence of 171 mM (10‰) ethanol substantially reduced the speck formation, and the diffuse cytosolic staining of ASC, comparable to control cells, remained in the presence of ethanol and nigericin (Study II, Figure 5C,D). Previously, it was demonstrated that, in unstimulated macrophages, ASC predominantly resides in the cytoplasm, mitochondria and nucleus, whereas NLRP3 localizes to the endoplasmic reticulum (Zhou *et al*. 2011, Misawa *et al*. 2013). Upon inflammasome activation, ASC and the attached mitochondria are transported along microtubules to the perinuclear area, where NLRP3 is present on the endoplasmic reticulum (Zhou *et al*. 2011, Misawa *et al*. 2013). Inflammasome activation is associated with the robust secretion of IL-1β. However, other inflammasome components, such as ASC, are also secreted simultaneously with IL-1β (Martinon *et al*. 2002, Baroja-Mazo *et al*. 2014). In fact, there have been reports of the release of whole NLRP3 inflammasome complexes, which can induce caspase-1 activation in the extracellular space and in neighboring macrophages upon its phagocytosis (Baroja-Mazo *et al*. 2014, Franklin *et al*. 2014). Indeed, the activation of the NLRP3 inflammasome with nigericin induced the secretion of ASC, which was blocked by exposing the cells to 171 mM (10‰) ethanol prior to the induction of inflammasome activation (Study II, Figure 5A,B). Furthermore, the activation of the NLRP3

and AIM2 inflammasomes has been demonstrated to induce the recruitment of caspase-8 to ASC via the PYD domain of ASC (Sagulenko *et al*. 2013), and this noncanonical caspase-8-mediated cleavage of IL-1β was also induced by the activation of the dectin-1 (Gringhuis *et al*. 2012). In the present study, ethanol was shown to specifically inhibit the activation of caspase-1 because no reduction in SAA-induced caspase-8 activation was observed in the presence of ethanol (Study II, Figure 6C,D). The results indicate that ethanol was capable of disrupting the CARD-mediated recruitment and activation of caspase-1 but not the PYD-mediated recruitment and activation of caspase-8. Overall, these results indicate that ethanol significantly reduced the nigericin-induced assembly of the NLRP3 inflammasome in THP-1 cells.

5.2 Hemin inhibits the assembly of the NLRP3 inflammasome

The finding that hemin and CoPP readily inhibited the secretion of IL-1β prompted us to study their effects on the assembly of the inflammasome. A nearly complete inhibition of ASC speck formation in response to CoPP or hemin treatment was observed in THP-1 cells activated with nigericin (Study III, Figure 5A,B). Surprisingly, diffuse cytoplasmic ASC staining was no longer detected in cells treated with CoPP or hemin prior to inflammasome activation. The results of immunofluorescence staining were confirmed by Western blots, which demonstrated reductions in ASC in cell lysates in the presence of CoPP or hemin, in both nonactivated and nigericin-activated cells (Study III, Figure 5C,D). To elucidate the mechanism mediating the dramatic reduction in intracellular ASC in response to hemin or CoPP treatment of the cells, we first assessed whether hemin or CoPP induced the secretion of ASC. However, neither hemin nor CoPP alone induced the secretion of ASC, and treating THP-1 cells with hemin or CoPP prior to their activation with nigericin actually diminished the secretion of ASC compared to the cells activated with nigericin (Study III, Figure 5E,F). Furthermore, ASC mRNA was shown to be expressed at a low basal level in THP-1 cells, and ASC expression was not induced in response to inflammasome activation. Importantly, CoPP did not reduce the expression of ASC (Study III, Suppl. Figure 3C). In conclusion, we infer that hemin and CoPP reduced the level of intracellular ASC, which led to diminished assembly of the NLRP3 inflammasome.

5.3 Ethanol and hemin inhibit the activation of the NLRP3 inflammasome

In the present study, we have shown that ethanol, hemin and CoPP reduce the secretion of mature IL-1β in response to many substances that activate the NLRP3 inflammasome via distinct mechanisms. The activation of caspase-1 is a hallmark of inflammasome activation and is required for the processing of proIL-1β into its mature, secreted form (Dinarello 1998, Davis *et al*. 2011). The incubation of THP-1 cells in the presence of 171 mM (10‰) ethanol reduced the SAA- and nigericin-induced cleavage of procaspase-1 to active caspase-1 (Study II, Figure 2F,G). Similarly the incubation of THP-1 cells in the presence of hemin and CoPP was shown to reduce the caspase-1 activation induced by nigericin or SAA (Study

III, Figure 2C,D). The observed inhibition of caspase-1 activation in the presence of ethanol, hemin and CoPP was in accordance with the results demonstrating the diminished secretion of IL-1β in response to several well-defined NLRP3 inflammasome activators.

Furthermore, in a model of NLRP3-dependent peritonitis, the injection of CoPP into the peritoneal cavity of wild-type mice prior to the administration of MSU crystals significantly reduced the secretion of IL-1β into the peritoneal cavity (Study III, Figure 2G). In addition, a slight, statistically insignificant reduction in leukocyte recruitment was observed in mice that received CoPP prior to the induction of peritonitis (Study III, Figure 2H). The reason for the modest inhibition of leukocyte recruitment could be related to the possible secretion of other proinflammatory mediators that are not inhibited by CoPP and may thus contribute to leukocyte recruitment. In conclusion, CoPP was shown to significantly inhibit the secretion of IL-1β in response to NLRP3 inflammasome activation *in vivo*.

Only a few studies have addressed the effects of ethanol on inflammasome activation. It seems that the responses of different cell types vary. Ethanol was shown to induce neuroinflammation and to inhibit neurogenesis in cultured slices of hippocampal-entorhinal cortex by inducing the activation of the NLRP1 and NLRP3 inflammasomes (Zou & Crews 2012). Zou *et al*. demonstrated that brain slices cultured in the presence of ethanol (100 mM) for 4 days exhibited increased staining of IL-1β, NLRP1 and NLRP3. Furthermore, the proinflammatory effects of ethanol could be reversed by an IL-1β receptor antagonist and neutralization of IL-1β (Zou & Crews 2012). In accordance with that finding, an *in vivo* study assessing the effects of a 5-week 5% ethanol diet reported increased expression of the inflammasome components NLRP1, NLRP3, and ASC, as well as enhanced caspase-1 activity and IL-1β secretion, confirming the inflammasome activation in the mouse cerebellum (Lippai *et al*. 2013). Furthermore, a recent study in a rat hepatocyte cell line reported that 24-48 h ethanol (250 mM) exposure induced the production of ROS and expression of NLRP3, ASC, and caspase-1 proteins, along with the secretion of mature IL-1β and other proinflammatory cytokines, and apoptosis (Xiao *et al*. 2015). Moreover, these proinflammatory responses to ethanol were reversed by a multitarget iron chelator (Xiao *et al*. 2015). In the above-mentioned studies, ethanol was reported to increase inflammasome activation, which is in contrast to the results of the present study. These contradictory results may be due to the significantly longer exposures to ethanol used in the studies reporting inflammasome activation.

Heme and hemin have been reported to both induce and inhibit NLRP3 inflammasome activation. In a recent study, Dutra *et al*. showed that heme, but not the other protoporphyrins, including CoPP, induced NLRP3 inflammasome activation in cells cultured in the presence of serum. However, in the absence of serum, heme had only a very modest activating effect (Dutra *et al*. 2014). Heme-induced inflammasome activation was mediated by the activation of Syk and was dependent on potassium efflux and the NADPH oxidase-dependent generation of ROS and mitochondrial ROS (Dutra *et al*. 2014). Heme-induced

inflammasome activation could be inhibited by NAC, the NADPH oxidase inhibitor apocynin, the deletion of the NADPH oxidase subunit *gp91phox*, and the pharmacological inhibition of mitochondrial ROS (Dutra *et al*. 2014). Furthermore, the harmfull effects of heme were demonstrated by the administration of heme to wild-type mice, which was not lethal, but induced organ damage (Larsen *et al*. 2010). However, heme exacerbated the production of free radicals in combination with TNFα but not alone (Larsen *et al*. 2010). In accordance with the results of Dutra *et al*., it was concluded that the increased ROS generation and cytotoxicity induced by heme were confined to the iron atom of heme (Dutra *et al*. 2014, Larsen *et al*. 2010). Because both elevated levels of free heme and TNFα can enhance inflammation, their combined cytotoxic actions potentially contribute to the pathogenesis of inflammatory diseases (Gozzelino *et al*. 2010).

Nevertheless, the proinflammatory effects of heme can be inhibited by the induction of HO-1 expression. HO-1 has well-acknowledged cytoprotective, anti-oxidant, and anti-inflammatory effects. HO-1-deficient mice have been shown to exhibit increased mortality in response to cecal ligation and puncture (Larsen *et al*. 2010). Furthermore, hemin has been reported to dampen the NLRP3 inflammasome activation induced by LPS in epithelial cells, by $_{D}$-Galactosamine and LPS *in vivo* in hepatic tissue, and by cecal ligation and puncture *in vivo* in lung tissue (Li *et al*. 2014a, Kim & Lee 2013, Luo *et al*. 2014). However, the three above-mentioned studies, which reported anti-inflammatory effects of hemin, also revealed reductions in the expression of inflammasome components, which may have also contributed to the observed inhibition. Nonetheless, Kim *et al*. and Luo *et al*. demonstrated that hemin reduced ROS generation, which contributed to the diminished assembly of the NLRP3 inflammasome reported by all three studies (Kim & Lee 2013, Luo *et al*. 2014, Li *et al*. 2014a). Diminished inflammasome activation was suggested to derive from the ability of hemin to induce HO-1. The most important difference between the study of Dutra *et al*. compared to the studies of Li *et al*., Kim *et al*., Luo *et al*. (Dutra *et al*. 2014, Li *et al*. 2014a, Kim & Lee 2013, Luo *et al*. 2014), and the present study is that in the latter three studies and in the present study, the effects of hemin were examined under proinflammatory conditions. In addition, the presence of serum may be critical for the proinflammatory actions of heme. However, the mechanisms by which serum modulates cellular responses to heme have yet to be elucidated.

6. Mechanisms of the hemin-induced inhibition of inflammasomes (Study III and unpublished data)

Heme/hemin is a potentially cytotoxic and proinflammatory compound due to its pro-oxidant properties (Balla *et al.* 1991, Kumar & Bandyopadhyay 2005). However, heme is also a prominent inducer of HO-1 (Abraham & Kappas 2008). The anti-inflammatory effects of HO-1 are generally attributed not only to heme catabolism, which reduces the level of free heme, but also to the

degradation products of heme: CO, biliverdin, and bilirubin (Constantin *et al*. 2012, Kapitulnik 2004, Gozzelino *et al*. 2010). Autophagy is a vesicular degradation system, and similar to HO-1, autophagy is activated in response to cellular stress in order to maintain homeostasis (Waltz *et al*. 2011, Parzych & Klionsky 2014). The multifaceted and converging signaling networks that mediate autophagy and inflammasome functions are not well understood (Deretic *et al*. 2012).

6.1 Inhibition of the NLRP3 inflammasome by hemin is partially dependent on HO-1 enzymatic activity

HO-1 is readily induced by hemin and CoPP (Abraham & Kappas 2008). To examine whether HO-1 mediates the inhibitory effects of hemin and CoPP, we studied the induction of HO-1 protein expression. In THP-1 cells, both CoPP and hemin significantly induced the expression of HO-1 at the 4 h time point (Study III, Figure 4A, Suppl. Figure 2A), and HO-1 protein expression was further increased at later time points. The possible involvement of HO-1 in the inhibitory effects of hemin and CoPP was further studied by inhibiting the enzymatic activity of HO-1 using its competitive inhibitor, tin mesoporphyrin (SnMP) (Abraham & Kappas 2008). SnMP partially reversed the inhibitory effect of CoPP on the nigericin-induced secretion of IL-1β in THP-1 cells (Study III, Figure 4B). However, SnMP could not increase IL-1β secretion when combined with nigericin to test whether the nigericin-induced IL-1β secretion would be limited by the possible induction of HO-1 expression during inflammasome activation. Thus, the lack of additional IL-1β secretion may imply that the nigericin-induced secretion of IL-1β was already maximal or that no HO-1 expression was induced during inflammasome activation.

CO and bilirubin, the degradation products of heme/hemin, have been shown to be cytoprotective. The effects of HO-1 and the degradation products of heme are similar, and they have been suggested to form positive feedback loops by which they increase each other's expression (Gozzelino *et al*. 2010). However, Brouard *et al*. demonstrated that the cytoprotective effect induced by HO-1 overexpression required its enzymatic activity because the antiapoptotic effect of HO-1 was abrogated by scavenging CO with hemoglobin (Brouard *et al*. 2000). In the present study, the water-soluble CO-generating molecule CORM-3 dose-dependently inhibited the secretion of IL-1β in human primary macrophages induced by ATP activation (Study III, Figure 4C). In accordance with our results, exposing macrophages to CO inhibited the LPS-induced secretion of IL-1β, TNFα, and macrophage inflammatory protein-1β, and it promoted the secretion of IL-10 (Otterbein *et al*. 2000). Furthermore, the inhalation of CO reduced the LPS-induced production of TNFα and increased the levels of IL-10 *in vivo* (Otterbein *et al*. 2000).

Another heme degradation product, biliverdin, which is rapidly further converted to bilirubin by biliverdin reductase activity, also possesses anti-inflammatory properties. Biliverdin

and bilirubin have been demonstrated to ameliorate LPS-induced lung inflammation and to reduce mortality in mice (Sarady-Andrews *et al*. 2005). In addition to reducing inflammatory responses, biliverdin promoted anti-inflammatory responses by increasing the expression of IL-10 (Sarady-Andrews *et al*. 2005). Similar results were obtained in a model of cecal ligation and puncture, in which biliverdin reduced neutrophil recruitment and the mRNA expression of the proinflammatory mediators IL-6 and monocyte chemoattractant protein-1, but promoted the expression of IL-10 (Overhaus *et al*. 2006). We added bilirubin to THP-1 cell cultures and subsequently activated the NLRP3 inflammasome with ATP. However, under our experimental conditions, only a very modest inhibition of IL-1β was observed (Study III, Suppl. Figure 2B). These contradictory results imply that the effects of bilirubin may be specific to certain models or cell types. Taken together, these results of the present study imply that the hemin- and CoPP-induced inhibition of NLRP3 inflammasome activation may be mediated by HO-1.

6.2 Inhibition of the NLRP3 inflammasome by hemin is partially attributed to Nrf2 and HO-1

The cellular level of HO-1 is regulated principally via the transcription of *HMOX1*, which can be induced by several signaling routes. Thus, several transcription factors, including Nrf2, NFκB, and AP-1 and the transcriptional repressor Bach1, regulate the expression of *HMOX1* (Paine *et al*. 2010). Oxidative stress activates the transcription factor Nrf2 (Paine *et al*. 2010). However, considering the pro-oxidant nature of the hemin and ROS generated during inflammation, it is conceivable that the primary route of HO-1 expression in response to heme and NLRP3 inflammasome activation proceeds via the induction of Nrf2 (Gozzelino *et al*. 2010, Paine *et al*. 2010). Thus, we studied the involvement of Nrf2 in the inhibitory effects of hemin and CoPP in macrophages derived from Nrf2 knockout mice ($Nrf2^{-/-}$) (Study III, unpublished data). In $Nrf2^{-/-}$ mouse bone marrow-derived macrophages (mBMDM), the expression of HO-1 was significantly reduced compared to wild-type macrophages (Figure 13A,B).

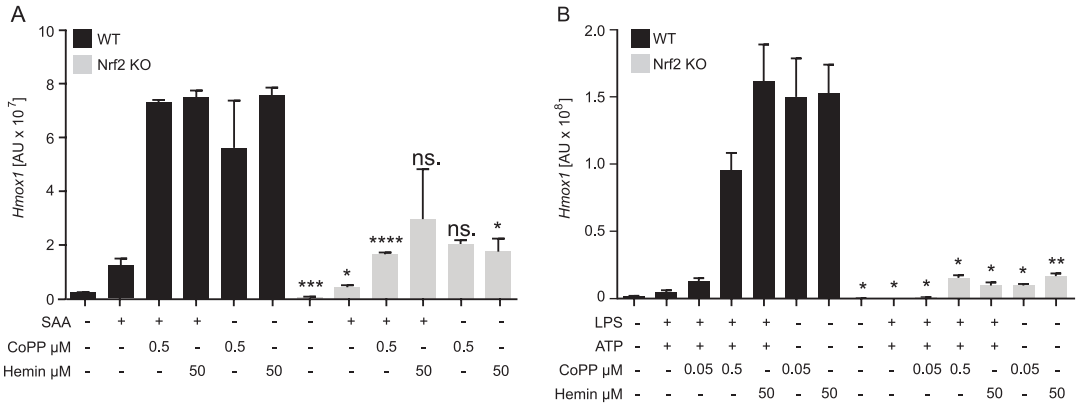

Figure 13. Deletion of Nrf2 reduces the expression of *Hmox1*. BMDMs derived from Nrf2 knockout (KO) and wild-type (WT) mice were incubated in the presence of CoPP or hemin at the indicated concentrations for

(A) 1 h or (B) 3 h, after which they were activated with (A) SAA (2 μg/ml, 18 h) or (B) ATP (5 mM, 30 min). (A, B) The expression of *Hmox1* was determined using quantitative real-time RT-PCR and is expressed in arbitrary units (AU). LPS-induced priming (3 h) of the cells (0.01 μg/ml) was as indicated. Statistical significancies were determined by comparing corresponding samples of wild-type and Nrf2-deficient macrophages. ATP, adenosine triphosphate; CoPP, cobalt protoporphyrin; LPS, lipopolysaccharide, SAA; serum amyloid A. The data represent the means ±SEM of 4 individual experiments.

Supporting the hypothesis that the inhibitory effects of hemin and CoPP are mediated by the induction of HO-1, CoPP and hemin were unable to inhibit the secretion of IL-1β in $Nrf2^{-/-}$ mBMDMs induced by 18 h SAA activation (Figure 14A). This finding contrasted with the results from their wild-type littermates, in which CoPP and hemin inhibited the secretion of IL-β. Furthermore, the treatment of $Nrf2^{-/-}$ mBMDMs with CoPP or hemin significantly increased the secretion of IL-1β in response to SAA compared to $Nrf2^{-/-}$ mBMDMs incubated in the presence of SAA alone. Because heme has been shown to promote ROS production in mBMDMs (Dutra *et al*. 2014), it is conceivable that during the 18 h incubation, hemin (and possibly CoPP) induced ROS production, which could not be inhibited in the $Nrf2^{-/-}$ mBMDMs. The increase in ROS production could therefore explain the increased secretion of IL-1β in the CoPP- and hemin-treated $Nrf2^{-/-}$ mBMDMs. The inhibitory effects of CoPP and hemin were also partially reversed during shorter ATP-induced inflammasome activation in $Nrf2^{-/-}$ mBMDMs (Figure 14B). However, in the ATP-activated $Nrf2^{-/-}$ mBMDMs, CoPP retained the ability to inhibit the ATP-induced secretion of IL-1β at the higher concentration of 0.5 μM, but not at the lower concentration of 0.05 μM. It is unclear why the inhibitory effect was not reversed at the 0.5 μM concentration. However, because the expression of *Hmox1* was slightly elevated at the higher 0.5 μM concentration compared to the lower 0.05 μM concentration, it is possible that the expression of *Hmox1* was sufficient for the inhibitory effect. Another possibility is that CoPP-induced inhibition is not fully mediated by Nrf2. Furthermore, reduced secretion of IL-1β was also observed in response to NLRP3 stimulation in $Nrf2^{-/-}$ BMDMs compared to wild-type mBMDMs (data not shown), which is in accordance with previous reports by Freigang *et al*. and Zhao *et al*. (Freigang *et al*. 2011, Zhao *et al*. 2014). In conclusion, because the deletion of Nrf2 was able to reverse the inhibitory effects of CoPP and hemin to a certain extent, it is plausible that the inhibitory effects of CoPP and hemin were mediated by the induction of Nrf2.

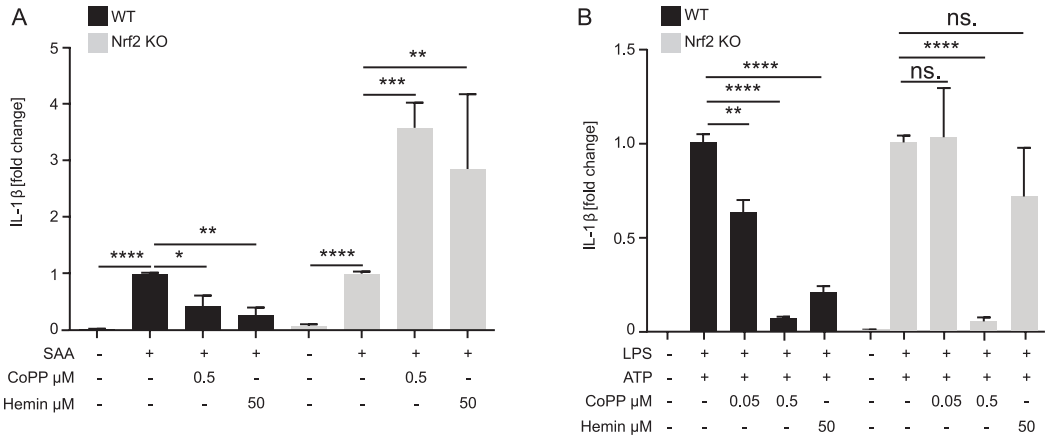

Figure 14. Hemin- and CoPP-induced inhibition of inflammasome depends on transcription factor Nrf2. BMDMs derived from Nrf2 knockout (KO) and wild-type (WT) mice were incubated in the presence of the indicated concentrations of CoPP or hemin for (A) 1 h or for (B) 3 h and were subsequently activated with (A) SAA (2 µg/ml, 18 h) or (B) ATP (5 mM, 30 min). (A, B) The secretion of IL-1β was determined using ELISA and is expressed as fold changes compared to control cells. LPS-induced priming (0.01 µg/ml, 3 h) of the cells was performed as indicated. ATP, adenosine triphosphate; CoPP, cobalt protoporphyrin; IL, interleukin; LPS, lipopolysaccharide; SAA, serum amyloid A. The data represent the means ±SEM of 4 independent experiments.

HO-1 protects against hypoxia-induced inflammation (Minamino *et al*. 2001). Reductions in the expression of proinflammatory mediators and reduced neutrophil recruitment have been observed in HO-1-overexpressing mice *in vivo* (Minamino *et al*. 2001). To further clarify the participation of HO-1 in the inhibitory effects of hemin and CoPP, we isolated thioglycollate-elicited peritoneal macrophages (TEPMs) from mice with a conditional deletion of *Hmox1* (LysMCre$^{+/-}$ HMOX$^{fl/fl}$) and stimulated the TEPMs with nigericin. TEPMs were selected for this study because they were used in the original study in which the HMOX1$^{fl/fl}$ mouse strain, which was also used in the present study, was generated (Tzima *et al*. 2009). The HO-1 deficient TEPMs (LysMCre$^{+/-}$ HMOX$^{fl/fl}$) exhibited an 80% overall reduction of *Hmox1* expression compared with the control TEPMs (LysMCre$^{-/-}$ HMOX$^{fl/fl}$) in nonactivated and nigericin-activated cells (Study III, Figure 4D). The activation of the HO-1-deficient TEPMs with nigericin significantly promoted the secretion of IL-1β compared to TEPMs derived from the control mice (Study III, Figure 4E). However, CoPP and hemin exhibited equal levels of inhibition of the secretion of IL-1β by TEPMs derived from both HO-1-deficient and control mice (Study III, Suppl. Figure 2C). A substantial leakiness of the Cre-LoxP-induced deletion of HO-1 was observed in HO-1-deficient TEPMs subjected to treatment with CoPP and hemin. In the HO-1-deficient TEPMs *Hmox1* expression was reduced by only 60% on average, which may well have contributed to the persistent inhibitory effect (Study III, Suppl. Figure 2D). Therefore, it could not be concluded that the inhibitory effect of CoPP and hemin was mediated by the induction of *Hmox1*.

Based on the results of the present study showing that 1) an enzymatic inhibitor of HO-1 reversed the inhibitory effect of CoPP; 2) a degradation product of heme/hemin, CO, inhibited the secretion of IL-1β; 3) Nrf2 partly reversed the inhibitory effects of hemin and CoPP; and

4) the deletion of *Hmox1* increased the secretion of IL-1β in response to nigericin activation, it seems plausible to propose that the inhibitory effect of hemin and CoPP on the activation of the NLRP3 inflammasome is partially mediated by HO-1. However, the inhibitory effect of CoPP and hemin was not affected by the deletion of HO-1. In addition, the expression of HO-1 was not fully upregulated at the 4 h time point, when the prominent inhibition of the inflammasome had already been established. In conclusion, the inhibitory effect of hemin and CoPP on the activation of the NLRP3 inflammasome was possibly mediated by HO-1, but the induction of HO-1 certainly seems to not be the only contributing factor.

6.3 Hemin inhibits the NLRP3 inflammasome via autophagy

Autophagy regulates the activation and outcome of inflammasome activation at multiple levels. Autophagy is an important gatekeeper of inflammasome activation because it reduces the generation and release of mitochondrial ROS and DNA by degrading damaged mitochondria (Zhou *et al*. 2011, Nakahira *et al*. 2011, Shimada *et al*. 2012). Furthermore, enhanced autophagy has been shown to lead to the degradation of inflammasome components, including ASC and IL-1β (Shi *et al*. 2012, Harris *et al*. 2011). In the present study, we have confirmed that CoPP and hemin reduce the levels of ASC in nonactivated and activated THP-1 cells. Because the decrease in the amount of intracellular ASC was not caused by the enhanced secretion or reduced expression of ASC, the involvement of autophagy was studied. During autophagosome formation, the conjugation of PE to cytosolic LC3-I is required for the expansion of the autophagosomal membranes, and if the rate of autophagosomal degradation remains unchanged, the increased ratio of PE-conjugated LC3-II to LC3-I reflects an increase in autophagosome formation (Kabeya *et al*. 2000, Mizushima 2004, Yang & Klionsky 2010). Compared to untreated cells, human primary macrophages treated with CoPP and hemin exhibited increased ratio of LC3-II to LC3-I, indicating that CoPP and hemin possibly increased autophagosome formation (Study III, Figure 5G, Suppl. Figure 3F).

Because the increased ratio of LC3-II to LC3-I may have also resulted from reduced degradation of autophagosomes (Klionsky *et al*. 2012), the level of cellular SQSTM1, which is a substrate of autophagosomal degradation, was measured. The cellular level of SQSTM1 reflects the degradation of autophagosomal content because it is constitutively expressed and it is degraded in autophagosomes (Klionsky *et al*. 2012). CoPP and hemin significantly reduced the level of SQSTM1 compared to control cells (Study III, Figure 5I, Suppl. Figure 3G). Furthermore, ATP-activated cells treated with CoPP exhibited a statistically nonsignificant reduction in SQSTM1 compared to ATP-activated cells. These results indicate that CoPP and hemin induce both autophagosome formation and degradation. Consistent with our results, HO-1 was previously reported to be required for the autophagy induced by cecal ligation and puncture and LPS in mouse hepatic tissue *in vivo* and in hepatocytes *in vitro* (Carchman *et al*. 2011). In addition, CO has been shown to increase autophagosome formation and the levels of cellular LC3 in mouse lung and cultured human alveolar and bronchial epithelial cells by increasing ROS generation (Lee *et al*. 2011). The

general ROS scavenger NAC and a scavenger of mitochondrial ROS prevented the CO-induced increase in the ratio of LC3-II to LC3-I (Lee *et al*. 2011).

To further confirm that the observed reduction in intracellular ASC was mediated by autophagy, a widely used pharmacological inhibitor of autophagy, 3-methyladenine (3-MA), was used to inhibit PI3K activity, which is necessary for autophagosome formation. As expected, 3-MA significantly inhibited the conversion of LC3-I to LC3-II in THP-1 cells at the timepoint used in the experiments (Study III, Suppl. Figure 4C,D). Importantly, the inhibition of autophagy by 3-MA reversed the CoPP-induced reduction in intracellular ASC (Study III, Figure 5J, Suppl. Figure 4E). In agreement with the results of Shi *et al*., 3-MA increased the secretion of IL-1β both alone and in nigericin-activated THP-1 cells, and it reversed the inhibitory effect of CoPP (Figure 15). The increased secretion of IL-1β in response to 3-MA may have been due to increased ROS formation and the subsequent inflammasome activation caused by the inhibition of autophagy (Zhou *et al*. 2011). The induction of autophagy has also been shown to not inhibit but, instead, to be required for the secretion of IL-1β (Dupont *et al*. 2011). These discrepant results may be explained by the different time points used in the experiments (1 h starvation in the study by Dupont *et al*. and 6 h starvation in the study by Shi *et al*.) (Dupont *et al*. 2011, Deretic *et al*. 2012). Thus, the induction of autophagy is hypothesized to be necessary for the secretion of IL-1β at the early time point, whereas at the later time point, the degradation of the activated inflammasomes and IL-1β result in the reduced secretion of IL-1β (Deretic *et al*. 2012). According to the model presented by Deretic *et al*., autophagy is necessary for the secretion of IL-1β while simultaneously acting to limit the activity of the inflammasome (Deretic *et al*. 2012).

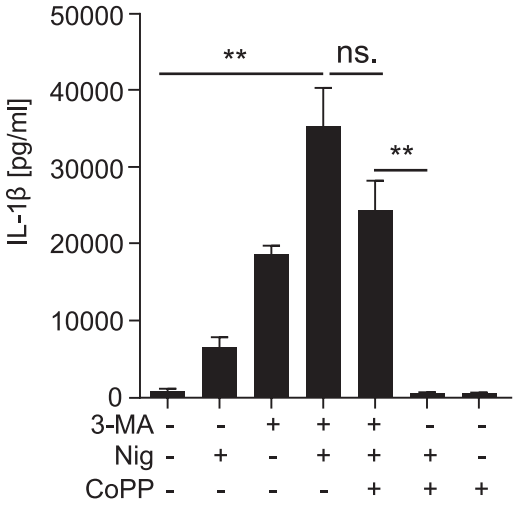

Figure 15. Inhibition of autophagy reverses the inhibitory effect of CoPP. THP-1 cells were incubated first in the presence of the autophagy inhibitor 3-MA (5 mM, 23 h) and then in the presence of CoPP (5 μM, 3 h) before finally being activated with nigericin (4 μM, 1 h). The secretion of mature IL-1β was determined using ELISA. The data represent the means of ±SEM of 3 individual experiments. CoPP, cobalt protoporphyrin; IL, interleukin; 3-MA, 3-methyl adenine; Nig, nigericin.

Lastly, we stained CoPP-treated THP-1 cells with antibodies against ASC and SQSTM1. At the 2 h and 4 h time points, CoPP promoted the oligomerization of SQSTM1 as shown by the increased formation of SQSTM1 specks (Study III, Figure 5H). The recruitment of SQSTM1 to ULK1 is an early step in the process of autophagosome formation, and the oligomerization of SQSTM1 in connection with ULK1 may define the autophagosome formation site (Itakura & Mizushima 2011). Untreated cells exhibited diffuse cytosolic ASC staining, but in the presence of CoPP, cytosolic ASC staining was reduced and ASC staining appeared to colocalize with DAPI staining in the nucleus. Recently, the nuclear localization of ASC was connected with the resting state of mBMDMs (Martin *et al*. 2014). Upon inflammasome activation the resting state was reversed and ASC was relocalized to the perinuclear area (Martin *et al*. 2014). Taken together, the results suggest that hemin and CoPP induced autophagosome formation and the degradation of autophagosomal contents, i.e., autophagic flux. Thus, the increased autophagic flux inhibited inflammasome activation by degrading the adaptor protein ASC.

VI Conclusions

1. Summary and conclusions

In this study, the mechanisms that contribute to the regulation of innate immune functions were explored. The observed inhibitory mechanisms may form a basis for the discovery of novel means of treating chronic inflammatory diseases.

Ethanol has well-acknowledged cardioprotective properties. Thus, an aim of this study was to elucidate the underlying mechanisms of this cardioprotective effect. Furthermore, hemin was identified as an immunoregulatory compound that strongly inhibits the activation of the inflammasome. The main findings from publications I-III and the Discussion section are summarized as follows:

1. Ethanol dose-dependently reduces the viability and proliferation of mast cells. Decreased mast cell viability and reduced proliferative potential could diminish mast cell numbers in response to acute alcohol exposure, especially in the mucosa of the alimentary tract, where the highest ethanol concentrations are encountered. The diminished mast cell viability was caused by the induction of immunologically silent cell death, apoptosis. Ethanol, but not its metabolite acetaldehyde, contributed to the reduction in viability and the increase in apoptosis. The induction of apoptosis proceeded via the activation of both the intrinsic and the extrinsic pathways, as modest inductions of the activities of both caspase-8 and caspase-9 were observed. The cellular pathways that mediate the induction of apoptosis in response to ethanol clearly warrant further study. In addition, a decline in TNFα secretion was observed in activated mast cells. It is feasible that the reduction in mast cell numbers slows the initiation of local immune responses and contributes to the immunosuppression associated with alcohol abuse. Because activated mast cells are considered mediators of cardiovascular events, the beneficial cardioprotective effects of moderate alcohol consumption could partly be explained by a decline in the numbers of coronary mast cells.

2. Ethanol, but not its metabolite acetaldehyde, dose-dependently inhibited the secretion of IL-1β and IL-18 induced by the activation of the NLRP3 inflammasome in human macrophages. Two activating signals are required for the activation of the NLRP3 inflammasome: the priming signal induces the expression of NLRP3 and proIL-1β, and the activating signal induces the assembly and activation of the NLRP3 inflammasome. Ethanol did not reduce TLR-induced priming. Therefore it was concluded that ethanol specifically inhibited the activation of the NLRP3 inflammasome. Ethanol inhibited lysosomal destabilization and the release of cathepsin B from disrupted and seemingly intact lysosomes. However, ethanol had no effect on potassium efflux, nor did the inhibition of ROS production reverse the inhibitory

effect of ethanol. Thus, the inhibitory effect of ethanol seems general, as the secretion of IL-1β induced by several NLRP3 inflammasome activators (ATP, cholesterol crystals, SAA, and nigericin) was inhibited in ethanol-treated cells despite differences in the mechanisms that mediate the inflammasome activation. Additionally, the secretion of IL-1β induced by AIM2 inflammasome activation was inhibited by ethanol. Ethanol inhibited inflammasome assembly and activation, which was reflected by the reduced oligomerization of ASC and the activation of caspase-1. However, ethanol did not inhibit the activation of the apoptotic caspase-8, thereby directing inflammasome-induced cell death toward apoptosis. Excessive NLRP3 inflammasome activation contributes to the pathogenesis of several common chronic inflammatory diseases. Thus, the reduced inflammasome activation may partly explain the reduced risk of RA associated with the moderate consumption of ethanol. Furthermore, the potential ethanol-induced inhibition of cholesterol crystal-induced inflammasome activation in atherosclerotic arteries may be partly responsible for the reduced risk of cardiovascular diseases associated with moderate ethanol consumption. These findings are presented in Figure 16.

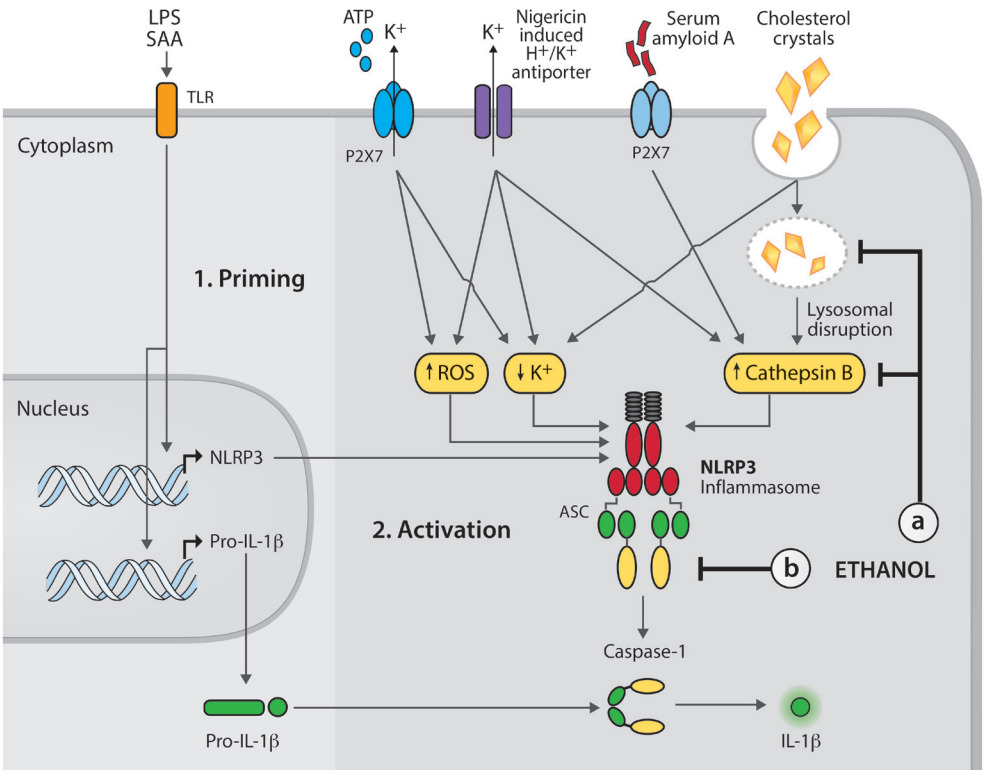

Figure 16. Schematic representation of the inhibitory effects of ethanol on NLRP3 inflammasome activation. The inflammasome activators used in Study II are illustrated; some steps are omitted for clarity. Priming is shown on the left and inflammasome activation on the right. (1) First, the activation of TLRs by LPS or SAA initiates the expression of NLRP3 and proIL-1β, which is called the priming step. (2) Second, stimuli induced by extracellular ATP, nigericin, SAA or cholesterol crystals induce NLRP3 inflammasome activation. Extracellular ATP binds $P2X_7$, which induces potassium efflux. Additionally, nigericin functions as an H^+/K^+ antiporter to induce potassium efflux. Furthermore, nigericin induces the release of cathepsin B from seemingly intact lysosomes (intact lysosomes are omitted for clarity) and ROS formation. SAA induces the release of cathepsin B from seemingly intact lysosomes by the activation of $P2X_7$ receptors. Phagocytosed cholesterol crystals induce lysosomal disruption, which results in the leakage of cathepsin B from lysosomes. Cholesterol crystals also induce potassium efflux. Decreased intracellular potassium concentration, increased ROS production, and the release of cathepsin B each alone or in combination induce the activation of the NLRP3 inflammasome. Caspase-1 is activated in the NLRP3 inflammasome, and it processes proIL-1β to its mature, secreted form. Ethanol reduces the activation of caspase-1 and the secretion of mature IL-1β by inhibiting the activation of the inflammasome. Two mechanisms were described: a) Ethanol inhibits lysosomal disruption and the release of active cathepsin B from disrupted and seemingly intact lysosomes, and b) ethanol inhibits the assembly of the inflammasome. ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; ATP, adenosine triphosphate; IL-1β, interleukin 1β; LPS, lipopolysaccharide; NLRP3, nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing 3; $P2X_7$, P2X purinergic receptor 7; ROS, reactive oxygen species; SAA, Serum amyloid A; TLR, Toll-like receptor. Figure adapted from (Nurmi *et al*. 2013).

3. Hemin and its derivative CoPP inhibited the secretion of IL-1β and IL-18 in human and mouse macrophages in response to several NLRP3 inflammasome activators. Additionally, the IL-1β secretion induced by the AIM2 inflammasome was inhibited, although the

exact mechanism of AIM2 inflammasome inhibition remains to be elucidated. The local NLRP3-induced secretion of IL-1β was further inhibited *in vivo* in mice. Hemin and CoPP specifically inhibited NLRP3 inflammasome activation because no reduction in priming was observed. CoPP did not affect potassium efflux but instead inhibited the release of active cathepsin B, which may reflect the diminished secretion of IL-1β along with other inflammasome components from autophagosomes. The inhibitory effect of hemin and CoPP was partially dependent on the induction of HO-1 by Nrf2 and the enzymatic activity of HO-1. Additionally, the degradation product of heme/hemin, CO, dose-dependently inhibited NLRP3 inflammasome activation. Hemin and CoPP blocked the assembly of the NLRP3 inflammasome by reducing the cellular level of the inflammasome adaptor protein ASC, which is required for the recruitment and activation of caspase-1. The reduction in ASC was caused by enhanced autophagy, which induced its degradation. In conclusion, the results of this part of the study indicate that endogenous hemin may function as an important negative regulator of the NLRP3 inflammasome. Thus, it is conceivable that hemin inhibits inflammasome activation under certain pathologic conditions that involve excessive hemolysis. The main findings of the study are illustrated in Figure 17.

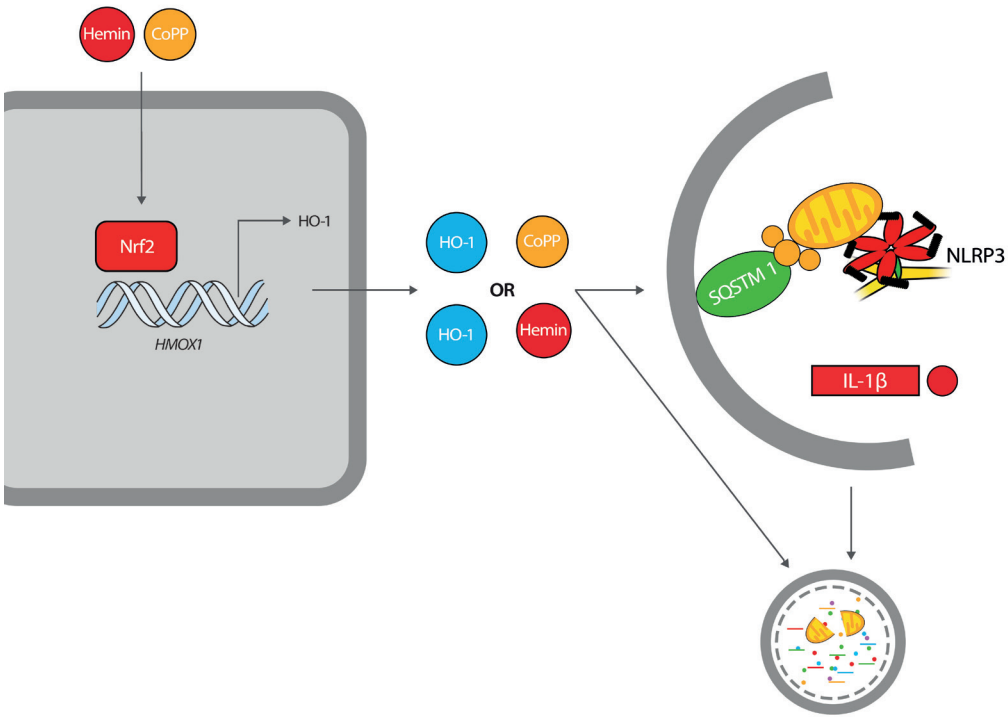

Figure 17. Hemin and CoPP enhance autophagy, which subsequently results in inhibition of the NLRP3 inflammasome. Hemin and CoPP induce the expression of HO-1 at least partially via the activation of Nrf2. Hemin and CoPP, or HO-1, enhance autophagosome formation, which sequesters ASC (and possibly other inflammasome components) for degradation in autolysosomes. Enhanced autophagic process results in the degradation of inflammasome components and thereby diminishes the secretion of mature IL-1β. The colocalization of the NLRP3 inflammasome with mitochondria (orange-yellow ellipse shape with cristae) was demonstrated by Zhou *et al.* and Misawa *et al.* (Zhou *et al.* 2011, Misawa *et al.* 2013). ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; CoPP, cobalt protoporphyrin; IL-1β, interleukin 1β; HO-1, heme oxygenase-1; Nrf2, NF-E2-related factor-2; SQSTM1, sequestosome 1.

2. Future prospects

Excessive inflammation is a key factor in the pathogenesis of several common diseases that are not limited to autoimmune diseases, such as RA and inflammatory bowel disease, but also include chronic inflammatory diseases, such as atherosclerosis and type 2 diabetes. A more thorough understanding of the function and regulation of the immune system is needed for the development of new therapeutic strategies to treat chronic inflammation. In the present study, the regulation of innate immune cell functions was studied, particularly the mechanisms that inhibit the inflammatory actions of two key players of innate immunity, i.e., mast cells and macrophages. Two novel factors, ethanol and hemin, were demonstrated to limit the proinflammatory actions of mast cells and macrophages.

Increases in the number and activation of mast cells in atherosclerotic coronary arteries contribute to the recruitment of immune defense cells and to cardiovascular events, such as plaque rupture (Bot *et al*. 2013, Laine *et al*. 1999). Chronic inflammation directs the progression of atherosclerosis. IL-1β is a prototypical proinflammatory cytokine that mediates the accumulation of phagocytes in atherosclerotic plaques, thereby indirectly contributing to foam cell formation and the further enhancement of plaque progression (Young *et al*. 2002, Kirii *et al*. 2003). In the present study, ethanol was shown to reduce mast cell viability by inducing immunologically silent apoptotic cell death. Further, the ability of ethanol to directly inhibit the activation of the NLRP3 inflammasome and the secretion of IL-1β from macrophages suggests that reductions in the functions of macrophages and mast cells may contribute to the cardioprotective effects of ethanol. Targeting chronic inflammation in atherosclerotic arteries could be a potent therapeutic strategy for the treatment of CHD. Indeed, the well-acknowledged cardioprotective effects of statins may be mediated in part by reduction in inflammation, and several anti-inflammatory drugs are currently being tested for their potential to reduce cardiovascular risk (Golia *et al*. 2014).

In the present study, we discovered that hemin prevents the assembly of the NLRP3 inflammasome and the secretion of mature IL-1β in macrophages. Highly proinflammatory conditions and the release of free heme/hemin caused by massive hemolysis induce the periodic fever observed during malarial infection (Olivier *et al*. 2014). Malaria is a severe infection caused by several species of the protozoan parasite *Plasmodium*. *Plasmodium* produces hemozoin, which is released to the circulation by the lysis of red blood cells. Hemozoin is further phagocytosed by macrophages, eliciting the strong activation of the NLRP3 inflammasome and the secretion of the pyrogenic cytokine IL-1β (Shio *et al*. 2009). In light of our results, it is conceivable that evolutionary pressure has induced the development of a negative regulatory loop in which enhanced levels of free heme/hemin inhibit NLRP3 inflammasome activation during malarial infection.

Our results suggest that hemin inhibits inflammasome activation by enhancing autophagy. However, the contribution of HO-1 in the inhibiton of inflammasome activation and enhancement of autophagy has yet to be fully elucidated. Another intriguing question is the role of autophagy during inflammasome activation. How does the induction of autophagy first drive the secretion of IL-1β? At which point does autophagic degradation become dominant and block the secretion of IL-1β by the degradation of inflammasome components (Deretic *et al*. 2012)? Based on our results, we propose a model in which hemin enhances the autophagic degradation of the critical inflammasome adaptor protein ASC. This inhibitory pathway also holds potential for the development of novel therapeutic approaches for the treatment both of severe autoinflammatory diseases and of more common chronic diseases characterized by excessive NLRP3 inflammasome activation.

VII Acknowledgements

This study was commenced in the historical settings of old Surgical Hospital, its main part was carried out at the soulful Salus hospital and the study was finalized in the dynamic atmosphere of Biomedicum. I wish to thank Wihuri Research Institute and its former director Petri Kovanen as well as the current director Kari Alitalo for the excellent research facilities. This thesis work has been financially supported by Jenny and Antti Wihuri Foundation, Ida Montin Foundation, Maire Lisko Foundation, Aarne Koskelo Foundation, Finnish Foundation for Cardiovascular Research, Paulo Foundation, Veritas Foundation, and Meilahti Foundation.

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