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FOLLOWING THE WHITE RABBIT:

RELEASE AND REGULATION OF HMGB1 IN INFLAMMATION

Agnieszka Sowińska

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Following the white rabbit: release and regulation of HMGB1 in inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ACADEMIC DISSERTATION

The public thesis defense for the degree of Ph.D. at Karolinska Institutet will take place on **Friday, March 15, 2019 at 9:00**

CMM Lecture Hall (L8:00, Karolinska University Hospital, Solna).

“The illiterate of the 21st century will not be those who cannot read and write, but those who cannot learn, unlearn, and relearn.”

— Alvin Toffler

To my family

ABSTRACT

Dysregulated inflammatory responses are characterized by excessive release of endogenous pro-inflammatory molecules, danger-associated molecular patterns (DAMPs). A prototypical DAMP, High Mobility Group Box 1 protein (HMGB1), is primarily bound to DNA in the nucleus of most eukaryotic cells. However, when HMGB1 exits the cell, it acquires novel functions and can trigger immune activation.

The aim of this thesis was to study the release of HMGB1, follow its fate at the site of inflammation and therapeutically block its activity. Multiple studies have reported that cells release HMGB1 in either a passive or an active manner. In order to understand the possible impact of cell death in inflammatory conditions, we performed well-controlled *in vitro* analyses of HMGB1 release under different cell death modes. Necrotic cells instantly released high levels of HMGB1, while apoptotic cells retained HMGB1 unless they advanced to secondary necrosis. The controlled immunogenic cell death types, such as pyroptosis and necroptosis, secreted HMGB1 along with other pro-inflammatory molecules in comparable or higher levels than cells activated with lipopolysaccharide (LPS) and interferon gamma (IFNγ).

Next, we investigated HMGB1 proteolytic regulation at the site of inflammation. Human neutrophil elastase (HNE), cathepsin G (CG) and matrix metalloproteinase 3 (MMP-3), endoproteases present in arthritis-affected joints, cleaved HMGB1 into smaller peptides *in vitro*. While HNE and MMP-3 truncated the acidic C-terminal tail domain of HMGB1, CG completely degraded the protein. We showed that the C-terminal tail truncation negatively regulated HMGB1 binding to Toll-like receptor 2 (TLR2). Only the protein form devoid the acidic tail domain bound TLR2 *in vitro*. Thus, truncation of HMGB1 by endoproteases might improve its affinity to receptors. TLR2 and HMGB1 interaction did not result in cytokine induction. However, both full-length and truncated HMGB1 formed complexes with potent TLR2 ligand peptidoglycan (PGN) to potentiate the inflammatory response in peripheral blood mononuclear cells (PBMCs).

Lastly, we developed a novel tool that could enable anti-HMGB1 therapy in the clinic by engineering a chimeric, partially humanized, monoclonal antibody (h2G7). The antibody displayed anti-inflammatory effects when tested in a mouse model of paracetamol-induced liver toxicity. Experiments with mutated variants, non-Fc receptor binding and non-complement binding variants, of the novel antibody suggested HMGB1 neutralization as the mode of action.

Taken together, results presented in my thesis increased the knowledge about HMGB1 biology regarding its release during immunogenic cell death, proteolytic regulation at the site of inflammation and interaction with its receptor TLR2. In addition, the generation of a partially humanized monoclonal antibody could promote the introduction of anti-HMGB1 therapy in the clinic.

LIST OF SCIENTIFIC PAPERS

I. Mode of cell death influences amounts of released HMGB1.
Agnieszka Sowinska, Peter Lundbäck, Daniel Applegren, Lena Klevenvall, Manoj Neog, Ulf Andersson, Helena Erlandssoon Harris
Manuscript

II. Proteolytic cleavage of the alarmin HMGB1 and its potential role in regulating HMGB1 activity in inflammatory disease.
Agnieszka Sowinska, Merlin Rensing, Lena Klevenvall, Manoj Neog, Peter Lundbäck, Helena Erlandsson Harris
Submitted for publication

III. Ligation of free HMGB1 to TLR2 in the absence of ligand is negatively regulated by the C-terminal tail domain.
Hannah Aucott, Agnieszka Sowinska, Helena Erlandsson Harris, Peter Lundbäck
Mol Med. 2018 May 4;24(1):19

IV. A novel high mobility group box 1 neutralizing chimeric antibody attenuates drug-induced liver injury and post-injury inflammation in mice.
Peter Lundbäck, Jonathan D. Lea, Agnieszka Sowinska, Lars Ottosson, Camilla Melin Fürst, Johanna Steen, Cecilia Aulin, Joanna I. Clarke, Anja Kipar, Lena Klevenvall, Huan Yang, Karin Palmblad, B. Kevin Park, Kevin J. Tracey, Anna M. Blom, Ulf Andersson, Daniel J. Antoine, Helena Erlandsson Harris
Hepatology. 2016 Nov;64(5):1699-171

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LIST OF ABBREVIATIONS

ACPA	anti-citrullinated protein antibodies
ADCC	antibody-dependent cell-mediated cytotoxic
ALI	acute liver injury
ALT	alanine aminotransferase
APAP	acetaminophen
AUC	area under the curve
CBP	calmodulin-binding protein
CDC	complement-dependent cytotoxic
CG	Cathepsin G
CHO	Chinese Hamster Ovary
CIA	collagen-induced arthritis
CLR	C-type lectin receptor
DAMP	danger-associated molecular pattern
DC	dendritic cells
DILI	drug-induced liver injury
DTT	dithiothreitol
E. coli	*Escherichia coli*
FcR	Fc receptor
GST	gold sodium thiomalate
HAMA	human-anti mouse antibody
HAT	histone acetyl transferase
HDAC	histone deacetylase
HIS	polyhistidine
HMGB1	High Mobility Group Box 1
HNE	Human Neutrophil Elastase
HSP	heat shock protein
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist

JIA	juvenile idiopathic arthritis
LBP	LPS-binding protein
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LRR	leucine-rich repeats
mAb	monoclonal antibody
MD-2	myeloid differentiation factor 2
MLKL	mixed lineage kinase domain-like pseudokinase
MMP-3	matrix metalloproteinase 3
MPRR	pattern recognition receptor
MS	mass spectrometry
MTX	Methotrexate
NAC	N-acetylcysteine
NAPQI	N-acetyl-p-benzo-quinoneimine
Nec-1	Necrostatin-1
NET	neutrophil extracellular trap
NLR	NOD-like receptor
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing
NLS	nuclear localization sequence
NSAID	nonsteroidal anti-inflammatory drug
PAMP	pathogen-associate molecular pattern
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PR3	proteinase 3
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end products
RIP1	receptor-interacting protein 1
RIP3	receptor-interacting protein 3
RLR	RIG-I-like receptor
RNS	reactive nitrogen species

ROS	reactive oxygen species
SDF-1/CXCL12	stromal cell-derived factor-1
SEAP	secreted embryonic alkaline phosphatase
SF	synovial fluid
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
sRAGE	soluble RAGE
STS	Staurosporine
TLR	Toll-like receptor
TNF	tumor necrosis factor
WB	western blotting

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

We live in a world full of various pathogens that constantly challenge our bodies. Injured skin and mucosal sites enable them to enter our system. Fortunately, the immune system has evolved to keep us safe from the environmental hazards to ensure physiological homeostasis [2]. Not only does it skillfully discriminate between endogenous molecules ('self') and molecules derived from pathogens ('non-self'), but it also participates in tissue repair and removal of dying cells [3]. Importantly, danger signals released by host cells under sterile conditions, as a result of cell death or stress, serve as potential triggers of immune activation [4]. Most of the immune processes occur smoothly and we are not aware of the molecular challenges that we are facing on a daily basis. However, certain illnesses and novel pathogens can compromise the immune system. Complex defense mechanisms are engaged to promote health and restore homeostasis.

1.1.1 Innate and adaptive immunity

The immune system consists of a complex network of specialized cells that is conventionally divided into two overlapping mechanisms to destroy pathogens: the innate immune system and the adaptive immune system [5, 6]. The innate system is evolutionarily older and serves as the first line of defense against infections and sterile injury, i.e. hazards of non-microbial origin [7]. It is rapidly activated and has the ability to recognize pathogens and products of injured cells. The innate immune cells actively scan the body and activate biochemical defense mechanisms upon danger recognition. However, these innate immune responses are not very specific and only activate the same mechanisms when subjected to repeated threats [8].

On the other hand, the adaptive immune system is slower to respond but much more efficient in the termination of the danger [9]. It is characterized by high specificity to a great variety of targets and the ability to create memory towards detected hazards. The defense mechanisms are fine-tuned towards previously encountered threats. Thus, the adaptive immune system responds more effectively and precisely compared to the innate immune system. All cells of the immune system secrete soluble proteins that activate, regulate and coordinate its many activities. It is evolutionarily beneficial for the body to have two interconnected systems for danger recognition and elimination as the innate and adaptive immune systems complement and communicate with each other ensuring proper immune function [10]. While this thesis focuses mainly on the mechanisms within the innate immune system, it is important to remember about its cooperation with the adaptive immunity.

1.1.2 Cells and receptors of the innate immune system

The innate immune system has passive and active components. The passive part consists of physical and chemical barriers as well as circulating proteins that protect the body against infections. The physical barrier of our innate immune system is visible to the naked eye as it consists of epithelial cells (i.e. skin) and epithelial mucosa that provide the first line of defense

against the harmful environment [11]. Anti-microbial molecules produced by the epithelium provide the chemical barrier [12]. Another passive part of innate immunity is a system of circulating plasma proteins termed the complement system [13]. These elements can directly bind to pathogens and alarm the immune system by recruiting innate immune cells or inducing direct lysis of the pathogen [14].

Cells compose the active component of the innate immune system. While the tissue-resident scavenger cells such as macrophages, granulocytes and dendritic cells (DCs) are the first ones to respond to danger in their proximity, the most efficient tissue-resident cells to fight against infections are professional phagocytes that actively engulf extracellular content [15]. These include neutrophils, monocytes, macrophages, mast cells and DCs, which are armed with pattern recognition receptors (PRRs) on their surfaces [16, 17]. Receptors are specialized protein molecules capable of sensing and responding to chemical signals received from outside the cell. PRRs have the ability to engage classes of molecules rather than individual ligands. Thus, they detect diverse molecular motifs such as microbial nucleic acids, lipoproteins and carbohydrates, which are collectively known as pathogen-associated molecular patterns (PAMPs). Many of the PRRs can also detect motifs released from injured cells, damage-associated molecular pattern (DAMP) molecules [18, 19]. Thus, the molecular sensing and response to tissue injury resembles mechanisms involved in the elimination of exogenous pathogens.

Four families of PRRs currently exist: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) [1, 20]. Members of the TLR family are major PRRs in cells. The discovery of TLRs was an important breakthrough for immunology research and the scientific community recognized that by awarding the 2011 Nobel Prize to Jules Hoffmann and Bruce Beutler [21]. TLRs are type I transmembrane proteins containing leucine-rich repeats (LRRs) that recognize PAMPs and certain DAMPs both in the extracellular environment (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 on cell surface) and intracellularly (TLR3, TLR7, TLR8, TLR9 and TLR10 in endosomes) [22]. C-type lectin receptors (CLRs), primarily expressed on membrane of macrophages and DCs, often collaborate with TLRs during an immune response [23]. PRRs also include two classes of cytoplasmic receptors: NLRs and RLRs. NLRs are best known for their ability to activate NF-κB leading to production and secretion of pro-inflammatory molecules. The NLR family members NOD2 and NLRP3 have received considerable attention as mutations in these proteins are linked to inflammatory disease [24]. Lastly, signaling through the RLRs activates intracellular pro-inflammatory processes in response to viral and bacterial nucleic acids in the cytoplasm. The PPR examples and their PAMP and DAMP ligands are illustrated in Figure 1.

Twenty-five years ago, Alan Shaw and colleagues described a novel scavenger receptor and called it receptor for advanced glycation end products (RAGE) [25]. It was recognized that besides binding advanced glycated proteins, RAGE detects a class of ligands through a common structural motif [26]. Interaction of RAGE on leukocytes or endothelial cells with its ligands results in the transcription of pro-inflammatory molecules and inflammatory cell recruitment [27]. All of the aforementioned characteristics classify RAGE as a PRR. There is,

however, one striking difference between RAGE and other PRRs, as there are no known exogenous ligands for RAGE.

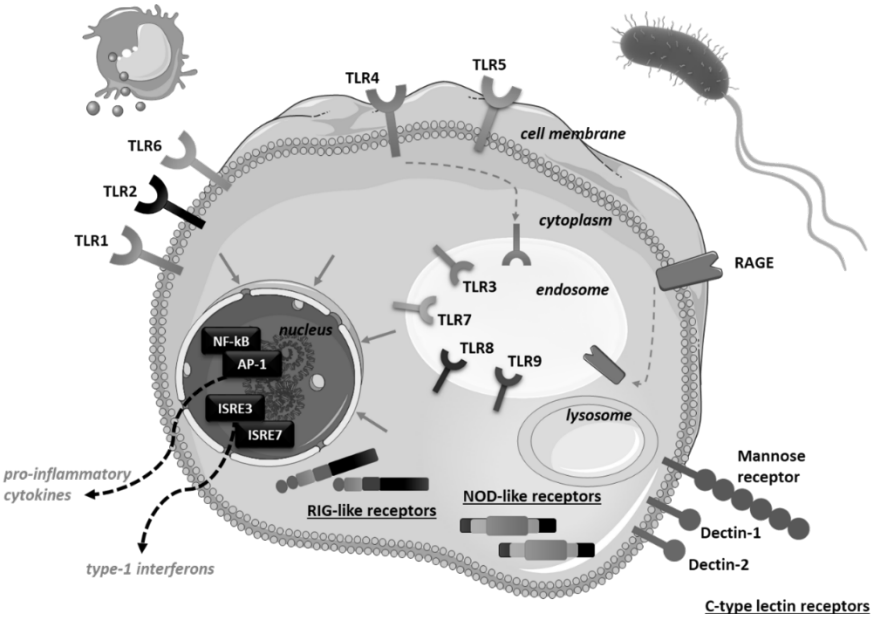

Figure 1. Pattern recognition receptors (PRRs). *PRRs are localized on the cell surface as well as in endosomes and cytosol. TLR4 is a membrane bound receptor and its ligands include LPS (PAMP) and HMGB1 (DAMP). TLR9 is expressed in endosome, where it recognizes non-methylated CpG motifs of bacterial and viral DNA (PAMPs) and extracellular genomic/mitochondrial DNA (DAMPs). NLRP3, a NOD-like receptor, binds bacterial muramyl dipeptide (PAMP) and extracellular ATP (DAMP) in the cytosol. More information about PRRs and their ligands and be found in review by Takeuchi et al. [1].*

1.1.3 The immune response

The body needs to regulate the activation of the immune system very tightly. A complex network of firmly regulated mechanisms accomplish this by utilizing numerous checkpoints controlling immune activation and ensuring that leukocytes are recruited and activated only in response to foreign invaders and dead tissues. Systemic activation of leukocytes could have detrimental consequences due to the potential cytotoxic properties of the immune cells.

The initiatory steps of immune responses involve activation of PRRs, which then assemble large complexes in order to transmit the danger signal. Next, the activated signaling cascades trigger the release of factors promoting recruitment of leukocytes to the region of infection. Some infections are easily cleared due to prompt phagocytosis by the tissue-resident cells, while others cause immune activation and lead to inflammation.

1.2 CELL DEATH AND IMMUNE ACTIVATION

Perhaps the most evident consequence of host-pathogen interactions is cell death. Although it was described as early as in the 19th century, it is only recently that cell death has been recognized as an important player at the site of inflammation [28, 29]. Depending on the mechanisms involved, cell death can contribute to tissue homeostasis or inflammation and disease pathogenesis. Host cell death may impair normal organ function and lead to associated signs of disease.

1.2.1 Homeostatic cell death

Every day billions of aged or faulty cells die which the body complements by generating the same number of new cells. The immune system does not react to such homeostatic cell clearance as it mainly proceeds by a controlled silent process called apoptosis [30]. The word apoptosis originates from Greek and denotes a “falling off” which refers to leaves falling from the tree in the fall [31]. This is an imaginative way to describe a harmless process of removing individual components of an organism without destruction or damage to the organism itself. This mode of cell death involves mechanisms leading to elimination of harmful and old cells. Apoptotic cells are engulfed by macrophages and successful absorption of the dying cells ensures that no intracellular components are released into the extracellular environment [32]. Apoptosis is often referred to as a type of a programmed cell death since it is either a genetically determined elimination of cells during normal development or a highly controlled cell disintegration during the immune response [33]. The mechanisms of apoptosis are highly complex, involving an ATP-dependent cascade of molecular events and the regulation of over thousands of proteins [34].

Apoptosis can be triggered by a variety of physiological agents signaling intrinsically as a response to damaged DNA or extrinsically through death receptors on the cell surface. The intrinsic pathway involves the Bcl-2 protein family members that can stimulate the release of cytochrome c from mitochondria. Hence, it is often referred to as the mitochondrial apoptotic pathway. Once cytochrome c relocates to cytosol, it binds to Apaf-1 to form a protein complex named the apoptosome, which in turn leads to the execution of apoptosis [35]. The extrinsic apoptosis pathway is triggered by death receptors of the tumor necrosis factor (TNF) family. Those membrane-bound proteins contain death domains, which transmit the death signal from the cell surface to the intracellular signaling pathways. The extrinsic and intrinsic pathways are linked so that molecules in one pathway can influence the other [36]. Both pathways are orchestrated by a distinct molecular mechanism led by cysteine proteases (caspases) acting in cascades and merging on the same execution pathway. This terminal pathway culminates in the cleavage of caspase 3 and the disposal of fragmented DNA and proteins in apoptotic bodies expressing ligands for phagocytic cell receptors [37].

Although apoptosis is considered a homeostatic cell death, overwhelming apoptosis or inefficient apoptotic cell clearance can also contribute to immune activation. When apoptotic cells are not efficiently engulfed by phagocytes, they undergo secondary necrosis and release intracellular immune-stimulatory materials including DAMPs. Such clearance deficiencies are major contributors to the development of autoimmune inflammation in systemic lupus erythematosus (SLE) [38].

1.2.2 Immunogenic cell death

Cell death that is not developmentally programmed is a sign of stress, injury or infection and is linked to tissue damage and disease pathogenesis. Contrary to homeostatic apoptosis, infected cells can die in an immunogenic manner (Figure 2). The classic example of a non-programmed cell death is necrosis, which denotes cell response to damage [39]. During necrosis, the sudden cell membrane rupture and loss of structural integrity results in passive release of intracellular components including cytosolic enzyme lactate dehydrogenase (LDH) and DAMPs into the extracellular space [40]. Necrotic cell debris acts as a potent trigger for

inflammation [29]. Despite the widespread use of the apoptosis-versus-necrosis paradigm, there is an increasing awareness of the complexity of processes prior to immunogenic death. Multiple immunogenic cell death modes have been described and recently classified as either necrosis or stress-induced cell death [41]. The latter is often referred to as regulated necrosis due to the distinct mechanisms that lead to cell membrane disintegration. Multiple cell death modes that occur during inflammation or infection are programmed or regulated by gene products. Stress-induced cell death includes several cell death modes such as necroptosis, pyroptosis and cell death associated with the release of extracellular traps, which is described as NETosis in neutrophils.

The term “necroptosis” was introduced in 2005 to indicate a new pathway of programmed necrosis [42]. Since then, plenty of molecules and processes have been characterized as initiators of necroptosis including toll-like receptors and the apoptosis-activating death receptors. However, there are multiple differences between apoptosis and necroptosis. Firstly, necroptosis does not proceed by the caspase-dependent intracellular signaling routes. Therefore, it can overpower apoptotic pathways in situations where energy supplies are limited such as ischemia. Secondly, execution of this cell death mode involves the loss of membrane integrity, release of DAMPs and a proinflammatory response [43, 44]. Although, the molecular pathways involved in necroptosis are still not fully understood, they depend on the kinase activity of receptor-interacting protein 1 (RIP1) and RIP3 with mixed lineage kinase domain-like pseudokinase (MLKL), a substrate of RIP1 kinase. Necroptosis contributes to disease pathogenesis and plays a central role in myocardial infarction, stroke, drug-induced liver injury and ischemia–reperfusion injury [45-47]. Blocking necroptosis has shown promising therapeutic results in the treatment of multiple animal disease models, such as myocardial ischemia-reperfusion injury and ethanol-induced liver injury. The blockade is possible through suppression of RIP1 kinase activity by a specific inhibitor, Necrostatin-1 (Nec-1) [48, 49].

Certain hazards induce the production of immature forms of inflammatory mediators and the formation of inflammasomes, which in turn leads to a cell death called pyroptosis. The term pyroptosis was introduced in 2001 to describe the process of pro-inflammatory chemical signals bursting out of a host cell (“pyro” means fire in Greek) [50]. Released DAMPs are sensed by Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP) protein family that are the core parts of a protein complex responsible for innate immunity activation of the inflammasome. The mechanism of the NLRP3 inflammasome activation is well described in macrophages and involves recruitment and subsequent cleavage of pro-caspase 1 to its active form caspase 1 [51-53]. Caspase 1 in its turn processes the pro-forms of the inflammatory mediators IL-1β and IL-18 that are subsequently released as mature inflammatory mediators [54]. Caspase-1 also cleaves gasdermin D leading to colloid-osmotic lysis and release of cytoplasmic contents including DAMPs and LDH [55]. Therefore, pyroptosis not only plays a protective role in the host response to infection but it can also promote pathogenic inflammation. It is associated with strong inflammatory conditions driven by Salmonella-infected macrophages, or during gout and rarer autoinflammatory conditions [56].

Another interesting immunogenic type of cell death can be observed in a few cell types including neutrophils. Since neutrophils are the most abundant leukocytes in the human body and they are the first cells migrating to the infected site, their deaths can significantly affect the

host immune system. Those short-lived cells have classically been known to die by apoptosis. However, a neutrophil-specific death leading to the release of extracellular traps (NETs) was discovered in 1996. NETs are composed of chromatin fibrils and antimicrobial proteins that can kill invading pathogens. Moreover, NETosis can be involved in noninfectious inflammatory events and has the ability to amplify the immune response. The main component of NETs is DNA. Nucleic acid can be sensed by some PRRs, such as endosomal TLR9 of plasmacytoid dendritic cells (pDCs) and monocytes. Activation of these receptors leads to the synthesis of pro-inflammatory mediators.

It is important to remember that cell death modes are not mutually exclusive and can co-exist independently or act in concert during an inflammatory response. Immunogenic cell death pathways, however, can drive the pathogenesis of certain disease phases. For example, inflammation during gout is propagated in a positive-feedback loop by inflammatory mediators released by pyroptotic macrophages [57]. In addition, the same triggers can induce different death pathways depending on the cell type as well as the intensity and persistence of the stimulus. At low doses, a variety of pathogenic stimuli such as hypoxia and cytotoxic anticancer drugs can induce apoptosis but these same stimuli result in secondary necrosis at higher doses [58, 59].

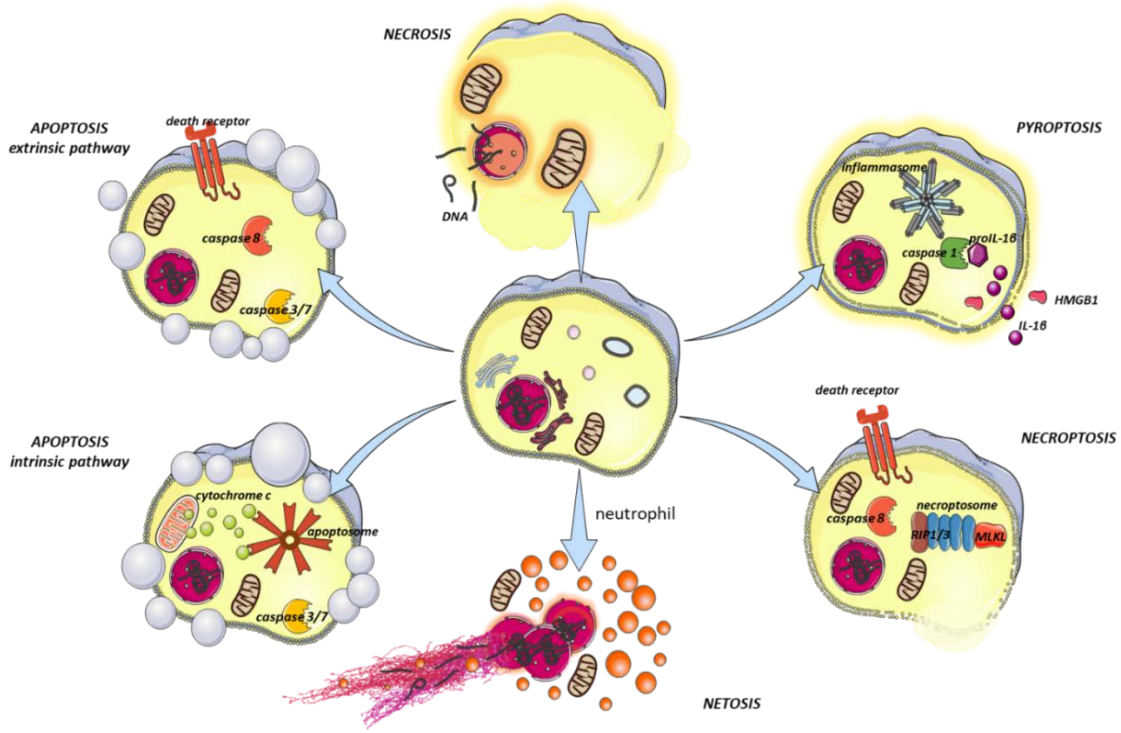

Figure 2. Myeloid cell undergoing homeostatic and immunogenic modes of cell death. *Apoptosis is a homeostatic type of cell death that can be induced by extrinsic or intrinsic pathways. Death receptor activation and downstream signaling marks the extrinsic pathway of apoptosis. The intrinsic pathway, on the other hand, is characterized by cytochrome c release and apoptosome formation. Both pathways are executed by ATP- dependent apoptotic effector caspases 3 and 7 and lead to the formation of apoptotic bodies. In contrast, necrosis is an abrupt cell membrane disintegration and results in the release of the intracellular contents. Pyroptosis is a stress-induced cell death mode characterized by inflammasome-mediated cytokine release. Similarly to extrinsic pathway of apoptosis, necroptosis is initiated by death receptors. However, the lack of ATP, e.g. due to starvation, directs cells to proceed with RIP1/3 pathway instead of energy-dependent apoptotic caspase cascade. NETosis is a cell death type characterized with rapid release of NETs containing intracellular molecules.*

1.3 INFLAMMATION

Ancient Greek scholars, such as Hippocrates, were among the first to report the reaction by the body to injury or stress. In AD 25, a Roman encyclopedist, Aulus Cornelius Celsus, summarized symptoms of the immune response as heat, redness, swelling, pain and loss of function [60]. Those are the cardinal signs of inflammation, the protective host response of the innate immune system to the injury or stress. The molecular events leading to systemic inflammation manifestations described by Celsus have been extensively studied over centuries leading to the identification of immune cells, specialized mediators and changes in blood vessels, needed to eliminate the initial cause of cell injury and to initiate the process of repair. Nowadays, we also understand that the cardinal signs of inflammation illustrate only the initiation phase of the inflammation. Equally important to the induction of inflammation is its resolution. Inflammation does not resolve in a passive way but as a result of active processes. Two thousand years after Celsus described symptoms of inflammation, Charles Serhan identified molecules that mediate the resolution of inflammation and promote homeostasis [61]. Regulated inflammation with both induction and resolution phases occur during acute inflammation (e.g. a burn). Without inflammation, the infection would go unchecked and lead to a more serious condition. Prolonged inflammation, however, can also be harmful [62]. When the resolution phase of inflammation fails, inflammatory cells and mediators continue to promote tissue destruction. This condition is called chronic inflammation and is a major cause of complex human autoimmune inflammatory pathologies such as arthritis [63]. My thesis work focuses on such dysregulated inflammation (Figure 3).

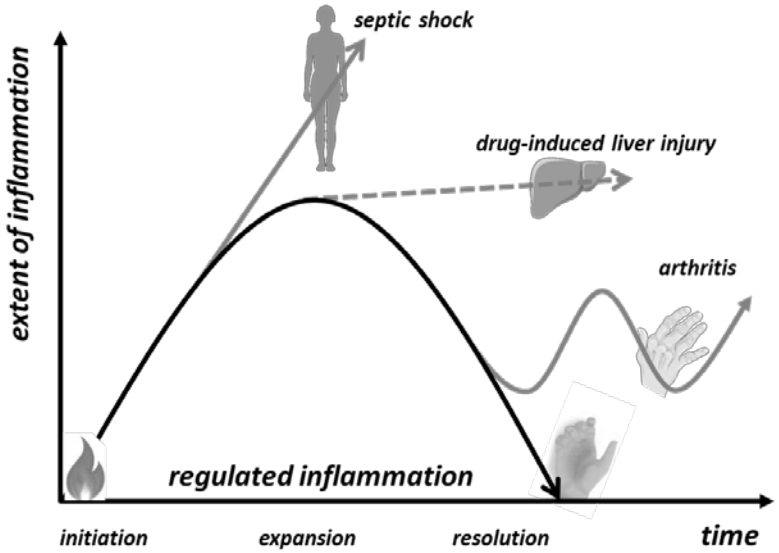

Figure 3. Acute vs. dysregulated inflammation. *PAMPs and DAMPs that are sensed by PRRs on cells can initiate the inflammatory response. Activation of PRRs results in expansion of the inflammatory response by chemokine and cytokine production. Chemokines recruit circulating cells to the site of injury and cytokines activate cells to remove the danger and initiate resolution of inflammation.*

In case of acute inflammation, such as a burn, the process follows the course of regulated inflammation and leads to repair and homeostasis. Dysregulated inflammation proceeds by different patterns: 1. overwhelming systemic expansion of inflammation, such as septic shock, 2. Uncontrolled local expansion of inflammation induced by mediators released from injured cell, such as drug-induced liver injury, 3. chronic inflammation where the balance between the expansion and resolution phase is not achieved, such as arthritis.

1.3.1 Pathogenesis of inflammatory diseases

Inflammation can be either local or systemic. However, this is not a binary process and local inflammation can lead to complex systemic effects in multiple human diseases. Here, the mechanisms involved in pathogenesis of arthritis and drug-induced liver injury (DILI) were studied.

DILI is the leading cause for acute liver failure in the Western world and results from injury to resident liver cells [64]. Although the injury is local, DILI represents a broad spectrum of liver manifestations including acute or chronic hepatitis and a critical condition called acute liver failure (Figure 4a). DILI often occurs due to cytotoxic hepatocyte death after an overdose of acetaminophen (APAP), either in an intended suicidal attempt or by and accidental harmful drug combination [65, 66]. The pathogenesis of APAP overdose has been well characterized and develops when a small proportion of the drug gets metabolized to a reactive metabolite, N-acetyl-p-benzo-quinoneimine (NAPQI) [67]. The toxic by-product acts both as a strong electrophile and as an oxidant that bind intracellular molecules and induce mitochondrial dysfunction. Downstream events lead to activation of the immunogenic cell death pathways and subsequent DAMPs release. The presence of pro-inflammatory molecules in the blood (IL-6 and IL-8) indicates the involvement of inflammatory pathways [68]. APAP overdose is problematic to treat due to a lack of reliable biomarkers and a short window of opportunity for patient treatment with the classical medication, N-acetylcysteine (NAC) [69, 70]. Since many patients do not reach the emergency room within the therapeutic time bracket, there is a great need to identify new therapy targets and more stable biomarkers than cytokines and liver enzymes. One promising marker is miR-122; miRNA accounting for 75 % of the total miRNA in the liver [71]. DAMPs and other inflammatory molecules released from dying liver cells could also serve as potential biomarkers.

Chronic inflammatory systemic diseases like arthritis develop over a span of years and once self-tolerance is broken, symptoms of local inflammatory events in the joints appear. While the etiology of arthritis is not fully understood, it involves environmental factors that trigger disease in genetically susceptible individuals, which is about 1% of the human population [72]. The disease manifests itself by chronic joint inflammation leading to joint disability both in adults (rheumatoid arthritis, RA) and children (juvenile idiopathic arthritis, JIA). The synovium, tissue surface in the joint, thickens so that the patient's mobility is disturbed. Circulating immune cells, such as monocytes and neutrophils, infiltrate the joint tissue and execute inflammatory processes by secreting specialized molecules (Figure 4b). An excess of synovial fluid accumulates and causes swelling of the joint. Interestingly, neutrophils are also present in synovial fluid of arthritis patients. The inflamed synovium invades adjacent cartilage and promotes articular degradation. Activated immune cells release pro-inflammatory mediators and regulatory enzymes as well as DAMPs. RA has a quite well defined inflammatory profile with autoantibodies to immunoglobulin G and anti-citrullinated protein antibodies (ACPAs) [73]. Due to disease heterogeneity, arthritis treatment is often crafted to the patient. Standard treatment involves nonsteroidal anti-inflammatory drugs (NSAIDs) complemented by inflammatory mediator blockers, such as anti-TNF agents [74, 75].

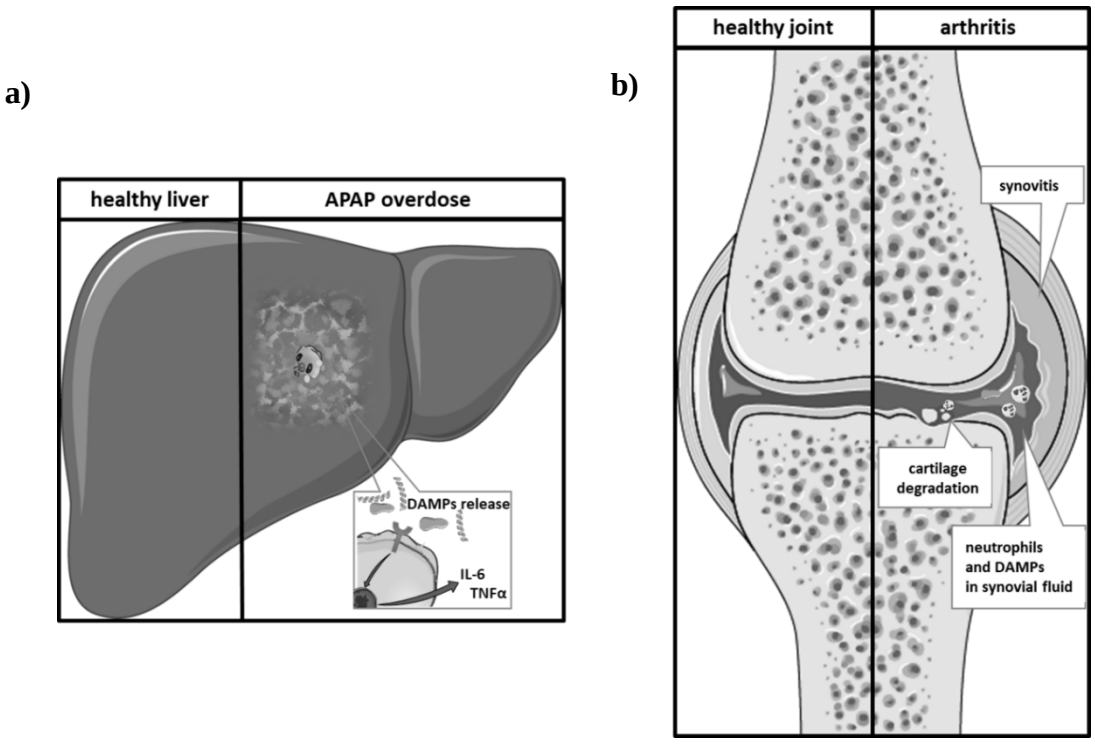

***Figure 4. Inflammatory diseases**. **a)** Drug-induced liver injury due to APAP overdose results in cytotoxic hepatocyte death and leads to a broad spectrum of liver manifestations including acute or chronic hepatitis and a critical condition called acute liver failure. **b)** Arthritis develops gradually and leads to thickening of the synovium and tissue infiltration by activated circulating immune cells.*

1.3.2 Soluble mediators in inflammation: PAMPs, DAMPs and cytokines

Activated, injured and dying cells present their status to the immune system utilizing several classes of soluble mediators. Initiatory steps of inflammation are triggered by agents either of exogenous or endogenous origin, PAMPs and DAMPs, which lead to immune activation and inflammatory signal propagation by cytokines.

Lipopolysaccharide (LPS) is a bacterial endotoxin coming from the outer membrane of Gram-negative bacteria. This is the most studied PAMP and is recognized by TLR4 [76]. LPS is an extremely potent immune activator and its systemic presence can lead to a critical inflammatory condition called septic shock. Another example of PAMP is peptidoglycan, a polymer forming the cell wall of Gram-positive bacteria, specifically recognized by TLR2.

1.3.3 Endogenous mediators of inflammation

Endogenous molecules can also initiate inflammatory responses. In 1994, Polly Matzinger concluded that the immune system is so concerned with the consequence of infection or injury that it can stop discriminating between ‘self’ and ‘non-self’[77]. She called this phenomenon “The Danger Theory” and defined DAMPs, the endogenous initiators of inflammation. Importantly, this class of soluble mediators comprises diverse group of molecules that have non-immune functions within the cell and become danger signals only when they reach the extracellular space. Stimulated leukocytes and epithelia actively secrete DAMPs while necrotic cells passively release them. All cell types in the body express certain DAMPs, such as heat

shock proteins (HSPs) and High Mobility Group Box 1 (HMGB1) protein. Moreover, S100 family members expressed in phagocytes are also considered DAMPs. Once those proteins enter the extracellular space, they are capable of binding TLRs and RAGE thanks to their hydrophobic parts revealed upon release [78]. DAMPs do not only include nuclear, cytosolic and extracellular matrix proteins but also organic molecules and metabolites such as ATP, uric acid crystals and nucleic acids. However, only the DAMP proteins were categorized as alarmins by Joost Oppenheim ten years after "The Danger Theory" was published [79]. Alarmins promote the activation of innate immune cells and the recruitment and activation of antigen-presenting cells, but also promote tissue repair. Due to their pro-inflammatory activities, alarmins may serve as useful diagnostic and prognostic biomarkers in inflammatory disorders. They may also serve as therapeutic targets.

After TLR binding, DAMPs or PAMPs can utilize the intracellular adaptor protein MyD88 and signal downstream leading to activation of NF-κB, which is a nuclear transcription factor [80]. The NF-κB activation leads to cytokine and chemokine transcription in macrophages, mast cells and endothelial cells. These small proteins are the basic units of the body's immunomodulatory language so that several different types of cells can secrete a given cytokine. Once secreted, cytokines act on the target cell and relay the pro- or anti-inflammatory message by binding to specific receptors. Cytokines amplify and direct the inflammation to combat the stress mainly by leukocyte recruitment. Historically, many cytokines are called interleukins (ILs) due to the belief that they could only carry information between leukocytes. Although a bit misleading, the name interleukins remained. Some cytokines are produced in a mature form while others require further processing within the cell. For example, IL-1β is synthesized in a pro-form and requires inflammasome and subsequent caspase-1 cleavage [81]. The major cytokines in acute inflammation are TNF, IL-1β, IL-6 and a group of chemoattractant cytokines called chemokines [82]. Acute-phase cytokines act locally and usually over a short range to activate endothelial cells and local tissue leukocytes, create positive feedback loops to amplify cytokine release and chemokine recruitment. They also increase vascular permeability allowing immune cell migration from circulatory system into tissue. Although TNF, IL-6 and IL-1β are secreted at sites of inflammation, they may enter the circulation and act at distant sites to induce the systemic acute-phase reaction that is often ssociated with infection and inflammatory diseases [83]. Measuring systemic levels of cytokines n patients provides good insight into the status of an inflammatory disease. Importantly, pro-inflammatory cytokine blockade turned out to be a successful therapy for dampening dysregulated inflammatory response in several diseases

Table 1: Key endogenous mediators of inflammation.

		Function in inflammation	Diagnostic use
DAMPs	S100 proteins	• S100B and S100Al2 interact with RAGE • S100A8, S100A9 and S100Al2 are secreted at sites of inflammation	• S100B is a marker of astrocytic activation • high levels of S100A8, S100A9 and S100Al2 present in several inflammatory disorders such as rheumatoid arthritis and chronic bronchitis
	HMGB1	• cytokine induction though TLR4 binding • internalization of pro-inflammatory mediators through RAGE • Synergy with other DAMPs • chemoattractant for neutrophils	• Aberrant levels in synovial fluid of arthritis patients
CYTOKINES	TNF	• major player in acute-phase response • activates neutrophils and endothelial cells • kills cells through death receptors	• TNF blockers have been approved for the treatment of several autoimmune diseases: rheumatoid arthritis, psoriasis, and Crohn disease
	IL-6	• induces acute-phase inflammatory response in liver • regulates maturation of B cells (adaptive immunity)	• anti-IL6 or anti-IL6R mAbs have clinical efficacy in Castleman disease and in inflammatory diseases such as rheumatoid arthritis or Crohn disease
	IL-1β	• activates endothelium in inflammation • induces fever and acute-phase response • stimulates neutrophil production	• current strategies target IL-1 expression using caspase-1/NALP3 inhibitors • IL-1 receptor blocker is a successful treatment for rheumatoid arthritis (Anakinra)
	IL-8	• chemoattractant for neutrophils and cells of adaptive immunity	• current effors to target the IL-8 receptors CXCR1/ 2 with a non-competitive inhibitor (Reparixin)

1.3.4 Regulation of soluble mediators

Since excessive immune activation could be harmful for the body, a dynamic and tight regulation of soluble mediators exists ensuring that inflammatory processes are run in a controlled manner. Certain molecules that counteract the pro-inflammatory events are already produced alongside inflammatory mediators during the induction phase of inflammation. Such anti-inflammatory molecules can ameliorate the immune response by modulating production, function and activation of pro-inflammatory agents. Thanks to tight regulation of the immune response, tissue damage is limited and the defense mechanisms turn into healing processes. Multiple systems have evolved in order to control and downregulate pro-inflammatory processes before the resolution of inflammation takes place.

Certain anti-inflammatory cytokines and specific cytokine inhibitors mediate suppression of inflammatory responses. The most prominent anti-inflammatory cytokines are IL-10 and TGF-β1, which are potent inhibitors and downregulators of pro-inflammatory cytokines produced by macrophages and cells of the adaptive immune system [84]. However, many cytokines have both pro- and anti-inflammatory properties. IL-6 is an example of a cytokine with dual functions. Although IL-6 was described as a pro-inflammatory mediator of acute inflammation in the chapter above, it is known to downregulate pro-inflammatory cytokines by promoting the synthesis of Interleukin-1 receptor antagonist (IL-1ra) and release of soluble TNF receptor, which dilutes TNF inflammatory activities [85]. Cytokines act as a double-edged sword and the net outcome of their actions depends on factors such as the timing of cytokine release, the availability of receptor and local milieu.

During the course of chronic inflammatory disease, recruited and activated neutrophils release serine proteases upon degranulation [86]. Two common neutrophil enzymes, elastase and cathepsin G, degrade the TNF precursor into biologically inactive fragments [87]. Moreover, serine proteases can downregulate immune responses by cleaving essential receptors on the surface of both adaptive and innate immune cells. For example, neutrophil elastase cleaves receptor for IL-8 on the surface of neutrophils, thereby preventing neutrophil activation and migration to the synovium [87, 88]. Other downregulatory mechanisms seen in inflammatory disease are reduced PRRs density on cell surface and receptor blockade by non-inflammatory agents. Actually, inducing natural IL-1ra shows therapeutic effects in RA treatment [89].

More research is needed to understand the downregulation of DAMPs. There are no known anti-inflammatory agents produced by cells that could counteract DAMPs in a similar way to how anti-inflammatory cytokines work. However, some regulatory mechanisms seen in cytokine downregulation also have effects on DAMPs. One of such similarity is the processing by proteases. For example, mast cell chymase and neutrophil cathepsin G can cleave and activate alarmin IL-33 [90-92]. DAMPs are also subject to soluble scavenger receptor binding which limits their pro-inflammatory capacity.

Many inflammatory mediators are subject to post-translational modifications (PTMs). Those covalent chemical modifications of amino acids occur after protein synthesis and provide a plethora of mechanisms for regulation of proteins produced by the cell [93, 94]. Protein function can be affected by PTMs so that molecules can gain or lose pro-inflammatory activities. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) post-translationally modify many thiol-containing redox-sensitive molecules, thus playing an important role in inflammatory disease pathogenesis.

2 HMGB1: A PROTOTYPICAL DAMP

As discussed in the previous chapter (§1.3.2), DAMPs, also known as alarmins, are non-microbial pro-inflammatory molecules released from dying or stressed cells. The highly conserved nuclear protein, HMGB1, acts as a prototypical alarmin once it exits the cell [95].

HMGB1 was first isolated and described in 1973 by Graham Goodwin and Ernest Johns [96]. Back then, the researchers only recognized it as a member of the chromatin-binding group of molecules, which migrated fast during electrophoresis. As HMGB1 is ubiquitously expressed by almost all eukaryotic cells, it was not too surprising that it turned out to be the most abundant protein in the discovered protein group. All four members of the isolated protein family contain HMG box domains that are DNA-binding motifs characterized by three α-helices [97]. HMGB1 contains 215 amino acids that form two subsequent HMG box domains (boxes A and B) followed by a C-terminal tail domain (Figure 5a). The consecutive boxes contain a high number of positively charged lysine residues while the C-terminus is formed by a stretch of 30 continuous negatively charged glutamic and aspartic acid residues. This unique bipolar charge of the molecule prompted some researchers name the protein "amphoterin" [98]. As the protein started to be researched more extensively over the past 20 years, the scientific community decided to prevent confusion and call the protein HMGB1 [99].

The first time HMGB1 was reported as a DAMP was in 1999, when the group led by Kevin Tracey discovered that it was present the in blood of mice during endotoxemia. It was proposed as a late mediator of lethality as mice showed increased serum levels of HMGB1 between 8 to 32 hours after endotoxin exposure [100]. Discovery of the nuclear protein as a pro-inflammatory factor in circulation was a breakthrough in HMGB1-related research and encouraged the scientists to study HMGB1 properties as a DAMP.

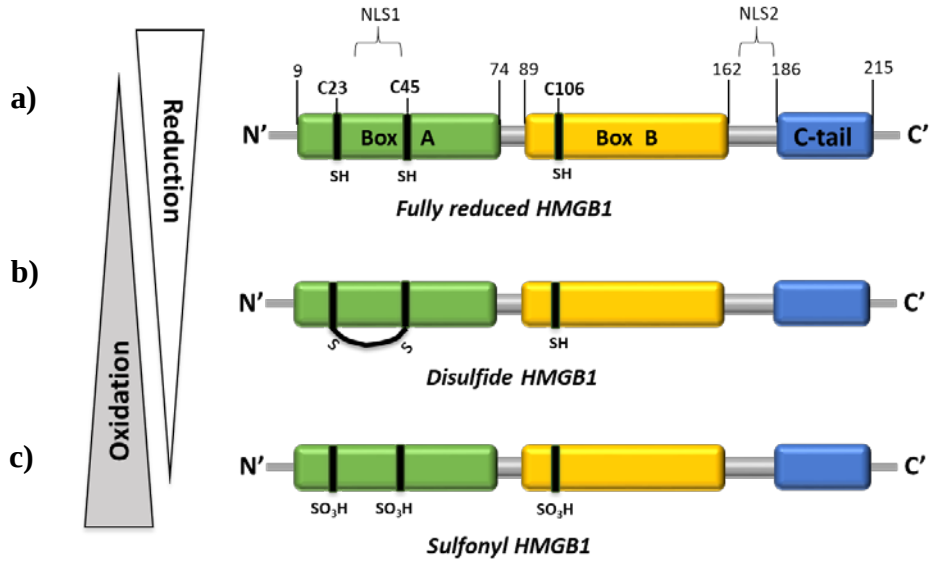

Figure 5. HMGB1 structure and redox isoforms. a) *HMGB1 consists of three domains: DNA-binding boxes A and B, and an acidic C-terminal tail. Two lysine-rich NLS regions are located within the structure. Fully reduced HMGB1 has thiol groups attached to cysteine residues at positions 23, 45 and 106 (C23, C45, C106) of the 215 amino acid sequence. The fully reduced HMGB1 isoform is localized in the cell nucleus or extracellularly in the reducing environment where it promotes chemotaxis.* ***b)*** *Disulfide HMGB1 has a disulfide bond between C23 and C45 while C106 has a thiol group attached. This isoform has a cytokine-inducing function.* ***c)*** *When the cysteine residues become oxidized HMGB1 loses its immunogenic functions.*

2.1 THE GENERATION AND RELEASE OF EXTRACELLULAR HMGB1

As mentioned above, HMGB1 is a highly abundant nuclear protein. A single cell is estimated to contain approximately 10 million HMGB1 molecules [101]. The box domains of HMGB1 bind to the minor groove of DNA and bend its structure, which results in enhanced accessibility of DNA to regulatory elements including transcription factors and nucleosomes [102]. Two lysine-rich nuclear localization sequences (NLSs) are present within the protein structure whose unmodified form determines nuclear localization of HMGB1 [103]. Lysine residues are subject to a PTM called acetylation. If no multiple acetyl groups are introduced to NLSs, HMGB1 intranuclear localization and function is favored. However, hyperacetylated NLS1 and NLS2 regions promote HMGB1 relocation to the cytosol by preventing nuclear reentry [104]. Cytosolic HMGB1 has been demonstrated to be an autophagy regulator and nucleic acid sensor. Hyperacetylation is associated with active HMGB1 secretion. This process is regulated enzymatically by the activity of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Studies show that HDAC inhibition promotes cytoplasmic translocation and the release of HMGB1 [105].

HMGB1 can be released in a passive or an active manner. The major source of extracellular HMGB1 is through necrosis. This rapid form of cell death is typically followed by inflammatory reactions due to passive release of HMGB1 and other alarmins. Necrotic cell debris are potent triggers of inflammation and necrotic cells lacking HMGB1 display greatly impaired pro-inflammatory properties [106]. HMGB1 is also actively secreted during pyroptosis and further escalates the inflammatory reactions [107-109]. The active secretion of HMGB1 is not fully understood since the protein lacks a classic leader peptide and its secretion is thus not routed through the classical protein secretion pathway via endoplasmic reticulum – Golgi.

Studies suggest that activated monocytes, macrophages, DCs and other immune cells actively discharge HMGB1 via two-step mechanism. First process requires HMGB1 translocation to the cytoplasm dependent on hyperacetylation of NLS sites as a result of JAK–STAT pathway-activation [110]. The JAK-STAT pathway is the principal intercellular signaling mechanism that can be activated by a variety of molecules, such as or IFNγ [111]. Hyperacetylated HMGB1 accumulate in the cytosol and becomes a subject of different secretory mechanisms. It has been shown that cytoplasmic HMGB1 can be packed into and released by secretory vesicles, similarly to members of the IL-1 family, in response to inflammatory stimuli [112]. Hyperacetylation of lysines was observed in HMGB1 released during pyroptosis as a consequence of NLRP3 and NLRC4 inflammasome activation [52, 113]. On the contrary, passive release of HMGB1 does not require NLS acetylation. Therefore, HMGB1 that is released rapidly due to disruption of cell membrane is not hyperacetylated. When HMGB1 is passively released during cell death or secreted by activated cells into the extracellular milieu, the molecule acquires a novel function to serve as a powerful mediator of inflammation [100, 114].

2.2 PRO-INFLAMMATORY FUNCTIONS OF HMGB1

There are thousands of scientific publications regarding HMGB1 functions and most of them focus on the pathogenic functions of the molecule. My thesis goes along with this trend and focuses on understanding the molecular events regarding the release and extracellular regulation of HMGB1 pro-inflammatory activities.

2.2.1 Impact of PTMs on HMGB1 function

Upon release to the extracellular space, HMGB1 acts as a prototypical DAMP and can initiate and perpetuate inflammatory responses by binding to different receptors. Studies using high-resolution mass spectroscopy and NMR have identified different functional isoforms of HMGB1 depending on the redox status of three conserved cysteine residues C23, C45 and C106 [115-117]. The cysteine thiolate represents a potent nucleophile containing a sulfur atom that can form bonds to another sulfur atom or to hydrogen, nitrogen, oxygen and carbon atoms [118].

The biochemical form of HMGB1 determines the receptor binding capabilities of the molecule. Certain isoforms are recognized as pro-inflammatory, while others have no known immune function. The reducing environment within the cells under physiological conditions promotes thiol cysteine side-chains of HMGB1. This redox isoform is commonly denoted “fully reduced HMGB1” (“HMGB1C23hC45hC106h”, Figure 5a). However, oxidation of the cysteine thiol groups at positions 23 and 45 facilitates the formation of a disulfide bridge resulting in a minor change in protein conformation but, strikingly, a major change in biological activity [119]. If the cysteine at position 106 remains in its thiol form while a disulfide bond forms between cysteines 23 and 45, HMGB1 function also changes. Such “disulfide HMGB1”-mediated signaling leads to NF-κB activation and proinflammatory cytokine release [115, 116]. In order to clarify the biochemical form of the cytokine-inducing HMGB1, a more specific nomenclature has been proposed: “HMGB1C23-C45C106h” or more simply “HMGB1C23-C45” (Figure 5b) [118]. In order to understand the relationship between different redox isoforms and put them into the context of inflammatory disease, a real-time kinetics study of HMGB1 isoform stability was performed with In Situ Protein NMR Spectroscopy [120]. The study concluded that the fully reduced HMGB1 half-life is regulated by the nature of the extracellular environment. While the half-life of fully reduced HMGB1 was only about 17 minutes in human serum and saliva, it extended to 3 hours in prostate cancer cell culture medium [120]. Considering ROS involvement is a typical phenomenon in an inflammatory disease, the pro-inflammatory isoforms of HMGB1 should instead be considered a molecule with a short half-life. One could speculate that the detection of endogenous disulfide HMGB1 implies an active production of such isoform. When HMGB1 undergoes further oxidation due to an increased oxidative environment, all three cysteines become oxidized to an irreversible sulfonyl form. This “sulfonyl HMGB1” (“HMGB1C23soC45soC106so”, Figure 5c) has not been shown to have any pro-immunogenic function [121]. Interestingly, this terminal oxidation of HMGB1 has been recorded in the resolution phase of inflammation and may be a natural occurring mechanism in downregulating pro-inflammatory activities of HMGB1 [115]. Sulfonyl HMGB1 has also been reported to increase cytotoxicity of chemotherapeutic agents in cancer therapy by inducing apoptosis via the mitochondrial pathway [122].

While the functional activity of HMGB1 seems to be solely dependent on its cysteine redox states, the extent of inflammation in disease is more strongly correlated with the acetylation status of the NLSs than the total levels of HMGB1 [104, 123]. We have recently shown by LC-MS/MS analysis that the levels of HMGB1 released into the synovial fluid of juvenile idiopathic patients strongly correlates with acetylation of NLS2 and that NLS acetylation correlates with the pro-immunogenic forms of HMGB1 [124]. However, it remains unclear whether HMGB1 is secreted in its intranuclear fully reduced form and undergoes functional shifts in the extracellular space, or whether these shifts occur within the cells before it is released.

2.2.2 HMGB1 binding PRRs

One way to assess immune mediator's function is by identifying the receptors it employs. To date, 14 different receptors have been described as targets of the extracellular HMGB1 [114, 125]. None of the identified receptors is unique to HMGB1. However, PRR systems were studied more in detail with regard to HMGB1 signaling and are considered the dominant receptors for HMGB1: TLR4 and RAGE.

2.2.2.1 *RAGE*

The first receptor that was identified as HMGB1 binder was RAGE [126]. There are two RAGE-binding sites within HMGB1 structure: within aa23-50 and 150-183 [125]. The latter one was the first reported binding site for RAGE and resulted in neurite outgrowth in the fetal mouse brain, while the other binding site was identified recently and its binding to RAGE reversed apoptosis-induced tolerance in dendritic cells [127, 128]. HMGB1 interaction with RAGE results in NF-κB activation followed by induction of chemotaxis in many cell types including dendritic cells and neutrophils [129, 130]. TLR4 deficient macrophages do not produce cytokines when activated by highly purified HMGB1 of any redox isoform [119]. As RAGE is normally expressed at low levels on the cell surface, the degree of RAGE expression needed for cytokine induction is still unknown. Alternatively, HMGB1-dependent cytokine release may not result from the direct RAGE-activated intracellular signal cascades. Research teams lead by Jie Fan and Timothy Billiar at the University of Pittsburgh recently showed that HMGB1 can be endocytosed via RAGE and later translocated into lysosomes in macrophages. After lysosomal rupture HMGB1 entered cytoplasm and led to pyroptosome formation, caspase-1 activation and cytokine production [131, 132].

2.2.2.2 *HMGB1 and TLRs*

Germline encoded TLRs are major PRRs in cells and several of them are recognizing and responding to HMGB1. Out of the suggested TLRs, the interaction between HMGB1 and TLR4 is best understood and is highly dependent on the redox state of HMGB1. Disulfide HMGB1 activates TLR4 in presence of MD-2 on the cell surface [133]. HMGB1 signaling through TLR4/MD-2 complex results in initiation of intracellular signaling events such as activation of ERK1/2, JNK, IKKα, IKKβ and p38 signaling pathways. The intracellular reactions culminate in NF-κB transcription factor translocation and subsequent pro-inflammatory cytokine production. Interestingly, recombinant box B, which contains the cytokine-inducing domain of HMGB1, also binds to TLR4 [119]. The disulfide HMGB1 form

can induce long-lasting hypersensitivity in muse arthritis models in a TLR4 dependent manner [134]

While the binding of HMGB1 to TLR4 has been extensively studied, binding to other TLRs has also been reported. However, neither the redox state of HMGB1 nor any other molecular requirements are presently known for other Toll-like receptor interactions. HMGB1 is a ligand for TLR2 and TLR5 and their interaction leads to activation of pro-inflammatory pathways. Similar to HMGB1-TLR4 interaction, binding of HMGB1 to TLR2 results in intracellular events leading to NF-κB activation [135]. Studies utilizing cell line overexpressing TLR2 confirmed the NF-κB-dependent signaling by IL-8 production. However, TLR2 blockade did not prevent TNF release by HMGB1 neither in stimulated primary macrophages nor whole blood cultures [119]. Thus, HMGB1 interaction with TLR2 is not considered as cytokine inducing. This was also demonstrated in this thesis work focused on the physical interaction between TLR2 and HMGB1 ([136], Paper III). TLR5 has only recently been identified as HMGB1 receptor. HMGB1 binding to TLR5 initiates NF-κB signaling pathway activation in a MyD88-dependent manner. The pro-inflammatory processes due to HMGB1 interaction with TLR5 result in allodynia in rats [137].

2.2.3 HMGB1: a partner in crime

HMGB1 acts in synergy with other pro-inflammatory mediators and as a partner molecule signals through a broader range of receptors than on its own. This unique feature of HMGB1 has created confusion over the years regarding the biological functions of the molecule and its pathological role in disease. Nowadays, HMGB1 researchers need to verify that they work with a pure protein but for many years the possibility of HMGB1 complex formation with pro-inflammatory agents was overlooked. Independently of its redox isoform, HMGB1 enhances pro-inflammatory functions of exogenous or endogenous pro-inflammatory molecules.

Let us start with the receptors mentioned in the previous chapter. A prototypical PAMP, LPS, signals through TLR4 [138]. LPS binding to the receptor is facilitated by LPS-binding protein (LBP), which transfers LPS to CD14 to initiate TLR4-mediated pro-inflammatory response. HMGB1 plays a similar role to LBP by transferring LPS to CD14, which results in an enhanced LPS-mediated inflammation [139]. HMGB1 has two high-affinity binding sites for LPS enabling complex formation [140]. Stimulation with preformed HMGB1-LPS complexes induces a 100-fold stronger response than stimulation with comparable levels of LPS alone [141]. The potent cytokine-inducing function of HMGB1-LPS complex has been demonstrated in multiple studies *in vitro* [141-144]. An exciting novel discovery regarding HMGB1-LPS complex function came out last year from a big joint study between Ben Lu's and Timothy Billar's labs. Researchers showed that the LPS-HMGB1 complex can be internalized via RAGE and lead to caspase-11-dependent lethality in sepsis [145].

HMGB1 pairs up also with other PAMPs and DAMPs to signal through TLRs. PGN from bacteria and Pam3CSK4, a synthetic triacylated lipopeptide, are exogenous TLR2 ligands that show enhanced stimulatory effect when complexed with HMGB1. Endogenous TLR2 ligands include nucleosomes, which can also for complexes with HMGB1 to stimulate cytokine production from primary macrophages in a TLR2-dependent manner [146]. TLR2 deficient mice produce less anti-dsDNA and anti-histone antibodies than wild type mice [147].

Nucleosomes bound to HMGB1 break immunological tolerance to double-stranded DNA and induce production of cytokines [146]. In 2007 an endosomal receptor, TLR9, was identified as a receptor for HMGB1 in a complex with DNA and same year was the direct binding of HMGB1 to TLR9 confirmed by immunoprecipitation [148, 149]. HMGB1-DNA complexes are recognized by RAGE and then transferred to endosomal TLR9 receptors mediating enhanced IFNγ production in plasmacytoid DCs and B lymphocytes [148].

When HMGB1 acts as a partner molecule, it can support signaling through few other receptors besides PRRs. It enhances the activity IL-1α and IL-1β and stromal cell–derived factor-1 (SDF-1/CXCL12) through their receptors [143, 150, 151]. While cytokine-inducing effects of pro-inflammatory molecule are enhanced by HMGB1 independently of its redox state, only fully reduced HMGB1 can interact with CXCL12 to act as a chemoattractant by activation of the CXCR4 [151-153]. In addition, interaction between fully reduced HMGB1 and RAGE induces upregulation of CXCL12 expression [154]. Thus, extracellular HMGB1 can play distinct roles at the same time: induce cytokine production on its own through TLR4 if in disulfide form or complex with LPS and recruit inflammatory cells to damaged tissue if in fully reduced form and bound to CXCL12. Interestingly, HMGB1-CXCL12 complex can also play a beneficial role by promoting tissue healing after solid organ injury [154]. Known PRRs of HMGB1 are presented in Figure 6 below.

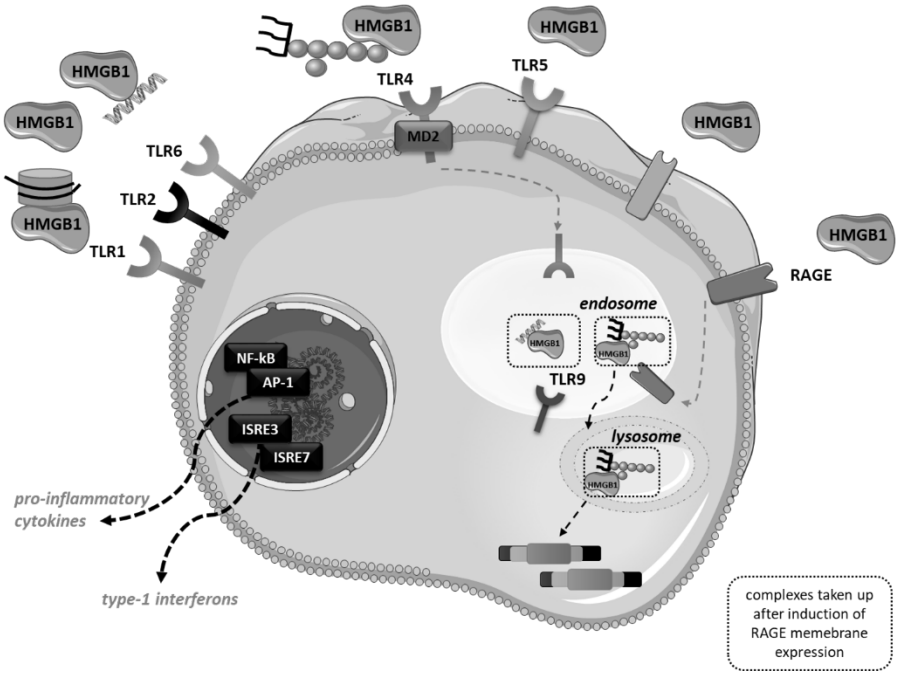

Figure 6. HMGB1 binding PRRs alone or in complex with other molecules*. HMGB1 is a ligand for TLR2, TLR4, TLR5 and RAGE. In addition, HMGB1 forms complexes to enhance other receptor-specific ligands, such as boosting LPS signaling via TLR4. HMGB1 and LPS complexes are internalized via RAGE, allowing HMGB1 to interact with endosomal and cytosolic PRRs.*

2.2.4 Downregulation of HMGB1

As many other soluble mediators of inflammation, HMGB1 is a subject for regulation that limits its pro-inflammatory activities. Several endogenous molecules bind and neutralize extracellular HMGB1. One of such agents is haptoglobin, a protein that binds free hemoglobin in blood plasma and directs it towards removal by the reticuloendothelial system. It has been shown that haptoglobin also forms complexes with fully reduced and disulfide HMGB1 (although with a lower affinity than hemoglobin) [155]. The haptoglobin-HMGB1 complexes are taken up by macrophages via the hemoglobin scavenger receptor CD163 and induce the production of anti-inflammatory agents, such as heme oxygenase-1 and IL-10. Moreover, plasma level of soluble CD163 is increased in a large spectrum of acute and chronic inflammatory disorders, further suggesting HMGB1 downregulation by haptoglobin and CD163 as an anti-inflammatory mechanism [155, 156].

There is another interesting relationship between HMGB1 and macrophages regarding the functional regulation. While disulfide HMGB1 directs monocyte differentiation towards pro-inflammatory (M1) macrophage phenotype, a complement protein C1q modulates HMGB1 effects on cell differentiation towards anti-inflammatory M2-like macrophages [157]. 24-hour exposure of monocytes to both HMGB1 and C1q leads to expression of classical M2 macrophage markers, such as Mer, PD-L1, IL-10 and CD163. However, C1q binding cannot completely cease pro-inflammatory functions of HMGB1 as it only mediates the reaction via RAGE inhibition while having no effect on HMGB1-TLR4 signaling [157].

As mentioned in §1.3.4, inflammatory mediators can also be downregulated by soluble receptors released to circulation. Such receptors bind extracellular HMGB1 and dilute its signaling. RAGE, which is a specific HMGB1 receptor, also exists in a soluble form lacking C-terminal and transmembrane domain. Soluble RAGE (sRAGE, an extracellular truncated form of RAGE that acts as a decoy receptor) disrupts HMGB1-RAGE signaling, thus dampening HMGB1 pro-inflammatory functions [158, 159]. Rheumatoid arthritis patients display low levels of sRAGE both in the blood and synovial fluid allowing pathogenic signaling of HMGB1 via RAGE [160].

One study reported that CD24, a membrane-bound protein modulating growth and differentiation signals received by mature granulocytes and B cells, also negatively regulates extracellular HMGB1 activity and inhibits NF-κB activation. Accordingly, CD24-deficient mice exhibit increased susceptibility to DAMPs. The process of HMGB1 downregulation by CD24 is partially mediated through Siglec G in mice or Siglec 10 in humans [161].

An interesting mechanism of HMGB1 downregulation utilizes activities of both an anti-inflammatory molecule and a protease. HMGB1 is degraded by thrombomodulin, an endothelial anticoagulant cofactor that promotes thrombin-mediated formation of activated protein C [162, 163]. Thrombomodulin binds and sequesters HMGB1 utilizing the N-terminal domain, which results in the proteolytic cleavage of HMGB1 by thrombin [162, 164].

Lastly, the NMR study of HMGB1 oxidation mentioned earlier (§2.2.1) also provided kinetic information about the clearance of the alarmin due to protease activities in extracellular fluids. Researchers noticed reduction of total HMGB1 but could not distinguish any smaller HMGB1

peptides. They concluded that once cleavage occurs, HMGB1 molecules become highly susceptible to complete digestion by extracellular proteases to an amino acid level [120].

2.3 PATHOLOGICAL ROLE OF HMGB1 IN DISEASE

Forming complexes with other pro-inflammatory mediators and signaling through different receptors enables HMGB1 to contribute to disease pathogenesis in multiple ways. The specific role HMGB1 plays in disease mechanisms depends on its release mode and the environment it enters. Factors such as the total amount of the alarmin, its redox state, availability of receptors and binding partners shape the net outcome of HMGB1 signaling.

HMGB1 signaling through TLR4 is mostly associated with negative effects. It has been implicated in the pathogenesis of ischemia/reperfusion injury of multiple organ systems including kidney, brain, heart, and liver [165-170]. Studies with experimental disease animal models of human disease also suggest a role for the HMGB1-TLR4 axis in lung inflammation and injury, inflammation following traumatic injury, as well as seizure generation and perpetuation in epilepsy [171-173]. HMGB1 is also a major player driving pathogenesis of sepsis and trauma, the leading causes of patient death in the Western world. Its negative role is also prominent in arthritis and DILI, which is described more in detail in the following chapters.

Interestingly, HMGB1 acts as a double-edged sword in cancer. The pro-inflammatory HMGB1-TLR4 signaling is crucial for effective responses to chemo-radiation in established tumors [174]. On the other hand, some studies suggest that HMGB1 can also contribute to immunosuppressive cancer microenvironment by association with receptor TIM-3 on DCs. TIM-3 actively competes with nucleic acids to bind the A box of HMGB1, thereby inhibiting antitumor efficacy of DNA vaccines and chemotherapy [175, 176].

Despite many deleterious activities of HMGB1 in the inflammatory disorders, there are also some positive effects of the alarmin. As explained in §2.2.3, fully reduced HMGB1 forms a heterocomplex with CXCL12 and acts via CXCR4 as an immunostimulatory chemoattractant. However, it also promotes tissue healing and regeneration. Injection of HMGB1 accelerates skeletal, hematopoietic, muscle and liver regeneration by acting on resident and infiltrating cells [154, 177]. Stem cells play a key role in the healing reactions as upon HMGB1-CXCL12 complex stimulation, quiescent stem cells that express CXCR4 transition from G_0 to G_{Alert} [154, 178]. Such primed stem cells respond to the activating factors released upon injury and readily enter the cell cycle, thereby promoting tissue repair.

2.3.1 HMGB1 during DILI

Liver cells, hepatocytes, express high levels of HMGB1. Thus, injury to the liver can cause massive HMGB1-induced inflammation. The mechanisms of HMGB1 release and inflammatory functions in liver diseases have been extensively studied in liver diseases, such as drug-induced liver injury (DILI). Actually, DILI is believed to be the best-characterized HMGB1-dependent condition in preclinical animal models and in patients.

As explained in §1.3.1 DILI can occur when paracetamol/APAP, a commonly used painkiller, is overdosed. The increase in drug dose or reduction of antioxidants in the liver increases the amount of a toxic by-product of paracetamol metabolism, NAPQI. The excess of metabolite is highly toxic for hepatocytes as it reacts with free cysteine residues, causes mitochondrial dysregulation, tissue hypoxia and decreased hepatic metabolism. Thus, the consequence of NAPQI challenge is cell function impairment followed by a combination of apoptotic and necrotic cell death in the liver. Necrotic hepatocytes passively release multiple DAMPs including the intranuclear fully reduced HMGB1, a ligand for RAGE. Moreover, the hypoxic environment is associated with increased expression of HMGB1 binding partner, CXCL12. The HMGB1-CXCL12 complex induces leukocyte recruitment to the liver via CXCR4 [152]. The leukocyte activation and influx into the liver results in a new phase of HMGB1 release. Now, immunogenic isoforms of HMGB1 are secreted by the activated immune cells and can intensify the inflammatory processes in the liver by signaling via TLR4 and RAGE. Animal studies with conditional knockout of HMGB1 indicate that the second wave of HMGB1 release is responsible for DILI-associated lethal inflammation [100, 167].

While more studies are needed to verify whether the mechanistic results from preclinical studies could be translated to the clinical setting, HMGB1 has already been proved as a useful biomarker for paracetamol-induced acute liver injury (ALI), an acute type of DILI. Interestingly, serum level of HMGB1 measured within 8 hours of drug overdose outperforms the commonly used liver injury marker alanine aminotransferase (ALT) as a predictor of ALI development [179]. This is extremely useful information for clinicians as it enables them to identify the first stage of APAP hepatotoxicity and adjust the patient treatment. Clinical symptoms of toxic drug ingestion typically present only in the third stage of the condition. Late patient arrival in the clinics is arguably the most challenging and devastating feature of DILI. It is during stage III of APAP hepatoxicity, when the maximal liver injury occurs leading to multi-organ failure [180]. This stage has the highest risk of mortality. Early biomarkers of DILI are of great need in order to recognize the pathological processes early and prevent patient mortality. HMGB1 is a good candidate for being such biomarker but not the perfect one. There are some disadvantages of the measurement of serum HMGB1 from APAP-overdose patients. Even though HMGB1 is expressed in high levels by hepatocytes, it is not specific for the liver cells. In fact, any dying or stressed cell in the body can release HMGB1. Therefore, measuring HMGB1 systematically can be misleading when the patient suffers from underlying inflammatory conditions. HMGB1 could be an informative biomarker when combined with liver-specific markers such as ALT and miR-122. There is also hope that HMGB1 isoform measurement could improve HMGB1 value as an early DILI biomarker.

2.3.2 HMGB1 in arthritis

HMGB1 also contributes to chronic inflammatory conditions, such as arthritis. While the cause of arthritis is not fully understood, the inflammatory reactions associated with the disease symptoms have been quite well defined. Probably the most striking evidence for HMGB1 as a key mediator driving the pro-inflammatory mechanisms in arthritis comes from animal studies. In a rat experimental arthritis model, HMGB1 was detected in all nuclear, cytoplasmic and extracellular compartments of synovial tissue while it was restrained in the nucleus in tissue of healthy rodents [181]. Moreover, researchers at Sahlgrenska University Hospital in Göteborg showed that intra-articular injection of recombinant HMGB1 into mouse joints

induced long-lasting destructive arthritis in healthy rodents [182]. They also complemented the study with detailed *in vitro* experiments showing HMGB1-induced activation of macrophages. Recently, another important discovery regarding HMGB1-driven disease symptoms came from animal studies. Signaling of disulfide HMGB1 through TLR4 induced long-lasting pain hypersensitivity associated with arthritis in mice [134].

Strong evidence of HMGB1 role in arthritis come from clinical studies. Examination of synovial fluid and synovial tissue biopsies indicate abnormal expression of HMGB1 in inflamed joints of RA and JIA patients. In fact, HMGB1 in the pannus tissue has been associated with the aggressive and destructive synovitis at the cartilage–bone interface [183]. *Ex vivo* assays with patient cells give insight in HMGB1 pro-inflammatory activities and the pathogenic mechanisms in arthritis. Endogenous HMGB1 interacts with TLR4 in synovial fluid extracted from arthritis patients most likely contributing to the severe disease profile [119]. Moreover, monocytes from RA patients require only a low concentration of HMGB1 to enhance CXCL12-induced migration indicating activity of HMGB1-CXCL12 heterocomplex is chronic inflammation associated with arthritis [184].

My research group has a special interest in understanding the mechanisms of JIA, arthritis in children. Our studies confirm that HMGB1 is significantly increased in the inflamed joints of JIA patients directly reflecting the extent of local inflammation. While other inflammatory mediators such as S100 proteins, IL-6 and IL-8 are increased at the disease onset, HMGB1 levels remain apparent during the active disease independent of disease duration [185]. Alarmin extracted from synovial fluid of JIA patients displays a pro-inflammatory profile. HMGB1 redox isoforms demonstrate mainly cytokine-inducing or chemotactic functions while the hyperacetylated form of the HMGB1 indicates its active secretion. Mono-methylation at lysine residue in box A of HMGB1 reveals its neutrophil origin [124].

While the analysis of tissue and fluid at the site of inflammation gives a valuable insight into disease mechanisms, molecules detectable in circulation are preferable as candidates for disease biomarkers. HMGB1 levels are significantly elevated in serum of RA patients and correlate with the disease activity score DAS-28 [186]. Therefore, HMGB1 measured in the joint provides detailed information about disease mechanism while blood measurement of HMGB1 is a general indicator of disease activity.

2.3.3 Dampening inflammation through anti-HMGB1 therapy

While HMGB1 can serve as diagnostic, prognostic and predictive biomarker of different inflammatory diseases, efforts are made to also evaluate it as a therapeutic target. Currently, there is no anti-HMGB1 therapy in clinics. However, targeting HMGB1 in experimental models shows promising results and highlights the need of introducing anti-HMGB1 therapy for patients suffering from conditions with inflammatory components. Different types of biologics have been developed in order to inhibit HMGB1 specifically, including antibodies, recombinant peptides of HMGB1 fragments and soluble receptors.

Several treatment strategies for targeting HMGB1 in DILI have been tested in mice by either inhibiting its release or function (or both). A classic method for targeting a molecule in circulation is developing recombinant antibody against it. This approach has been successful both with polyclonal and monoclonal antibodies. APAP- challenged mice have significantly

decreased serum HMGB1 concentrations and reduced gut bacterial translocation by 85% after treatment with polyclonal HMGB1 antibody [187]. Even more exciting results come from studies with monoclonal antibody against aa 53-63 of HMGB1, so called m2G7. Besides improving mouse survival, antibody reduced the liver injury size and APAP- induced inflammation [133]. Other promising treatment options targeting HMGB1 release in DILI include chemical interventions with ethyl pyruvate and therapy with naturally derived compounds such as glycyrrhizin [188, 189]. Currently, small peptides are receiving considerable attention as an alternative strategy to target HMGB1. A tetrameric peptide FSSE (named P5779) blocks HMGB1 interaction with myeloid differentiation factor 2 (MD-2), thereby preventing disulfide HMGB1 signaling through TLR4. P5779 shows therapeutic effects by reducing elevation of hepatic serum enzymes and TNF, liver necrosis, and by improving survival in mouse model of DILI [133].

HMGB1 blockade has also been successful in arthritis models. Multiple experimental studies demonstrate improved outcome after treatment with HMGB1-specific antagonists. Systemic administration of polyclonal neutralizing HMGB1 antibody and recombinant box A in collagen-induced arthritis (CIA) and a spontaneous chronic, destructive polyarthritis significantly reduced the mean arthritis score, the disease-induced weight loss, and protected against the devastating destruction of cartilage and bone. Importantly, HMGB1 suppressed arthritis associated inflammation in rodents [190, 191]. The monoclonal HMGB1 antibody, m2g7, was also tested in those two mouse arthritis models. Therapeutic anti-HMGB1 intervention significantly ameliorated the clinical disease course and partially prevented joint destruction, as demonstrated by histological examination [192]. Monoclonal HMGB1 antibody has also reduced pain hypersensitivity associated with arthritis [134].

Whilst the mechanism of action for certain drugs used in the treatment of arthritis patients is not fully understood, studies suggest that they may target HMGB1. Methotrexate (MTX), a standard disease-modifying antirheumatic drug, is a good example. The majority of patients responding to MTX experience less pain, joint swelling and lower disease activity overall [193]. Recent studies show that MTX binds to HMGB1 and prevents its association with RAGE. *In vitro* studies on murine macrophage-like cells confirmed that MTX inhibit TNF release dependent on specific RAGE stimulation with HMGB1. Therefore, HMGB1 inhibition is part of the mechanism of action for the anti-inflammatory effect of MTX [194]. Another example is gold sodium thiomalate (GST), a gold compound that has been administered as injections to treat RA for many years. Although GST is not a popular treatment any more, its inhibitory effect on HMGB1 is noteworthy. Activated macrophages do not release HMGB1after GST stimulation as GST interferes with intracellular HMGB1 transport mechanisms [195].

Currently, more therapeutic tools against HMGB1 are being developed and tested in different experimental disease models. They include administration of recombinant HMGB1 downregulators, such as haptoglobin, thrombomodulin and soluble RAGE. As these molecules have a broad set of substrates, monoclonal HMGB1 antibody and small recombinant molecules may a better approach to target HMGB1 specifically.

2.4 BIG CHALLENGES IN HMGB1 FIELD

While experimental studies show very promising results, there are still numerous barriers to cross to bring anti-HMGB1 therapy to clinics. Ubiquitous expression and complex biology of HMGB1 have been discouraging pharmaceutical companies to examine HMGB1 as a target in disease. Therefore, there is a big need to understand HMGB1 biology in terms of its release, structure and PTMs. The academic research centers have taken on the responsibility to expand knowledge.

The most desired goal for anti-HMGB1 therapy would be a straightforward method to identify and inhibit pro-inflammatory isoforms of HMGB1. Unfortunately, the isoform identification is currently dependent on a time consuming and expensive mass spectrometry analysis. There are only a few laboratories worldwide capable of measuring HMGB1 isoforms and the central one has been dealing with scientific misconduct. Several recently published studies based on collaboration with this lab need verification. Lastly, there is also limited knowledge regarding total levels of HMGB1 in the healthy individuals [196]. Lifestyle factors, such as smoking, alcohol drinking and Western-style diet can potentially increase the extracellular systemic HMGB1 levels [197, 198]. It is therefore unclear what levels of the alarmin are not necessarily alarming. All these factors decelerate progress in clinical HMGB1 research.

Addressing the big challenges in HMGB1 field requires a good collaboration of dedicated researchers all over the world and can take long years of hard work. My efforts to expand knowledge about HMGB1 biology and its role in inflammation resulted in the experimental studies described in the following chapters and attached to this thesis. While the presented work is a just a small puzzle in the big HMGB1 field, it is the small pieces that make the big picture.

3 AIMS OF THE THESIS

It is well established that HMGB1 plays a major role as a DAMP in the pathogenesis of inflammatory diseases. However, it is not fully understood how the protein exerts its functions and how it is regulated in the extracellular space. The aim of this thesis was to characterize HMGB1 release patterns from immune cells and its pro-inflammatory activities. Moreover, we set out to investigate endogenous down-regulatory mechanisms controlling HMGB1 extracellular functions.

Blockade of HMGB1 has shown promising results in multiple animal disease models. In order to evaluate the HMGB1 potential as a therapeutic target and facilitate anti-HMGB1 therapy development in clinical setting, we decided to develop a novel therapeutic tool and test it in an established animal disease model.

The specific aims of this thesis are to:

- Understand how and to which extent monocytic cells release HMGB1 during immune activation and different modes of cell death

- Elucidate whether HMGB1 is a substrate for proteases associated with chronic inflammatory conditions; and whether such processing results in formation of functional fragments and /or HMGB1 functional downregulation

- Investigate HMGB1 interaction with TLR2 and determine the functional outcome of the receptor binding

- Develop a humanized anti-HMGB1 antibody as a potential therapeutic option and test its effect in an inflammatory disease model

4 METHODOLOGICAL CONSIDERATIONS

While the methods used in each study are listed in the Material and Methods sections of the publications and manuscripts attached to this thesis, my reasoning behind their selection and concerns regarding their use are discussed below. One should remember that all methods have advantages and flaws. The researcher has the responsibility to select procedures and materials appropriate for testing the scientific hypothesis. Despite constant advances in molecular biology tools and methods, the reality of basic research in academia is that one often needs to focus on the resources available in lab.

4.1 RECOMBINANT PROTEIN PRODUCTION

My studies relied heavily on recombinant HMGB1 expressed and purified from *Escherichia coli* (*E. coli*). The standard protocol for recombinant protein production involves the construction of an expression plasmid followed by a pilot experiment to assess the yield and solubility of the target protein. Successful completion of the optimization steps is followed by large-scale protein expression and purification. Experienced researchers in our lab constructed the plasmids used in these studies before my experiments commenced. For publication purposes, our HMGB1 proteins contained either a calmodulin-binding protein (CBP; rat HMGB1) or a cleavable 6-residue polyhistidine (HIS; human HMGB1) tag at the N-terminus of the protein. Importantly, both of these tags have no known effect on HMGB1 function [199].

In my studies, I have used both rat and human HMGB1 proteins. HMGB1 is a highly conserved protein, which exhibits 99% sequence homology across species. There are only two amino acids differences between rodent and human HMGB1: D189E and E202D. Considering that these substitutions occur within the C-terminal tail of the protein, and that glutamic and aspartic acid residues have similar properties, the slight sequence difference is highly unlikely to influence protein function. However, other factors could affect the activity of lab-produced HMGB1. These include purity, mismatches in expression and PTMs.

As discussed in the introduction of this thesis (§2.2.3), HMGB1 forms highly active pro-inflammatory complexes by binding both LPS and DNA. Endotoxin and DNA contamination can result in misjudgment of HMGB1's cytokine-inducing activity, a common problem that affected the field in the early years of HMGB1 research. When producing the recombinant proteins used in this these experiments, LPS was eliminated using Triton-X114 purification and bacterial DNA was removed by DNase I treatment. To confirm that these removal methods were efficient, endotoxin and DNA levels were measured using the limulus amoebocyte lysate assay and agarose gel electrophoresis, respectively.

Our group, and other researchers, have observed that bacteria is inefficient in producing full-length HMGB1 protein due to the long stretch of repeating glutamic and aspartic acid residues present in the acidic C-terminal tail domain [199]. Therefore, recombinant HMGB1 purified from *E. coli* usually consists of a mixture of full-length and C-tail truncated HMGB1 proteins. While partial loss of the C-terminal tail can be visualized using SDS-PAGE gel electrophoresis, smaller differences in the protein sequence can only be assessed by mass spectrometry analysis.

For the experiments included in Paper III, we used the different HMGB1 proteins (full-length HMGB1, full C-tail truncated HMGB1 and partially C-tail truncated HMGB1) to investigate the effect of the tail domain on the interaction with TLR2. C-tail domain is believed to control access to the DNA binding boxes of HMGB1 [200]. Therefore, it is important to analyze the output of the protein production.

I will conclude this section with a brief discussion about the most important factor that influences HMGB1 function: the redox state of the cysteine residues. After the HMGB1 protein is purified, additional tests must be performed to examine the biological activity of the synthesized protein. To determine the cytokine-inducing activity of our recombinant proteins, we measured pro-inflammatory cytokine production (e.g. TNF) after addition to peripheral blood mononuclear cells (PBMCs). To generate the fully reduced HMGB1 protein, we add a redox reagent, dithiothreitol (DTT), to the purification buffers. *E. coli* expresses a periplasmic enzyme that is required for catalyzing disulfide bonds [201]. However, production of disulfide HMGB1 remains challenging.

4.2 *IN VITRO* EXPERIMENTS

My thesis work was also heavily based on *in vitro* experiments: immortal cell lines (Papers I and III), primary PBMC cultures (Paper III) and enzymatic reactions in Eppendorf tubes (Paper II).

Immortal cell lines come from tumorous cells or cells with artificially introduced mutations in order to proliferate indefinitely. The most commonly used and oldest example of such a cell line are HeLa cells derived from cervical cancer cells of a deceased patient in 1951 [202]. Possibility of culturing a homogenous cell population over several generations is the major reason for choosing immortal cell lines over primary cells in the scientific studies. In addition, there are more advantages of immortalized cell lines. Standard well-characterized lines help to obtain reproducible results and bypass ethical concerns associated with the use of animal and human tissue. Thus, they are a common thread in experimental research performed in labs worldwide. The main goal in selecting appropriate methodology for Paper I was to create an optimized well-controlled system for systematic comparison of cell death modes. THP-1 is a spontaneously immortalized cell line isolated from the peripheral blood of a child suffering from acute monocytic leukemia and can be cultured for about 3 months without changes of cell sensitivity and activity [203, 204]. Although THP-1 cell became the gold standard for studying immune responses of monocytes and macrophages, it also has downsides [204]. While the use of cultured THP-1 cells as a model for primary human monocytes *ex vivo* constitutes the basic concept of translational research, multiple studies show significant differences between them [205]. THP-1 is a monocyte-like cell line and should not be confused with monocytes as immortalized cell lines can express unique gene patterns not found in any cell type *in vivo*. On the other hand, once the cells are differentiated to macrophages, they express similar markers to PBMCs. Another issue is the spontaneous mutation leading to THP-1 cell immortalization that could affect responses to induction of different cell death modes in my experiments. In order to overcome this issue, doses of death inducing agents were optimized (Paper I, data not shown). Lastly, immortalized cells are studied in a controlled setting that is different from their

natural complex environment. Therefore, it is desirable to compare the results with experiments in primary cells before drawing strong conclusions.

Another advantage is a possibility of creating a cell line expressing a gene of interest. This was the basis of choosing human embryonic kidney cell line (HEK293) as experimental model in Paper III. HEK293 cells are widely used in cell biology research due to high success rate of transfection. In order to scrutinize the relationship between HMGB1 and its receptor TLR2, HEK293 were transfected either only with TLR2 or along TLR1/TLR6. Depending on the stimulant, TLR2 forms heterocomplexes with TLR1and TLR6. Utilizing three separate systems based on the same cell line can provide specific information about the ligand. On the other hand, such a straightforward approach creates a risk of oversimplification of complex cellular processes. Therefore, we also performed experiments in a mouse alveolar macrophage (MH-S) cell line, which expressed all TLRs and the CD36 receptor important for TLR2 activation [206, 207].

Lastly, the lack of positive results in the cell lines lead us to perform experiments in more complex primary systems, PBMCs and whole blood of healthy volunteers (Paper II). Primary cultures are formed by culturing cells directly obtained from blood or tissue biopsy. PBMCs are isolated from peripheral blood and consist of lymphocytes, monocytes, natural killer cells and dendritic cells. Thus, PBMCs are the major cells in human immunity. While the frequencies of cell populations differ between donors and even can be vary in same donor on different days due changes in the donor's physiological status, consistency of results obtained from separate donors strengthens the scientific observation. One drawback in using PBMCs in our studies is their skew towards the adaptive immunity. Lymphocytes are the dominating cell population in PBMCs while cells of the innate immune system we focus our studies on constitute only up to 20% of the collected cells. Another drawback of primary cells is short life span of the cultures. It is not possible to culture PBMCs over several passages. As researchers most often need to work on freshly isolated cells, the number of donors and extracted blood volume create limiting factors in experiments. Similar issues apply to whole blood assays, where freshly collected blood is stimulated and cultured *ex vivo.* Although cells are not separated in such assay, they might respond to the environmental change by losing their structural and functional characteristics.

In summary, cell lines provide an elegant system to study detailed immune reactions and understand processes taking place in more complex settings. However, one should always verify the results in a native primary cell setting. It is crucial to select the appropriate experimental system and think about readouts.

4.3 EXPERIMENTAL ANIMAL MODELS

Animal models of human disease provide a practical tool for studying disease-driving immune reactions *in vivo*. They also constitute an important step towards translation of basic research into human disease and help to predict the outlook for novel therapeutic targets. On the other hand, there are many drawbacks and ethical controversies regarding use of animals in research. Most of the animal models represent only one or several symptoms of a condition but do not address the complete human disease pathogenesis. Therefore, the researcher needs to specify which aspect of the disease is the study focus before selecting an animal model. Moreover, the

study results need to be validated in different pre-clinical disease models before clinical trials could be allowed. Even such a stringent approach is not guaranteeing success in human therapy. Unfortunately, many therapies show promising results in experimental models but fail in clinical trials [208]. Another major concern regarding animal use in research is ensuring their ethical use in studies. In order to support animal welfare, the "three Rs" rule was introduced in 1959: Replacement, Reduction and Refinement [209]. This rule was followed in the study design for evaluation of a humanized, chimeric anti-HMGB1 antibody effects in DILI (Paper IV).

Replacement

Efforts are made to avoid animal testing and use *in vitro* models. While traditional cell culture models fail to predict hepatotoxicity of the tested drugs, the novel 3D culturing systems could be a solution for pharmacological studies [210]. However, our study focus was not on the drug hepatoxicity but the HMGB1-mediated inflammation. Liver tissue culture models do not reflect the role of the immune system in DILI development. As there was no replacement possibility in order to carry out a pre-clinical study, we chose to utilize the standard dose-dependent mouse model of DILI with well-characterized involvement of HMGB1.

Reduction

Our strategy was to use the minimum number of animals needed for statistical power in results analysis. Unfortunately, this approach forced us to reduce the quality of life of the experimental animals by fasting them 15 hours prior to APAP challenge. Putting the mice in such metabolic state allowed us to reduce inter-subject variation and see stronger effect in DILI, and thus reduce the number of animals needed to obtain sufficient data to answer our research question. Another way to minimize the number of experimental animals is to maximize the information obtained per study object. We analyzed liver and blood for established biomarkers of DILI and the presence of inflammatory mediators.

Refinement

We chose the acute model of DILI with respect to animal welfare. Staff at Karolinska animal facility ensured the animal quality of life and independently evaluated whether the experimental procedures aimed to minimize pain and distress.

In summary, we chose an experimental mouse model of DILI with already described HMGB1-mediated inflammation. APAP-induced liver toxicity in mice is a straightforward approach for pre-clinical evaluation of HMGB1-blocking therapies and does not harm animal welfare.

4.4 CLINICAL SAMPLES

While my thesis work focused on the mechanisms of HMGB1 signaling in inflammatory conditions in general, it also involved examination of human samples. My research group, together with the Pediatric Rheumatology division at Karolinska University Hospital, has established a biobank of JIA patients' samples, which include serum and synovial fluid (SF). While many assays in research facilities and clinics are optimized for plasma and serum measurements, only a few are compatible with SF. This is due to the very viscous physical state

of SF and the limited sample volume that can be obtained from patients only during a disease flare.

For the study described in Paper II, I selected SF samples with complete clinical data and characterized isoforms of HMGB1 [124]. The samples contained high total levels of HMGB1 with chemotactic and a cytokine-inducing function. Additionally, monomethylation of lysine 43 of the protein indicated that the HMGB1 present in the sample originated from neutrophils [124]. Therefore, those samples were suitable material for investigating the effect of neutrophil enzymes (elastase and cathepsin G) and other disease-associated enzymes present in the joint (matrix metalloproteinase 3) on the pathogenic extracellular HMGB1.

The SF samples were chosen to study HMGB1-regulating processes at the local site of inflammation. However, one cannot extrapolate the results to the pathogenesis of JIA. The sample size was small and heterogenous with regard to JIA sub-diagnosis and treatment. In addition, the sample selection did not follow the 2:1 female-to-male patient ratio apparent in the clinic.

4.5 DETECTION AND QUANTIFICATION OF HMGB1

In order to allow for a proper comparison and to increase the reproducibility of the findings, it was that we had a reliable HMGB1 detection method. The HMGB1 ELISA developed by Shino-Test Corporation was first introduced onto the market approximately 10 years ago and it is regarded as the gold-standard assay for measuring HMGB1 in both cells supernatants and biological samples [211]. Therefore, I chose to use this assay as the primary detection method for measuring total HMGB1 levels throughout my thesis work. While it is preferable to use a standard detection assay, it is important to remember that every method has also its limitations. As mentioned in the introductory chapters of this book, HMGB1 forms complexes with other DAMPs and immune mediators. Although the epitope for the antibody used in the HMGB1 ELISA is confidential, it is most probably located within/close to box B of the protein. We noticed that recombinant box A is undetectable in the assay (unpublished data). It is also possible that molecules bound to HMGB1 interfere with HMGB1 detection in biological samples due to epitope masking [212, 213].

In addition to using the commercial ELISA, I have also used western blotting analysis, another standard method for protein detection, to measure HMGB1. Most of the epitopes of the antibodies used in these experiments are known. Detecting HMGB1 with antibodies against both the N- and C-terminal domains is superior over ELISA as it confirms presence of full-length protein. In contrast, the Shino-Test ELISA does not distinguish between full-length and truncated protein. Using western blotting analysis with multiple anti-HMGB1 antibodies allowed us to map the different peptides formed after enzymatic cleavage of HMGB1 (Paper II). However, the technique also has its drawbacks as it requires a relatively high concentration of HMGB1 in small sample volume and assay optimization for each individual antibody.

While the abovementioned methods are useful for measuring total HMGB1 levels, they do not detect PTMs, and thus it is not possible to distinguish between the different redox

isoforms or identify hyperacetylated protein using this assay.s To date, mass spectrometry is the only technology that allows such analysis. During my PhD studies, LC-MS/MS was used for characterization of targeted PTMs. The output of this technique was area under the curve (AUC) values for specific peptides, which allowed identification of redox isoforms and hyperacetylation of NLS2 in a qualitative manner. However, access to this method is very limited. As HMGB1 is a major player driving the pathogenesis of multiple inflammatory diseases, efforts should be made to develop a straightforward and high throughput technique to measure the different functional isoforms of HMGB1.

4.6 MAPPING PROTEASE CLEAVAGE SITES IN HMGB1

I separated the peptide/protease mixture on a SDS-PAGE gel in order to identify peptides resulting from HMGB1 proteolytic cleavage (Paper II). The standard Tris/glycine gels 4-20% gradient gels were used for that purpose. I expect I would have obtained an improved resolution of small peptides if I used Tris/tricine gels, which slow down the migration rates of peptide-SDS complexes. On the other hand, I was primarily interested in the initial products of the proteolytic cleavage and Tris/glysine gels were sufficient for the separation.

The samples were investigated by western blotting as described above. In addition, selected gel bands containing HMGB1 peptides were analyzed by mass spectrometry, nanoLC-MS/MS. This method allowed identification of peptide sequences. However, the method required in-gel trypsin digestion of the fragments in question. Trypsin preferentially cleaved peptides on the C-terminal side of lysine and arginine amino acid residues. High number of these residues in HMGB1 sequence (over 20%) made it difficult to identify the exact protease cleavage sites. Therefore, we could only confirm the coverage of peptides detected with SDS-PAGE and western blotting using this method. Mass spectrometry assay that could analyze the peptides without enzymatic digestion would be preferred. Alternatively, an enzyme with fewer cleavage sites within HMGB1 sequence could be chosen for in-gel digestion of the protein fragments.

A higher resolution of the peptide/protease mixture could also be obtained by 2D gel electrophoresis. The method could be successful due to the dipolar nature of HMGB1 [214]. The negatively charged aspartate and glutamate amino acid repeats make the C-terminal tail domain prone to protonation at low pH [215]. The separated differentially charged peptides could be then analyzed by mass spectrometry.

Published literature and specialized online tools were helpful with identification of the predicted cleavage sites. We used PROSPER (https://prosper.erc.monash.edu.au/), a webserver for *in silico* prediction of protease substrate in order to speculate about the cleavage sites. However, the protease cleavage sites predicted by the abovementioned methods would require further verification. Site-directed mutagenesis of specific residues could confirm the expected cleavage sites.

5 RESULTS AND DISCUSSION

5.1 HMGB1 IN INFLAMMATION: REGULATION OF HMGB1 RELEASE FROM THE IMMUNE CELL

As a prototypical DAMP, extracellular HMGB1 is associated with stress or injury in the body [106]. Multiple studies have reported that circulating HMGB1 is a result of immune cell activation and cell death. However, it has never been shown in a well-controlled manner how a single immune cell contributes to the extracellular levels of HMGB1 under different physiological conditions. Therefore, we decided to induce different modes of cell death in vitro in THP-1 monocyte-like cells; a cell line that is commonly used to study functions of human monocytes and macrophages.

In Paper I, we confirmed that pure apoptosis does not lead to HMGB1 release while necrosis results in a passive release of intranuclear HMGB1. Other types of immunogenic cell death also result in HMGB1 release, although to a lesser extent. Our results suggest that dying cells release HMGB1 with different kinetics. Alamin release happens in a controlled manner unless cells become necrotic. In addition to measuring the extent of HMGB1 release during different physiological conditions, we looked at other markers of cellular immunogenicity. While the release of the cytosolic enzyme LDH and alarmin HMGB1 without active production of pro-inflammatory cytokines during necrosis was expected from previous studies, interesting information about immunogenicity of different cell death modes could be derived from other experimental conditions [106].

Cell populations maintain homeostasis by removing aged and impaired cells from tissues by homeostatic mechanisms of apoptosis. We activated intrinsic pathways of apoptosis by two different inducers: the clinically relevant topoisomerase II inhibitor, Etoposide, and an unspecific prototypical ATP-competitive kinase inhibitor, Staurosporine (STS). Subsequently, we monitored apoptosis induction for up to 24 hours by analysis of activation of apoptotic effector caspases and cellular membrane permeability. Extracellular HMGB1 was detected only after membrane disintegration, as confirmed by LDH release. Being the homeostatic type of cell death, apoptosis was neither associated with release of cytosolic components nor secretion of pro-inflammatory molecules. Our data confirmed that STS is a potent inducer of apoptosis displaying a more rapid kinetics than topoisomerase II inhibition. However, cells released LDH and HMGB1 even before the peak of the apoptotic effector caspase activity. Lack of TNF and IL-1β in the supernatant suggests that the cells underwent secondary necrosis. No pro-inflammatory molecules were detected in Etoposide-induced apoptosis, which suggest successful formation of apoptotic bodies and further supports use of Etoposide as cancer drug in the clinics. This result complements our earlier study, where patients suffering from macrophage activation syndrome treated with Etoposide showed reduced levels of systemic HMGB1. Detected HMGB1 was only in the sulfonyl redox form in contrast to the fully reduced and disulfide HMGB1 isoforms present in serum before the treatment [216].

Lack of ATP hinders caspase activity and prevents cells from undergoing apoptosis. If cells are simultaneously subjected to stress, the intracellular processes lead to the activation of necroptotic pathways. Necroptosis is a cell death type, which serves as an intrinsic host defense

against viral infection. This cell death mode is also associated with certain ischemic conditions. Metabolism in cells is affected by the oxygen shortage resulting from the restricted blood supply. Limited energy supplies impede function of caspases that normally signal towards homeostatic apoptosis. When the distressed cells sense an additional stressing factor, such as acute-phase pro-inflammatory cytokine TNF, they activate receptor-interacting protein kinase 1 (RIPK1) and RIPK3 that lead to JNK activation, ROS production and the induction of necroptosis. I induced necroptosis by blocking apoptotic caspase cascades and stimulating cells with LPS, a potent inducer of TNF transcription. We then followed the release of HMGB1 for 24h in a kinetic manner and noticed the slight increase of HMGB1 levels 18 hours after necroptosis induction. Interestingly, Necrostatin 1(Nec-1), a potent inhibitor of RIPK1, did not affect the alarmin release. High dose of LPS caused constant release of TNF and likely subsequent activation of the cell death receptor, TNFR1. However, there was no difference in the readouts upon blocking RIPK1 with its allosteric inhibitor Nec-1. This compound was originally discovered as a blocker of necrotic cell death in human and murine cells [49]. In the initial optimization experiments, Nec-1 reduced cytoxicity associated with necroptosis (data not shown). Since the Nec-1 effect on cytotoxicity could not be replicated in the study described in Paper I, Nec-1 inhibition of cytotoxicity would require further verification. In addition, experiments using other and more stable specific RIPK1 inhibitors might explain the poor effects of Nec-1 [217].

While HMGB1 release from pyroptotic cells was reported before, the exact release mechanism has never been clarified in relation to the activated inflammasome. We noticed that HMGB1 was released from stimulated PMA-differentiated THP-1 cells independently of NLRP3-inflammasome-dependent pyroptosis. The two-hit system for inflammasome activation with LPS and nigericin, a bacterial-derived potassium ionophore, was needed to induce IL-1β production associated with NLRP3 inflammasome but not necessary for HMGB1 induction. The high sensitivity of cells was likely caused by PMA differentiation. There is a possibility that HMGB1 would only be released from the pyroptotic cells after a longer post-differentiation resting period. LPS and nigericin as well as nigericin alone caused high release of cytosolic LDH. Thus, nigericin not only induced formation and activation of the NLRP3 inflammasome but also induced cell death.

Cellular activation in the pyroptosis experiments inspired us to further investigate HMGB1 release from the challenged cells. We decided to take a step back and use undifferentiated cells in order to assess their ability to secrete HMGB1 upon activation. THP-1 cells stimulated with 100 ng/ml LPS produced induced secretion of approximately 40 ng/ml HMGB1 after 24 hours. However, LDH release and low level of IL-1β in the supernatant indicated ongoing cell death, possibly pyroptosis. Interestingly, co-stimulation with IFNγ did not affect neither HMGB1 release nor the cytotoxicity. Our results suggest that immunogenic cell death pathways such as necrosis and pyroptosis produce more extracellular HMGB1 than the monocyte-like cells activated with LPS + IFNγ.

Surprisingly, no HMGB1 was detected in the NETs of neutrophils undergoing NETosis. Previous report showed that neutrophils carried HMGB1 predominantly in the cytoplasm instead of nucleaus, which was associated with methylation of lysine at position 42 of HMGB1 [218]. The PTM within box A possibly weakened HMGB1 binding to DNA. As NETs were

primarily composed of DNA, HMGB1's absence could be expected. I could speculate that HMGB1 would be released by activated neutrophils before they proceed to NETosis. HMGB1 that remained in the cell would also be released during NETosis but would not attach to the NETs.

5.2 EXTRACELLULAR HMGB1 AT THE SITE OF INFLAMMATION

After demonstrating that HMGB1 is released from the monocytic cells into the extracellular space though multiple mechanisms (Paper I), we decided to follow the fate of the alarmin (Paper II and Paper III).

5.2.1 Proteolytic processing of HMGB1: new regulatory mechanism

While pro-inflammatory signaling is necessary for the activation of defense and repair systems, the downregulatory mechanisms should operate in concert to limit result in acute inflammation. Extracellular HMGB1 is present not only during the acute immune response but also during dysregulated chronic inflammation, for example in the synovial fluid of arthritis patients. Therefore, we designed a study to investigate HMGB1 regulation at the site of inflammation (Paper II). Previous study showed that mast cells, abundantly present in synovium, secrete chymase, serine peptidase stored in their secretory granules, to degrade HMGB1 and other alarmins [92, 219, 220]. Our study further explored the potential effect of synovial endopeptidases on HMGB1. Neutrophils are found in high numbers in the inflamed rheumatic joints and undergo degranulation to secrete proteases upon activation. Serine proteases such as Human Neutrophil Elastase (HNE), Cathepsin G (CG) and proteinase 3 (PR3) are the major group of endopeptidases abundantly secreted by those cells to the synovial fluid. Liberated enzymes are important regulators of inflammation due to their ability to process multiple chemokines, cytokines and cell surface receptors in order to activate or deactivate them. For example, HNE and PR3 cleave the precursor of TNF to its mature form. Both HMGB1 and neutrophils are found in synovial fluid of arthritis patients. We thus speculated that neutrophil serine proteases could affect the pro-inflammatory features of the released alarmin.

We studied the published literature and utilized PROSPER software for *in silico* prediction of protease substrates and their cleavage sites to identify whether HMGB1 was a substrate of HNE and CG. We confirmed the presence HNE and CG in synovial fluid of JIA patients at average levels of 278.1 ng/ml and 60.2 pg/ml, respectively. After performing an initial optimization and screening, we saw a consistent cleavage pattern of HMGB1 by HNE in SDS-PAGE. Initial cleavage lead to the formation of a peptide with molecular weight close to the one of the full-length protein. We hypothesized that such proteolytic cleavage could potentially lead to the formation of HMGB1 fragments with altered features. Multiple studies with recombinant peptides suggest that box B of HMGB1 alone can act as pro-inflammatory agent while box A can act as an HMGB1 antagonist. However, no reports exist that could confirm the presence of such fragments *in vivo*. Western blotting and mass spectrometry analysis indicated an initial and gradual truncation of the C-terminus tail of HMGB1 upon HNE processing. Based on the prediction model, we also suspected that HMGB1 could be cleaved at the N-terminus later in the process. While the loss of the acidic C-terminal tail could expose the receptor binding sites

within HMGB1, N-terminal cleavage could also serve as a regulatory switch. Most proteolytic events that activate members of the IL-1 cytokine family and multiple chemokines take place within a limited region at the N-terminus. Partial protein degradation could also result in improved accessibility of receptor binding sites and thereby improve the binding of HMGB1 to its receptors. The C-terminal tail domain was previously reported to make extensive contacts with the DNA-binding surfaces of both HMG boxes [221]. One could speculate that only this initial cleavage of HMGB1 by HNE might take place *in vivo* as the proteomic analysis of the synovial fluid of RA patients reveled upregulation of HNE inhibitory complex [222].

On the contrary, it was not possible to identify any fragments of HMGB1 after its processing by CG. At molar ratio 1:80 CG completely degraded HMGB1 within 5 minutes. Hence, we concluded that CG inhibits pro-inflammatory activities of HMGB1 by protein disintegration. This result strongly suggests that the CG's proteolytic cleavage is a novel mechanism of HMGB1 clearance at the local site of inflammation during arthritis. However, CG levels did not correlate with HMGB1 in the synovial fluid of JIA patients. Due to the lack of direct relationship between CG and HMGB1 in the patients' samples and identified broad spectrum of CG substrates, CG might not be a promising therapeutic agent for HMGB1 downregulation. It is also unclear whether redox and acetylation of HMGB1 affect proteolytic regulation. We used recombinant HMGB1 while the endogenous HMGB1 in JIA patients had different PTMs [124]. Nevertheless, our results provide an insight into endogenous clearance of HMGB1 by activated neutrophils at the inflammatory site.

In addition to studying the neutrophil serine proteases' activity on extracellular HMGB1, we also examined whether other arthritis-associated enzymes could process the alarmin. Matrix metalloproteinase 3 (MMP-3) is a cysteine endoprotease driving the cartilage degradation in arthritis. MMP-3, expressed in synovial tissue by endothelial cells and fibroblasts, is upregulated both in synovial fluid and tissue of RA patients [223]. Moreover, clinical studies indicate a direct correlation between MMP-3 serum levels and the progression of joint destruction in patients with early RA [224, 225]. MMP-3 was detected in all SF samples at significantly higher levels than serine proteases, with an average of 31.5 μg/ml. Similar to HNE, MMP-3 cleaved HMGB1 at the C-terminal tail. We analyzed two peptide products of the MMP-3 cleavage by WB and MS (fragment I and fragment II in Fig. 4 of Paper II). Although the fragments differed in size on the SDS-PAGE and WB, the cleavage mapping assays showed identical results. Both C-terminal tail and its linker region were missing in the examined fragments. Therefore, we proposed that the higher molecular weight peptide (fragment I, Fig. 4, Paper II) consisted of both box A and box B. Such fragment could retain the pro-inflammatory activities of the full-length HMGB1. As it is not possible for a lower molecular weight fragment to have exactly the same sequence, we suggest that fragment II in Fig. 4 of Paper II is actually a mixture of two peptides of equal molecular weight. It is difficult to speculate about the possible functions of such peptides. It is, however, apparent that both HMGB1 fragments contain amino acids 89-109, the minimal sequence needed for cytokine activity.

Our results were based on HMGB1 produced in *E. coli*, which is a common system of protein expression and usually results in high and homogenous protein yields. However, the results should be verified in studies with mammalian expressed HMGB1, for example Chinese Hamster Ovary (CHO) cells. It was shown that only HMGB1 expressed CHO cells had

glycosyl modification [199]. Therefore, a possibility exists that there are other PTMs in mammalian expressed HMGB1 that could not be retained in the bacterial system. PTMs could affect the affinity of the proteases to their cleavage sites. As discussed earlier, the actively secreted HMGB1 at the inflammation site is hyperacetylated at its NLSs. In order to strengthen our findings about proteolytic regulation, experiments should be reproduced in acetylated HMGB1.

In conclusion, we were able to identify a novel mechanism of HMGB1 regulation at the local site of inflammation by demonstrating the rapid degradation of the alarmin by and removal of the C-terminal tail domain by HNE and MMP-3. Protein truncation could liberate functional fragments of HMGB1 that were shown to exhibit either pro- or anti-inflammatory functions as recombinant peptides in the previous *in vitro* studies. It is not known whether PTMs of HMGB1 could affect the proteolytic regulation.

5.2.2 Understanding HMGB1 interaction with its receptor TLR2

Although we never cloned and expressed any of the predicted enzymatic cleavage products, we received a recombinant HMGB1 truncated at the C-terminal tail as a "gift" from bacteria during the protein production. Our lab and others noticed that truncated protein is a common by-product of HMGB1 production in *E. coli* when N-terminal tags are used for protein purification [226, 227]. We decided to investigate whether the protein truncation could affect the receptor binding ability of HMGB1.

TLRs are key components of the innate immune response. Several members of the TLR family have been reported to interact with HMGB1 including the transmembrane receptors TLR2, TLR4 and TLR5 [119, 137, 228]. Previous studies reported that the C-terminal tail presence is dispensable for TLR4 binding but essential for activating TLR5-mediated pro-inflammation [119, 137]. Conflicting data in the literature exists regarding HMGB1 binding with TLR2 and no data exists pointing towards HMGB1 structure as a factor in this interaction [119, 135, 229, 230]. Therefore, we set up a study to clarify the opposing results regarding the receptor binding and investigate whether protein truncation at C-terminal tail could affect the interaction. We specifically focused on the cytokine-inducing function of HMGB1 as the expected result of its interaction with TLR2.

Firstly, we tested if the different protein constructs bound to TLR2 in a plate-based assay, where we coated the wells with HMGB1 or C-terminal tail truncated HMGB1 (Δ30). The truncated HMGB1 form bound to TLR2-Fc in a dose dependent manner, while the full-length HMGB1 protein did not interact with the receptor. Interestingly, partial loss of the C-terminal tail, which is a common result of HMGB1 protein production, also showed binding to TLR2. This initial result suggested that the C-terminal tail inhibited the interaction with TLR2. As such, we wanted to investigate whether the Δ30 binding with TLR2 could have a functional outcome. HMGB1 interactions with different TLRs had been previously tested in HEK293 cells that were selectively transfected with individual receptors. HEK293 cells expressing TLR2 and stimulated with full-length HMGB1 showed NF-κB-induced secreted embryonic alkaline phosphatase (SEAP) upregulation [137]. However, TLR2 is known to form heterodimers with TLR1 or TLR6 in order to enable differential recognition of lipopeptides [231]. We therefore set up an *in vitro* system with HEK293 cells transfected with TLR2 alone, TLR2 and TLR1 or

TLR2 and TLR6. We stimulated cells with full-length HMGB1 and Δ30 as well as the known ligands of the receptor heterocomplexes, PGN and Pam3CSK4. Surprisingly, neither of the HMGB1 ligands induced IL-8 production in any of the HEK293 cell lines. This is in contrast to previous report showing HMGB1 interaction with TLR2 RAW264.7 macrophages by fluorescence resonance energy transfer. [135]

TLRs often require co-receptors for a proper reaction to sensed danger. CD36 is a glycoprotein found in lipid rafts of the cell membrane. It has been shown that CD36 enhances immune responses to some TLR2–TLR6 ligands [232]. Thus, we decided to preform analogous experiments in cells which highly express CD36, the mouse alveolar macrophage cell line MH-S. Again, neither HMGB1 nor Δ30 induced pro-inflammatory cytokine production. Finally, we confirmed the lack of functional outcome of interaction between C-tail truncated HMGB1 and TLR2 in the whole blood system.

Extracellular HMGB1 can form complexes with TLRs' ligands such as LPS and DNA. Our lab has previously demonstrated that HMGB1 is able to interact with Pam3CSK4 to enhance its pro-inflammatory activity [141, 144]. Thus, we decided to investigate whether HMGB1 enhances PGN-induced cytokine production via TLR2. We let the full-length HMGB1 and Δ30 pre-form complexes with PGN before we stimulated PBMC to discover that both protein complexes induced cytokine production dose-dependently and at similar levels. These comparable results suggest that the binding site(s) for PGN and TLR2 are located within the HMG boxes or the linker residues.

In summary, we purified HMGB1 lacking the C-terminal tail and confirmed its binding to TLR2 *in vitro*. However, we could not record cytokine induction as the result of the interaction. Both the full-length and truncated HMGB1 proteins formed complexes with the TLR2 ligand, PGN, and enhanced cytokine production in PBMCs. The C-terminal tail truncated HMGB1 could be a result of HMGB1 regulation by endopeptidases as explained in Paper II. The gradual truncation of the C-terminal tail by enzymatic cleavage could promote HMGB1/TLR2 interaction *in vivo*.

5.3 ANTI-HMGB1 THERAPY: FROM BASICS TO CLINIC

Understanding release and regulation of HMGB1 is important for establishing it as a therapeutic target. While HMGB1 biology is complex, blocking the total levels of extracellular HMGB1 might be beneficial for the patients suffering from inflammatory conditions. Therefore, we decided to make a step towards anti-HMGB1 therapy by designing a new clinical tool.

5.3.1 Developing h2G7 as a new therapeutic tool

Monoclonal antibodies (mAbs), i.e. epitope-specific antibodies produced from a single B-lymphocyte clone, gained the attention of pharmaceutical industry quite fast after their generation. The technology was developed in 1975 and led to the approval of the first murine therapeutic antibody OKT3 (muromonab) as a treatment of kidney transplant rejection ten years later [233, 234]. To date, more than 40 mAbs have been approved by the U.S. Food and Drug Administration as effective therapy in different diseases, for example the TNF-blocking

mAb, infliximab, for treatment of RA [235]. Furthermore, around 300 mAbs are currently in clinical testing and at the current approval rate around 70 monoclonal antibody products will be on the market by 2020 [236]. While the pre-clinical testing of therapeutic mAbs showed promising results, the clinical tests revealed few shortcomings. The human immune system recognizes proteins from different species as foreign. Thus, the patient's immune system eliminates the murine antibody in the human anti-mouse antibody (HAMA) response [237, 238]. Besides the short serum half-life, murine mAbs often fail to trigger human effector functions. Since the first mAb was approved in therapy, the advancing antibody engineering techniques have resulted in reduction of immunogenicity and the HAMA response. Chimeric antibodies were developed by replacing the constant domains of the mouse antibody with human sequences [239]. As the chimeric antibodies are approximately 70% human, they are not readily cleared by the patient's immune system. Moreover, humanized antibodies were developed which have an even stronger human backbone than the chimeric antibodies, where only the antigen-binding site in the variable region of the humanized antibody originates from mouse.

Monoclonal antibody 2G7 was produced by fusing spleen cells from immunized mice with myeloma cells. It showed promising results in multiple disease models of inflammation of both sterile and infectious origin. Our research group and collaborators have showed that mouse 2G7 (m2G7) reduced the clinical signs in mouse models of arthritis and DILI [133, 192]. We decided to make the 2G7 antibody more suitable for clinical use by applying rational methods of antibody humanization [240]. We generated a human chimeric 2G7 antibody (h2G7) by hybridization with human IgG1, a common feature of clinically approved therapeutic antibodies (Paper IV). The IgG1 backbone does not affect the antibody's mechanism of action and permits both the antibody-dependent cell-mediated cytotoxic (ADCC) activity and the complement-dependent cytotoxic (CDC) activity. We confirmed antibody specificity using *in vitro* studies and the affinity by surface plasmon resonance (SPR). In order to verify that m2G7 mode of action was retained in h2G7, we took a rational approach of creating and testing effector function-deficient variants of h2G7 incapable of binding complement or Fc receptors (FcRs). Our approach is described in the Materials and Methods section of Paper IV.

5.3.2 HMGB1 blockade in pre-clinical model of DILI

After we successfully created a potential new therapeutic tool for HMGB1 neutralization, we decided to test its therapeutic properties. In order to facilitate anti-HMGB1 therapy with h2G7 in the clinics, we set out to test h2G7 in a known pre-clinical disease model and compare its activity to the original mouse antibody.

The major role of HMGB1 as a pro-inflammatory mediator in DILI pathogenesis was explained in the previous chapters. The therapeutic efficiency of m2G7 in APAP-induced hepatic inflammation, a common type of DILI, was shown before [106, 241]. Thus, we decided to use this disease model to verify that the humanization had no negative effect on the therapeutic efficiency. We successfully induced DILI in mice as demonstrated in Figure 2 of Paper IV (PBS group). Both m2G7 and h2G7 displayed similar therapeutic effects in APAP-challenged mice. While there was only limited protection from the liver injury itself, the post-injury hepatic inflammation was suppressed. Prevention of the HMGB1-mediated propagation of inflammation is a desirable therapeutic outcome in DILI since the post-injury hepatic

inflammation negatively correlates to clinical outcome of APAP overdose patients. The strong effect on the inflammatory aspect of DILI pathogenesis with little or no effect on the hepatic injury, suggests 2G7 blocks HMGB1-induced cytokine production and immune cell infiltration.

In conclusion, we created a chimeric monoclonal antibody that blocks total levels of HMGB1 and prevents post-injury inflammation in mouse DILI. The antibody is partially humanized and, according to experiments with mutant versions of the mAb, most likely acts through HMGB1 neutralization, which could prevent HAMA and other adverse effects in patients. However, more detailed studies on antibody effects are needed before h2G7 could be tested in chronic complex diseases.

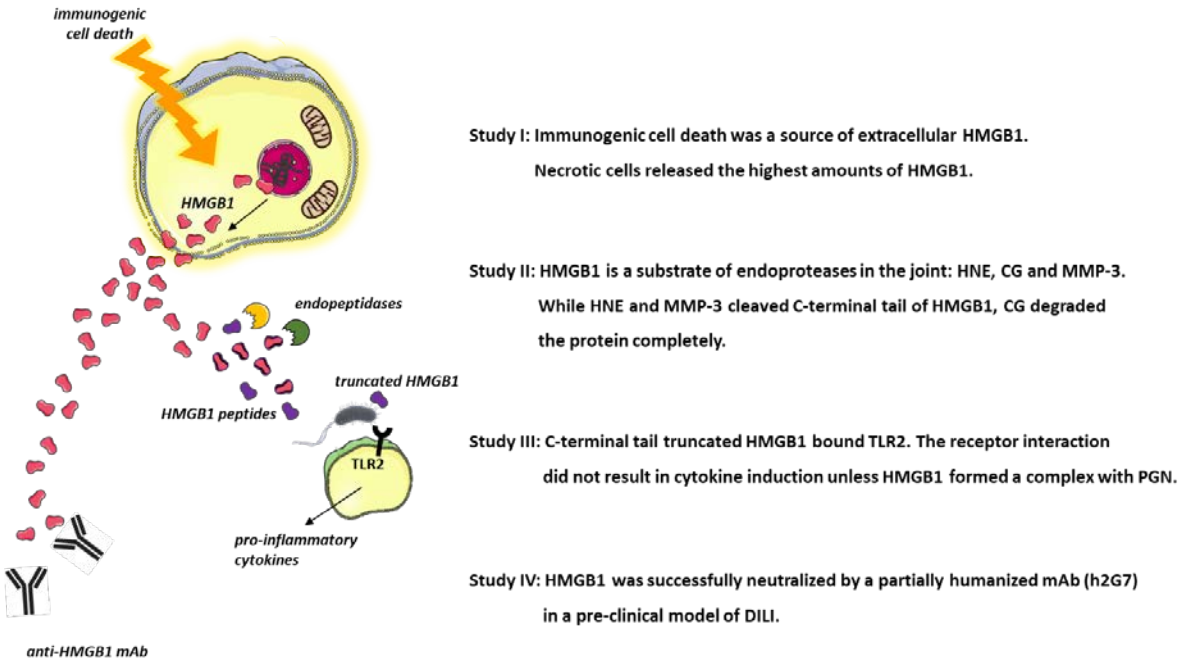

Figure 7. Summary of the main results.

6 CONCLUDING REMARKS

In conclusion, my PhD work expanded the knowledge regarding extracellular HMGB1, its function and regulation during inflammation. I studied the release of HMGB1 from a monocytic cell line, THP-1, investigated its processing by inflammation-associated endopeptidases, clarified interactions with its receptor, TLR2, and developed partially humanized monoclonal antibody against HMGB1 that could be a successful therapy in pre-clinical DILI. The specific conclusions are listed below:

Immunogenic cell death modes are major contributors of extracellular HMGB1

Our results confirmed that cells undergoing controlled apoptosis do not release HMGB1 unless the apoptotic process proceeds to an autolytic necrotic outcome. High cytotoxicity and HMGB1 release without production/release of other inflammatory markers indicated that necrosis is associated with passive release of nuclear fully reduced HMGB1, as previously reported [116]. Pyroptosis is another immunogenic cell death type associated with high HMGB1 release. Both DAMPs and pro-inflammatory cytokines are produced by pyroptotic cells, which indicates active secretion of HMGB1. Necroptosis is also associated with HMGB1 release, although at a slower rate. Although HMGB1 does not form NETs of human neutrophils undergoing NETosis, I speculate that activated neutrophils release HMGB1 upon activation and/or that HMGB1 is released during NETosis but detaches from DNA. Methylation of lysine 42 in box A of HMGB1 found in synovial fluid of arthritis patients suggests its neutrophil origin.

Extracellular HMGB1 is a novel substrate for the inflammation-associated proteases: HNE, CG and MMP-3

Neutrophils are immune cells recruited to the sites of inflammation, where they contribute to propagation of inflammation and tissue damage. Our group has previously shown that neutrophils are major source of extracellular HMGB1 found in synovial fluids of JIA patients [124]. My PhD work now suggests that besides releasing the alarmin, activated neutrophils might be involved in regulation of HMGB1 activity via proteases released from their azurophilic granules. A possible outcome is downregulation of HMGB1 and formation of functional peptides. HMGB1 is also a substrate for a highly abundant tissue-degrading protease in synovium, MMP-3. It was previously shown that the levels of HMGB1 and MMP-3 correlate in synovial fluid of JIA patients [185]. Such correlation strengthens the hypothesis that MMP -3 cleaves HMGB1 at the site of inflammation. The initial cleavage of HNE and MMP-3 occurs at the acidic C-terminus.

The C-terminal tail domain of HMGB1 regulates TLR2 binding

While the purified full-length HMGB1 is not a ligand of TLR2, the C-terminal tail truncation enables the receptor binding. Therefore, the epitope for TLR2 binding is within HMG boxes or the linker region of HMGB1, and can be masked by the C-terminal tail. However, we could not detect any cytokine production as a result of HMGB1 interaction with TLR2.

Both the full-length and C-terminal tail truncated HMGB1 formed pro-inflammatory complexes with PGN, a known ligand of TLR2. Therefore, we hypothesized that truncated HMGB1 formed after proteolytic cleavage (e.g. by HNE or MMP-3) could enhance TLR2-mediated inflammatory activities. In fact, earlier reports showing HMGB1-mediated induction of cytokine production via TLR2 could actually be due to endotoxin impurities in recombinant protein preps.

The results from Paper II and Paper III suggest a novel mechanism that could affect HMGB1 extracellular functions. Proteolytic cleavage of HMGB1 might lead to higher affinity of HMGB1 to its receptors. Therefore, proteases can enhance pro-inflammatory functions of HMGB1. On the other hand, there is also a possibility of anti-inflammatory outcome of the proteolytic cleavage by exposing box A of the protein, HMGB1 antagonist, or by complete protein degradation.

A novel humanized anti-HMGB1 antibody serves as a successful therapy in pre-clinical DILI

A mAb against HMGB1, 2G7, has shown therapeutic effects in animal models of human diseases. As many mAbs are produced in mice, human-anti mouse antibody responses are a significant problem in the clinics. We made a step towards designing 2G7-based patient treatment by the antibody humanization. In order to confirm therapeutic properties of the novel chimeric antibody, we compared its performance along with the original 2G7 in a mouse model of DILI. Both antibodies successfully blocked hepatic post-injury inflammation. The antibody performance was neither dependent on complement nor Fc mediated cytotoxicity. Therefore, we concluded that the h2G7 mAb had therapeutic value and its mode of action of 2G7 was HMGB1 neutralization.

7 FUTURE PERSPECTIVES

While my PhD work expanded on previous knowledge about the activity of extracellular HMGB1 during inflammatory conditions, it also created new questions.

Is immunogenic cell death the major source of the extracellular HMGB1?

My data suggest that immunogenic cell death is the major producer of the extracellular HMGB1 and that the released levels exceed the amounts of HMGB1 secreted by the activated cells. These results should be verified in other *in vitro* systems; especially in cells where the activity of JAK-STAT signaling pathway, associated with active release of HMGB1, is validated. In order to maintain the controlled conditions, comparisons could be made with experiments on the macrophage-like differentiated THP-1 cells before a more challenging verification in PBMCs.

The next important step needed for understanding the role of cell death in inflammation would be to identify which functional redox isoforms of HMGB1 are released. It is known that necrotic HMGB1 has thiol adducts at all three cysteine residues. Thus, necrotic HMGB1 promotes leukocyte recruitment to the site of inflammation. The redox state of HMGB1 released after pyroptosis is believed to be in the highly pro-inflammatory disulfide form. Thus, lower total HMGB1 levels during pyroptosis do not mean that pyroptosis is less immunogenic than necrosis. Our quantitative analysis should be followed by a qualitative study in order to have a more complete picture of HMGB1 extracellular activity at the site of inflammation. It would also be interesting to follow the isoform formation in a kinetic manner to understand whether the fate of HMGB1 is determined within the cell or formed as a net effect of the factors in the extracellular milieu. The qualitative analysis is currently only possible with mass spectrometry, an expensive and laborious technique. Due to high costs and limited availability of the mass spectrometry, an indirect analysis could be performed based on *in vitro* assays, where cells in homeostasis are stimulated with the supernatants from dying cells. While such analysis could only suggest but not determine HMGB1 redox isoforms, it could provide more insight into the impact of cell death on healthy tissue.

What is the outcome of proteolytic regulation of HMGB1 the site of inflammation?

Redox regulation has been a hot topic in HMGB1 research for the past few years. However, other regulatory mechanisms for HMGB1 activity exist and contribute to HMGB1 activity during inflammation. My PhD work pointed towards proteases as a novel endogenous regulatory mechanism of HMGB1 activity at the local site of inflammation. In order to understand the effect of inflammation-associated endopeptidases HNE, CG and MMP-3 on HMGB1, the predicted peptides formed after proteolytic cleavage could be synthesized. The

HMGB1 fragments could be then tested in functional assays, such as *in vitro* stimulation of macrophage cell cultures. It would also be interesting to test different redox isoforms of these peptides by oxidizing them with H_2O_2 and reducing with DTT. In addition, my data in combination with a previous report from our lab suggests an interesting feedback loop mechanism in which activated neutrophils downregulate the HMGB1 that they release [124]. Therefore, more efforts should be made to study neutrophils found in the synovial fluid of JIA patients. I believe that my initial results could open a new direction in studying extracellular HMGB1 regulation and more inflammation-associated endopeptidases should be studied as potential regulators of HMGB1, e.g. PR3 expressed not only in the primary granules but also on the cellular membrane of neutrophils.

JIA is a heterogeneous disease. Analyses of higher number of patient samples and/or stratification od samples according to patients sub-diagnosis could reveal potential correlation between levels of HMGB1 and endoproteases. More insight into HMGB1 regulation at the site of inflammation could also be derived from levels of protease inhibitory complexes, such as alpha1 protease inhibitor and alpha2 macroglobulin.

Our studies showed that C-terminal tail truncated HMGB1 is a ligand of TLR2. Unfortunately, we could not detect functional outcome of this interaction. This is in contrast to some previous studies suggesting a pro-inflammatory effect of the receptor interaction. For example, HMGB1 signaling through TLR2 on myeloid dendritic cells resulted in an effective anti-glioblastoma immune response [242]. It is possible that the interaction is cell-type dependent and that more factors are involved in the signaling *in vivo* that could be elucidated from controlled *in vitro* experiments with highly purified HMGB1.

Is HMGB1 blockade a promising therapy for inflammatory diseases?

Lastly, we created and tested a novel therapeutic tool for HMGB1 blockade: a partially humanized mAb specific for HMGB1. The antibody performed well in a pre-clinical mouse model of DILI by inhibiting post-injury hepatic inflammation. Thus, it can serve as a basis for further development of a clinical HMGB1-specific treatment option. HMGB1 is a late mediator of lethal systemic inflammation compared to TNF and IL-1 [241, 243]. Therefore, HMGB1 blockade could open a new therapeutic window for treatment of inflammatory conditions.

On the other hand, multiple studies have reported a protective role for the fully reduced HMGB1 for. It has important functions in wound healing, muscle regeneration and repair of multiple tissues. Therefore, creating a therapeutic tool targeting only the disulfide isoform of HMGB1 would be an idealanti-HMGB1 therapy.

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