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DUAL CYTOKINE INDUCING PROPERTIES OF HMGB1

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To my wonderful family

ABSTRACT

High mobility group box protein 1 (HMGB1) is a nuclear protein that can be released either passively by necrotic cells or actively by stimulated cells. Extracellular HMGB1 is a potent inducer of inflammation and the importance of HMGB1 as a mediator in a number of inflammatory diseases including rheumatoid arthritis, sepsis and ischemia-reperfusion injury, has been demonstrated by successfully targeting the protein in preclinical models. The aim of my thesis was to characterise the cytokine-inducing properties of HMGB1 and to study which receptors were required for cytokine induction.

Several studies have indicated that HMGB1 can co-operate with other pro-inflammatory molecules to induce inflammation. To further study this mechanism we formed complexes of HMGB1 together with the exogenous TLR ligands LPS, Pam_3CSK_4 and CpG-ODN or the endogenous ligands IL-1α and IL-1β. Stimulation of macrophages or synovial fibroblasts with these different HMGB1 complexes resulted in significantly enhanced cytokine production as compared to stimulation with each ligand alone (**papers I and II**). Importantly, HMGB1 selectively enhanced the stimulatory activity of certain molecules as it did not display this activity with all tested ligands. In **papers II and III** the receptor requirements of HMGB1 complexes were studied. HMGB1 in complex with LPS, Pam_3CSK_4 and IL-1α/β stimulated cytokine release via the TLR4, TLR2 and IL-1RI receptors, respectively, demonstrating that cytokine induction by HMGB1 complexes is dependent on the receptor for the respective partner molecule.

In **paper IV** we demonstrated that cytokine release stimulated by uncomplexed HMGB1 was dependent on TLR4 but not on RAGE or TLR2, and that a direct association of HMGB1 and TLR4 was detected both *in vitro* and *in vivo*. Using site-directed mutagenesis we furthermore determined that the cysteine in position 106 of HMGB1 was required for both binding to TLR4 and for cytokine induction.

In summary, in this thesis I have demonstrated that HMGB1 has the ability to induce cytokine production in two ways: through forming complexes with certain danger molecules and thereby increasing their stimulatory activities, and through direct interaction with the TLR4 receptor. The TLR4-mediated endogenous cytokine-inducing capacity of HMGB1 requires a cysteine in position 106, while the enhancing capacity of HMGB1-partner ligand complexes is independent of HMGB1 ligation to TLR4 but dependent on signalling via the partner molecule receptor. Neither mechanism involved an interaction of HMGB1 with its suggested receptor RAGE. These results are of value for designing HMGB1-targeting therapies that focus on blocking only certain HMGB1 functions or certain receptor interactions.

LIST OF PUBLICATIONS

I. **The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation**
Hulda Sigridur Hreggvidsdottir*, Therese Östberg*, Heidi Wähämaa, Hanna Schierbeck, Ann-Charlotte Aveberger, Lena Klevenvall, Karin Palmblad, Lars Ottosson, Ulf Andersson, Helena Erlandsson Harris
Journal of Leukocyte Biology 2009 Sep;86(3):655-62.

II. **HMGB1 in complex with LPS or IL-1 promotes an increased inflammatory phenotype in synovial fibroblasts**
Heidi Wähämaa, Hanna Schierbeck, Hulda Sigridur Hreggvidsdottir, Karin Palmblad, Ann-Charlotte Aveberger, Ulf Andersson, Helena Erlandsson Harris
Submitted for publication

III. **HMGB1-partner molecule complexes enhance cytokine production by signaling through the partner molecule receptor**
Hulda Sigridur Hreggvidsdottir, Anna Lundberg, Lena Klevenvall, Ulf Andersson, Helena Erlandsson Harris
Manuscript

IV. **A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release**
Huan Yang, Hulda S. Hreggvidsdottir, Karin Palmblad, Haichao Wang, Mahendar Ochani, Jianhua Li, Ben Lu, Sangeeta Chavan, Mauricio Rosas-Ballina, Yousef Al-Abed, Shizuo Akira, Angelika Bierhaus, Helena Erlandsson-Harris, Ulf Andersson, Kevin J. Tracey
Proc Natl Acad Sci USA 2010 Jun 29;107(26):11942-7.

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LIST OF ABBREVIATIONS

α7nAChR	alpha7 subunit of nicotinic acetylcholine receptors
DC	Dendritic cell
C106	Cysteine in position 106 of HMGB1
ERK1/2	Extracellular signal-related kinase 1/2
HMGB1	High mobility group box 1 protein
HSP	Heat shock protein
IFN	Interferon
IKK	I-kappa B kinase
IL	Interleukin
IL-1RI	IL-1 receptor type I
iNOS	Inducible nitric oxide synthase
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
MAP kinase	Mitogen activated protein kinase
MIP-1	Macrophage inflammatory protein 1 / CCL3
MyD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear factor κB
ODN	Oligodeoxiribonuleotides
PAMP	Pathogen associated molecular pattern
Poly(I:C)	polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RANKL	Receptor activator of the nuclear factor κB ligand
ROS	Reactive oxygen species
sRAGE	Soluble RAGE
TGF-β	Transforming growth factor-β
TIR domain	Toll/IL-1R homology domain
TIRAP	TIR domain-containing adaptor protein
TNF	Tumour necrosis factor
TLR	Toll-like receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor-inducing IFN-β

1 INFLAMMATION

Inflammation is the body's response to damage caused either by microbial pathogens or by mechanical or chemical injuries. Cells and proteins of the innate immune system work together in a complex way to eliminate the pathogen and the damaged tissue, to promote tissue repair and restore tissue homeostasis. In this chapter selected part of inflammation are discussed.

1.1 THE IMMUNE SYSTEM

The primary role of the immune system is to protect the body from infectious agents, but also to mount an appropriate response to tissue damage. The immune system is divided into the non-specific first response termed innate immunity and the more specific response termed adaptive immunity. Innate immunity is the first line of defence against foreign particles in the body, it is rapidly engaged but does not give prolonged protection. Activation of the innate immune system by pathogens or by tissue injury sets into motion an inflammatory response during which innate immune cells and effector molecules work together to eliminate the pathogen or clear the injured tissue. Only if a pathogen is able to break through the defences of the innate immune system will adaptive immunity be activated. The adaptive immune system is based on antigen-specific cells, recognising the specific antigen, and immunological memory, which protects the host from re-infection with the same pathogen [1].

1.2 CELLS IN INFLAMMATION

Phagocytes are the first cells that respond to infection or injury. Monocytes and neutrophils circulate in the blood and are recruited to a site of infection or injury whereas macrophages are resident cells in almost all tissues. Phagocytes are recruited to sites of infection or injury where their role is to ingest and kill pathogens or to clear cell debris, respectively.

Neutrophils are the first cells recruited to the site of infection or injury in tissues. There they play an important role in phagocytosing and killing pathogens but they are short-lived cells and die few hours after they enter the tissue. Monocytes

circulate in the blood but mature into macrophages as soon as they leave the bloodstream and enter non-vascular tissues. Macrophages are long-lived cells that reside in tissues and have the ability to recognise pathogens that manage to penetrate the epithelium and to recognise tissue injury. In addition to phagocytosing and killing pathogens, macrophages secrete inflammatory mediators that initiate and coordinate the inflammatory response. Finally, macrophages clear up apoptotic neutrophils and dead tissue and secrete mediators that promote inflammation resolution and tissue remodelling, and are therefore involved in all stages of the inflammatory process.

In addition to the innate immune cells mentioned above, non-professional cells such as epithelial cells, endothelial cells and fibroblasts also contribute to the inflammatory response [1].

1.3 SOLUBLE MEDIATORS IN INFLAMMATION

Immune cells secrete cytokines and other immune mediators in response to pathogens or other activating stimuli. Cytokines are soluble proteins that mediate immune and inflammatory reactions. Cells of the immune system use cytokines to communicate with each-other and with other cell types. Cytokines mediate their responses by binding to specific receptors on the target cell and although they usually act autocrinely or paracrinely some are stable enough to act in an endocrine manner. The prototypic cytokines that activated macrophages secrete are TNF, IL-1β and IL-6. These cytokines mediate many of the early actions and symptoms of inflammation such as endothelial activation, increased vascular permeability, local tissue destruction, lymphocyte activation, acute-phase protein production, fever and shock.

Chemokines are chemoattractant cytokines that recruit cells and guide them to the sites of inflammation. MCP-1 (CCL2) and IL-8 (CXCL8) are chemoattractants for monocytes and neutrophils, respectively, and they function in two ways to recruit these cells. First, they enhance the binding of monocytes and neutrophils to endothelia, allowing them to cross the blood vessel wall and migrate into the tissue. Second, the chemokines form a gradient in the tissue to guide the cells to the site of inflammation. In addition to cell recruitment, MCP-1 and IL-8 also activate their respective target cell type.

As noted above the cytokines TNF, IL-1β, IL-6 induce the production of acute-phase proteins, which are plasma proteins produced by the liver. Many of these proteins recognise and bind carbohydrate-specific structures on pathogens, leading to aggregation and facilitating subsequent killing. Other important mediators of inflammation that macrophages release in response to pathogen stimulation are the lipid mediators prostaglandins, leukotrienes and platelet-activating factor. Phagocytes do not only kill pathogens by engulfing them, they also produce reactive oxygen species that are toxic to the pathogens and antimicrobial peptides that are thought to kill bacteria by damaging the bacterial cell membrane.

A number of mediators are involved in the inflammatory response and co-ordinate the process in a complicated manner. Some are pro-inflammatory in nature while others are anti-inflammatory or promote healing. Furthermore, some molecules can act either pro- or anti-inflammatory depending on the timing and context [1].

1.4 THE INFLAMMATORY RESPONSE

Inflammation can be divided into infectious and sterile inflammation depending on if it is initiated in response to infection or tissue injury without involvement of microbial agents.

Both infection and tissue damage are of danger to the body and have to be eliminated. The immune system has evolved to recognise both these types of danger and to initiate an inflammatory response to remove them and then heal the tissue. Infection or injury in the tissues is recognised by resident macrophages. Upon recognition of the danger macrophages become activated and secrete cytokines and other inflammatory mediators that initiate a number of events. The first of these are dilution of local blood vessels, resulting in increased blood flow and upregulation of adhesion molecules on endothelia. This allows neutrophils and monocytes, which are recruited by chemokines, to attach to the endothelium and migrate into the tissue. Once in the tissue the phagocytes engulf and kill the pathogens and remove dead cells and cellular debris. Additionally, they promote inflammation by secreting cytokines and other mediators that assist directly or indirectly in killing of the microbes. Cytokines also cause increased vascular

permeability, leading to leakage of fluid and plasma proteins into the tissue, and some of the plasma proteins help with killing the pathogens. Finally, in order to avoid spread of infection the cytokines induce clotting of microvessels. These initial events cause the classical characteristics of inflammation; pain, redness, heat and swelling. The cells and soluble mediators of the innate immune system therefore work together in a complex reaction to eliminate the danger. Only if the innate immune system is unable to eliminate the danger will it activate the adaptive immune system to establish a specific response against the pathogen [1].

After the infection and tissue damage has been removed the inflammation has to be resolved and the tissue healed. Damage of host cells and tissues can be caused by the actions of pathogens or directly by mechanical or chemical assault, but the inflammatory response itself will also cause damage to the surrounding tissue. The inflammatory response therefore includes a resolution process, leading to remodelling and regeneration of the tissue. When neutrophils and macrophages have entered the tissue and started killing the pathogens, they will also start to produce mediators with anti-inflammatory and pro-resolution activity. As a consequence neutrophil recruitment ceases, they die by apoptosis and are taken up by macrophages [2]. Phagocytosis of apoptotic neutrophils by macrophages triggers release of both anti-inflammatory mediators and growth factors, such as IL-10 and TGF-β. Macrophages also produce proteases such as matrix metalloproteases that degrade the extracellular matrix to allow tissue remodelling. Next, the growth factors, cytokines and other mediators released by macrophages will lead to tissue repair by promoting growth of new blood vessels, fibroblast proliferation, collagen synthesis and formation of fibrous tissue [3]. The inflammatory response finally ends with emigration of macrophages via the lymphatics [4]. In summary, the resolution of inflammation is not achieved simply through the absence of pro-inflammatory mediators but needs active function of both anti-inflammatory and resolution-promoting mediators.

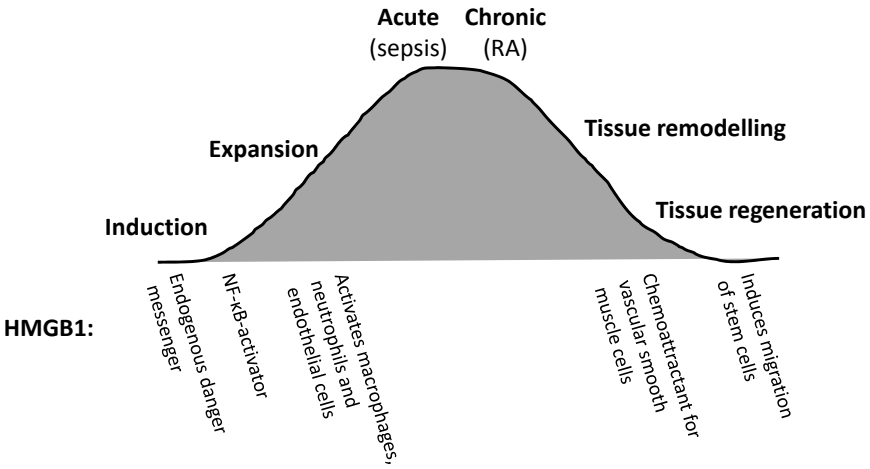

Figure 1: The inflammatory response. *The involvement of the prototypic DAMP HMGB1 in the inflammatory response is marked in as an example.*

1.5 PATTERN RECOGNITION RECEPTORS

To avoid unnecessary and potentially harmful activation of the immune system, it is not enough that the immune system distinguishes between self and non-self. It also needs to make a distinction between harmful and harmless events. In order to do so, the immune system has evolved danger-sensing systems. The presence of pathogens are sensed via so-called pathogen-associated molecular patterns (PAMPs), which are specific structures conserved among pathogens. PAMPs bind to a group of receptors that are collectively termed pattern recognition receptors (PRRs) and stimulate induction of an inflammatory response. Recent studies demonstrate that PRRs can also recognise endogenous molecules from dying cells, DAMPS and this will be discussed in the next chapter. Besides being expressed on innate immune cells such as macrophages and neutrophils, PRRs are found on various non-immune cells.

Four families of PRRs have been identified to date: RIG-I-like receptors (RLRs) are located in the cytoplasm and involved in viral recognition; NOD-like receptors (NLRs) are also located intracellularly and respond to bacterial peptidoglycans; and C-type lectin receptors (CLRs) are transmembrane receptors stimulated by carbohydrates from different microbes [5]. The best known family of PRRs are the Toll-like receptors (TLR), transmembrane receptors that are located on the cell

membrane or intracellularly on endosomes [6]. To date 10 TLRs have been identified in humans and 12 in mice. TLRs recognise different parts of pathogens; for example, TLR4 recognises LPS, a component of the outer membrane of Gram-negative bacteria, while TLR3 binds viral double-stranded RNA.

TLRs consist of extracellular leucine-rich repeats, a transmembrane region and a cytoplasmic Toll/IL-1R homology (TIR) domain. Via the TIR domain TLRs recruit adapter molecules to start the signalling cascade [7]. All TLR receptors except TLR3 signal through the MyD88 adaptor protein to induce NF-κB translocation, MAPK activation and production of pro-inflammatory cytokines. TLR7 and TLR8 additionally induce type I IFNs in a MyD88-dependent manner. Conversely, TLR3 signals through the TRIF adaptor leading to phosphorylation of IRF3 and IRF7 and subsequent production of type I IFNs, in addition to NF-κB activation and pro-inflammatory cytokine induction. TLR4 is unique in this regard as it can both signal through the MyD88-dependent pathway and the TRIF-dependent pathway.

1.6 ALARMINS / DANGER ASSOCIATED MOLECULAR PATTERNS (DAMPS)

As mentioned above the immune system senses infection through conserved molecular patterns on microbes, but the immune system also has to be able to sense and respond to tissue injury caused by non-microbial mechanisms. Only recently has it become clear that the immune system does this by recognising specific molecules that have been termed alarmins or danger-associated molecular patterns (DAMPs). Alarmins/DAMPs are endogenous molecules that are released during injury from stressed cells and cells undergoing necrotic death, and alert the immune system of danger [8-10]. In addition to DAMPs from intracellular sources, molecules that are released from the extracellular matrix during injury can act as DAMPs. In this way DAMPs give a signal of a potential danger to the host. A number of molecules, representing a very diverse group, have been suggested to function as DAMPs. These include HMGB1 [11], heat shock proteins (HSPs) [12], S100 proteins [13] and IL-1α [14]. DAMPs promote similar inflammatory responses as PAMPs although the mechanisms underlying activation are not fully understood. However, emerging evidence indicate that many DAMPs are recognised by PRRs and induce inflammation through similar

mechanisms as PAMPs. Several other receptors have been proposed to mediate the pro-inflammatory activity of DAMPs, including RAGE, CD44 and integrins [9].

2 HMGB1

2.1 INTRODUCTION

High mobility group box protein 1 (HMGB1) belongs to the superfamily of HMG proteins that are DNA-binding proteins that mediate structural and functional changes to chromatin. HMGB1 is highly conserved with 99% amino acid sequence homology in mammals. The gene codes for a 215 amino acid protein of 25 kDa. The protein comprises of two DNA binding domains, named the A-box and B-box, which are highly positively charged, and a strongly acidic carboxyl tail (Figure 2).

In 1973 Johns and colleagues purified a group of non-histone chromatin proteins that they named *high mobility group proteins* because of their ability to migrate quickly during electrophoresis [15,16]. One of these proteins was HMG-1, which became HMGB1 following revision of the nomenclature for the HMG protein family in 2001 [17].

HMGB1 is present in the nucleus of all mammalian cells where it plays an important role [18]. Even though the expression levels can be modified it is an abundant protein with about 10^6 molecules in each cell. The DNA binding domains of HMGB1 bind to the minor groove of DNA and bend the DNA structure [19]. No DNA sequence specificity has been determined for HMGB1 and it seems that structure specificity rather determines its binding sites. By binding to DNA and changing its structure HMGB1 participates in a number of activities in the nucleus, including transcription, replication and DNA repair [20]. The importance of HMGB1 functions in transcription is reflected by the fact that HMGB1 knockout mice are not viable. They are born alive but die within 24 hours after birth due to hypoglycaemia [21].

HMGB1 has been rediscovered several times through the years and been studied under different names. “Differentiation enhancing factor” was shown to be secreted from murine erythroleukemia cells and enhance their rate of differentiation [22,23] and the Rauvala group has for many years studied its ability to stimulate neurite outgrowth under the names “p30” and “amphoterin” [24,25]. In 1999 Tracey and colleagues discovered that activated macrophages secreted HMGB1 and identified the protein as a late mediator of endotoxin

lethality [26]. Since then the extracellular functions of HMGB1 have been extensively investigated and will be the focus of this thesis.

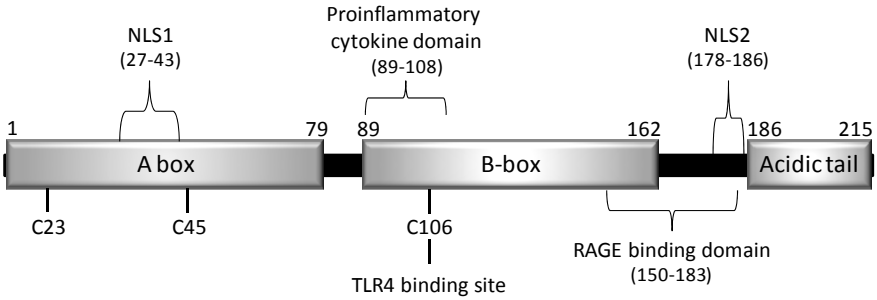

Figure 2: Amino acid structure of HMGB1. *HMGB1 contains two DNA binding domains, A-box and B-box and an acidic C-terminal tail. Some functionally important regions are illustrated in the figure. NLS, nuclear localisation signal; C, cysteine.*

2.2 POST-TRANSLATIONAL MODIFICATIONS

HMGB1 undergoes extensive post-translational modifications in the form of glycosylation, acetylation, phosphorylation, methylation and oxidation. HMGB1 contains 43 lysines and mass spectrometry analysis reveals that at least 17 of them can be phosphorylated *in vivo* [27]. Either acetylation or phosphorylation of the two nuclear localisation signals (Figure 2) of HMGB1 will inhibit nuclear import of the protein leading to its accumulation in the cytoplasm and subsequent extracellular secretion [27,28]. In neutrophils, a portion of HMGB1 is mono-methylated at lysine in position 42 which localises it to the nucleus [29]. Interestingly, no methylation was evident in HMGB1 derived from lymphocytes. While the functional relevance of these modifications on extracellular HMGB1 are not yet understood redox changes of HMGB1 have been a focus recently, demonstrating its effect on the extracellular activity of HMGB1.

HMGB1 contains three cysteine residues at position 23, 45 and 106 (Figure 2). Disulfide bridges between the two first cysteines, at position 23 and 45, have been demonstrated [30,31]. Studies of oxidation status of HMGB1 from viable and dead cells did not detect the oxidised form in viable cells whereas HMGB1 from apoptotic cells was oxidised [32]. Further studies have demonstrated that oxidation of HMGB1 during apoptosis leads to inactivation of its

immunostimulatory function [33]. This process involves a caspase-dependent production of reactive oxygen species which in turn oxidised HMGB1. Mutation experiments revealed the cysteine at position 106 (C106) to be the critical residue that needed to be oxidised in order to disable the immunostimulatory properties of the protein. Supporting this notion are the findings from a mouse model of liver injury, showing that inhibition of caspase-dependent apoptosis is associated with release of the reduced form of HMGB1 and recruitment of inflammatory cells [34]. In mice in which apoptosis was allowed, the major form of HMGB1 had oxidised residue C106. These recent findings connecting oxidation of C106 to loss of cytokine-inducing activity concord well with our discovery that C106 is necessary for binding of HMGB1 to TLR4/MD2 receptor complex and subsequent cytokine induction [paper IV].

2.3 EXTRACELLULAR RELEASE

HMGB1 is located in the nucleus of all cells in the body. Under certain conditions it is secreted, either in a passive or an active way, into the extracellular environment. These two different release pathways differ in many aspects such as kinetics and posttranslational modifications of the released protein.

Many inflammatory cells such as monocytes, macrophages, dendritic cells (DCs) and natural killer cells secrete HMGB1 actively [26,35-37], but a number of other cell types have also demonstrated this ability: pituicytes, hepatocytes, endothelial cells, platelets, neurons, astrocytes and a number of tumour cells [23,38-45]. Active HMGB1 release can be induced in response to PAMPs or endogenous pro-inflammatory cytokines such as LPS, Poly(I:C), lipoteichoic acid, TNF, IL-1β, IL-2, IFNγ and IFNα [26,35,37,45-47]. Furthermore, hypoxia-induced HMGB1 release is an active process that needs TLR4-dependent production of ROS and calcium-mediated signalling [39,48,49]. Finally, both necrotic and apoptotic cells can stimulate macrophages to release of HMGB1 [50].

HMGB1 does not contain a secretory signal peptide and is therefore not secreted in a classical way through the endoplasmic reticulum or the golgi apparatus. Instead it is secreted through a non-classical pathway similar to the leaderless cytokine IL-1β, although the process might differ between cell types. Under normal conditions HMGB1 is continuously shuttled between the nucleus and the

cytoplasm with the balance strongly tilted towards nuclear accumulation [27]. Stimulation of monocytes leads to both acetylation and phosphorylation of HMGB1, blocking it from re-entering the nucleus and leading to its accumulation in the cytoplasm [27,28]. Via an unknown mechanism HMGB1 is then packaged into secretory lysosomes that transport it to the extracellular milieu through exocytosis [51]. The exocytosis of HMGB1-containing secretory lysosomes is induced by lysophosphatidylcholine, a bioactive lipid that is produced several hours following monocyte activation. This is reflected in the delayed secretion kinetics of HMGB1 as observed in LPS-stimulated macrophages in which extracellular HMGB1 is first detected after 6 hours and still increases after 30 hours [26,51]. This is a considerately delayed response compared to the canonical early pro-inflammatory cytokines TNF and IL-1β. Most HMGB1 protein released during the first 12 hours post-macrophage activation is preformed and only thereafter is release of newly synthesised HMGB1 detected [26].

Any cell type undergoing necrosis will release HMGB1 in a passive way [52,53]. Scaffidi and colleagues reported in 2002 that while wild type necrotic cells induced inflammation, $HMGB1^{-/-}$ necrotic cells were incapable of doing so [53]. This identified HMGB1 as a DAMP or alarmin. Primary apoptotic cells release very little HMGB1 because the protein binds tightly to the chromatin during apoptotic death [53]. However, if these apoptotic cells are not cleared they may go through secondary necrosis and consequently release HMGB1 [54,55]. Interestingly though, this HMGB1 will not induce inflammation. This is due to an oxidation of C106 that occurs during the apoptotic process and that inactivates the immunostimulatory activity of HMGB1, explaining why apoptotic cells do not induce inflammation despite releasing HMGB1 [33].

Passive release happens rapidly, in contraast to the active process that requires more time. This is reflected in HMGB1 being a *late mediator* in diseases where it is actively secreted but an *early mediator* in diseases with major necrosis or cell damage (as will be discussed further in later chapters). Another difference between these two release pathways is the acetylation status of the protein. During active secretion HMGB1 is acetylated but there is no evidence of acetylation during passive release of HMGB1 [27]. It is still unclear if the acetylation status has any effect on the extracellular functions of HMGB1.

2.4 FUNCTIONS OF EXTRACELLULAR HMGB1

In the extracellular environment HMGB1 is a versatile protein with many reported functions. HMGB1 induces a strong pro-inflammatory response through among others activating macrophages and DCs and inducing production of number of cytokines. The protein is however, also involved in the healing process of inflammation by inducing tissue regeneration through attracting stems cells to the site of injury. Several receptors have been proposed to mediate the different functions of HMGB1 including RAGE, TLR2 and TLR4. Receptors and their role in mediating the diverse functions of HMGB1 will be discussed in detail in next chapter.

2.4.1 HMGB1 as a pro-inflammatory protein

The first discovery of HMGB1 as an inducer of inflammation came in 1999 when Kevin Tracey and colleagues detected high levels of HMGB1 in mice suffering from lethal endotoxemia, a model of sepsis [26]. Administration of anti-HMGB1 antibodies to these mice protected them from lethality, illustrating the important role of HMGB1 in this model. They furthermore demonstrated that administration of purified HMGB1 was lethal to the mice. For the last decade the pro-inflammatory function of HMGB1 has been explored extensively revealing diverse roles for the protein in inducing inflammation.

HMGB1 is a potent inducer of a number of cytokine and chemokines as is observed in human monocytes cultures which produce TNF, IL-1α, IL-1β, IL-1RA, IL-6, IL-8, MIP-1α and MIP-1β in response to HMGB1 stimulation [56]. Further studies have reported that in addition to monocytes, HMGB1 stimulates pro-inflammatory cytokine production in macrophages and neutrophils, and induces increased nuclear translocation of NF-κB in these cells [53,57-63]. HMGB1 also causes iNOS expression and nitric oxide release from macrophages as well as reactive oxygen species (ROS) from neutrophils [57,64,65]. Besides activating these important innate immune cells, HMGB1 can works as a chemoattractant for them. HMGB1 mediates transendothelial migration of monocytes and *in vivo*

studies have demonstrated recruitment of neutrophils in response to HMGB1 injection [66-68].

Another pro-inflammatory effect of HMGB1 is activation of endothelial cells. HMGB1 induces cytokine and chemokine secretion from endothelial cells and upregulation of adhesion molecules, which in turn leads to increased adhesion of neutrophils to the endothelial cells [69,70]. HMGB1 is suggested to have a role in central nervous system inflammation as it activates astrocytes and stimulates them to secrete pro-inflammatory factors [71]. Moreover, HMGB1 increases permeability of the epithelial monolayer *in vitro* and of the intestine *in vivo*, thereby leading to dysfunction of the gut barrier [72].

HMGB1 has a substantial effect on both the maturation and activation of DCs. Besides acting as a chemoattractant for immature DCs, HMGB1 causes their maturation, leading to secretion of pro-inflammatory cytokines and chemokines [11,37,73,74]. Activated DCs will also themselves secrete HMGB1 that in turn acts autocrinely to sustain cell maturation [36]. HMGB1 matured DCs are able to stimulate T cells and to polarise them towards a Th1 phenotype [11,73]. Moreover, HMGB1 secretion from DCs is required for the proliferation, survival and polarization of T cells [36]. Further studies revealed that although HMGB1 on its own did not induce proliferation of T cells the protein significantly enhanced the proliferation of T cells receiving suboptimal anti-CD3 stimulation [75]. These findings demonstrate that HMGB1 does not only play a role in the innate immune reaction but can also promote an antigen-specific immune response.

The cytokine-inducing ability of HMGB1 can be recapitulated by the B-box domain of the protein and by using synthetic peptides the cytokine-inducing function has been further mapped to a 20 amino acid sequence in the B-box (Figure 2) [58,59]. The B-box has ability to reproduce many other pro-inflammatory functions of full length HMGB1 such as increasing the permeability of epithelial cells and activation of endothelial cells [69,72].

In summary, HMGB1 acts in several ways to promote inflammation. HMGB1 induces secretion of chemokines from neutrophils, monocytes and DC and can also itself function as a chemoattractant for these cells. It activates endothelia, allowing leukocyte adhesion and transendothelial migration. When inflammatory cells have entered the site of inflammation HMGB1 will then activate them and

perpetuate the inflammatory response. Finally, HMGB1 matures DCs and plays a role in T cell polarization and proliferation, providing the link to adaptive immune activation.

2.4.2 HMGB1 as a regenerative protein

As mentioned above, HMGB1 can stimulate migration of monocytes, neutrophils and DCs, thereby promoting inflammation. However, HMGB1 also induces migration and proliferation of stem cells and other cell types that are involved in tissue regeneration, and in that way is involved in the healing process.

In concordance with its role as a danger signal, HMGB1 can induce migration and proliferation of stem cells. Mesangioblasts are vessel associated stem cells that are able to home to damaged muscle tissue and participate in regeneration. Palumbo and colleagues showed that mesangioblasts both migrate and proliferate in response to HMGB1 [76]. This was valid both for embryonic and adult mesangioblasts and moreover HMGB1 could stimulate mesangioblast homing *in vivo*. HMGB1 has similar effect on endothelial progenitor cells, as it induces their migration and homing to ischemic tissue [77]. Additionally, HMGB1 activates integrins on these cells and is able to promote angiogenesis [77-79]. Since HMGB1 is released in ischemic tissue, the protein could play an important role in neovascularisation of the ischemic area by recruiting endothelial progenitor cells. HMGB1 furthermore induces migration of mesenchymal stem cells, but contraty to its effects on mesangioblasts, HMGB1 inhibited the proliferation of these cells [80]. This could be because of different experimental settings of the proliferation assay, or due to a true difference in the effects of HMGB1 on different stem cells.

HMGB1 not only induces migration of stem cells but also works as a chemoattractant for embryonic fibroblasts and smooth muscle cells [52,81]. Changes in cell structure and cytoskeleton reorganisation were observed in smooth muscle cells after exposure to HMGB1 [52]. In addition, HMGB1 functions as a proliferative signal to fibroblasts [60]. In the brain HMGB1 induces neurite outgrowth of neurons and plays an essential role in migration of brain tumour cell lines [24,25,42-44].

Beneficial effects of HMGB1 following myocardial infarction have been demonstrated in two independent studies. Overexpression of HMGB1 in cardiac cells or local administration of HMGB1 induced myocardial regeneration, restored cardiac function and improved survival in a mouse model of myocardial infarction [82,83]. These effects of HMGB1 were mediated via proliferation and differentiation of cardiac stem cells and induction of angiogenesis [82,83].

In order for cells to be recruited from the blood to tissues they have to travel through the blood vessel wall. Consistent with its role as a chemoattractant of stem cells and immune cells, HMGB1 mediates migration of mesangioblasts and monocytes across endothelial monolayers [66,76]. This action is mediated through loss of cell-cell contact and barrier function of the endothelial monolayer [76].

2.4.3 HMGB1 in co-operation with other molecules

Recent studies of the pro-inflammatory properties of HMGB1 reveal that besides promoting inflammation on its own, HMGB1 can bind to other pro-inflammatory molecules and enhance their function.

Several studies have been published during recent years in which recombinant HMGB1 induces little or no cytokine production from macrophages or DCs [31,84-86,87, paper I-III]. However since HMGB1 has been demonstrated to possess pro-inflammatory properties in settings where recombinant HMGB1 is not involved this led to investigation on the interaction of HMGB1 with other pro-inflammatory molecules. HMGB1 forms complexes with various TLR ligands and enhances their cytokine-inducing function. Stimulation of cells with HMGB1 in a complex with low amounts of LPS, CpG-ODN or Pam_3CSK_4 (a synthetic TLR1-TLR2 ligand) results in strongly enhanced cytokine production as compared to stimulation with each ligand alone [paper I-II, 84,86-88]. HMGB1 does not only interact with bacterial components, it can also bind to the endogenous proteins IL-1α, IL-1β and CXCL12 to enhance their function [paper II, 85,89,90]. Furthermore, HMGB1-nucleosome complexes may break immunological tolerance to dsDNA and induce cytokine production and DC maturation, which HMGB1-free nucleosomes are unable to do [91]. Interestingly, HMGB1 was not able to bind and enhance the stimulation of TNF, RANKL, IL-18 or Poly(I:C) [paper I].

It therefore seems that HMGB1 can bind a number of different molecules and thereby enhance their immunostimulatory capacities. As the role of HMGB1 in the nucleus is to bind DNA it is not surprising that it has the ability to bind other nucleotides. Besides binding DNA, HMGB1 binds many different proteins in the nucleus and is suggested to play a central role in stabilisation and/or assembly of multifunctional complexes through protein-protein interaction [92]. It is therefore likely that extracellular HMGB1 also can bind a number of different proteins and evolution has probably selected for binding and functional effect on certain molecules. The reasons for loss of pro-inflammatory activity by many recombinant HMGB1 preparations might be a result of the purification process. As mentioned earlier HMGB1 can undergo different posttranslational modifications, and as demonstrated for oxidation this can affect the protein functions.

These results indicate that in addition to promoting inflammation on its own, HMGB1 has an important role in increasing the sensitivity of the immune system to certain pro-inflammatory molecules.

2.4.4 Other functions

Several functions of HMGB1 have been demonstrated that cannot be categorised as either pro-inflammatory or regenerative functions. HMGB1 has been implicated in cancer as it induces tumour growth and invasion of tumours [93]. Neovascularisation is an important step in tumour growth in order to sustain oxygen supply and as mentioned above HMGB1 promotes angiogenesis and furthermore induces homing of endothelial progenitor cells to tumour tissue [77-79].

HMGB1 has been reported to possess antibacterial activity, comparable to other antibacterial peptides [94]. A synthetic peptide of the C-terminal acidic tail of HMGB1 can reproduce this activity; however, the mechanism underlying the antibacterial activity of HMGB1 is still unclear [95].

2.5 HMGB1 RECEPTORS

RAGE was the first receptor described for HMGB1. RAGE-HMGB1 interaction has been extensively studied revealing that RAGE mediates many extracellular

functions of HMGB1, especially those regarding cell motility. During the last years HMGB1 has been demonstrated to interact with several other receptors, including TLR2, TLR4, TLR9, CD24, MAC-1 and TREM1.

2.5.1 RAGE

The receptor for advanced glycation end products (RAGE) is a transmembrane receptor and a member of the immunoglobulin superfamily. It is a promiscuous receptor that binds a variety of ligands, including advanced glycation products, S100 proteins and HMGB1 [96]. RAGE also has a secretory truncated form, sRAGE, comprising the extracellular domain that functions as a decoy receptor.

RAGE was the first receptor for HMGB1 identified in 1995, and the binding site has been mapped to amino acids 150-183 (Figure 2) [97,98]. Since then we have learned that RAGE mediates many of the extracellular functions of HMGB1. HMGB1 induced migration is in most if not all cases mediated through RAGE. Migration of immune cells (DCs, monocytes, neutrophils) [11,66,67], stem cells (endothelial progenitor cells, mesenchymal stem cell) [77,80] and smooth muscle cells [52] are mediated by RAGE. The *in vitro* migration of mesangioblasts was RAGE-dependent; however, homing of these cells to muscle *in vivo* did not depend on functional RAGE, suggesting that RAGE is sufficient but not necessary for migration of these cells [76]. Furthermore, HMGB1 promotes neurite outgrowth through RAGE [97].

HMGB1 induces neutrophil adhesion to endothelial cells in a RAGE dependent manner [69]. RAGE is suggested to mediate HMGB1-stimulated DC maturation [36] and RANKL-induced osteoclastogenesis is dependent on HMGB1-RAGE interaction [94]. HMGB1-induced permeability of endothelial monolayer is RAGE-mediated and RAGE deficiency protects mice from mucosal hyperpermeability in the intestine following hemorrhagic shock and resuscitation, indicating that HMGB1-RAGE interaction is important in this model [48,72]. HMGB1-RAGE interaction is furthermore involved in proliferation and invasion of tumour cells as well as in tumour growth [93].

A few studies have reported partial or complete dependence for RAGE in HMGB1-induced cytokine production while other data indicate no role of RAGE in this

process [57,68,70,99,100, papers III-IV]. A possible role for RAGE in HMGB1-mediated cytokine production is therefore controversial.

Ligand binding to RAGE can activate multiple intracellular signalling cascades [96]. RAGE regulates cytoskeleton changes and cell motility through Cdc42 and Rac, which are Rho-family small GTPases (Figure 3) [101]. HMGB1 stimulation of RAGE also activates the MAP kinases p38 and JNK and leads to nuclear translocation of NF-κB [93,101]. Indeed, both involvement of Cdc42 and Rac as well as NF-kB activation is required for HMGB1-induced cell motility [81,101].

2.5.2 TLR4

A direct physical interaction between HMGB1 and TLR4 has been demonstrated with immunoprecipitation, fluorescence resonance energy transfer (FRET) and surface plasmon resonance [102, paper IV]. Interaction of endogenous HMGB1 and TLR4 has furthermore been detected in synovial fibroblasts from arthritis patients that spontaneously secrete HMGB1 [paper IV]. The B-box, which contains the cytokine-inducing domain of HMGB1 (Figure 2), can also bind to TLR4 [paper IV].

It was unclear in the beginning what receptor HMGB1 uses to induce cytokine production, but compiling evidence indicates that the TLR4 receptor mediates this activity. Stimulation of macrophages deficient in RAGE, TLR2 or TLR4, or wild type macrophages treated with blocking antibodies to the same receptors revealed dependence of TLR4, but not TLR2 or RAGE, for cytokine production [100, paper IV]. Further studies of the HMGB1-TLR4 interaction have revealed a need for cysteine in position 106 of the HMGB1 protein, both for binding to TLR4 and for cytokine induction [paper IV]. As mentioned earlier, oxidation of this same C106 inactivates the immunogenic properties of HMGB1, reinforcing the importance of this amino acid for immune induction by HMGB1 [33].

Besides being important in HMGB1-induced cytokine production TLR4 is also involved in HMGB1-mediated tissue damage. HMGB1-TLR4 signalling mediates liver damage following hepatic ischemia-reperfusion injury [103]. ROS production by neutrophils after hemorrhagic shock and resuscitation is important in mediating lung inflammation and injury and this process is dependent on HMGB1-

TLR4 signalling [65]. In tumour biology, HMGB1-TLR4 signalling is important for cross-presentation of tumour antigens and establishment of cytotoxic T cell response towards tumours during radio- or chemotherapy [104,105]. Furthermore, HMGB1 signalling via TLR4 and the subsequent inflammatory response is suggested to be involved in skin tumour development [106].

HMGB1 and LPS initiate many similar intracellular events such as activation of ERK1/2, JNK and p38, as well as translocation of NF-κB (Figure 3) [61,70]. However, HMGB1 stimulation leads to activation of both IKK-α and IKK-β while LPS stimulation only activates IKK-β [62] and gene array studies have demonstrated different gene expression profiles after stimulation with HMGB1 and LPS [61]. Thus even though HMGB1 and LPS signal through the same receptor they do not stimulate identical intracellular responses.

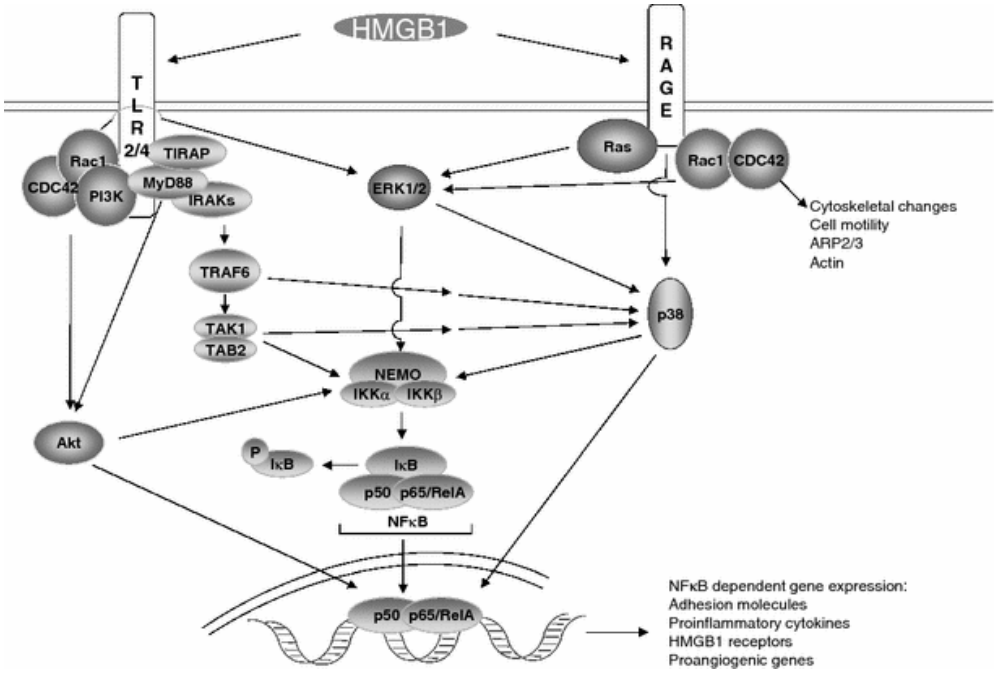

Figure 3: Intracellular signalling of TLR2, TLR4 and RAGE [107].

2.5.3 TLR2

Physical interaction between TLR2 and HMGB1 has been demonstrated and binding of HMGB1 to TLR2 leads to cellular activation as measured by NF-κB dependent transcription [102]. HMGB1-stimulated TNF production was unaffected in primary macrophages deficient in TLR2 and in whole blood cultures treated with TLR2 neutralising antibody, indicating that HMGB1-TLR2 interaction is not involved in cytokine induction [paper IV, 100]. However, human embryonic kidney 293 cells overexpressing the TLR2 receptor effectively produced IL-8 in response to HMGB1 stimulation suggesting a possible role for TLR2 in HMGB1-induction of pro-inflammatory mediators, depending on the cell type [100].

As mentioned in earlier chapters, nucleosomes containing HMGB1 break immunological tolerance to dsDNA and induce production of cytokine [91]. Nucleosome-HMGB1 complexes stimulate cytokine production from primary macrophages in a TLR2- and MyD88-dependent manner, but deficiency of TLR4, TLR9 or RAGE had no effect on this function. TLR2 also mediated the immunogenicity of HMGB1-containing nucleosomes *in vivo*, as $TLR2^{-/-}$ mice produced significantly lower titers of anti-dsDNA and anti-histone antibodies then did wild type mice.

2.5.4 TLR9

TLR9 was recently identified as a receptor for HMGB1 in a complex with DNA, and a direct association between HMGB1 and TLR9 was detected using immunoprecipitation [87,88]. HMGB1-CpG-ODN complexes stimulated enhanced cytokine production in a TLR9- and MyD88-dependent manner, but independent of TLR2 and TLR4 [87,88]. Interestingly, RAGE is also important for this process and RAGE associates directly with TLR9 after stimulation with HMGB1-CpG-ODN [87]. Further studies of this mechanism revealed that HMGB1 accelerates the association of CpG-ODN to TLR9, and moreover accelerates transfer of TLR9 to early endosomes, this representing an essential step for TLR9 activation [88]. These effects of HMGB1 probably explain the mechanism underlying the enhanced cytokine production in response to HMGB1-CpG-ODN complexes.

2.6 PATHOLOGICAL ROLE OF HMGB1 AND HMGB1-TARGETED THERAPIES

Being a pro-inflammatory protein released during both infection and injury HMGB1 is logically involved in a number of inflammatory conditions. Elevated levels of HMGB1 are found in many sterile and infectious diseases and the importance of HMGB1 as a mediator of these diseases has been demonstrated by successful targeting of the protein in preclinical models. The most studied conditions regarding HMGB1 involvement will be discussed below.

2.6.1 Therapeutic targeting of HMGB1

Different strategies can be used to inhibit the effect of extracellular HMGB1:

- Inhibit secretion of HMGB1
  - Nuclear or cytoplasmic retention of HMGB1
- Neutralise extracellular HMGB1
  - Anti-HMGB1 antibodies
  - HMGB1 binding factors
- Block HMGB1 receptors
  - Blocking antibodies
  - Receptor antagonists

Oxaliplatin is a DNA-platinating compound that generates DNA adducts that bind HMGB1 tightly, resulting in nuclear retention of HMGB1 and inhibition of its release [108]. Ghrelin, an endogenous orexigenic peptide and a potential anti-inflammatory factor, inhibits HMGB1 secretion from macrophages and has been used to target HMGB1 in preclinical models [109]. The cholinergic anti-inflammatory pathway works via release of acetylcholine and its binding to the alpha7 subunit of nicotinic acetylcholine receptors (α7nAChR). Activation of this pathway by stimulation of the vagus nerve or usage of α7nAChR agonists leads to inhibition of HMGB1 release [110,111].

Neutralisation of pro-inflammatory cytokines with antibodies or antibody fragments has been used in the clinic for over ten years. Anti-TNF antibodies have proven to be very successful in diseases such as rheumatoid arthritis (RA)

[112,113] and antibodies against IL-1β and IL-6 are beneficial in many diseases [114-116]. A number of studies have reported the beneficial effects of polyclonal or monoclonal anti-HMGB1 antibodies in preclinical models of inflammatory conditions. Another strategy to neutralise HMGB1 is using molecules that bind to it with high affinity. Thrombomodulin is an endothelial anticoagulant that binds HMGB1 and prevents it from mediating its pro-inflammatory activities [117]. sRAGE, the soluble extracellular domain of RAGE, acts as a decoy receptor, competing with RAGE for binding to HMGB1 [97]. Thrombomodulin and sRAGE compete for binding of HMGB1, indicating that these molecules have the same binding site on HMGB1 [117].

Blocking the receptors that HMGB1 signals through might be a more specific approach, allowing blockage of only certain functions of the protein. This can be achieved either by using blocking antibodies against the receptors or by using receptor antagonists. A possible downside of this strategy is that most of the known receptors for HMGB1, such as RAGE, TLR4 and TLR2 mediate functions of many other ligands and blocking these receptors might cause harmful side effects.

The HMGB1 A-box has been identified as an antagonist for HMGB1 *in vivo* and *in vitro*. The A-box competes with full length HMGB1 for binding to macrophages, but the mechanism underlying this inhibition is still unclear [118].

2.6.2 Sepsis and endotoxemia

HMGB1 has been linked to endotoxemia and sepsis both in humans and in experimental models. HMGB1 is not detected in the blood of healthy individuals whereas high levels are recorded in critically ill septic patients. HMGB1 levels range from a few ng/ml to 150 ng/ml and non-survivors have significantly higher levels than do survivors [26,119,120]. A direct role of HMGB1 as a mediator in sepsis was demonstrated in animal models of endotoxemia and sepsis in which treatment with anti-HMGB1 antibodies or A-box peptide significantly increased survival and prevented organ damage [38,50,118]. HMGB1 is strongly elevated in these models and increased levels are detected from 18 hours subsequent to disease induction, remaining significantly elevated up to 72 hours [26,118]. This is a considerately later release than of the early pro-inflammatory cytokines TNF and IL-1β and identifies HMGB1 as a late mediator in sepsis. This is mirrored by

the fact that antibody treatments targeting TNF and IL-1β have not been successful, whereas anti-HMGB1 antibody administered as late as 24 hours after sepsis induction significantly improves survival and organ damage [50,118].

Administration of thrombomodulin is another way to target HMGB1 and results in increased survival of mice receiving a lethal endotoxin dose [117,121]. Vagus nerve stimulation or administration of different agonists of the α7nAChR inhibits HMGB1 release *in vivo* and provides protection against lethal endotoxemia and sepsis in animal models [110,111,122]. *In vitro*, these agonists inhibit HMGB1 release from macrophages, probably explaining the mechanism for their effect *in vivo*.

2.6.3 Arthritis

A number of studies during recent years have identified HMGB1 as a key mediator of arthritis. Synovial fluid from RA patients contains high levels of HMGB1 [49,57,123]. HMGB1 is extracellularly expressed in the joint both in experimental models of arthritis and in RA patients, and co-localises with hypoxic areas [49,57,123]. Intraarticular injection of HMGB1 to mice causes destructive synovitis [124] and RA patients responding to cortisone treatment have decreased levels of extranuclear HMGB1 in the joints [125].

The prototypical model for arthritis in rodents is collagen type II-induced arthritis. Using this model administration of several molecules targeting HMGB1 or HMGB1 release have been shown to reduce severity of arthritis and joint destruction, including polyclonal and monoclonal anti-HMGB1 antibodies [49,126, Schierbeck H, submittet manuscript], recombinant HMGB1 A-box protein [126], recombinant thrombomodulin [127], sRAGE [128], oxaliplatin [108], α7nAChR agonists [129] and ghrelin [109]. In a newly described spontaneous model of arthritis in DNaseII$^{-/-}$xIFN-IR$^{-/-}$ mice, extracellular HMGB1 is significantly increased and treatment with either monoclonal anti-HMGB1 antibodies or A-box protects the animals against arthritis and joint destruction [130].

Besides stimulating production of pro-inflammatory cytokines HMGB1 induces expression of metalloproteinases and promotes osteoclastogenesis, this possibly

explaining the mechanism of HMGB1-mediated inflammation and tissue destruction during arthritis [89,94, paper II].

2.6.4 Ischemia-reperfusion injury

Ischemia-reperfusion (I/R) activates the innate immune system leading to cytokine-driven inflammatory response and local tissue injury. HMGB1 has been identified as an early mediator of inflammation and tissue injury following I/R in multiple organs including the liver, heart and brain, and is suggested to be important in kidney I/R [99,103,131-133]. HMGB1 is released into the extracellular space immediately after ischemia induction in animal models and elevated levels are detected for at least 24 hours [99,103,132]. In agreement with animal data patients with cerebral or myocardial ischemia have dramatically increased HMGB1 levels in serum compared to healthy controls [134,135].

Treatment with anti-HMGB1 antibodies or A-box reduces the inflammatory response, tissue damage and infarct size in a myocardial I/R model [99,131]. Similar effects are apparent in cerebral ischemia models in which both polyclonal and monoclonal antibodies as well as A-box treatment have been shown to ameliorate ischemic brain damage [135,136]. Finally, administration of anti-HMGB1 antibodies to mice after hepatic I/R decreases liver damage [103].

Since many studies have demonstrated the importance of TLR4 signalling in I/R injury it has been suggested that HMGB1 is the important ligand in this setting [39,131,133]. In a mouse model of liver I/R anti-HMGB1 antibodies provided protection against liver damage in wild type (C3H/HeOuj) mice but not in TLR4-defective (C3H/Hej) mice, supporting the idea of HMGB1-TLR4 signalling during I/R [39]. Other studies have identified RAGE as the receptor mediating the effect of HMGB1 in I/R [99,135].

3 AIMS

There has been an intense discussion during recent years regarding the cytokine-inducing properties of HMGB1. After the original discovery of HMGB1 as a mediator of sepsis in 1999 both the pro-inflammatory and cytokine-inducing properties of HMGB1 became well established. Through the years the cytokine-inducing function of HMGB1 has been questioned. The aim of my thesis work was therefore to re-evaluate the cytokine-inducing properties of HMGB1, with or without co-operation with other pro-inflammatory molecules and, furthermore, to study which receptor/receptors HMGB1 uses to stimulate cytokine production.

The specific aims of the thesis were to:

- Investigate if HMGB1 could bind and co-operate with endogenous and exogenous pro-inflammatory molecules to induce cytokine production.
- Study the receptor usage of HMGB1 in a complex with pro-inflammatory molecules.
- Explore the cytokine-inducing ability of highly purified HMGB1 and which receptor it signals through.

4 RESULTS AND DISCUSSION

4.1 HMGB1 binds endogenous and exogenous danger signals and enhances their pro-inflammatory activities (papers I and II)

The cytokine-inducing function of HMGB1 has been an area of controversy during recent years. Many groups have reported a significant cytokine-inducing capacity of the protein whereas several reports conclude that HMGB1 is unable to stimulate cytokine production [26,31,56,85-87]. Because of the highly charged nature of HMGB1 it easily binds to other molecules. This has proven to be an issue during purification of the protein and it has been suggested that the cytokine-inducing capacity of recombinant HMGB1 proteins is caused by contamination with bacterial products. We hypothesised that rather than being just a contamination it is a biological role of HMGB1 to bind different pro-inflammatory molecules, such as bacterially derived TLR ligands, and thereby to enhance inflammation.

We tested four different batches of highly purified, endotoxin-free HMGB1, two recombinant batches produced in *E. coli*, one batch produced in a eukaryotic baculovirus system and HMGB1 purified from thymic tissue. None of these batches stimulated IL-6 production in PBMCs. However, when each of these batches was pre-incubated with small amounts of LPS that itself induced low amounts of IL-6, high levels of IL-6 were stimulated from PBMCs. Formation of a complex between HMGB1 and LPS was necessary for this synergistic cytokine production since HMGB1 and LPS added simultaneously to the cultures did not stimulate IL-6 production and an increased pre-incubation time of the molecules yielded increased IL-6 levels. Immunoprecipitation further demonstrated the formation of a complex between HMGB1 and LPS. PBMCs from different donors respond very heterogeneously to LPS stimulation, but independent of the IL-6 levels stimulated by the low dose of LPS, pre-incubation with HMGB1 always enhanced the cytokine production in a synergistic manner, as demonstrated using PBMCs from 12 different donors. In addition to co-operation with LPS, HMGB1 in a complex with either the TLR1/TLR2 ligand Pam_3CSK_4 or the TLR9 ligand CpG-

ODN synergistically enhanced cytokine production. HMGB1 can therefore act in complex with multiple TLR ligands to enhance their pro-inflammatory activities.

We further investigated if HMGB1 could also co-operate with endogenous pro-inflammatory molecules. HMGB1 pre-incubated with IL-1α or IL-1β significantly enhanced cytokine and chemokine production by synovial fibroblasts.

In summary, I have demonstrated that HMGB1 co-operates with multiple exogenous and endogenous danger signals to enhance their stimulatory activities. Importantly, HMGB1 did not enhance cytokine induction by Poly(I:C), TNF, IL-18 or RANKL, demonstrating the specificity of this function.

Recently, other groups have reported similar enhancing effects of HMGB1 on the stimulatory activities of danger signals, including LPS, CpG-DNA and IL-1β [84-88]. Youn and colleagues showed that HMGB1 enhances LPS-mediated TNF production by disaggregating LPS and transferring it to CD14, the co-receptor for TLR4 [84]. HMGB1 has a similar effect on CpG-DNA as it accelerates the association of CpG-DNA to TLR9, leading to enhanced pro-inflammatory cytokine secretion [88]. The mechanism by which HMGB1 enhances the stimulatory activity of TLR ligands and IL-1β was not addressed in these studies but will be discussed in the next chapter in which receptor usage of HMGB1 complexes and subsequent intracellular signalling was investigated.

Pre-incubation of HMGB1 with LPS or IL-1β led to about 10-fold increase in cytokine production compared to stimulation with the ligands alone. The enhancement of HMGB1 in a complex with either Pam_3CSK_4 or CpG-ODN was not as strong. This could be caused by the fact that both of these molecules are synthetic ligands whereas LPS and IL-1β are natural ligands. Furthermore, higher doses of HMGB1 were needed for synergistic effects in a complex with Pam_3CSK_4, although these are still within physiological concentrations measured during pathological conditions. Serum HMGB1 levels in patients with inflammatory conditions are a few hundred nanograms per millilitre and it is plausible that much higher HMGB1 levels are apparent at sites of inflammation such as in synovial fluid from RA patients in which HMGB1 levels have been measured up to 10 μg/ml [26,119,123,137].

An important question regarding HMGB1 complexes is how HMGB1 binds its partner molecules and if it can bind more than one molecule at a time. Even

though this was not addressed directly in our studies we demonstrated that pre-incubation of either A box or B box with LPS induced enhanced cytokine production, indicating that HMGB1 has at least two binding sites for LPS [paper I]. The A box and B box are both DNA binding domains, indicating that HMGB1 can also bind at least two DNA molecules at the same time. HMGB1 is able to interact with a number of very structurally diverse molecules, making it probable that HMGB1 uses different binding sites and chemical bonds to bind these molecules.

My studies and others suggest that complex formation of HMGB1 is not a general feature since HMGB1 is unable to enhance the stimulatory activity of several pro-inflammatory proteins, including TNF [85, paper I]. In my experience it is important to use the right concentrations of both HMGB1 and partner molecule to record the synergistic effects in *in vitro* systems and the enhancing effect could possibly be detected by further titrations of the ligands. However, three different research groups have reported the synergistic effects of HMGB1-IL-1β complexes and two independent publications demonstrate a lack of synergy between HMGB1 and TNF, providing compelling evidence that HMGB1 selectively enhances the stimulatory activity of certain cytokines [85, paper I,89]. Why HMGB1 has evolved to enhance the function of IL-1β but not of TNF is unclear but it could be speculated that TNF would cause too much damage if its functions were enhanced.

HMGB1 in complex with TLR ligands or cytokines has not been investigated *in vivo*, whereas HMGB1-nucleosome complexes have been detected in blood from systemic lupus erythematosus patients. Formation of HMGB1-IL-1β complexes *in vivo* is likely since both of these molecules are highly expressed in synovial joint of RA patients and it would be interesting to look for this type of complex in the synovial fluid [123]. Complex formation between HMGB1 and bacterial products might occur at sites of infection in the tissues, making them difficult to detect. However, during sepsis, a condition characterised by high serum levels of HMGB1, bacteria enter the bloodstream, opening up the possibility for HMGB1 to form complexes with bacterial molecules.

4.2 HMGB1-partner molecule complexes promote inflammation by signalling through the receptor of the respective partner molecule (papers II and III)

HMGB1 has been proven to interact with multiple receptors in order to mediate its activity, the most studied ones being RAGE, TLR4 and TLR2. However, the receptor usage of HMGB1 in complex with other molecules is largely unknown.

We started by using peritoneal macrophages from mice deficient in TLR2, TLR4 or RAGE receptors. Stimulation of these cells with HMGB1 in a complex with LPS or Pam_3CSK_4 demonstrated that HMGB1-LPS complexes signal through TLR4 and that HMGB1-Pam_3CSK_4 complexes signal through TLR2 to stimulate cytokine production. No requirement was found for the RAGE receptor, which has previously been suggested to be partly involved in HMGB1-induced cytokine production [57,68]. That HMGB1-LPS complexes signal through TLR4 was further supported by antagonist blockade of the TLR4 receptor significantly inhibiting synovial fibroblast production of pro-inflammatory mediators. The same principle was apparent when receptor usage of HMGB1-IL-1 complexes was studied. Blocking the IL-1 receptor type I (IL-1RI) with an IL-1R antagonist completely abolished the cytokine production stimulated by HMGB1-IL-1α or HMGB1-IL-1β complexes. Furthermore, TLR4 was not involved in HMGB1-IL-1β complex signalling. In conclusion, my data demonstrate that HMGB1-partner molecule complexes signal through the cell surface receptor of the respective partner molecule.

A dual receptor hypothesis for HMGB1 complex signalling was proposed by us and other groups, suggesting that HMGB1 complexes augment cytokine production by activating two receptors, possibly RAGE and the receptor of the respective partner ligand [138, paper I,139]. In concordance with this hypothesis Ivanov and colleagues showed that both TLR9 and RAGE are needed for HMGB1-CpG-DNA stimulation and Orlova *et al* demonstrated that co-operation between RAGE and Mac-1 was needed for HMGB1-mediated neutrophil recruitment [67,88]. However, this is not supported by my results and those of others as cells lacking RAGE responded in the same way to HMGB1-TLR ligand complexes as did wild type cells, and RAGE is not required for the immunostimulatory activity of nucleosome-HMGB1 complexes [91, paper III]. Furthermore, stimulation of TLR2$^-$

$^{-/-}$ cells with HMGB1-Pam_3CSK_4 complexes and of $TLR4^{-/-}$ cells with HMGB1-LPS complexes completely abolished IL-6 production, which does not indicate involvement of another receptor in the signalling of HMGB1 complexes. It thus seems that both RAGE and another receptor are required for cell activation by some HMGB1 complexes, but this does not explain the general mechanism underlying the enhancing effect of the complexes.

A few studies have indicated partial or complete requirement for RAGE in HMGB1-induced cytokine production [57,68,99]. This is in contradiction to my conclusions that neither HMGB1 in complex with TLR ligands nor un-complexed HMGB1 requires RAGE for cytokine induction [papers III-IV]. These differences could possibly be caused by different experimental setups such as different activation states of cells that probably have different expression levels of the receptors. Kokkola *et al* and van Zoelen *et al* used 8 and 2 hour stimulations, respectively, compared to a 16 hour stimulation period in my studies [57,68]. One could speculate that RAGE is involved in the initial cytokine production but that in a longer cell culture the TLR4-mediated cytokine production overrides the need for RAGE. In one of these reports the *in vivo* effects of intraperitoneal HMGB1 injection were investigated, demonstrating lower TNF and IL-6 levels as well as reduced neutrophil recruitment in the peritoneum of $RAGE^{-/-}$ and $TLR4^{-/-}$ mice [68]. *In vivo* systems are complex systems with more factors involved than in cell culture. RAGE is for instance involved in HMGB1-stimulated neutrophil recruitment and the reduced neutrophil count in peritoneum of $RAGE^{-/-}$ mice could therefore account for the reduced cytokine levels [67].

Several studies have used soluble RAGE to investigate the role of RAGE in HMGB1-mediated functions. For example, Qin *et al* used RAGE-Fc to block the enhancing effect of HMGB1-LPS complexes on cytokine production and concluded that RAGE was the receptor mediating the enhancing effect, in contradiction to my results [86]. Binding of HMGB1 to sRAGE has been demonstrated [97,117], although this binding is very likely to also block HMGB1 from binding to other receptors. Indeed, my unpublished results indicate that RAGE-Fc significantly inhibits HMGB1-stimulated IL-6 production from peritoneal macrophages. However, HMGB1 induced as strong IL-6 production in $RAGE^{-/-}$ cells as in wild type cells.

These results clearly demonstrate that blocking HMGB1 by soluble RAGE does not necessarily indicate a role for RAGE in the signalling.

Since engagement of two receptors by HMGB1 complexes did not explain the enhanced cytokine induction, I went on to study the intracellular signalling. In order to investigate if the complexes altered the intracellular signalling compared to signalling with the TLR ligand alone I used peritoneal macrophages from mice deficient in the adaptor molecules for TLR2 and TLR4; MyD88, TIRAP, TRIF and TRAM. Deficiency of these adaptor molecules had similar effects on HMGB1-LPS or HMGB1-Pam_3CSK_4 complex stimulation as on stimulation with LPS or Pam_3CSK_4 alone. These results indicate that complex stimulation increases intracellular signalling without making any qualitative changes in adaptor molecule activation.

When searching for mechanisms underlying the increased cytokine induction by HMGB1 complexes my data do not suggest that the complexes mediate their effects through engaging additional receptors or by making changes in the intracellular signalling. I hypothesise that HMGB1 instead mediates its synergistic effects by making the ligand more accessible to its receptor or by aggregating it at the cell surface, leading to engagement of more receptors and increased intracellular signalling. This is supported by data from other groups showing that HMGB1 enhances the stimulatory effect of LPS and CpG-DNA by on the one hand transferring LPS to CD14 and on the other hand by accelerating the association of CpG-DNA to its receptor, TLR9 [84,88].

4.3 Cytokine-inducing ability of pure HMGB1 is mediated via TLR4 and is dependent on cysteine at position 106 (paper IV)

As mentioned above, there has been much debate in the field about the ability of HMGB1 to induce cytokine production. Many early reports showed prominent cytokine-inducing function of HMGB1 [26,56], but recently several groups have demonstrated lack of this function [31,85-87]. I demonstrated in paper I that different batches of HMGB1 from prokaryotic, eukaryotic or native source were unable to induce cytokine production and suggested that HMGB1 instead mediated inflammation by binding to other danger molecules and enhancing their activities.

Several studies, however, support the notion that HMGB1 without any bacterial contamination is able to stimulate cytokine production. HMGB1 purified from mammalian CHO cells induces TNF production in macrophages [59,60] and cytokine induction by necrotic cell supernatants is dependent on the presence of HMGB1 in necrotic cells [53]. In these studies we can still not exclude that HMGB1 forms complexes with endogenous ligands for its function. The strongest evidence for the cytokine-inducing ability of pure HMGB1 without any cofactors derives from studies in which a synthetic peptide containing the 20 first amino acids of the B-box stimulated considerable cytokine production from macrophages [58]. These contradictory results led me to further investigate the cytokine-inducing function of HMGB1.

CHO cells were genetically engineered to secrete HMGB1. In agreement with previous reports, conditional medium from these cells stimulated TNF release from macrophages that could be blocked with neutralising anti-HMGB1 antibody. Next, recombinant HMGB1 with cytokine-inducing ability was produced and purified from *E. coli*. Importantly, this HMGB1 batch was not exposed to the reducing agent dithiothreitol (DTT), as were the prokaryotic batches in paper I. This HMGB1 batch was used to study which receptors uncomplexed HMGB1 uses to induce cytokines. Stimulation of peritoneal macrophages deficient in RAGE, TLR2 or TLR4, or double deficient for RAGE/TLR2 or RAGE/TLR4 revealed that HMGB1-induced cytokine production is only mediated via TLR4 with no involvement of TLR2 or RAGE. Direct interaction between HMGB1 and the TLR4/MD2 complex was demonstrated using surface plasmon resonance and furthermore the B-box, which contains the cytokine-stimulating domain, bound to the TLR4/MD2 complex. Binding of endogenous HMGB1 to TLR4 was also evident in a cellular system in which HMGB1-TLR4 interaction was detected using proximity ligation in synovial fibroblasts spontaneously secreting HMGB1, demonstrating that HMGB1 and TLR4 interact *in vivo*. Thus HMGB1 interacts with TLR4/MD2 to induce cytokine production in macrophages.

Recently oxidation of cysteine at position 106 (C106) within HMGB1 was shown to inactivate its pro-inflammatory properties [33]. The first 20 amino acids in the B-box, which include C106, have previously been reported to encompass the cytokine-inducing activity of HMGB1 [58]. We therefore decided to investigate the

role of this cysteine in HMGB1 binding to TLR4 and induction of cytokines. A recombinant HMGB1 protein with C106 mutated to alanine was unable to stimulate TNF release from macrophages or to bind to the TLR4/MD2 complex. The importance of this cysteine was further discerned by using a synthetic peptide of the first 20 amino acids in the B-box. While the wild type peptide induced TNF production from macrophages the same peptide sequence with C106 replaced for serine did not. In summary, HMGB1 induces cytokine production through the TLR4 receptor, and cysteine in position 106 is necessary for this function.

In 2008 Kazama and colleagues concluded that oxidation of C106 in HMGB1 was necessary for tolerance induction by apoptotic cells [33]. This process was dependent on activation of caspases and blocking of caspase activation or mutating the C106 lead to immune induction by the apoptotic cells. A recent article by Antoine *et al* supports the importance of C106 in the pro-inflammatory activity of HMGB1 [34]. In a liver toxicity model in mice, in which HMGB1 is an important mediator as evidenced by using neutralising antibodies, liver necrosis was associated with inflammation induction and HMGB1 with C106 in a reduced form. Conversely, mice with apoptosis in the liver had reduced inflammation and HMGB1 with oxidised C106. These data provide compelling evidence that reduced C106 is necessary for the pro-inflammatory function of HMGB1. Taking this together with our finding that C106 is required for HMGB1 to bind TLR4 and induce cytokine production, it is likely that C106 needs to be in a reduced form to allow binding of HMGB1 to the TLR4 receptor and subsequent cytokine induction.

A recent study suggests that HMGB1 from necrotic cells is oxidised; however, it has previously been established that HMGB1 from necrotic cells can stimulate cytokine induction [32,53]. On closer scrutiny half of the oxidised HMGB1 from necrotic cells had one oxidised cysteine and the other half had two oxidised cysteines, raising the possibility that the critical C106 remains in a reduced form when released from necrotic cells and able to promote inflammation. Mass spectrometric analysis of recombinant HMGB1 batches from different laboratories, with or without cytokine-inducing ability, have indeed revealed that a disulphide bridge between C23 and C45 as well as a reduced C106 associates with the ability of recombinant HMGB1 to induce cytokine production *in vitro* (Daniel Antoine unpublished results). In agreement with these results, HMGB1

associated with necrosis and inflammation in the liver toxicity model contains a disulphide bridge between C23 and C45 in addition to reduced C106 (Daniel Antoine unpublished results). Taking these results together, the oxidised HMGB1 found in necrotic cells most likely contains oxidised cysteines at positions 23 and 45 and reduced C106. This same protein would be referred to as reduced HMGB1 if one only considered the oxidation status of C106 and implicates the importance of stating which cysteine is being referred to when discussing the redox status of HMGB1.

Due to the reducing environment inside a cell, nuclear HMGB1 will have all its cysteines reduced. During necrotic release or active secretion HMGB1 should therefore be in a reduced form when entering the extracellular environment. During inflammation the extracellular milieu is oxidative and will oxidise the cysteines. Redox analysis of HMGB1 has revealed that under mild oxidative conditions C23 and C45 form a disulphide bond but that C106 remains in a reduced form [30]. If the extracellular environment is mildly oxidative HMGB1 will therefore have a redox status allowing it to induce inflammation, whereas a highly oxidative milieu would probably oxidise C106 and inactivate its pro-inflammatory function. The oxidative state of the extracellular environment can in this way control the pro-inflammatory activity of HMGB1.

HMGB1 undergoes many post-translational modifications that might influence its biological activity, as has been proven for its oxidative status. The post-translational modifications of recombinant or tissue purified HMGB1 that is used in experiments by different groups are largely unknown and modifications of extracellular HMGB1 *in vivo* under different conditions is still an unexplored area. This most likely gives the explanation for the diverse results obtained from different groups studying the cytokine-inducing function of HMGB1(s). We have indeed confirmed by mass spectrometry that two of the HMGB1 batches used in paper I that did not induce cytokines lack the disulphide bridge between C23 and C45, probably because they are kept in a buffer containing DTT. If these assumptions are true there is a need to revise the literature with this new knowledge of the importance of redox status of C106 in mind. Unfortunately the oxidation status of HMGB1 in earlier reports investigating the cytokine-inducing function is usually unknown, making it hard to interpret the data.

5 CONCLUDING REMARKS

The main findings of my PhD project represented in this thesis are as follows:

- HMGB1 increases the sensitivity of the immune system to danger signals
  - HMGB1 forms complexes with endogenous and exogenous danger signals and enhances their pro-inflammatory activities
  - HMGB1 complexes signal through the receptor of the respective partner molecule

- HMGB1 induces cytokine production by signalling through the TLR4 receptor
  - A cysteine in position 106 in HMGB1 is necessary for both binding and signalling through TLR4
  - RAGE and TLR2 were not involved in cytokine production induced by HMGB1 alone

HMGB1 is an important inflammatory protein as is demonstrated primarily by the beneficial effect that HMGB1-targeted therapy has in many different experimental models of inflammatory diseases. The pro-inflammatory properties of HMGB1 have, however, been a matter of recent debate. It has been controversial whether HMGB1 can induce production of cytokines or if this function is caused by contamination in preparation of the recombinant protein. Different groups have used HMGB1 either purified from tissue or produced in prokaryotic or eukaryotic cell systems and in addition, different purification methods have been used. This has made comparison between experiments very difficult. Thanks to reports that have been published during the last few years by me and other groups we now better understand the cytokine-inducing activity of HMGB1 and are starting to unravel the reasons behind the contradictory results that have been earlier reported.

Based on the results of my studies and others I propose the following hypothesis in a few steps (summarised in Figure 4):

1) HMGB1 with reduced C106 can react with the TLR4 receptor to induce cytokine production.

2) HMGB1 with oxidised C106 is unable to bind TLR4 and induce cytokine production but is able to induce migration of cells through RAGE and can thereby mediate tissue regeneration.

3) Independent of its TLR binding status HMGB1 can bind endogenous and exogenous danger signals and enhance their pro-inflammatory activities, and these complexes signal through the receptor of the respective danger molecule.

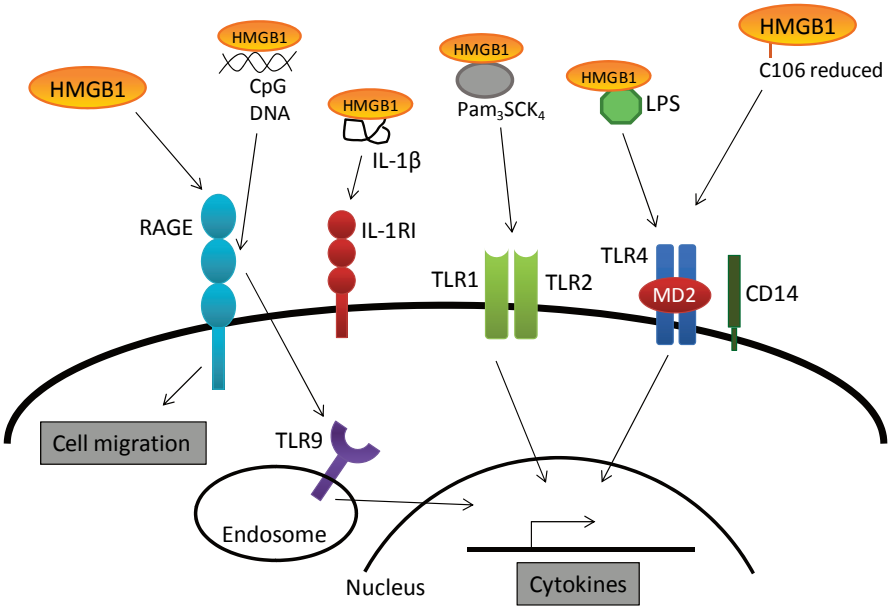

Figure 4: Interaction of extracellular HMGB1 with receptors and pro-inflammatory molecules. *HMGB1 with reduced C106 interacts alone with TLR4 to induce cytokine production. HMGB1, independent of its TLR binding status, can form complexes with certain pro-inflammatory molecules and induce cytokine production. Complex-induced cytokine production is mediated via the receptor of the respective partner molecule and in the case of HMGB1-CpG-DNA complexes RAGE is also involved. Finally, independent of its redox status, HMGB1 can mediate cell migration via RAGE.*

I and others have identified HMGB1 as a protein that binds and enhances the pro-inflammatory activity of other molecules. This information will be important when developing blocking therapies against HMGB1 as it might be possible to block relevant binding sites and therefore inhibit interaction of HMGB1 with certain molecules. However, before this can be done more studies are needed to address the physical interaction and binding sites of HMGB1 and its partner molecules.

A favourable therapy for inflammatory diseases would be to block the pro-inflammatory properties of HMGB1 while leaving its tissue regenerative properties intact. Blocking the interaction of HMGB1 to specific receptors might give the possibility to target certain functions of HMGB1. In general, RAGE mediates HMGB1-induced cell migration and tissue regeneration while TLR4 seems to mediate the pro-inflammatory properties of HMGB1, especially its cytokine-inducing activity. However, it is important to mention that RAGE may also contribute to the pro-inflammatory effects of HMGB1 by mediating for example migration of innate immune cells and DC maturation. The RAGE binding domain of HMGB1 has been identified, however, my results are the first indicating which area of HMGB1 is important for TLR4 binding, and this information will be valuable in designing antibodies targeting HMGB1-TLR4 interaction

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