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THE ROLE OF THE DAMP MOLECULE HMGB1 IN NEUROINFLAMMATION AND MACROPHAGE ACTIVATION

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The role of the DAMP molecule HMGB1 in neuroinflammation and macrophage activation

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By

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To mom, dad, Jonna and Riku

ABSTRACT

High mobility group box 1 (HMGB1) is a highly conserved nuclear protein expressed in all eukaryotic cells. While its nuclear functions are vital, we know today that HMGB1 also functions as a Damage Associated Molecular Pattern (DAMP).

When HMGB1 is released from cells to extracellular space due to cell death or stress, it induces migration of immune cells and secretion of pro-inflammatory cytokines. These functions are regulated by reduction or oxidation of cysteines in the HMGB1 structure. When all cysteines are reduced, fully reduced HMGB1 (frHMGB1) induces cell migration and proliferation. HMGB1 with a disulfide bond (dsHMGB1) induces cytokine secretion. These two functions are lost when HMGB1 is terminally oxidized (oxHMGB1).

The first aim of this thesis was to determine the functions of extracellular HMGB1 as an inducer of neuroinflammation. As excessive neuroinflammation can worsen neuronal damage in diseases like stroke, HMGB1 neutralization in brain is an interesting therapeutical strategy. To develop a therapeutic against HMGB1 in neuroinflammation.

We started by determining if frHMGB1 and dsHMGB1 intracortical injections in healthy rat brains could induce neuroinflammation without any underlying neuroinflammatory state. This was to confirm the causative effect of HMGB1. Both frHMGB1 and dsHMGB1 induced local neuroinflammation and blood brain barrier (BBB) leakage. However, dsHMGB1 was more potent as it lead to activation of major histocompability complex II (MHC-II).

Next, we set out to investigate neuroinflammation in a rat ischemic stroke model, M2 middle cerebral artery occlusion model (M2CAO), and simultaneously tested an Affibody molecule (ABY) as a potential therapeutic targeting HMGB1. Neuroinflammation was still present in M2CAO after four days from the induction of ischemia-reperfusion injury and an anti-HMGB1 ABY binding to HMGB1 could cross the BBB, however, no reduction of the ischemic lesion nor neuroinflammation could be detected.

Both HMGB1 injections and M2CAO induced microglia activation and macrophage infiltration and activation in the brain. Macrophages are among the immune cells recruited to brain in response to tissue injury and can obtain roles either in inducing inflammation or repairing damaged tissue. The second aim of this thesis work was to determine if and how macrophages polarize in response to HMGB1 stimulation.

To characterise the macrophage activation, frHMGB1 and dsHMGB1 were used to stimulate mouse bone marrow derived macrophages. Both dsHMGB1 and frHMGB1 activated a motile phenotype. However, only dsHMGB1 induced macrophages to obtain a pro-inflammatory phenotype including cytokine secretion whereas induction with frHMGB1 only had minor effects on the macrophage gene expression profile outside of migratory mechanisms. The results in this thesis work have expanded the understanding of the role of HMGB1 redox in inflammation and improved the understanding of HMGB1 as a therapeutic target for neuroinflammation.

LIST OF SCIENTIFIC PAPERS

I. Aucott H, Lundberg J, **Salo H**, Klevenvall L, Damberg P, Ottosson L, Andersson U, Holmin S, Erlandsson Harris H. *Neuroinflammation in Response to Intracerebral Injections of Different HMGB1 Redox Isoforms*. J Innate Immun. 2018;10(3):215-227. doi: 10.1159/000487056. Epub 2018 Feb 23. PMID: 29478057; PMCID: PMC6050639.

II. **Salo H**, Little P, Linthorst N, Jussing E, Lu L, Chireh A, Wahlberg E, Bianchi E M, Andersson U, Frejd Y F, Holmin S, Erlandsson Harris H. *Treatment of experimental M2CAO ischemia reperfusion stroke with an HMGB1-specific Affibody® molecule.* Manuscript

III. **Salo H**, Qu H, Mitsiou D, Aucott H, Han J, Zhang XM, Aulin C, Erlandsson Harris H. *Disulfide and Fully Reduced HMGB1 Induce Different Macrophage Polarization and Migration Patterns. Biomolecules*. 2021 May 28;11(6):800. doi: 10.3390/biom11060800. PMID: 34071440; PMCID: PMC8229957.

IV. Qu H, **Salo H**, Heinbäck R, Ewing E, Espinosa A, Aulin C, Erlandsson Harris H. *Transcriptome profile of mouse bone marrow derived macrophage upon stimulation with disulphide high mobility group box 1 (dsHMGB1) and LPS/IFN-γ reveals distinct polarization characters*. Manuscript

CONTENTS

1 INTRODUCTION....1
2 BACKGROUND....3
2.1 HMGB1 is a prototypical alarmin....3
2.1.1 (I) HMGB1 is released from cells undergoing immunogenic cell death....4
2.1.2 (II) HMGB1 is secreted from activated cells via non-classical secretion pathway....4
2.1.3 (III) Role in inflammation and in the immune system – HMGB1 binds to PRRs and activates inflammatory functions....5
2.1.4 (IV) Promotion of tissue regeneration....7
2.1.5 (V) Sensing and separating damage and danger....7
2.2 The role of HMGB1 in neuroinflammation....8
2.2.1 HMGB1, macrophage recruitment and polarization....9
2.3 Clinical reality of stroke....11
2.4 Rodent models of stroke....12
2.5 Pathogenic and protective role of HMGB1 in stroke....13
2.6 Targeting HMGB1 in stroke and other neuroinflammatory diseases....15
3 RESEARCH AIMS....17
4 RESULTS AND DISCUSSION....19
4.1 Paper I: HMGB1 induces neuroinflammation....19
4.2 Paper II: Neuroinflammation in ischemic stroke....20
4.3 Paper II: HMGB1 as a target for ischemic stroke therapy....21
4.4 PAPER III and IV: role of HMGB1 in macrophage polarization....22
4.4.1 dsHMGB1 induced macrophage phenotype....23
4.4.2 frHMGB1 polarized phenotype....24
5 RESULTS AND DISCUSSION OUTSIDE OF CONSTITUENT PAPERS....25
5.1 Macrophage gene expression patterns in response to stimulation with HMGB1 redox isoforms....25
5.2 TLR4 signalling in macrophage polarization....28
6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES....32
7 ACKNOWLEDGMENTS....35
8 REFERENCES....39

LIST OF ABBREVIATIONS

2G7	Monoclonal antibody against A-box of HMGB1
BBB	Blood brain barrier
C	Cysteine
CNS	Central nervous system
CXCR4	C-X-C motif chemokine receptor 4
DAMP	Damage associated molecular pattern
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
GFAP	Glial fibrillary acidic protein
HMGB1	High Mobility Group Box 1
Iba1	Ionized calcium-binding adapter molecule 1
IF	Immunofluorescence
IHC	Immunohistochemistry
IPA	Ingenuity Pathway Analysis
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycation end products
RNAseq	RNA sequencing
ROS	Reactive oxygen species
tPA	Tissue plasminogen activator

1 INTRODUCTION

HMGB1 protein was originally discovered in the 1970s as a part of DNA chromatin structure in cell nucleus (1), where it has homeostatic functions. During the 1980s and the 1990s HMGB1 was also found to have several additional functions such as extracellular induction of neurite outgrow and inflammation (2–4). The importance of HMGB1 in inflammation was further understood when it was recognized to be a Damage Associated Molecular Pattern (DAMP) and later further defined to be an alarmin, a dual function protein with roles both in homeostatic state and in stressed conditions, then as a pro-inflammatory mediator (5). When writing this thesis, the search term "HMGB1" gives 29 588 results in the US National Library of Medicine National Institutes of Health (PMC) database– the knowledge of HMGB1 has grown exponentially.

This thesis work focuses on understanding the role of HMGB1 in neuroinflammation and stroke. Dampening the post-stroke neuroinflammation has been suggested as a potential therapeutic strategy that could improve the outcome and recovery for patients. HMGB1 inhibition has shown promising results in stroke animal models, however no candidate drugs have yet made it to clinical use. Development of drugs targeting HMGB1 requires in depth knowledge of HMGB1 regulation and function in neuroinflammation.

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2 BACKGROUND

2.1 HMGB1 IS A PROTOTYPICAL ALARMIN

Inflammation is the complex process that is initiated when the immune system has recognized danger. What the immune system recognizes as danger can come in many forms, it can be molecules recognized from foreign pathogens, referred to as pathogen associated molecular patterns (PAMPs) or danger can be molecular damage or stress signals from host cells, damage associated molecular patterns (DAMPs). This "Danger theory" was first introduced by Polly Matzinger in 1994, and although certain parts of the original danger theory can be criticized, the existence of PAMP and DAMP molecules are undeniable, as there are soluble molecules recognized as danger, damage or pathogenic by the immune system (6,7).

While the concept of PAMPs and DAMPs are currently widely taught in immunology, the concept of alarmins, a sub-group of DAMPs, is less well known. The focus of this thesis is a protein called high mobility group box 1 (HMGB1), which in the thesis title is referred to as a DAMP, but it would be more accurate to refer to it as an alarmin. Whereas DAMPs are any endogenous molecules that signal damage and danger, for instance extracellular ATP or DNA, alarmins are endogenous dual function proteins that perform their main task inside cells, but have a secondary task outside of cells in mobilizing the immune system and initiating inflammation (8). Alarmins are divided into three groups depending on their homeostatic cellular localization: granule derived (antimicrobial peptides), nuclear (regulating gene transcription and DNA repair) and cytoplasmic (protein folding, calcium binding) (9). HMGB1 is a nuclear alarmin, with the homeostatic task in DNA architecture, and its alarmin task is to activate cytokine secretion, cell migration and proliferation (10,11).

The name "alarmin" was originally proposed by Joost Oppenheim (12) and HMGB1 was defined to be the prototypic alarmin by Marco Bianchi in 2007 (5), based on the characteristics of HMGB1 as well as criteria from other (suspected) alarmin molecules. The criteria are the following:

(I) *Passive release from necrotic, but not apoptotic cells*
(II) *Active non-classical secretion*
(III) *Role in inflammation or immune system*
(IV) *Promotion of tissue regeneration*

Additional criteria have been suggested, according to which alarmins "sense" and induce a different signal for either danger or damage, by regulating post-translational modifications (13). In this thesis, I discuss my results in the light of this argument and underline adding a fifth criteria:

(V) *Signalling damage and danger*

The following five chapters will give examples of HMGB1s functions regarding the listed criteria and give more detail on more recent discoveries done since the original criteria was defined.

2.1.1 (I) HMGB1 is released from cells undergoing immunogenic cell death

HMGB1 is released from necrotic cells. However, it is also released from pyroptotic and necroptotic cells. As the original statement defines, HMGB1 originating from necrotic cells is passively released and apoptotic cells do not release HMGB1 unless apoptosis turns into secondary necrosis, which occurs if the apoptotic cells are not cleared by phagocytes. The amount of HMGB1 released from necrotic cells correlates directly to cytotoxicity levels (14).

The most in depth studied example of HMGB1 release can be found in a study performed on trauma patients. Ottestad *et al* measured HMGB1 concentration from patient blood directly after entering hospital and then frequently every hour until 24 h, when the source of HMGB1 is most likely necrotic cell death. The levels were high in most patients, in some patients reaching over 100 ng/mL, however there was no correlation between the outcome of trauma and initial high HMGB1 levels. Repeated blood samples were collected and they found that the HMGB1 release was biphasic for part of the patients, as they had a second peak of HMGB1 release a few hours after trauma, where a higher peak predicted a worse outcome (15). The second peak in HMGB1 release was interpreted to be from active secretion leading to the next criteria: *(II) Active non-classical secretion.*

2.1.2 (II) HMGB1 is secreted from activated cells via non-classical secretion pathway

The classical pathway for secretory proteins is to transfer from the endoplasmic reticulum into the Golgi, from where it is secreted to the extracellular environment. However, alarmins including HMGB1 are lacking the leader peptide to signal transfer of the protein to Golgi for secretion.

A major source of secreted HMGB1 are cells of monocytic lineage, monocytes, macrophages and dendritic cells. Hepatocytes, platelets, endothelial cells and cancer cells can also actively secrete HMGB1 depending on disease context (16,17). When monocytes are activated by a pro-inflammatory signal, the JAK-STAT pathway is activated, leading hyperacetylation of the nuclear localization sequences in HMGB1 (see Fig 1.) (18). Hyperacetylated HMGB1 is transported to cytoplasm where it accumulates into secretory vesicles for further release into the extracellular space where HMGB1 performs pro-inflammatory functions (19). The passive release and active secretion when hyperacetylated are the main described mechanisms for HMGB1 release, however there is possibility for unknown mechanisms especially with other alarmins. Active secretion can also happen via pyroptosis, a programmed lytic cell death that takes place in immune cells like monocytes (20).

The biphasic release of HMGB1 in trauma patients can also be detected in sepsis and skeletal muscle injury, indicating that the same events may take place in stroke (21). The second peak

of HMGB1 release seen in the trauma patients (15), was almost certainly HMGB1 secreted by activated immune cells. Importantly, unlike the first peak, the second peak did predict a worse outcome in patients, where the patients developed critical systemic inflammation. These results are supported by findings made in sepsis studies where HMGB1 is secreted from activated cells and inhibiting HMGB1 reduces lethality (4,15).

2.1.3 (III) Role in inflammation and in the immune system – HMGB1 binds to PRRs and activates inflammatory functions

Extracellular HMGB1 causes inflammation by binding pattern recognition receptors (PRRs) on cell surfaces, either alone or in complex with other pro-inflammatory molecules such as lipopolysaccharides (LPS) and DNA. There are currently over ten receptors associated with HMGB1 binding, including toll like receptor (TLR) 4, Receptor for advanced glycation end products (RAGE), Mac1, TLR2/3/5/7/9, interleukin 1 receptor1 (IL1-R1) and C-X-C Motif Chemokine Receptor 4 (CXCR4) (22,23). Most of the receptors are binding to HMGB1 in complex with other molecules. This thesis work has mostly involved the receptors: CXCR4, TLR4 and RAGE, since they are the most well characterized and confirmed in molecular studies with endotoxin free HMGB1.

HMGB1 binding to CXCR4 and TLR4 is regulated by its redox status. HMGB1 has three cysteines in its structure, C23, C45 and C106 (numbers corresponding to amino acid order), which can be reduced or oxidized (see Fig 1). When the three cysteines are fully reduced (frHMGB1, HMGB1C23hC45hC106h), HMGB1 forms a complex with CXCL12 and binds to CXCR4, promoting cell migration and proliferation. Mild oxidation of HMGB1 forms a disulfide bond between C23 and C45, whereas C106 remains in fully reduced form. Disulfide HMGB1 (dsHMGB1, HMGB1C23-C45C106h) loses the ability to bind to CXCL12, instead it binds to MD-2 and TLR4, activating NF-κB signaling pathway which leads to a downstream secretion of pro-inflammatory cytokines like TNF-α, IL-1α/β, IL-6 and IL-8. (10,24). According to current knowledge, HMGB1 loses its inflammatory functions when all three cysteines become oxidized (oxHMGB1, HMGB1C23soC45soC106so), without the ability to bind to CXCR4 or to TLR4 (10,25). Intracellular oxHMGB1 is an inducer of apoptosis (26).

HMGB1 can bind to RAGE inducing MAPK pathway activation (27). Both frHMGB1 and dsHMGB1 can bind to RAGE, however dsHMGB1 binds with a higher affinity (17). Several studies show that HMGB1 binding to RAGE induces pro-inflammatory cytokine release (28). However, it appears that the effect could be more indirect. MAPK activation will translocate TLR4 to the cell surface, which in turn will lead to cytokine secretion as described in the previous paragraph (27). Though TLR4 activation can also happen intracellularly also leading to cytokine secretion (29). Importantly, HMGB1 facilitates the access of DAMPs to their intracellular receptors when RAGE is endocytosed with its ligands, which leads to a full-blown inflammatory reaction (30,31). HMGB1 has also been shown to form a complex together with DNA, RAGE and TLR9 (32). This suggests that a potential mode of action of RAGE is also heterocomplex formation with other receptors.

OxHMGB1 is believed to be present during resolution of inflammation (33). Due to difficulties in defining HMGB1 redox states in biological samples it has not been shown to be true or false. However, the terminal oxidation of cysteine residues to sulfonic forms is typically impairing the protein function (34), making the statement of terminally oxidized HMGB1 likely to be true.

The results from the Ottestad *et al* (2019) trauma study can be interpreted in the light of HMGB1s proinflammatory abilities (15). Possibly the first wave of HMGB1 released originally from the necrotic cells has been frHMGB1 – recruiting immune cells towards the injury site without causing cytokine release. This is supported by a report indicating that necrotic cells release non-oxidized HMGB1, which means it is most likely frHMGB1 (35). However, in patients having a second wave of HMGB1 release predicting malignant prognosis, the HMGB1 has most likely been dsHMGB1 secreted by immune cells. The experiments performed on activated mouse bone marrow derived macrophages (BMDMs) indicate that a disulfide bond is formed already in the nucleus before active secretion of HMGB1 (36). The redox isoforms also play a role in the next function of alarmins – tissue regeneration.

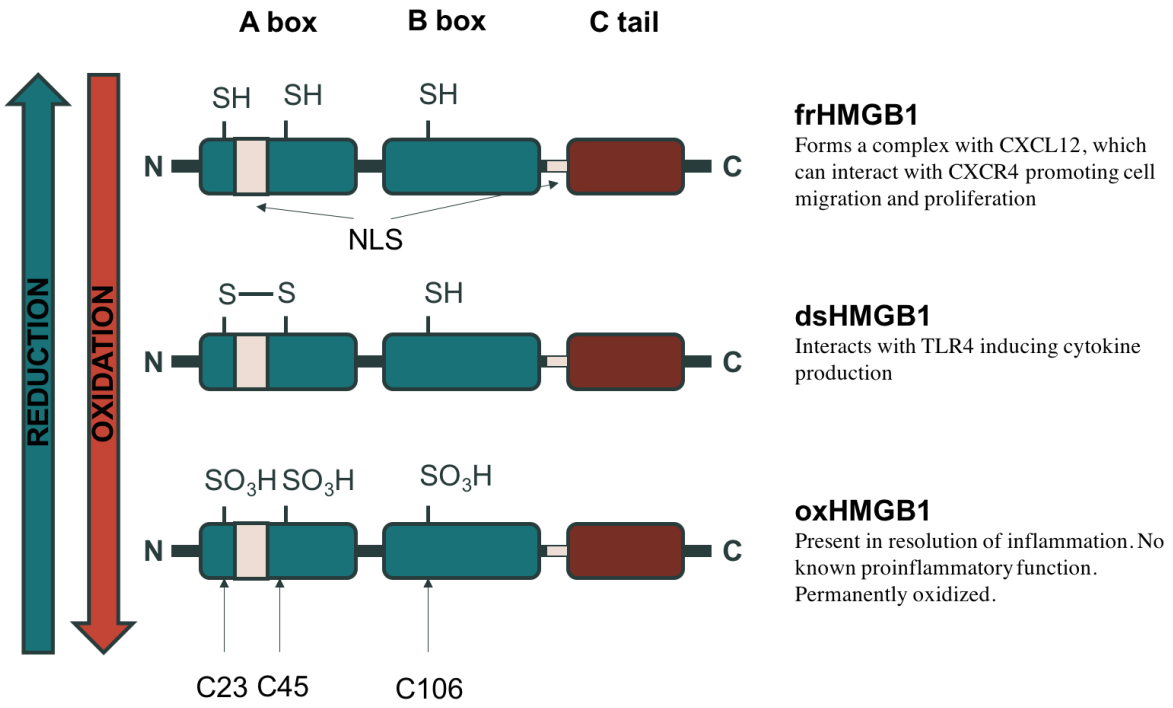

Figure 1. The structure and extracellular functions of HMGB1. HMGB1 functions are dependent on post-translational redox modifications that can occur both intra- and extracellularly. HMGB1 exists in three different redox isoforms: fully reduced (frHMGB1) when all cysteines are in thiol form, disulfide (dsHMGB1) where C23 and C45 form a bond and C106 is still in thiol form and fully oxidized (oxHMGB1) where all cysteines are in sulfonyl form. The cysteines C23, C45 are in the A box and C106 is in the B box. The cysteines can be oxidized or reduced and it will change the function of HMGB1. The structure of HMGB1 contains two nuclear localization sequences (NLS) that can be hypo- or hyperacetylated to regulate the localization of HMGB1 between nucleus and cytoplasm.

2.1.4 (IV) Promotion of tissue regeneration

The first extracellular function determined for HMGB1 was in neurite outgrowth in the developing nervous system, which hints that HMGB1 might also play a role in regeneration (37). During the last few years, HMGB1 was confirmed to have abilities in regenerating tissue after injury. So far, the function appears to be specific for frHMGB1, whereas dsHMGB1 has more of a destructive effect. Several different type of stem cells have been found to enter G_{alert} state, a cell cycle resting state where stem cells still have an improved regenerative function in comparison to G_0 phase when frHMGB1 is binding to CXCR4. In agreement with this, pre-treatment with frHMGB1 improved fracture healing by inducing the G_{alert} state. (38)

Interesting findings have been made in a model of muscular dystrophy, where injections of the non-oxidizable 3S mutant of HMGB1, in which the three cysteines are replaced with non-oxidizable serines thus mimicking frHMGB1, induced regeneration of muscle tissue. HMGB1 appeared to be oxidized to dsHMGB1 in the inflamed muscle and balancing the redox forms by 3S mutant improved the dystrophy (39).

Returning to the example of trauma patients (15), it is interesting to speculate the complex role of HMGB1 in injury and inflammation. If one would only focus on the initial high values of HMGB1 in the patient's blood circulation HMGB1 plays no role in injury, it is just an outcome. Based on the second peak, it appears that HMGB1 is detrimental. In the study, they stopped following the HMGB1 levels in blood circulation after 48 h, when the HMGB1 levels were already back to normal levels. As much as we should not compare mice to humans too directly, the HMGB1 released from original injury appears to have purpose also in recovering from the injury. It is interesting that a single molecule can have all these, almost reverse, functions. The next paragraph is discussing previously introduced topics of how HMGB1 could signal showing between damage and danger.

2.1.5 (V) Sensing and separating damage and danger

The fifth criteria, the ability of alarmins sensing between damage and danger was not part of the original list proposed by Marco Bianchi. Rider *et al* suggested that alarmins can differentiate between damage and danger after discovering that a classical cytokine, IL-1α, can translocate to a DNA damage site, become acetylated and then enter the cytoplasm to signal DNA damage. IL-1α plays a role in skin healing from UV damage (13,40). HMGB1 is also translocated to the cytoplasm after hyperacetylation as explained earlier in chapter 2.1.2 and it shares some structural similarities to IL-1α. The idea that alarmins could differentiate between damage – "*needs repairing*" and danger – "*needs to be destroyed*" is hence another way of regarding the pro-inflammatory HMGB1 functions.

HMGB1 translocates to cytoplasm in response to different types of cell stress. The classical way to induce stress is LPS stimulation, which would signal a presence of a pathogen, meaning danger. If we believe that actively secreted HMGB1 is dsHMGB1, that would be the post-translational modification signaling danger and result in cytokine secretion. Whereas frHMGB1, released from necrotic cells, is inducing cell migration and proliferation as well as

regeneration of the tissue, is the signal for damage. Thus, the purpose of frHMGB1 is to recruit immune cells to the site of injury. Then cells decide based on the other DAMPs, PAMPs or cytokines from the environment, if there is a reason to secrete dsHMGB1 or not. HMGB1 oxidation can occur both in cytoplasm and in the extracellular space (41). Redox regulation is also suggested to regulate the function of the alarmins S100 proteins (42). Cysteines in the structure of alarmins are a shared feature. For instance, proIL-1α has three cysteines like HMGB1. The focus of this thesis work has been the redox modifications of HMGB1, however HMGB1 undergoes other PTMs, like acetylation, phosphorylation and glycosylation, which might also play a role in regulating HMGB1 signaling (41).

Considering the philosophy of alarmins differentiating between damage and danger and signaling this to the immune system, one can consider the initial trauma during ischemic stroke result in release of frHMGB1 to signal damage that should be repaired (38). The recruitment of immune cells to the site of injury is only a signal of damage until the cells come in contact with a danger signal like dsHMGB1, that could form in the extracellular space due to oxidative stress or be purposely oxidized in cells for active secretion (43,44). Apoptotic cells contain oxHMGB1, which can be secreted to prolong the survival of cell (26) and does not alert the immune system to induce cytokines.

2.2 THE ROLE OF HMGB1 IN NEUROINFLAMMATION

Neuroinflammation, the activation of the immune system in the central nervous system (CNS), is present in different types of diseases and conditions, from injury with an acute neuroinflammatory response to neurodegenerative diseases which manifest with chronic neuroinflammation. HMGB1 participates neuroinflammation in ischemia (45), hemorrhage (46), traumatic brain injury (TBI) (47), epilepsy (48), Alzheimer's amyloidpathy (49), Multiple sclerosis (50) and Parkinson's disease (51). Inhibiting HMGB1 in animal models of these diseases has shown beneficial results and suggests that HMGB1 could be a therapeutic target in several different diseases.

General neuroimmune mechanisms are shared between the different diseases and can be characterised by the activation of glial cells (microglia and astrocytes) and the release of cytokines and chemokines. Microglia are the resident macrophages of the brain. HMGB1 has been shown to activate microglia as well as astrocytes via TLR4 and RAGE upregulating markers indicative of a pro-inflammatory phenotype (52,53). For microglia, this includes changes in cell morphology, which can be detected by increased Iba1+ area, and most importantly expression of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 and of HMGB1 (54–56). Microglia activation via TLR4 and secretion of cytokines is an indication of dsHMGB1 involvement.

Astrocytes are supporting endothelial cells that help form part of the blood–brain barrier (BBB). Astrocyte activation is characterized by hypertrophy detected in glial fibrillary acidic protein (GFAP) expression (57), and they can be activated by both IL-1β, which is secreted from activated microglia, and as previously mentioned by HMGB1. HMGB1 activation leads

to expression of inducible nitric oxide synthase (iNOS) (52). Astrocyte activation will modify blood brain barrier (BBB) permeability.

A major distinction of neuroinflammation from "normal" peripheral inflammation is the BBB, which regulates cells and molecules entering and leaving CNS as well as blood circulation. The BBB is closed by junction molecules between the endothelial cells. During ischemic stroke several proteins are upregulated, including Matrix metalloproteinase-9 (MMP-9), which is suspected to cleave extracellular matrix components and tight junction proteins between the endothelial cells to ease the opening of the BBB (58). A second upregulated protein is aquaporin-4 (AQP4), a water channel molecule, which is expressed in astrocytes that surround the endothelial layer of the BBB. The water passing through the AQP4 to astrocytes can lead to cell swelling and formation of edema (59). HMGB1 is associated to both upregulation of MMP-9 and AQP4 and to BBB leakage (60).

A consequence of increased BBB permeability is an increased access to the CNS for the peripheral immune system. HMGB1 is known to recruit and activate both lymphoid and monocytic cells (61). Thus, upregulation of HMGB1 facilitates access to the site of injury for immune cells by increasing BBB permeability and inducing cell migration. The adaptive immunity plays a role not only in neurodegenerative diseases, but also in traumatic events, for instance T-lymphocytes are activated by HMGB1 in ischemic stroke (62). The outcome of cell infiltration is complex, as infiltrating lymphocytes can potentially worsen the outcome of stroke (63) while infiltrating monocytes can have a dual role depending on their polarization towards pro-inflammatory or repairing phenotypes (64).

The role of HMGB1 in neuroinflammation is complex. As microglia and infiltrating monocytes constitutes a major part of neuroinflammation, this thesis work has investigated the effect of HMGB1 and its redox isoforms on microglia and macrophage phenoypes in detail.

2.2.1 HMGB1, macrophage recruitment and polarization

Macrophages are heterogeneous cells that perform homeostatic functions in different tissues. They are also key players of immune defense where they acquire different roles depending on the tissue, their niche and the inflammatory state of the body. Tissue resident macrophages, like microglia, find their niches during embryonic development (65). Microglia, as well as migrating monocytes are considered to be the critical regulators of the outcome and recovery from stroke (66,67). In neuroinflammation, bone marrow originated monocytes enter the blood circulation and migrate to the site of injury (68). Monocyte migration to the inflamed site is regulated by the balance in CXCR4 and CXCR7 expression and the chemokine CXCL12 (69). CXCR4-CXCL12-frHMGB1 complex induces the migration towards the site of injury whereas CXCR7-CXCL12-frHMGB1 may inhibit it (10,70).

When monocytes enter the brain, they mature to macrophages either to perform a task in clearing danger, damage and in repair. Infiltrating macrophages can even overtake an empty microglia niche and acquire microglia like functions (71). Macrophages acquire the needed phenotype by responding to stimuli with cytokines, chemokines, DAMPs and PAMPs. To

achieve that, macrophages undergo both gene expression and metabolic changes known as polarization. Macrophage polarization has historically been described by discrete polarization models, presently used to describe *in vitro* macrophages being pro-inflammatory (M1) or resolving (M2). However, the polarization is better described as a spectrum, which reflects the *in vivo* situation where macrophages are considered to move in a spectra between pro-inflammatory and anti-inflammatory phenotypes depending on their status and the microenvironment (72). In healthy brain microglia are in "resting" state, where their role is to constantly prune synapses and maintain homeostatic functions in brain. In response to stress signals, microglia become activated and can express cell surface markers considered to be M1 or M2 signature (73).

For polarization of macrophages to occur, a shift in metabolism to produce energy and building blocks for their different functions is needed. M1 cells need to increase ATP production and shift from aerobic oxidative phosphorylation to anaerobic glycolysis. M2 cells can use glycolysis as an energy source to some extent, but they mainly upregulate the oxidative phosphorylation pathway. M1 macrophages phagocytose opsonized cell particles, produce reactive oxygen species (ROS) and secrete pro-inflammatory cytokines, like TNFα, IL6, and HMGB1. To produce ROS, the M1 cells activate the nitric oxide synthesis pathway and use L-arginine to produce nitric oxide. M2 cells metabolize L-arginine to urea and ornithine and eventually citrulline. M2 macrophages participate in immune system functions like wound healing and regeneration and they can limit the function of effector T cells (74).

HMGB1 has been shown to polarize macrophages, however some reports claim that HMGB1 induces an M1 phenotype (75–78) and in different conditions an M2 phenotype (75,79). According to Son *et al*, HMGB1 alone polarizes macrophages towards M1 phenotype and in complex with C1q, while the M2 phenotype is induced by HMGB1 and RAGE (75). Schaper *et al* reported a shift from M2 macrophage phenotype towards M1 in response to HMGB1 (77). Shia *et al* defined HMGB1-TLR2 binding leading to M2 phenotype (80). Comparison of these studies is complicated as the source of macrophages is different, they most likely express different PRRs and may already be polarized either towards an M1 or an M2 phenotype before HMGB1 is added to the experiment, not to mention the lack of information regarding the HMGB1 redox status.

In vivo, it is important to understand that multiple subtypes of microglia and macrophages exist simultaneously even in the same organ and microenvironment and that they perform different functions simultaneously. Macrophages should always be regarded as diverse functional subpopulations that perform different functions encompassing destroying dead and injured tissue and helping in the regeneration and repair of tissue and maintaining tissue homeostasis. After ischemia both "M1" and "M2" glia populations are upregulated, showing that monocytes are activated to perform both tasks (81–83). The timeline of HMGB1 release and microglia and macrophage activation is presented in Figure 2. Since macrophage and microglia polarization can determine the outcome of how neuronal tissue recovers from neuroinflammation and since

it is so unclear how HMGB1 induces M1 and M2 phenotype the connection between HMGB1 and macrophage polarization is important to determine more in depth.

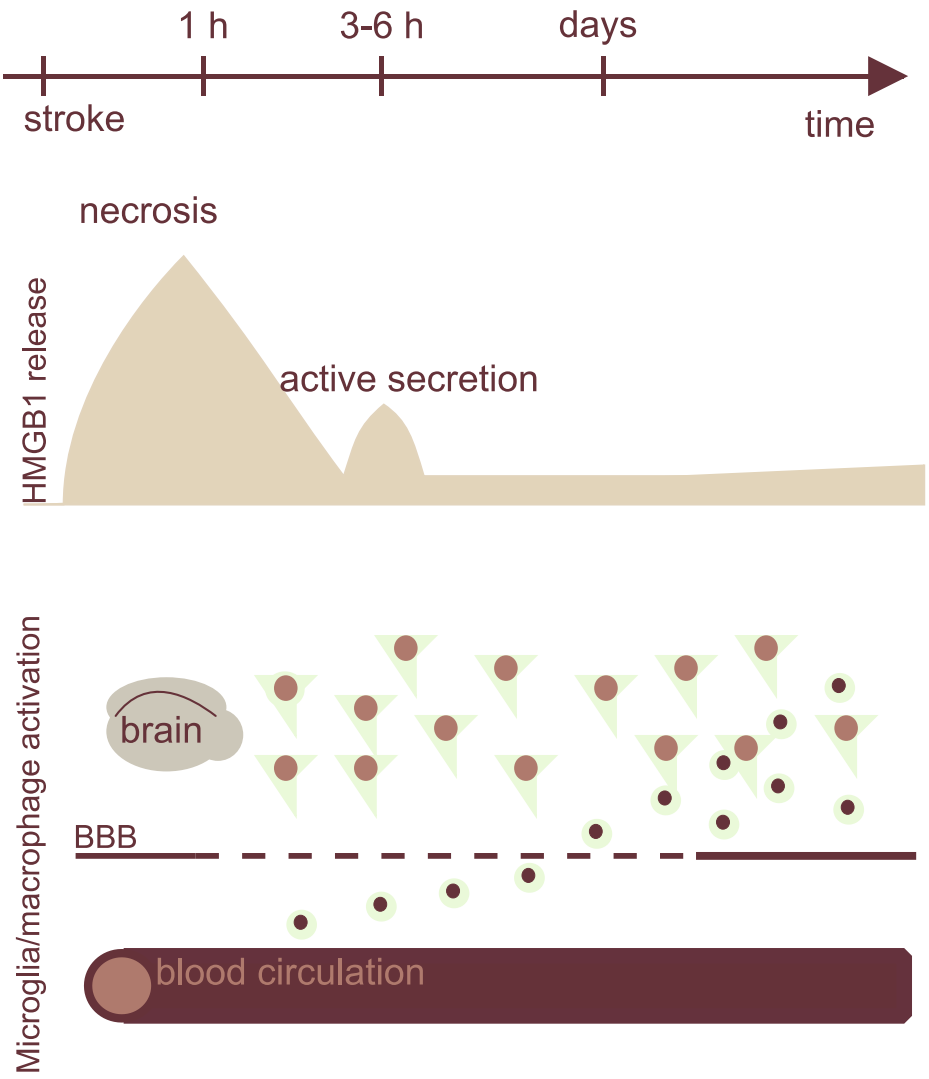

*Figure 2. The suggested timeline of HMGB1 release, microglia activation and macrophage recruitment in stroke. The initial **HMGB1 release** to blood circulation occurs during the first hour from the initiation of stroke leading to the highest HMGB1 concentration. The source of the HMGB1 in the first phase is necrotic cells. The HMGB1 levels decrease, until a second phase in HMGB1 occurs within 3-6 hours. The second peak in HMGB1 secretion is predicted to be from immune cells actively secreting HMGB1. **Microglia** becomes activated in response to stress molecules from the lesion and polarize towards "M1" and "M2" phenotypes. Activated microglia can actively secrete HMGB1, which also plays a role in opening the BBB. **Monocytes** can infiltrate to the lesion site through BBB and mature into macrophages, where they contribute to the balance of M1 and M2 microglia and macrophages.*

2.3 CLINICAL REALITY OF STROKE

Stroke is the third leading cause of death in the world (84). There are two subtypes of stroke: ischemic – caused by blood clot, or hemorrhagic – a bursting blood vessel in brain. Ischemic stroke is more, consisting nearly 87% of the patients, whereas hemorrhagic stroke is often more lethal (85,86). Common causes for stroke are hypertension, dyslipidemia, small and large artery disease and atrial fibrillation (85).

When a patient is suspected to have stroke, they undergo MRI imaging to identify the type of stroke. The severity and location of the stroke is estimated using National Institutes of Health

stroke scale (NIHSS), which scores for instance difficulties in speech, language movement of face, limbs and eyes. Based on the time from the initial symptoms, MRI imaging and NIHSS scoring a suitable treatment option will be selected.

The treatment options for ischemic stroke and hemorrhagic stroke differ from each other as in the case of ischemia, one attempts to dissolve the clot, whereas in hemorrhage the aim is to stop bleeding. Common treatment options for ischemic stroke are to dissolve the clot by tissue plasminogen activator (tPA) or by performing thrombectomy in case of a larger occlusion. The time window for tPA treatment to be an option is very short, preferably it should be administrated within 4 hours from the onset of stroke. Hemorrhagic stroke is mostly treated by surgery.

Survivors of stroke often suffer from severe disabilities including cognitive dysfunction and motoric problems. Patient's life expectancy is dependent on the early phase recovery and amount of inflammation incited. (87,88). Depending on the severity and location of the stroke, patients would suffer from combinations of speech, cognitive and physiological disabilities and would need assistance in recovery and managing life with the disabilities. Often, the lost functions are not recovered and currently there is no drug that could be used as a treatment option.

The tissue damage in brain ischemia can be divided to two areas: "ischemic core" where neuronal cell death occurs and "ischemic penumbra", where tissue is showing sign of damage, stress and neuroinflammation, but no actual necrosis is ongoing. The penumbral region is of interest in developing anti-inflammatory therapies against due to the large amount of neuroinflammation. In comparison, during hemorrhagic stroke the neuroimmune response of microglia happens within minutes, as the pro-inflammatory components of the blood enter the brain (89).

2.4 RODENT MODELS OF STROKE

No animal model can perfectly describe the human stroke starting from differences between brain anatomy between rodents and humans, to the different conditions that cause the stroke. Whereas the average patient is usually an elderly person with underlying conditions like hypertension or diabetes, the rodents used in ischemia injury models are young and healthy. When selecting a model to study the effect of a potential drug or therapy, each model has their own upsides and downsides. Choice of a model is dependent on the specific research question at hand.

Middle cerebral artery occlusion (MCAO)

The most commonly used model for ischemic stroke is induction of thrombosis in the middle cerebral artery by occluding it with a guidewire and surgical filament. Most of the time the model is performed either as a permanent blockade or as a transient ischemia reperfusion injury, meaning that occlusion is removed after set time period allowing reperfusion. 80% of ischemic strokes in humans occur in this artery making it clinically relevant for studying focal

brain ischemia. The severity of the infarct can be varied by changing the time of occlusion. In 30 min, damage occurs only in striatum whereas by extending the time to over one hour the damage spreads to cortex. The model can induce necrosis in ~40% of the brain (90).

M2 middle cerebral artery occlusion (M2CAO)

M2CAO is a subtype of MCAO which was used in this thesis (Paper II). Instead of occluding the middle cerebral artery, the M2 segment, the insular segment further in the artery, is occluded. The second most common location for ischemia to occur in humans after MCA is the M2 segment which is suitable for acute intervention. The volume of the necrotic area in this model is marginal in comparison to MCAO. The benefit of this model in developing therapeutic strategies is the smaller ischemic core and larger penumbra, which can be rescued by intervention, making it a good model for anti-inflammatory therapy development. (91)

Embolism

This model is also modeling thrombosis and can be performed either by injecting a preformed fibrin clot or by photocoagulation, where a dye is injected to circulation and is coagulated in response to light. The fibrin clot method is interesting as it mimics human stroke well and can be used for studying thrombolytic agents, however, it has high variability of lesion size, which can make conclusions difficult when testing potential drugs. The benefit of photocoagulation model is its high reproducibility and accurate location of the stroke. The downside is the formation of vasogenic edema, which is uncharacteristic for human stroke and is unsuitable for testing neuroprotective drugs (92).

Endothelin 1 injection

Endothelin 1 is vasoconstrictor and it can be used to induce ischemic lesions in cortical or subcortical regions by a direct intracerebral injection. The method is easy to perform and the mortality in this model is very low, however the duration of ischemia is not controllable and the reproducibility of infarcts is low (92).

Spontaneous stroke

The stroke prone spontaneously hypertensive rat (SHRSP) is a genetic model that develops strokes spontaneously due to hypertension, accurate to human disease in a sense that stroke develops due to the long term underlying condition. The model is interesting for studying the genetic component of developing hypertension and stroke, however it is complicated to use for testing therapeutics as the stroke develops spontaneously and it cannot be used for acute intervention (93).

2.5 PATHOGENIC AND PROTECTIVE ROLE OF HMGB1 IN STROKE

Elevated HMGB1 levels are detected in patient blood circulation after stroke, even a month later (94,95). Meta-analysis comparing HMGB1 levels in several different studies suggests that HMGB1 levels may correlate to a more severe condition (96). The causative effect of HMGB1 in brain tissue damage and as an inflammatory mediator has been confirmed in rodent models,

where HMGB1 inhibitors in animal models improve the outcome of stroke by reducing the size of the ischemic lesion. Moreover, intracerebral injection of HMGB1 in experimental stroke increases the volume of ischemic injury (97,98).

As seen in trauma patients (15), the release HMGB1 in stroke occurs biphasic. At the ischemic site, HMGB1 translocates from nucleus to cytoplasm immediately after ischemia and 3 hours later most of the HMGB1 has been released to extracellular space from neurons (99). HMGB1 is massively released within first few hours from stroke (98). 2 days later, HMGB1 expression increases in microglia and astrocytes, which suggests active secretion of HMGB1. The expression of HMGB1 in the ischemic core peaks around 4 days after ischemia (99). During the early phases of post-stroke neuroinflammation, microglia and astrocytes obtain mostly pro-inflammatory and damaging phenotypes. HMGB1 activated microglia damage neurons and release cytokines (described more in depth in chapter 2.7.), which end up activating astrocytes to express AQP4 and opening of BBB (100).

The amount of initial HMGB1 released to circulation and CNS correlates with the severity of ischemic injury, originating mostly from necrotic cells (101). The serum HMGB1 levels increase again after several days. It is interesting that second phase of HMGB1 happens a few days later in stroke, unlike in trauma patients where it occurred already within few hours (15). The slower kinetics can most likely be explained by the induction of BBB permeability needed to occur before immune cells can be recruited to the site of injury. Circulating immune cells: neutrophils, macrophages, dendritic cells and T cells typically require 1-2 days to access the site of stroke, whereas B-cells need 4-7 weeks (102).

The BBB leakage is initiated 3 h after ischemic injury and inhibition of HMGB1 decreases BBB leakage. These mechanism include dissociation of tight junctions between vascular endothelial cells, contracting pericytes lying on top of endothelial cells and reperfusion around capillary vesicles (103,104). MMP-9, a protein breaking extracellular matrix involved in BBB leakage, is associated with HMGB1 and poor outcome (105). BBB leakage can be considered as a damaging event, as it also leads to the formation of cytotoxic edema (106).

The circulating immune cells can be recruited by HMGB1 to the ischemic site through the opened BBB. Infiltrating macrophages and glial cells might play a major role based on evidence from permanent the MCAO ischemia model, where both global RAGE KO and bone marrow transplant from RAGE KO mice, reduced the size of the ischemic lesion (107). However, the model can be questioned for the clinical relativity as it is lacking the reperfusion stage, though this mechanism might not be as relevant in human stroke, especially since another study where transient MCAO was used reported no reduction of ischemic lesion size, instead RAGE and HMGB1 were inducing post-stroke sickness behavior in MCAO model, suggesting that the role of RAGE is systemic, not directly damaging the ischemic lesion site (98).

When considering other outcomes than lesion size, HMGB1 may also display a beneficial role. Examples of HMGB1's potentially beneficial role in the recovery from stroke can be acquired outside of stroke models. In models of traumatic brain injury, which in some ways is

comparable to ischemic stroke, conditional knock-out of HMGB1 displayed reduced loss of brain tissue. Despite this, there was lack of benefit regarding BBB leakage, edema formation and cognitive recovery of the mice (108). Roussel *et al* go as far as to suggest that modified recombinant HMGB1 lacking pro-inflammatory properties, should be used as a therapy as their *in vitro* experiments displayed a clot lysing effect by binding to tPA (109). Li *et al* also studied HMGB1 and its thrombolytic therapy, and found that inhibiting HMGB1 was ameliorating the neurovascular complications (110).

All this evidence supports the fact that more effort in research to develop HMGB1 inhibitors are needed in order to find suitable therapeutic treatments. However, as HMGB1 also plays a protective and regenerative role in ischemic injury, it is important to study HMGB1 regulation and mechanisms in depth to find a correct balance of which HMGB1 isoform and to what extend should be inhibited for beneficial outcome.

2.6 TARGETING HMGB1 IN STROKE AND OTHER NEUROINFLAMMATORY DISEASES

The most successful HMGB1 inhibitor in neuroinflammation is a monoclonal antibody recognizing an epitope in the C-tail of HMGB1. The monoclonal antibody against the C-tail of HMGB1 was used in a rat MCAO model, and the antibody had a protective effect: the infarct volume reduced as well as BBB leakage, expression of iNOS and TNFα. Mice that received the monoclonal antibody treatment had better neurological score compared to their counterparts without treatment (97). Additionally the antibody has been shown to be beneficial in experimental epilepsy (111). However, neutralizing antibodies often create secondary problems in clinical use, such as generation of anti-drug-antibodies. Antibodies also have difficulties in passing through the BBB.

Glycyrrhizin is a general anti-inflammatory molecule able to bind to HMGB1, which has successfully been used in several different animal models for stroke (112,113). Unfortunately, glycyrrhizin is very unlikely to be useful in clinical settings, as most stroke patients suffer from hypertension while glycyrrhizin itself can induce hypertension, which could be dangerous to give to a patient who has suffered from stroke (114).

Another monoclonal HMGB1 neutralizing antibody directed against an epitope in the B box, 2G7, has been beneficial in other inflammatory disease models, including sepsis and arthritis (115). When 2G7 was investigated as a potential drug in mouse MCAO model it was not reducing lesion volume nor motoric deficiency of mice. However, the paper had an interesting finding regarding measured expression of pro-inflammatory cytokines in spleens of MCAO mice, they saw a size reduction in mice which had received 2G7 treatment (98). Spleen is a source of secreted cytokines after stroke that could have an effect on the development of the necrotic lesion (116). The results from this study suggest that anti-HMGB1 therapy could be developed to rescue some patients from death even if it did not protect the neuronal tissue from death.

Recombinant A-box, which antagonizes RAGE, was beneficial in a permanent MCAO model implying that RAGE plays a role especially in microglia and monocyte activation. However, no effect on RAGE was seen when the transient ischemia model was used, suggesting that the role of RAGE is not significant when using a stroke model mimicking human stroke (98).

Inhibiting HMGB1 as therapy for stroke requires understanding of the dual function of HMGB1 and its relation to the redox isoform of the protein. Even though there is clear evidence showing that HMGB1 promotes neuroinflammation and increases the size of injury, HMGB1 also plays an important role in neurovascular repair and remodeling. A traumatic brain injury model performed in HMGB1 knockout (KO) mice supports the findings with HMGB1 inhibitors in stroke models, as the lesion volumes in the HMGB1 KO mice were significantly lower compared to wild type (wt) mice. Even though more neurons were preserved, the KO mice had more cognitive decline compared to wt mice, clearly illustrating that the balance of how much HMGB1 is neutralized and which isoform of HMGB1 that is targeted is very important for therapy development (108).

3 RESEARCH AIMS

The existence of HMGB1 redox isoforms was discovered over a decade ago and HMGB1 has been established as an inducer of neuroinflammation in stroke, however the role of the different HMGB1 redox isoforms has not been determined in brain. HMGB1 inhibition has been studied in different experimental models of ischemic stroke with beneficial outcomes. Yet, however, there is no drug candidate in clinical trials. In this PhD thesis we investigated a new type of drug molecule for use in an ischemic stroke model – an Affibody molecule - binding both fully reduced HMGB1 and disulfide HMGB1. Among the cells recruited to the site of inflammation by HMGB1 are macrophages. In this thesis we investigated for the first time the role of HMGB1 redox isoforms in macrophage migration and polarization.

The specific aims of my thesis work were to:

Paper I

Dissect the role of fully reduced HMGB1 and disulfide HMGB1 in neuroinflammation

Paper II

Investigate neuroinflammation and anti-HMGB1 Affibody molecule as a therapeutic option in a model of ischemic stroke

Paper III

Study the macrophage polarization and migration patterns induced by fully reduced HMGB1 and disulfide HMGB1

Paper IV

Compare differences in gene expression in pro-inflammatory macrophages polarized by LPS/IFNγ stimulation - mimicking infection - and disulfide HMGB1 stimulation - mimicking sterile inflammation

4 RESULTS AND DISCUSSION

4.1 PAPER I: HMGB1 INDUCES NEUROINFLAMMATION

HMGB1 was established as an inducer of neuroinflammation in neuronal diseases and sterile injuries, like ischemic stroke (50,98). HMGB1 is regulated by its redox status. frHMGB1 binds CXCL12 and CXCR4 to promote cell migration whereas dsHMGB1 induces cytokine secretion via TLR4 (10). Although the importance of HMGB1 redox status is well established, the impact of the different HMGB1 redox isoforms in brain tissue had not been studied, not to mention confirming the assumption that HMGB1 alone without the involvement of other damage signaling molecules could induce neuroinflammation.

Paper I had two aims. The first aim was to determine if HMGB1 alone without the involvement of other alarmins or signaling molecules from a cerebral injury, could induce neuroinflammation. Previously published studies investigated the role of HMGB1 in the context of injury or disease. The second aim was to compare inflammatory features of the two pro-inflammatory redox isoforms – frHMGB1 and dsHMGB1 – in brain.

The study was performed by injecting frHMGB1 and dsHMGB1 directly in the cerebral cortex of healthy Dark Agouti male rats. Saline and LPS were injected as negative and positive controls respectively. Cerebral images were taken with MRI after 24 h to measure edema formation and BBB damage. Gadolinium enhanced T1 signal volumes were significantly higher in both frHMGB1 and dsHMGB1 injected animals when compared to saline controls, indicating that the BBB leakage inducing effect of HMGB1 is independent of redox isoform.

Neuroinflammation was characterized by performing immunhistochemistry (IHC) and immunofluorescence (IF) staining on brain tissue. Both frHMGB1 and dsHMGB1 injected animals had increased IL-1β expression around the injection site, indicating that both frHMGB1 and dsHMGB1 can induce early neuroinflammatory activation. dsHMGB1 induced significantly more apoptosis, as measured by TUNEL staining, and MHC-II expression than saline control, suggesting that antigen presentation is activated. The effect could not be recorded for frHMGB1. Systemic inflammation was quantified by measuring serum α1-acid glycoprotein, however no increase was detected. The main conclusion we made from this study was, that HMGB1 is a potential therapeutic target for neuroinflammatory conditions.

No other study has to our knowledge investigated the functions of HMGB1 redox isoforms in neuroinflammation. A major novel finding has been made after the publication of Paper I regarding the ability of HMGB1 to induce neuroinflammation. When injected intravenously to healthy mice, mimicking HMGB1 in blood circulation after peripheral inflammation, HMGB1 caused microglia activation (117). However, the redox status of the HMGB1 used in this study was not determined. Furthermore, it is unknown if peripheral HMGB1 can cause BBB leakage or if its mechanism in promoting neuroinflammation is mediated via other mediators. Taken together, our study demonstrated that both HMGB1 redox isoforms has the

ability to induce neuroinflammation in healthy tissue with dsHMGB1 being somewhat more potent.

4.2 PAPER II: NEUROINFLAMMATION IN ISCHEMIC STROKE

Reduction of post-stroke neuroinflammation has long been studied as a possible therapeutic option for sparing neurons from additional damage, but so far no drug has made it to clinical use (118). There is strong evidence that post-stroke neuroinflammation exists in humans (119), however most of the discoveries regarding kinetics and mechanisms have been made in experimental models. A commonly used model is MCAO, mimicking occlusion in middle cerebral artery with extensive damage. As we know that mice are not men and each stroke model also differs from one another, it is important to carefully characterize neuroinflammation in each model used to understand in which aspects they mimic human stroke. M2CAO is mimicking human M2 segment occlusions, the second most common site for ischemic stroke in humans. Unlike MCAO, M2CAO results in a large penumbra and a smaller ischemic core making interventions more likely to succeed (91).

The role of neuroinflammation has not been fully characterized in human M2 segment occlusions, making it important to carefully document neuroinflammation in the M2CAO rodent model to eventually understand how it compares to human ischemic lesion in M2 segment. The M2CAO model has previously been characterised for classical markers of neuroinflammation, as well as secondary damage such as: phagocytic activity around neurons, reactive astrocytosis and microglia/monocyte activation during the first two weeks after induction of ischemic stroke (120). When the kinetics of neuroinflammation were compared between M2CAO and MCAO, they were not displaying the same pattern.

When Tóth *et al*. discuss how kinetics of neuroinflammation compares between M2CAO and MCAO, they point out that the models do not follow the same pattern. For instance, TSPO (an 18 kDa translocator protein), which upregulation is a general signal of activated glia cells and infiltrated monocytes (121), is reaching its peak few days earlier in M2CAO in comparison to MCAO. In **Paper II**, we focused on signs of neuroinflammation induced by HMGB1 and characterized those 4 days after M2CAO ischemia-reperfusion injury.

Paper II had two aims. The first was to detect markers or neuroinflammation: BBB-leakage, Iba1, CD68 and MHC-II – all previously associated with HMGB1 (paper I in this thesis) and to confirm that changes in brain HMGB1 expression was evident in the M2CAO model. The second aim was to test a new type of anti-HMGB1 therapeutic, an Affibody molecule (discussed in paragraph 4.3).

BBB-leakage was induced by the ischemia-reperfusion injury and had increased in approximately half of the M2CAO animals 4 days after injury. The difference was not significant for the group as a whole. Immunohistochemistry performed on brains collected 4 days after injury demonstrated a higher expression of HMGB1 at the ischemic site compared to healthy controls. HMGB1 was expressed both in nuclei and extranuclearly, evident when comparing to the nuclear staining pattern with DAPI. Iba1 and CD68 are microglia and

monocyte markers highly expressed in activated microglia. As expected, Iba1 was expressed in cerebral cortex of healthy controls as well as at the ischemic lesion site of M2CAO animals. The cell morphology of Iba1+ cells in M2CAO-subjected brains was different, indicating activation of microglia. CD68 could only be detected in M2CAO lesion sites which confirmed that activated microglia and infiltrating monocytes are present. MHC-II is expressed by activated microglia, monocytes/macrophages and by dendritic cells and could implicate ongoing antigen presentation.

To conclude, HMGB1 related neuroinflammatory changes can be detected in the M2CAO model, which suggests that it is a suitable model for pre-clinical experiments testing anti-HMGB1 therapy.

4.3 PAPER II: HMGB1 AS A TARGET FOR ISCHEMIC STROKE THERAPY

The second aim of **Paper II** was to investigate the effect of HMGB1 inhibition by an Affibody molecule on the development of the ischemic lesion and penumbra. Thus, M2CAO was selected for having a small ischemic core and a large size rescuable penumbral tissue, instead of the more common model MCAO, which mimics a large stroke.

Previous reported studies exploring HMGB1 inhibition in experimental stroke have reached contradictory results. Using a monoclonal antibody targeting HMGB1 experimental ischemic stroke was treated with a successful outcome (97). This supported the hypothesis that HMGB1 inhibition could be used for developing a therapy for ischemic stroke. Other types of inhibitors have been successfully tested in pre-clinical research since the first promising evidence, including the small molecule glycyrrhizin.

Potential problems with using antibodies specific for HMGB1 or glycyrrhizin as a candidate drug for ischemic stroke is for antibodies their poor penetrance across the BBB. Glycyrrhizin is unsuitable as a candidate drug, as it increases blood pressure, which could be detrimental in stroke patients. Therefore, the type of HMGB1 inhibitor we opted to test in paper II was a novel type of molecule in the context of stroke, an Affibody molecule (ABY). ABYs belong to a group of antibody mimetics, however they have a small molecular weight, consisting only from three helices and are well tolerated by humans and have excellent tissue penetrating abilities (122).

The results of Paper II demonstrate that the anti-HMGB1 ABY binds with high affinity to both pro-inflammatory redox isoforms of HMGB1, frHMGB1 and dsHMGB1. To confirm that the anti-HMGB1 ABY can cross the BBB, we produced the anti-HMGB1 ABY radiolabelled with Gallium-68 that once injected could be detected by autoradiography. This study demonstrated the physical functionality of the ABY as a potential drug molecule as the ABY accumulated in the region of the ischemic lesion. However, the anti-HMGB1 ABY treated rats did not show any improvement in the size of the ischemic lesion, BBB leakage or neuroinflammation, suggesting that the HMGB1 inhibitor used in this study was not improving stroke outcome.

The variability of stroke models and protocols of how HMGB1 inhibitors have been given to experimental animals, is making comparison between different studies difficult. Regardless, it still appears that the epitope targeted in HMGB1 by the inhibitor plays a role. It is still undefined if some HMGB1 inhibitors work because they prevent interaction to a certain receptor or to several receptors or specific complex formation. Inhibitors binding directly to the C-tail region of HMGB1 have been successful in alleviating ischemic lesion volume in the MCAO model. These include the anti-HMGB1 antibody used by the Nishibori laboratory (97), a small molecule binding HMGB1 C-tail, celastrol, also showed similar protective effects although it is not specific as an HMGB1 inhibitor. In addition to binding HMGB1, it inhibits NF-κB and increases IL1R expression (123). The C-tail of HMGB1 is not known to directly bind to a receptor, but is reported to regulate HMGB1 binding to DNA and preventing direct binding to TLR2 (124,125), indicating that inhibition targeting C-tail could prevent certain functions of HMGB1. When a monoclonal antibody binding to the A-box of HMGB1, 2G7, was used, there was no reduction in ischemic volume or neuroinflammation (98). The result was surprising as 2G7 is beneficial in experimental arthritis models, suggesting that there is a specific HMGB1 interaction ongoing in stroke, which could not be prevented by 2G7.

Treatment with Glycyrrhizin also decreases the size of ischemic lesion. Glycyrrhizin binds between the A- and B-boxes in HMGB1, and it can be used to inhibit both cytokine secretion and cell migration (126,127). This suggests that it is inhibiting both the frHMGB1 and dsHMGB1 forms similarly to the anti-HMGB1 ABY used by us, and that C-tail might not be the epitope *per se* which should be targeted. On the other hand, glycyrrhizin is also preventing HMGB1 translocation from nucleus to cytoplasm (128). It is currently not clear if its beneficial effect is due to inhibiting HMGB1 release or by preventing the binding of extracellular HMGB1 to its receptors.

In my opinion, it is evident that HMGB1 is inducing neuroinflammation in ischemic stroke and inhibiting HMGB1 is a beneficial strategy to reduce neuroinflammatory mediated damage in brain. However, it is not well defined how a drug against HMGB1 should be developed in the context of stroke; what are the events, receptor binding, specific complex formation, that make HMGB1 malignant and what possibly makes it protective in recovery from ischemic stroke.

4.4 PAPER III AND IV: ROLE OF HMGB1 IN MACROPHAGE POLARIZATION

Macrophage polarization is an important regulator of inflammation. Historically, macrophage polarization has been described with a discrete *in vitro* model: pro-inflammatory M1 and anti-inflammatory M2. In reality, macrophages polarize in a spectrum obtaining different functions (129).

HMGB1 has been shown to induce macrophage polarization, although the evidence is conflicting: some papers report HMGB1 to induce an M1 phenotype and some to induce an M2 phenotype (77,130). In **Paper III,** I wanted to compare the macrophage-polarizing features of frHMGB1 and dsHMGB1 in parallel using the same experimental set-up. This had never been reported previously.

4.4.1 dsHMGB1 induced macrophage phenotype

In **Paper III**, we defined the dsHMGB1 induced macrophage phenotype. The macrophages secreted the pro-inflammatory cytokines IL6 and TNFα and the anti-inflammatory cytokine IL10. As expected, gene expression of these cytokines were also upregulated in response to dsHMGB1 stimulation. In comparison, M1 macrophages induced by LPS/IFNγ and dsHMGB1 stimulated macrophages were displaying similar expression patterns.

It was clear that dsHMGB1 induced a phenotype that was not identical to the M1 phenotype, as dsHMGB1 stimulated macrophages did not secrete nitric oxide (NO). Interestingly, *Nos2* expression, the gene coding for inducible nitric oxide synthase, was recorded for both M1 and dsHMGB1 stimulated macrophages. dsHMGB1 induced substantial gene expression although not to the same extend as M1 macrophages.

M1 cells were generated by stimulating mouse BMDMs with LPS/IFNγ. LPS is a bacteria cell wall component recognised by TLR4 which would occur *in vivo* during bacterial infection. dsHMGB1 binds to TLR4 as well. Presence of dsHMGB1 alone is mimicking a sterile inflammation. We decided to compare the differences reported in **paper III** more in depth by performing an RNAseq analysis and compare the transcriptomic profiles of the two macrophage polarization phenotypes in **Paper IV.**

In general, **Paper IV** supported the results of **Paper III** that M1 macrophages and dsHMGB1 stimulated macrophages share many pathways and functional enrichments revealed by Ingenuity Pathway Analysis (IPA), with 10/12 significant pathways being shared. The differing pathways were M1 upregulation of HMGB1 signaling and Crosstalk between Dendritic cells and Natural killer cells. The two pathways only activated by dsHMGB1 were Role of PKR in Interferon Induction and Antiviral Response and VDR/RXR activation. However, the pathways defined by IPA are quite generalized and without any functional assays performed, do not provide strong explanations of differences between phenotypes.

Another interesting finding made in **Paper III** was regarding the migratory properties of macrophages with a dsHMGB1 induced phenotype. Cell motility in scratch assays is a signature property of M2 macrophages, while M1 macrophages did not migrate more than the baseline control. dsHMGB1 stimulated macrophages showed significantly increased migration although not reaching the motility of M2 macrophages. M1 macrophages are obtained by stimulating BMDMs with LPS/IFNγ. Both LPS and dsHMGB1 are mainly binding to TLR4, however it is apparent in our study that dsHMGB1 stimulated macrophages migrated, whereas LPS/IFNγ stimulated did not.

RAGE is a receptor that can endocytose HMGB1. HMGB1 in a complex with C1q and LAIR and binding to RAGE is suggested to induce the M2 phenotype (31,75). We investigated the migratory properties of dsHMGB1 stimulated macrophages lacking RAGE. RAGE deficient macrophages demonstrated higher migratory capacity that RAGE expressing macrophages, suggesting that RAGE was not a requirement for the dsHMGB1 stimulated migratory phenotype. When dsHMGB1 was used to stimulate macrophages in which TLR4 was inhibited,

the macrophages were less prone to migrate suggesting that TLR4 is needed for the migratory macrophage phenotype.

In **Paper IV**, we compared the induced gene expression pattern in M1 macrophages and dsHMGB1 stimulated macrophages by RNA sequencing. We found that macrophages of both phenotypes upregulated genes with annotations on migration, cell movement and chemotaxis. When we compared the genes included in these annotations more carefully, 13 genes were identified which differed in either dsHMGB1 stimulated macrophages or M1 macrophages. The expression pattern of these genes could possibly provide an explanation for the difference in migratory properties reported in **Paper III**. However, functional assays should be performed to find an explanation for the role of these genes and their products.

The general dogma in the HMGB1 field since 2012 defines that dsHMGB1 stimulates cytokine secretion via TLR4 and frHMGB1 induces cell migration and proliferation via CXCR4. Importantly, the functions are regarded as mutually exclusive (10). At first, it might appear as if the results in **Paper III** challenges the dogma of mutual exclusivity reported by Venereau *et al*. My interpretation of our results presented in Paper III is that dsHMGB1 induced a macrophage phenotype prone to migrate towards injured cells or unoccupied areas (as in our scratch assay). Venereau *et al* investigated cell migration through a transwell assay, which mimicks cell migration towards a chemokine.

4.4.2 frHMGB1 polarized phenotype

In **Paper III** we identified that frHMGB1 had only minor effect on direct macrophage polarization, inducing cells neither towards a pro-inflammatory nor an anti-inflammatory phenotype. HMGB1 redox isoforms are often understudied, especially in older reports before the impact of HMGB1 redox isoforms on function was clearly defined. A few studies have reported that HMGB1 induces M2 polarization without defining its redox state. For instance, hepatocellular carcinoma derived HMGB1 and HMGB1 in a complex with C1q were inducing macrophages to polarize towards M2 (80,130). We hypothesised that dsHMGB1 could induce M1 polarization, and frHMGB1 would induce M2 polarization. However, our results from **Paper III** indicate that frHMGB1 does not induce macrophage polarization.

frHMGB1 was not inactive regarding macrophages. It had a detectable effect on macrophages as it was inducing cell migration in a scratch assay. Proliferation and migration inducing properties have already been defined for frHMGB1, as explained earlier. In order to study potential additional effects not measured in **Paper III** we later performed RNAseq analysis for frHMGB1 stimulated macrophages. These results are presented in the next chapter "5. Results and Discussion Outside of Constituent Papers"

5 RESULTS AND DISCUSSION OUTSIDE OF CONSTITUENT PAPERS

5.1 MACROPHAGE GENE EXPRESSION PATTERNS IN RESPONSE TO STIMULATION WITH HMGB1 REDOX ISOFORMS

In **Paper III**, we investigated the effects of frHMGB1 and dsHMGB1 stimulation on macrophage polarization. We concluded that dsHMGB1 induced an M1 like phenotype and that both redox isoforms trigged macrophage migration in an *in vitro* scratch assay. In **paper IV**, we expanded the analysis by comparing the transcriptome patterns of dsHMGB1 stimulated macrophages and M1 macrophages. This expanded analysis demonstrated that dsHMGB1 stimulated macrophages and M1 had highly overlapping expression profiles. Function related enrichment indicated that migration related genes were upregulated in both M1 and dsHMGB1 stimulated cells, but interestingly the migration related gene expression patterns differed. As a continuation of **paper III and IV**, we went on and explored the impact of frHMGB1 stimulation on the macrophage transcriptome by RNAseq. A comparison with dsHMGB1 was performed. These data are presented and discussed below.

As shown in the PCA plot, the transcriptomes of frHMGB1 and dsHMGB1 stimulated macrophages separated from PBS stimulated controls (Fig. 1A), suggesting that frHMGB1 and dsHMGB1 triggered different transcriptional changes. PC1 captured 58.7% of the variation. Whereas frHMGB1 and PBS align together, the dsHMGB1 stimulation induced a substantially different gene expression pattern. These findings were reinforced by a heatmap analysis (Fig. 1B). Differentially expressed genes (DEG) were selected by using the cut-off of log2fold change larger than 1 or smaller than -1. As the samples were technical replicates, no p-values were calculated. The top 20 upregulated and downregulated genes from frHMGB1- and dsHMGB1 stimulated macrophages are listed in Fig. 1C and 1D. dsHMGB1 resulted in stronger transcriptome changes than frHMGB1, which supports the result obtained in the initial PCA (Fig. 1A). Only a few genes overlapped in the transcriptome changes of the two groups. 5 genes (*Rpl23a-ps3, Tnf, Serpinb2, Rpl17-ps10* and *Nfkbie*) were upregulated in both frHMGB1- and dsHMGB1 stimulated cells (Fig. 1E). 16 genes were downregulated in both frHMGB1- and dsHMGB1 stimulated cells as compared to PBS controls. Noteworthy is that all of them are pseudogenes or ribosomal genes (Fig. 1F).

Among the DEG, we found 4484 currently known genes when comparing dsHMGB1 stimulated BMDMs with PBS controls. The top upregulated genes encoded cytokines (e.g. *Il2b*, *Il6* and *Il1b*), chemokines (e.g. *Cxcl1* and *Ccl5*) and other proteins involved in an inflammatory response (e.g. *Saa3* and *Mx1*). On the other hand, genes that suppress inflammatory response (e.g. *Acod1* and *Socs3*) were also found to be upregulated, suggesting a possible activation of a negative feedback loop. Furthermore, analysis of the most downregulated genes, we found several genes encoding DNA binding proteins and transcription factors (e.g. *E2f2* and *Zfp395*), which further confirmed an altered transcriptome. Other downregulated genes include genes involved in Wnt-signaling (e.g. *Frat2*, *Frat1* and *Shisa3*).

Fewer known genes were found to be differentially expressed following stimulation with frHMGB1, even though our previous results showed a positive effect on BMDM migration (131). Only four protein-encoding genes were found to be upregulated, including *Tnf*, *Bc1*, *Serpinb2* and *Nfkbie*, whereof all except *Bc1* were upregulated following dsHMGB1 stimulation as well. Brain cytoplasmic RNA 1 (Bc1) is mostly known to be expressed in the nervous system. It is a long noncoding RNA (lnRNA) that controls gene expression. It has been shown to enhance invasion and migration of cells and is suggested to be involved in cancer pathology (132). Among the downregulated genes, we found 27 protein-coding genes, involved in processes such as metabolism (e.g. *Gpi-ps*, *mt-Co2/3*, *Aldoart1* and *mt-Atp6/8*) and antigen presentation (e.g. *H2-q2* and *Nfyc-ps*).

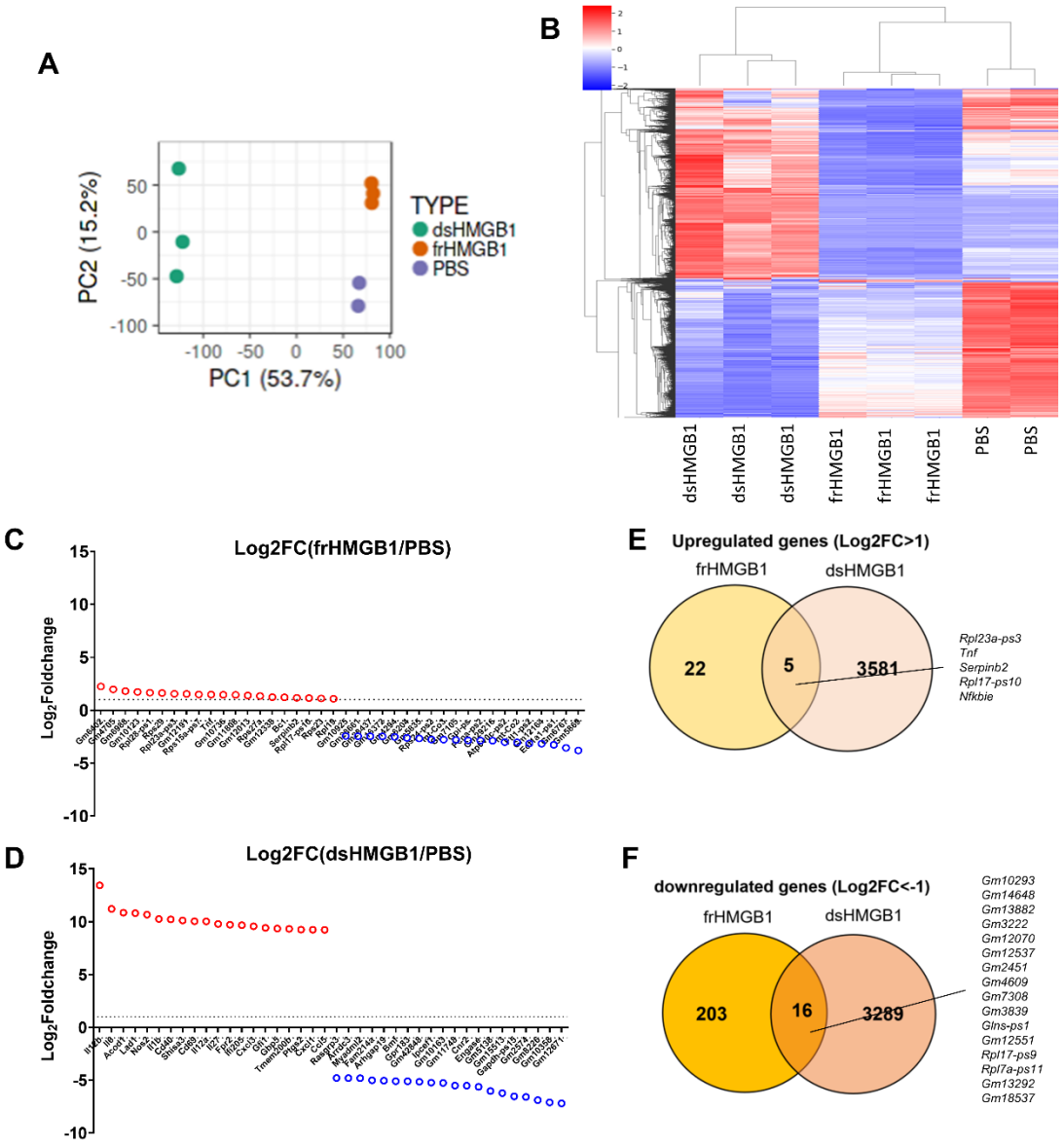

Figure 1. Gene expression patterns of frHMGB1- and dsHMGB1 stimulated macrophages compared to PBS-treated controls. (A) PCA plot of HMGB1 redox isoform stimulated cells and PBS-treated controls. (B) Heatmap clustered based on normalized counts from RNA-seq analysis of HMGB1 redox isoform stimulated cells and PBS-treated controls. (C) Top 20 up

and downregulated genes in frHMGB1- (D) and dsHMGB1 stimulated macrophages. The cut-off of log2Fold changes is 1 and -1. Overlapping of the upregulated (E) and downregulated (F) genes between the two HMGB1 groups and the PBS group.

Downstream pathway and gene set enrichment analysis were performed in Ingenuity Pathway Analysis (IPA) and Gene Set enrichment analysis (GSEA) separately. dsHMGB1 stimulation resulted in activation of 16 canonical pathways suggested by IPA (Fig. 2). No pathway activation or suppression was identified based on the DEG in the frHMGB1 stimulated group by IPA. This was potentially due to the smaller fold changes and fewer numbers of DEG being recognized by IPA. As IPA analysis uses a p-value cut-off for inclusion of DEG in the pathway analysis and has a bias towards already found results, we speculated that we might have missed signaling pathways. We thus continued to analyze the data with GSEA, which uses GO ontology database as a resource. GSEA revealed more detailed gene ontology enrichment of biological processes and molecular functions (Fig. 3). dsHMGB1 stimulation resulted in more abundant enrichment than frHMGB1 stimulation, especially biological functions related to a pro-inflammatory response. frHMGB1 stimulation significantly impacted molecular functions related to chemokine activity and biological processes related to plasma membrane fusion, suggesting its potential effect on cell motility.

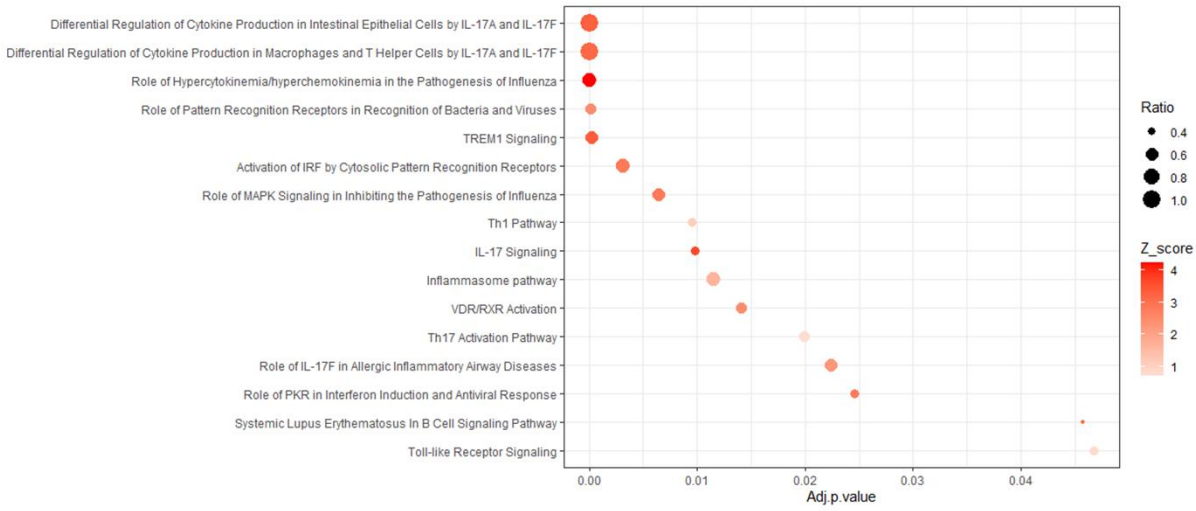

Figure 2. Canonical pathway activation in dsHMGB1 stimulated macrophages compare to control. Expression core analysis was based on the Log2Fold Change (with cut-off>1 or <-1) of the statistically significantly separated genes, including direct and indirect relationships, interaction and causal networks, all node types and data sources, experimentally observed confidence, restricted to bone marrow cells and mouse bone marrow derived macrophages. Z-scores represent the predicted activation state of upstream regulators using the expression patterns of the downstream factors, based on relationships published in the literature. Z-scores larger than 2.00 or smaller than -2.00 were regarded as significant changes. Statistical analysis was performed using Fisher's exact test right-tailed within the IPA software.

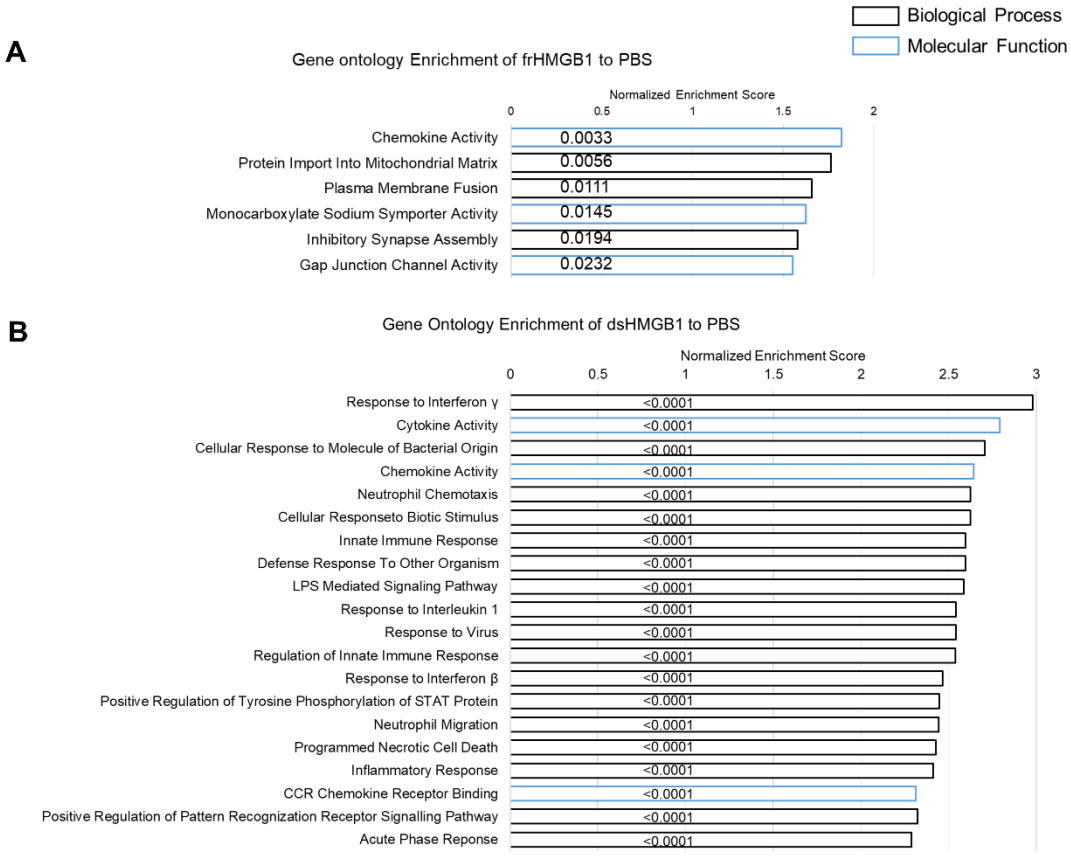

Figure 3. Gene set enrichment analysis for differentially expressed genes in HMGB1 stimulated macrophages. Gene Ontology (GO; http:// geneontology.org/) enrichment analyses for frHMGB1 (A) and dsHMGB1 (B) versus PBS stimulated control macrophages (p-values are indicated within the bars) were carried out by GESA. There were six gene set enriched by frHMGB1 stimulation, whereas the number for dsHMGB1 was 449. Due to the limitation of space, the top 20 enriched gene set were selected for demonstration.

5.2 TLR4 SIGNALLING IN MACROPHAGE POLARIZATION

In **paper III** dsHMGB1 induced a different phenotype from the classic M1 cells, which was induced by LPS and IFNγ. Both dsHMGB1 and LPS are ligands for TLR4, however dsHMGB1 is an endogenous alarmin whereas LPS is a bacterial component, which is a PAMP. We performed additional experiments to understand if a TLR4 ligand could signal differently whether it is of bacterial origin requiring inflammatory response against infection or as an alarmin indicating tissue trauma. Mouse BMDMs were stimulated with dsHMGB1 and ultrapure LPS, which only binds to TLR4, and not to other TLRs. Total RNA was bulk sequenced and gene expression patterns compared.

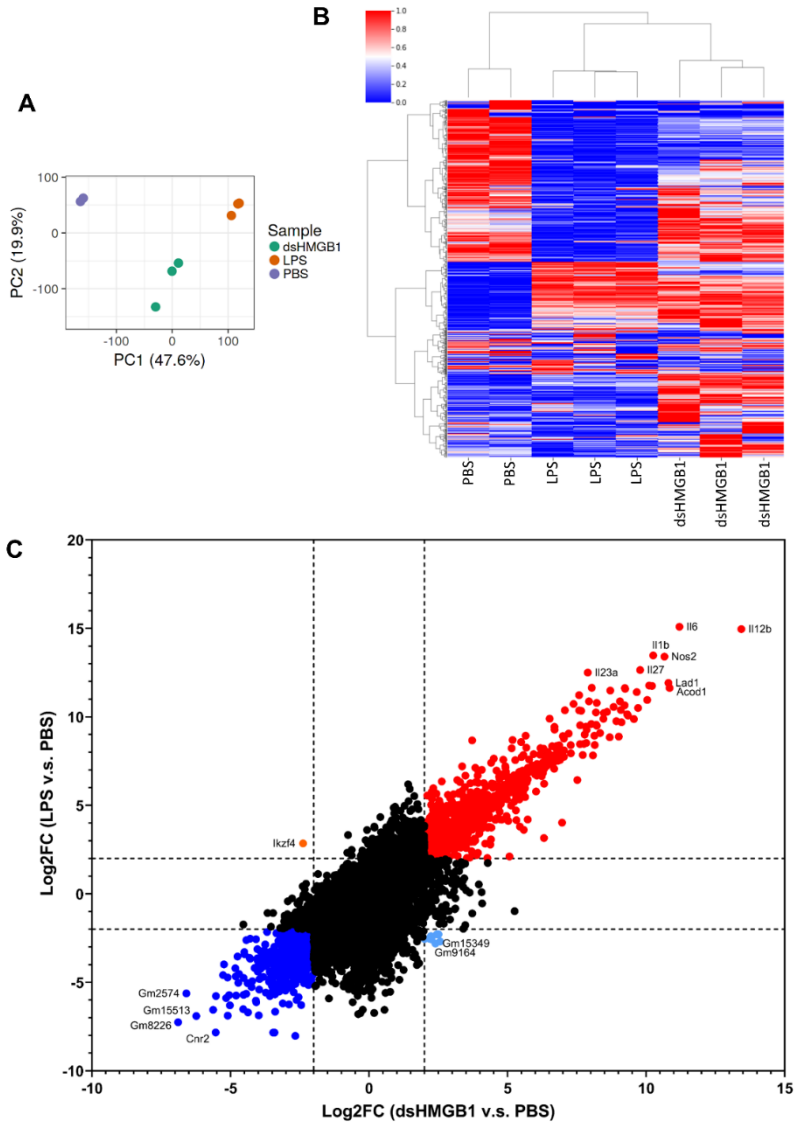

Figure 4. Gene expression patterns of dsHMGB1- and LPS stimulated macrophages compared to PBS-treated controls. (A) Principal component analysis (PCA) plot (B) and cluster heatmap reveal that the three comparison groups are separated. (C) Log2Fold Change versus Log2Fold Change plot of dsHMGB1 stimulated macrophages vs. control on the x-axis and LPS-treated macrophages vs. control on the y-axis highlighting common up-regulated genes in red (1181 genes) common down-regulated genes in dark blue (571 genes), LPS up- and dsHMGB1-down regulated genes in orange (1 gene) and dsHMGB1-up and LPS-down regulated genes in light blue (8 genes). Dashed lines represent a ± 2.0 Log2Fold Change cut-off.

As shown in the PCA plot, LPS- and dsHMGB1 stimulated macrophages were separated from PBS stimulated controls (Fig. 4A), suggesting that LPS and dsHMGB1 triggered different transcriptome changes. These findings were reinforced by a heat map analysis (Fig. 4B). Gene expression values were plotted in a Log2FoldChange versus Log2Fold Change plot (Fig. 4C). Differentially expressed genes (DEG) in this section were defined by an absolute log2fold Change difference larger than 2. 1181 co-upregulated (Fig. 4C, red) and 571 co-downregulated (Fig. 4C, dark blue) genes were identified between LPS- and dsHMGB1 stimulated macrophages.

We firstly focused on genes upregulated in one condition but downregulated in the opposite condition. There were 8 genes (*Gm9164, Rpl21-ps10, Gm11198, Gm15349, Gm5139, Gm18953, Gm7847* and *Gm5652*) upregulated in dsHMGB1 stimulated macrophages, which were reciprocally downregulated in LPS stimulated macrophages (Fig. 4C, bottom right quadrant, in light blue). Likewise, one gene named *Ikzf4* was upregulated in LPS-treated macrophages but downregulated in dsHMGB1 macrophages (Fig. 4C, upper left quadrant, in orange).

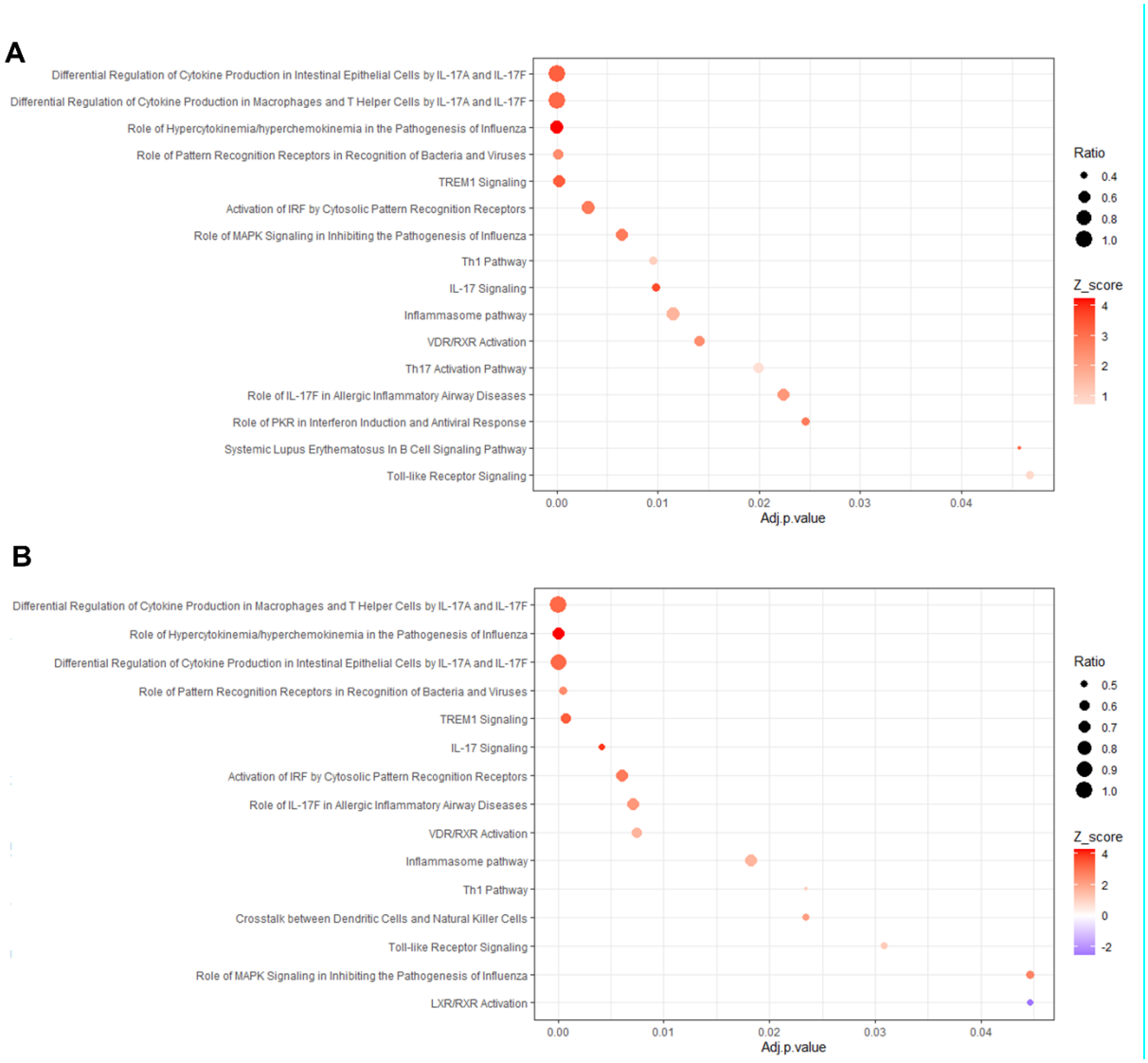

Figure 5. Canonical pathway activation by dsHMGB1- and LPS stimulation in macrophage. (A) dsHMGB1 (B) LPS. Expression core analysis was based on the Log2FoldChange (with cut-off>2 or <-2) of the statistically significantly separated genes, including direct and indirect relationships, interaction and causal networks, all node types and data sources, experimentally observed confidence, restricted to bone marrow cells and mouse bone marrow derived macrophages. Z-scores represent the predicted activation state of upstream regulators using the expression patterns of the downstream factors, based on relationships published in the literature. Z-scores larger than 2.00 or smaller than -2.00 were regarded as significant changes. Statistical analysis was performed using Fisher's exact test right-tailed within the IPA software.

Downstream canonical pathway analysis revealed that LPS and dsHMGB1 resulted in similar pathway activation (Fig. 5). The top five significant pathways were the same in both groups. The one pathway only significantly activated by LPS was Crosstalk between Dendritic Cells

and Natural Killer Cells (z-score = 2.111); the two pathways only activated by dsHMGB1 were Systemic Lupus Erythematosus In B Cell Signaling Pathway (z-score = 3.128) and Role of PKR in Interferon Induction and Antiviral Response (z-score = 2.828). There is one pathway, LXR/RXR Activation, was only suppressed by LPS with z-score -2.530. These results correspond to the gene expression pattern that compared to PBS stimulated control group, that most of the differentially expressed genes in M1 and dsHMGB1 were co-upregulated or co-downregulated (Fig. 4C).

In summary, the two TLR4 ligands, dsHMGB1 and LPS, triggered similar gene expression pattern and pathway activation. One limitation of the experiment so far was that only technical replicates were available, so that no statistical analysis was performed. Biological and functional verification are warranted before drawing the final conclusions.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis work has added novel findings to the HMGB1 research field with a focus on HMGB1 redox isoforms. Earlier reports indicated that HMGB1 plays a role in inducing neuroinflammation and in BBB leakage. **Paper I** confirmed that HMGB1 can induce neuroinflammation directly, without involvement of injury or other inflammatory mediators as the injections of pure HMGB1 were performed in healthy animals. **Paper I** was also the first report comparing the effect of HMGB1 redox isoforms and shows that BBB leakage and expression of IL-1β are induced by both HMGB1 redox isoforms. This led us to test an inhibitor that can bind both frHMGB1 and dsHMGB1 as a treatment for ischemic stroke in **Paper II.**

The candidate drug, anti-HMGB1 ABY was evaluated in a mild cerebral ischemia-reperfusion injury model, M2CAO. It could successfully penetrate BBB, however there was no difference in the outcome of stroke recorded as no reduction in neuroinflammation was evident when measured 4 days after surgery. Why the ABY used, proven to bind HMGB1 in *in vitro* assays, did not suppress stroke outcome is not known. It could be one of many reasons; treatment protocol, kinetics and dosing, targeting a wrong epitope in HMGB1 to give a few.

The whole HMGB1 field is still waiting for the first anti-HMGB1-specific drug to be approved for clinical use. The focus of this thesis has been ischemic stroke but developing a drug that can target and neutralize HMGB1 in the CNS could impact the disease burden of several acute and chronic neuroinflammatory diseases. Based on previous results obtained in different disease models, like the functionality of A-box and 2G7 in terminal ischemic stroke, sepsis model and arthritis mode, but not on transient ischemic stroke one can speculate that there is a different mechanism in “mild” ischemic stroke, which patient can recover from, and a stroke with more severe and chronic inflammation.

Sepsis and arthritis models, both involve inflammatory stimulation with LPS, which HMGB1 can bind with its B box (79). Obviously LPS is not present in the terminal stroke models, but TLR4, the receptor for LPS and dsHMGB1 is expressed and associated to worse outcome in stroke (133). However, TLR2, which can bind to HMGB1 without C-tail, is also expressed in stroke and associated to poor outcome (124,133). To my knowledge, no experiment has been performed to compare the difference in balance in TLR2 and TLR4 expression in different stroke models, to determine if TLR4 would be more dominant in terminal MCAO. I speculate that a possible mechanism that the C-tail binding antibody could have is to enhance HMGB1 binding to TLR2. It would then prevent the binding of other TLR2 ligands, thus functioning as an inhibitor. Another possibility is a difference in HMGB1 redox isoform balance in different disease models. Unfortunately, there is a gap in research regarding the HMGB1 redox isoforms and other regulatory post-translational modification (PTMs) in biological samples.

A setback for the field was the realization that several studies had to be retracted and data re-evaluated. In these publications, measurements of HMGB1 redox isoforms in biological fluids using mass spectrometry had been falsified (134). As the HMGB1 research community had been dependent on one researcher, Daniel J Antoine, who supposedly had developed a method which later was proven not to be true, the is no existing mass spectrometry method that could be used to study HMGB1 PTMs in biological fluids (134). Ferrara *et al* published their extensive work last year, where they confirm the existence of HMGB1 redox regulation in different injured and inflammatory tissues using Western Blot and thus prove that redox regulation exist for HMGB1 regardless of Antoine's fabricated data (43).

At present, the existing method for defining the redox states of HMGB1 is detecting the shift in HMGB1 migration on reduced and non-reduced gel followed by Western Blotting. However, this method is difficult to use for biological fluids like serum as the abundance of other proteins like albumin, may conceal the signal from other less abundant proteins like HMGB1. The detection of disulfide containing proteins as well as thiol groups in a mixture of proteins is possible on mass spectrometry, however it has not been established for HMGB1 (126,127). Developing a method, whether with mass spectrometry, ELISA or other techniques, to reliably detect HMGB1 redox states in biological fluids and cell supernatants, would be a great tool for better understanding of HMGB1 functions in different disease conditions.

The HMGB1 inhibitor research would benefit from characterizing the different redox isoforms in different diseases to determine if it is better to develop a HMGB1 inhibitor which could block the cytokine secretion, but not disrupt the healing processes later. Problems that should be addressed are also the fast occurring oxidation of frHMGB1 to dsHMGB1 in vivo, which could support using a pan-isoform inhibitor (like the anti-HMGB1 ABY used in Paper II) (128). Another problem that characterizing the different redox isoforms in different diseases is also understanding, at which stage HMGB1 obtains the healing functions and if HMGB1 should only be inhibited in a short time window to prevent cytokine release.

An interesting therapy option not yet investigated in ischemic stroke models is the FSSE peptide, a small molecule which prevents the binding of HMGB1 to MD-2 and thus inhibiting HMGB1-TLR4 signaling, without interfering with LPS binding to TLR4. This peptide was improving the survival of APAP, liver I/R injury and sepsis (129). Most likely the dsHMGB1 is involved in the destructive events, whereas frHMGB1 might play a role in the repairing of damaged tissue.

The second focus of this thesis work was the role of HMGB1 in macrophage polarization. HMGB1 was already known to induce macrophage polarization, however the field was very conflicting. Some studies stated that HMGB1 induces an M1 phenotype and some studies demonstrated the induction of the M2 phenotype. The redox status of HMGB1 used in the studies was most occasions undefined. In **Paper III** and **IV** we show that dsHMGB1 induces a pro-inflammatory macrophage phenotype, which differs from M1 macrophages by its migratory and NO secreting properties. dsHMGB1 induced the polarization towards a migratory phenotype by binding TLR4. We also concluded that neither dsHMGB1 nor

frHMGB1 induced an M2 phenotype. Most likely the HMGB1-induced M2 phenotype requires HMGB1 to form complexes with other stimulants.

Next step in macrophage polarization and activation studies should be the "easy to forget" third HMGB1 redox isoform, the terminally oxidized oxHMGB1, believed to be present in the resolution of inflammation. Confirming successful terminal oxidation of HMGB1 is difficult and might be an obstacle for such studies. Fully reduced recombinant HMGB1 can be recognized by its pro-migratory properties in transwell migration assay and absence of cytokine secretion from cells containing TLR4. For dsHMGB1, the readout is opposite, lack of migration in transwell assay, but induction of cytokine secretion. Regarding oxHMGB1 it would be the absence of both of these properties and still confirming that oxidation did not degrade the protein structure, only oxidized the cysteines. A way to investigate the potential functions of extracellular oxHMGB1 would be to stimulate cells and perform RNAseq or multiplexed proteomic study to see if oxHMGB1 can stimulate changes in gene expression or proteome.

In conclusion, my thesis work has added some pieces to the intricate puzzle of HMGB1 functions in inflammatory conditions both at cellular level and *in vivo*. It is my hope that I have contributed knowledge of use for the development of therapeutics around HMGB1, by providing novel findings on the role of HMGB1 redox isoforms in brain and on macrophage polarization. I hope that my report on and discussion of our trial of inhibiting HMGB1 in ischemic stroke model will be of use for future studies. Although the anti-HMGB1 ABY turned out not to be a therapeutic option in the M2CAO model, it may be a promising HMGB1 inhibitor in other inflammatory conditions.

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