

From the DEPARTMENT OF
WOMEN'S AND CHILDREN'S HEALTH

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**MULTIPLE ROLES OF HMGB1 IN CLINICAL
AND EXPERIMENTAL ARTHRITIDES**

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To my family

SUMMARY

Inflammation can be infectious and/or sterile depending on the initiating event. The proinflammatory mediator High mobility group box protein 1 (HMGB1) is a nuclear protein released from cells during both sterile and infectious inflammation and once extracellular, initiates and potentiates inflammation by inducing cytokine production and by recruiting inflammatory cells.

Autoimmune diseases are characterised by chronic sterile inflammation leading to tissue destruction. HMGB1 has been implicated in the pathogenesis of several autoimmune diseases including rheumatoid arthritis (RA), systemic lupus erythematosus, multiple sclerosis and myositis. The involvement of HMGB1 in arthritis has been shown by overexpression of HMGB1 in RA synovial tissue and synovial fluid, by beneficial outcome of therapeutic HMGB1-blockade in several experimental arthritis models and by the induction of arthritis by intra-articular injection of recombinant HMGB1 into mice.

In this thesis work I set out to investigate the potential role of HMGB1 in juvenile idiopathic arthritis (JIA), to further delineate mechanisms by which HMGB1 can contribute to arthritis pathogenesis and to study the means by which HMGB1 activity can be suppressed.

I could report for the first time that HMGB1 levels were increased in synovial fluid as compared to plasma during JIA. HMGB1 levels in synovial fluid did not correlate to disease duration. In contrast, the recorded levels of IL-8 and S100 proteins were higher in synovial fluid during early phases of disease. This indicates a change in the inflammatory phenotype during the progression of JIA. High HMGB1 levels in synovial fluid correlated with early JIA onset, suggesting differences in immunopathogenesis between patient groups.

I have also demonstrated that HMGB1 may form complexes with the exogenous TLR ligand LPS or the endogenous inflammatory mediators IL-1 α and IL-1 β , respectively. Compared to each mediator alone such complexes stimulated synovial fibroblasts from arthritis patients to enhanced production of cytokines and tissue degrading enzymes. This enhancement is mediated via the reciprocal receptor for each HMGB1-partner molecule. Since all the studied mediators are present in arthritic joint during inflammation, this is a potential mechanism through which HMGB1 enhances ongoing inflammation and destruction during rheumatic diseases.

Finally, I have demonstrated that the proinflammatory activity of HMGB1 can be therapeutically targeted, either by inhibiting its active release by clinically approved anti-rheumatic drugs or by neutralization with a HMGB1-specific monoclonal antibody. Extracellular secretion of HMGB1 from LPS+IFN- γ stimulated human primary monocytes was inhibited by dexamethasone, chloroquine and gold sodium thiomalate *in vitro* as recorded using an ELISPOT assay. Therapeutic administration of an HMGB1-specific HMGB1 monoclonal antibody ameliorated arthritis in two separate experimental models.

In conclusion, my thesis work has added to the growing evidence that HMGB1 is involved in the pathogenesis of arthritis, has revealed a potential mechanism for its proinflammatory function and has demonstrated a means by which HMGB1-mediated activities can be counteracted.

LIST OF PUBLICATIONS

- I. **HMGB1 levels are increased in JIA patients, correlate with early onset of disease and are independent of disease duration**
Hanna Schierbeck, Rille Pullerits, Chris Pruunsild, Marie Fischer, Dirk Holzinger, Erik Sundberg, Helena Erlandsson Harris
Submitted for publication
- II. **High mobility group box protein 1 in complex with lipopolysaccharide or IL-1 promotes an increased inflammatory phenotype in synovial fibroblasts**
Heidi Wähämaa, Hanna Schierbeck, Hulda S Hreggvidsdottir, Karin Palmblad, Anne-Charlotte Aveberger, Ulf Andersson, Helena Erlandsson Harris
Arthritis Research and Therapy. 2011, 26;13(4):R136
- III. **Immunomodulatory drugs regulate HMGB1 release from activated human monocytes**
Hanna Schierbeck, Heidi Wähämaa, Ulf Andersson, Helena Erlandsson Harris
Molecular Medicine. 2010, 16(9-10):343-351
- IV. **Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models**
Hanna Schierbeck, Peter Lundbäck, Karin Palmblad, Lena Klevenvall, Helena Erlandsson-Harris, Ulf Andersson, Lars Ottosson
Molecular Medicine. 2011, 17(9-10):1039-44

RELATED PUBLICATIONS

Pivotal advance: inhibition of HMGB1 nuclear translocation as a mechanism for the anti-rheumatic effects of gold sodium thiomalate

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J Leukoc Biol. 2008, 83(1):31-8

The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation

Hulda S. Hreggvidsdottir, Therese Östberg, Heidi Wähämaa, Hanna Schierbeck, Ann-Charlotte Aveberger, Lena Klevenvall, Karin Palmblad, Lars Ottosson, Ulf Andersson, Helena Erlandsson Harris
J Leukoc Biol. 2009, 86(3):655-62

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LIST OF ABBREVIATIONS

ACPA	Antibodies to citrullinated protein antigen
ANA	Antinuclear antibodies
APC	Antigen presenting cell
ATP	Adenosine triphosphate
CBA	Cytometric bead array
CIA	Collagen-induced arthritis
COX	Cyclooxygenases
DAMPs	Damage-associated molecular patterns
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
GST	Gold sodium thiomalate
HLA	Human leukocyte antigen
HMGB1	High mobility group box protein 1
HSPs	Heat shock proteins
IFN	Interferon
IL	Interleukin
JIA	Juvenile idiopathic arthritis
LPS	Lipopolysaccharide
MAS	Macrophage activation syndrome
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MRPs	Myeloid-related proteins
MSU	Monosodium urate
MTX	Methotrexate
NLRP3	NLR family, pyrin domain containing 3
NLS	Nuclear localisation signals
OA	Osteoarthritis
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PKR	Double-stranded RNA activated protein kinase
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RF	Rheumatoid factor
ROS	Reactive oxygen species
SF	Synovial fluid
SLE	Systemic lupus erythematosus
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 IMMUNOLOGY

We are living in a dangerous world and the immune system is crucial in the maintenance of the host's physiological homeostasis that can be disrupted by tissue injury caused by infectious as well as non-infectious stimuli. The immune system combats a wide range of pathogens and comprises of a variety of effector cells and molecules. Immune responses can be either *innate* or *adaptive* based on sensing and effector mechanisms. Until the 1990s immunological research was primarily focused on adaptive immunity as a key regulator. Adaptive immune responses were considered more important since the cells recognise specific antigens from pathogens, and innate immune responses were considered to be primitive. This view started to change following the discovery that Toll-like Receptors (TLR) play an essential role in immunity in *Drosophila melanogaster* by Hoffman and colleagues [1]. Subsequently, Beutler and colleagues discovered TLR4 as a receptor for the bacterial product lipopolysaccharide (LPS) that mediates septic shock [2]. For these two discoveries Jules A. Hoffmann and Bruce A. Beutler were rewarded the Nobel Prize in Medicine or Physiology in 2011.

These findings have provided important insights into the activation and regulation of the immune response and illustrate a link between innate and adaptive immunity. More recently, it has become understood that self-derived danger signals from injured tissues are recognised by TLRs or other receptors and induce an important immune response. The stimulation of cells in the innate immune system subsequently activates the adaptive immunity in similar ways as it is triggered by pathogens [3]. This sterile inflammation is important in maintaining health by healing of damaged tissues but can also cause tissue destruction if it is dysregulated. Failure of the regulation of the immune response can lead to chronic inflammation that is very destructive and may cause inflammatory diseases including allergy and autoimmunity.

1.1 INNATE IMMUNITY

The innate immune system is native and always available for a fast and effective action regardless of what kind of danger is threatening our body. It recognises both endogenous and exogenous molecular patterns and is not specific for any individual antigen.

1.1.1 Danger signals – molecular patterns

Alarmins are classified as cellular components that can stimulate the immune system when they leave their normal intracellular location and are released from the cell upon activation, injury or cell death. As potent activators of inflammation they play key roles in diseases characterised by sterile or infection-induced inflammation [4]. The term ‘alarmin’ was first proposed by Oppenheim [5], but they are also denoted danger signals or DAMPs (Damage-associated molecular patterns). The term DAMP was coined by Polly Matzinger as a part of her Danger model, and was based on extracellular release of molecules from cells damaged by pathogens, toxins or mechanical damage but not from cells undergoing normal physiological deaths (reviewed in [6]).

These DAMPs trigger the immune system in the same way as foreign (non-self) exogenous signals such as PAMPs (pathogen-associated molecular patterns) [6]. Examples of DAMPs are hyaluronan, IFN- α , monosodium urate (MSU) microcrystals, Adenosine triphosphate (ATP), heat-shock proteins (HSPs) (reviewed in [7]), S100 proteins [8] and High Mobility Group Box protein 1 (HMGB1) [9]. Uric acid is normally present in biological fluids but it is thought only to be immunogenic when it has formed MSU microcrystals that arise when dying cells release their intracellular pool of uric acid into the surrounding tissue. There is evidence that MSU crystals but not soluble uric acid induce immune responses *in vitro* and *in vivo* [10]. The cell death-induced immune response but not the microbial-induced immune response was significantly reduced in uric-acid depleted mice [11]. ATP can stimulate the production of proinflammatory cytokines such as IL-1 and through blocking ATP or its receptor P2X₇ then liver injury-induced inflammation could be inhibited [12]. S100 proteins are cytoplasmic proteins of neutrophils and monocytes that in response to activation are released and mediate inflammation via TLR4 [4]. The involvement of these proteins in experimental model of sepsis has been demonstrated by increased survival in deficient mice [13]. HSPs are upregulated in response to stress but can actually be beneficial in arthritis since HSP60 upregulates regulatory T cells that inhibit inflammation in experimental arthritis [14]. Conversely, an inhibitor of HSP90 reduced experimental arthritis [15].

1.1.2 Pattern recognition

Innate immune cells in the tissue have different cell surface receptors that recognise danger signals of both microbial and non-microbial origin and that yield a fast inflammatory response against cell damage caused by both sterile and infectious triggers.

This family of receptors are named pattern recognition receptors (PRRs) and are expressed on innate immune cells such as macrophages and dendritic cells as well as on non-immune cells. There are several classes of PPR families: Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are transmembrane proteins, and RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) are cytoplasmic proteins. Ligation of the receptor generates an intracellular signal cascade that finally upregulates the transcription of genes involved in the inflammatory processes e.g. proinflammatory cytokines, type 1 interferon, chemokines and antimicrobial proteins [16].

1.1.3 Immune activation

Innate immune activation is important during both sterile and infectious inflammation. Figure 1 illustrates the component of a typical inflammatory response to tissue damage. The presence of pathogens is sensed via PRRs that can recognise PAMPs that can be e.g. LPS, peptidoglycans and unmethylated CpG DNA, and represent regular patterns not specific for a certain pathogen. However, the innate immune system can also be activated via PRRs under absolutely sterile conditions and by molecules of non-microbial origin including those from the host itself (discussed in the previous section). The endogenous DAMPs HMGB1, S100 proteins, DNA and HSPs can be recognised by TLRs and thereby induce inflammation [17-20]. The acute neutrophilic inflammatory response is partly dependent on TLRs since the stimulation with dead cells of TLR2/TLR4 double-deficient mice was significantly reduced but not completely abolished. The responses in the other investigated gene-deleted mice, single knockout for TLR2 and TLR4 as well as TLR1, TLR3, TLR6, TLR7, TLR9, TLR11, respectively, were not significantly reduced. In contrast, mice deficient for IL-1R exhibited a markedly reduced response to dead cells and tissue injury *in vivo* as well as greatly decreased collateral damage from inflammation, thus demonstrating an important role of IL-1 in this system [21]. This indicates that IL-1 is produced in response to

cell death and activation via DAMPs. The production of IL-1 requires synthesis of pro-IL-1 β and caspase-1 cleavage that is mediated by cytoplasmic, multimolecular protein complexes termed *inflammasomes*. It has been suggested that the NLR family, pyrin domain containing 3 (NLRP3) inflammasome can be activated by sterile stimuli and does not require priming by pathogenic stimuli, thereby implying its important role during sterile inflammation [3].

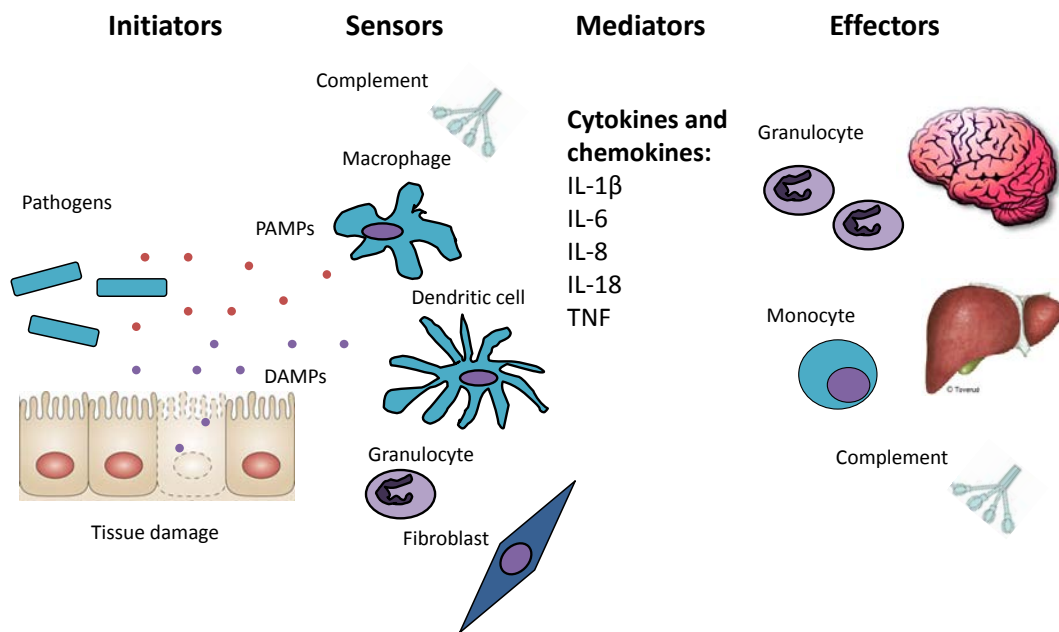


Figure 1: The inflammatory response. The inflammatory response can be initiated both by pathogens (by PAMPs) and by tissue damage (by DAMPs). PAMPs and DAMPs are sensed by PRRs expressed on macrophages, dendritic cells, granulocytes and certain tissue cells such as fibroblasts. The PRR-activation results in secretion of cytokines and chemokines that coordinate the body's inflammatory response. Chemokines recruit cells from the bloodstream to the site of injury/infection. Cytokines activate cells in the vicinity and also act on peripheral organs such as hypothalamus to induce fever and liver to induce synthesis of acute phase proteins. The complement system consists of a large number of proteins and can be both sensors, ie opsonizing pathogens, and effectors, ie inducing a series of inflammatory responses that help to fight infection and reduce tissue injury.

1.1.4 Mediators of the innate immune system

In the protection against infections, one important defence mechanism to prevent microbes from entering the body is the physical barriers as the skin and mucosal surfaces and the chemical barriers such as anti-microbial proteins. Phagocytes in the tissue are the first cells to respond if a pathogen breaks the barriers or if the tissue is injured. There are three different classes of phagocytes. One includes macrophages, which derive from monocytes in the blood that have

migrated into the tissue. Macrophages phagocytose and kill pathogens that have penetrated the physical barrier and can also clear dead cells and debris. Engulfed material is subsequently processed by antigen-presenting cells (APCs) and antigens are exposed on the cell surface to activate the adaptive immune system. Macrophages induce inflammation since they secrete inflammatory mediators such as cytokines and chemokines when they are activated.

The second class of phagocytes is the granulocytes that can be divided into three types: neutrophils, eosinophils and basophils, of which the former are the most abundant. They can leave the blood and migrate to the site of inflammation induced by infection or injury but are short-lived and only survive for hours. They can phagocytose a variety of microorganisms and destroy them by use of degrading enzymes. The third class of phagocytes is the tissue dendritic cells that engulf microbes, but their primary role is not intracellular killing of pathogens. Instead, they process the microbe and generate antigen fragments that can activate cells of the adaptive immune system. NK-cells are lymphocytes assigned to the innate immune system that can kill infected cells or activate macrophages to kill phagocytosed microbes [22].

1.1.5 Innate cytokines

Cytokines are proteins that function as signal molecules for communication between cells. They are secreted from cells upon activation and mediate many features of the immune response and inflammation by interacting with reciprocal specific surface receptors. IL-1, IL-6, IL-8, IL-12 and TNF are some well-known cytokines and chemokines secreted by macrophages upon activation.

The large group of cytokines named interleukins (IL) was initially thought to only be secreted by and to act on leukocytes. However, it is now known that they can be produced by and act on most cell types, which makes the name confusing. The IL-1 family contains many members, where the most prominent are IL-1 α , IL-1 β and IL-18. Several IL-1 subtypes are produced as inactive precursor molecules that need to be cleaved in order to become mature.

These cytokines have a wide spectrum of biological activities that coordinate the inflammation including act on the hypothalamus that mediates fever and stimulate bone marrow endothelium to release neutrophils. IL-6 acts on hepatocytes to induce synthesis of acute-phase proteins [22].

In many inflammatory diseases there is an imbalance between levels of pro- and anti-inflammatory cytokines. It is therefore beneficial to therapeutically block certain cytokines in autoimmune diseases as Rheumatoid arthritis (RA) in order to suppress the chronic inflammation. Examples of such biological therapeutics will be given in section 3.3.4.

1.2 ADAPTIVE IMMUNITY

The adaptive immune system is continuously developed during the lifetime of an individual. It consists of antigen-specific lymphocytes that recognise and respond to specific antigens. Billions of lymphocytes with individual highly specialized antigen receptors on the cell surface, form an effective broad defence against many antigens that the body can be exposed to. T cells are normally first activated by a signal derived from the pathogen and antigen is presented via the major histocompatibility complex (MHC) by APCs. Exogenous and endogenous danger signals are involved in T cell activation by activation of the APCs to provide the second signal for the T cell. T cells with specificity to endogenous autoantigens are reported in autoimmune diseases [23].

T cells circulate around the body and are inactive until they meet their specific antigen, but when they do they differentiate into functional lymphocytes. T lymphocytes (T cells) are divided into helper T cells that assist B cells and phagocytes in their effector functions and cytotoxic T cells that can kill target cells. B lymphocytes (B cells) produce antibodies that recognize specific target epitopes. The adaptive immune response is not as fast as innate immunity and takes days rather than hours to develop. When a lymphocyte has been exposed to an antigen and the stimuli are cleared, an immunological memory is generated in order to respond faster and more effectively when exposed to the same antigen [22].

1.3 INFLAMMATION

Inflammation is the body's response to tissue damage and can be triggered either by an infection or by sterile injury. Acute inflammation is normally a beneficial process initiated to remove the injurious stimulus and after a successful resolution to initiate healing and tissue repair. However, the inflammation process may itself cause damage or produce mediators that lead to disease, as occurs in septic shock or in autoimmune diseases. Chronic inflammation generally reflects physiological failure.

Inflammation is initiated by cells already present in the tissue, mainly macrophages, that are alert to pathogens and to cell damage. Once activated macrophages release inflammatory mediators that affect the local blood vessels, resulting in recruitment of plasma proteins and leukocytes. This process yields the cardinal signs of inflammation described by the latin words *calor* (heat), *dolor* (pain), *rubor* (redness), *tumor* (swelling) and *function laesa* (impaired function). Neutrophils attack the invading pathogen by releasing toxic contents from their granules. The potent effector mechanisms in inflammation cause damage to the host tissue, requiring a repair phase following elimination of the injuring stimulus and this process requires stem cells, fibroblasts and macrophages. If the elimination of the causative agent fails the acute inflammation can become chronic and the process persists, possibly acquiring new characteristics when T cells become involved [24]. The initial inducers of a strong inflammatory response can be exogenous microbial agents, exogenous non-microbial mediators (e.g. allergens, irritants and toxic compounds), but also endogenous inducers produced due to stressed or damaged tissue (discussed in section 1.1.1).

However, low-grade inflammation can occur if the tissue is stressed and the homeostasis and tissue functionality is disturbed. Stressed tissue can signal to macrophages that respond with release of growth factors or other signals that support the tissue. The persistence of tissue malfunction can result in chronic inflammation. Some human lifestyle diseases are associated with this condition, including obesity, type 2 diabetes and atherosclerosis [24].

1.4 CELL DEATH AND IMMUNOGENICITY

Another important task of the immune system is to remove dead cells. It is estimated that up to 10^6 cells die in the human body every second, most as a result of normal tissue turnover (silent death) and this process does not activate the immune system[25]. However, cells can die in different ways and thereby generate different immunological responses. *Apoptosis* is programmed cell death and is mediated by intracellular enzymatic cascades that require ATP. Cells can die through apoptosis from several reasons and the macrophages that are responsible for phagocytosing apoptotic cells may act differently during the degradation process. Apoptosis as a normal part of homeostasis does not induce an inflammatory response. If apoptosis is induced by stress or injury, normally a

tissue-repair response is initiated. Inflammation-induced apoptosis may generate anti-inflammatory pathways in order to down-regulate the inflammation [24].

Necrosis is an unprogrammed, accidental form of cell death. Necrotic cell death is usually associated with tissue damage and causes an inflammatory response. *NETosis* is a form of cell death that neutrophils may undergo. It is a rapid process and is based on release of DNA in a net formation containing proteins such as histones, HMGB1 and LL37 in order to entrap bacteria. These NETs promote inflammation by inducing high amounts of interferon [26,27].

Pyroptosis is a caspase 1-dependent programmed cell death that causes inflammation. Apoptosis and pyroptosis are thus both programmed processes with divergent effects on inflammation. Pyroptosis is induced by activated cytoplasmic inflammasomes that will cleave pro-caspase 1 to mature caspase-1 that will force the cell to commit suicide and release HMGB1, IL-1 β and IL-18 [28]. During pyroptosis, in contrast to apoptosis, the plasma-membrane breaks and proinflammatory content is released [29].

1.5 AUTOIMMUNITY

There are several mechanisms that serve to teach lymphocytes to discriminate between self and non-self, thereby protecting normal tissues from immune reactions. Autoimmune diseases represent a state when this regulation fails and the immune system starts to attack the body's own tissue. About 5% of the population in Western countries develop an autoimmune disease [22]. The diseases are classified according to the damaged organ or tissue and are divided into two categories: organ-specific and systemic autoimmune diseases. Examples of organ-specific diseases are multiple sclerosis (CNS), diabetes mellitus type 1 (pancreas) and Crohn's disease (gut). Systemic lupus erythematosus is an example of a systemic autoimmune disease in which the patients develop autoantibodies against DNA, RNA, nucleoproteins and systemic and organ-specific symptoms affecting almost any tissue ensue. Rheumatoid arthritis (RA) is also a systemic autoimmune disease that affects the joints but that also has systemic manifestations.

The causes of autoimmune diseases are currently unknown, but it has been demonstrated that people with certain genetic variants are more susceptible for disease and that environmental factors play a role. Autoimmune diseases are also

more common in females than in males. The three gene regions that are consistently associated with several autoimmune diseases are human leukocyte antigen (HLA), the gene encoding cytotoxic T lymphocyte-associated 4 (CTLA-4) and the gene PTPN22 coding for lymphoid tyrosine phosphatase (LYP). All these genes encode molecules that are involved in the immune cascade and in T cell activation, indicating that polymorphisms in these regions might trigger autoimmunity. Environmental factors such as infections and smoking can also trigger autoimmunity (reviewed in [30]).

2 HMGB1

The “High Mobility Group proteins” were first discovered in the seventies as nuclear proteins that were easy to recognize based on their high electrophoretic mobility in protein gels [31]. More recently, the name has been proven to be very suitable since these proteins indeed are also very mobile in the nucleus where they have an important function as architectural proteins and to facilitate DNA-dependent activities [32]. This chapter will focus on one of these proteins, High mobility group box protein 1 (HMGB1) that also has many important extracellular functions.

2.1 HMGB1 IN THE NUCLEUS

HMGB1 is an abundant protein predominantly located in the nucleus of all eukaryotic cells. The conservation of its sequence is unique since only 2 of 215 amino acids are substituted in all mammalian species evaluated to date [33]. The fact that HMGB1-deficient animals die shortly after birth demonstrates that HMGB1 plays a vital role during embryogenesis [34]. Depending on cell type and specific circumstances, HMGB1 can function both as a positive and negative regulator of gene transcription, chromatin replication and DNA repair [32].

HMGB1 consists of two DNA-binding domains (boxes), the A box and the B box, and an acidic C-terminal tail. The boxes comprise of approximately 80 amino acids and the tail consists of a continuous stretch of 30 glutamic and aspartic amino acids (Figure 2). The boxes recognize DNA structures rather than DNA sequences and facilitate targeting of transcription factors and other nuclear proteins to their DNA binding sites. HMGB1 thus functions as an architectural protein and provides structural support [33]. HMGB1 also interacts with other proteins such as transcriptional activators, repressors and co-repressors, indicating that HMGB1 plays a role in the stabilization of DNA binding protein complexes [35]. HMGB1 acts as a chaperone for p53 binding to DNA since it bends DNA, thereby enhancing the binding of p53. Once p53 has bound to DNA HMGB1 dissociates [36]. Furthermore, DNA chaperone activity of HMGB1 is also involved in nucleosome assembly, and HMGB1 levels dictate the number of nucleosomes in cells. The variation in nucleosome number is an emerging theme in the epigenetic field with implications for ageing, cancer and inflammation [37,38].

HMGB1 is also involved in DNA repair processes and can either inhibit or increase these. HMGB1 recognizes and binds to damaged DNA and modulates repair. Cells lacking HMGB1 are hypersensitive to DNA damage [39]. Conversely, it has also been demonstrated that HMGB1 can bind to damaged DNA sites caused by anti-cancer platinated drugs such as cisplatin- This prevents DNA from being repaired and thereby mediates the therapeutic effect of this group of pharmaceutical agents [40,41].

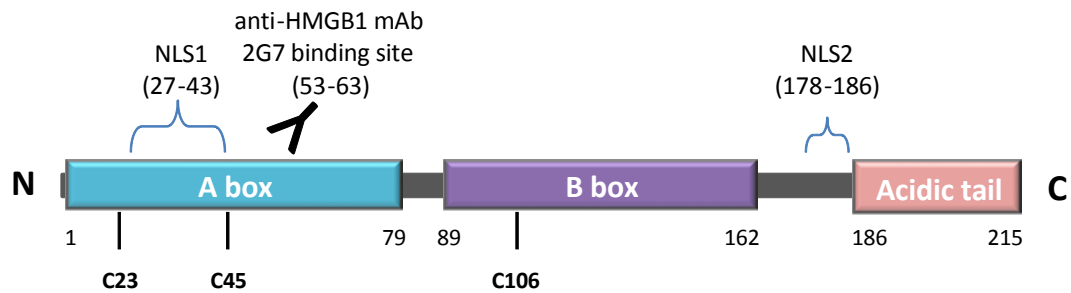


Figure 2: Amino acid structure of HMGB1. HMGB1 contains two DNA-binding domains (the A box and the B box) and an acidic C-terminal tail. The three cysteines are important for HMGB1s function. A disulfide bond between C23 and C45 and C106 in a thiol form are required for the cytokine inducing activity of HMGB1. The reduced form of C106 is required for the binding to TLR4 which mediates the cytokine-inducing activity. For the chemoattractant activity of HMGB1, all cysteines are required to be reduced 'all-thiol'. The acetylation of lysine residues in the NLS-regions regulates intracellular shuttling of HMGB1 between the cytoplasm and nucleus. The anti-HMGB1 mAb 2G7, used in paper IV binds to amino acid 53-63 in the A-box. Abbreviations: NLS = nuclear localization signal, C = cysteine.

2.2 HMGB1 IN THE CYTOPLASM

As the binding of HMGB1 to DNA is reversible, HMGB1 a very dynamic protein and it can very rapidly be transported out to the cytoplasm. The protein has two non-classical nuclear localisation signals (NLSs) that enable transport between the cytoplasm and the nucleus, although HMGB1 is predominantly located in the nucleus in resting cells. There are several mechanisms that have been described to regulate the localisation of HMGB1 in the cell; acetylation, phosphorylation, methylation and ribosylation. After translocation to the cytoplasm the acetylation of two clusters of lysines inhibits the re-entry of HMGB1 into the nucleus [42]. The phosphorylation [43] and activation of poly(ADP)-ribose polymerase (PARP) that occurs as a result of DNA-alkylating damage regulates the translocation of HMGB1 from the nucleus to the cytoplasm and prevents its import to the nucleus. It is also been reported that HMGB1 has a

mono-methylated lysine at position 42 (K42) in neutrophils, but not in lymphocytes, that also regulates the cytoplasmic translocation of HMGB1 and its subsequent release [44]. Qualitative and quantitative analysis of these post-translational modifications could potentially be used to determine the source of HMGB1 within clinical samples and as biomarkers for different disease conditions.

In the cytoplasm HMGB1 plays an important role in the nucleic-acid sensing system that is crucial in anti-viral immunity. The binding of immunogenic RNA to HMGB1 is required for the recognition and binding to specific PRRs and induction of an immune response. HMGB1^{-/-} cells express less type-1 IFN, IL-6 and RANTES compared to wild type cells when stimulated with DNA and RNA [45]. Viral infection or poly I:C stimulation are also known to induce a translocation of HMGB1 from the nucleus to the cytoplasm [28,46].

2.3 EXTRACELLULAR RELEASE OF HMGB1

HMGB1 can be passively leaked out from any cell type during injury or cell death when the plasma membrane breaks and release then occurs instantly. It can also be released from activated cells in response to trauma, infection, stress or other inflammatory stimuli and is then a slower process (Figure 3). This kinetic pattern makes HMGB1 an early mediator in diseases with massive cell damage and necrotic cell death and a late mediator in diseases with HMGB1 release due to cell activation.

When nuclear HMGB1 has been translocated to the cytoplasm (as described in the previous section) it can be released into the extracellular space. HMGB1 release can be induced either by PRR activation or activation by endogenous inflammatory mediators such as TNF, IL-1 β , IFN γ , nitric oxide, IFN α and IFN β [47-51]. The ability to actively secrete HMGB1 has been reported for a number of innate immune cells including monocytes, macrophages, dendritic cells, NK-cells [paper III, 47,48,52,53]. A number of other cells including pituicytes, hepatocytes, endothelial cells and platelets [49,54,55] are also capable of active HMGB1 secretion, but it is less studied whether they use the same secretory pathways as utilized by cells in the immune system.

HMGB1 lacks a secretory signal peptide and is therefore not transported via the ER-Golgi system. Instead, HMGB1 is accumulated in cytoplasmic vesicles that secrete their content extracellularly by exocytosis induced by the bioactive lipid

lysophosphatidylcholine (LPC) that has been shown in monocytes. LPC is generated later during inflammation since it is derived from phosphatidylcholine through the action of the secretory phospholipase sPLA2. IL-1 β is also released through secretory lysosomes but release is induced by ATP and hence occurs earlier than HMGB1 release [56-119]. Similar to the requirements for IL-1 β secretion, HMGB1 release from mouse macrophages stimulated with LPS depends on NALP3 inflammasome and caspase-1 activation [57]. The activation of PKR (double-stranded RNA activated protein kinase) is important for NLRP3 inflammasome-induced HMGB1 release when LPS-primed cells were activated together with typical danger signals such as poly I:C, ATP, MSU, adjuvant aluminium or live *E. coli*. In response to inflammasome activation caspase-1 is activated and cells will then undergo programmed, pro-inflammatory cell death, also known as pyroptosis (described in section 1.4). Pyroptotic-released HMGB1 is hyper-acetylated in the NLS regions in contrast to HMGB1 from necrotic or apoptotic cells [28, Nyström et al. EMBO J, in press]. It is the acetylation state of NLS that distinguishes passively released from actively released HMGB1 [42]. Assessment of hyper-acetylated HMGB1 is the first, and so far only marker that identifies actively released HMGB1, and may therefore be used as a biomarker for pyroptotic biological processes. It may be used as a systemic biomarker to discriminate necrosis, apoptosis and pyroptosis.

During apoptosis nuclear HMGB1 becomes tightly bound to condensed chromatin and remains inside the apoptotic bodies [58]. No HMGB1 will be extracellularly released provided that the apoptotic material is properly engulfed and degraded by the phagocytic cells. Insufficient clearance of apoptotic cells will lead to secondary necrosis and significant release of HMGB1 [59]. The HMGB1 that is released from apoptotic cells is not pro-inflammatory due to irreversible oxidation of the critical cysteine at position 106 (as will be discussed further in section 2.5.3) [60]. However, whether this terminally oxidised C106 HMGB1 exerts any biological activity is currently unknown. During necrosis HMGB1 is released in large amounts and is highly inflammatory [58]. HMGB1 can also be extracellularly released bound to DNA within neutrophil extracellular traps (NETs). NETs are released from neutrophils that die through NETosis. These NETs are highly anti-bacterial and pro-inflammatory and induces the release of type I interferons [27].

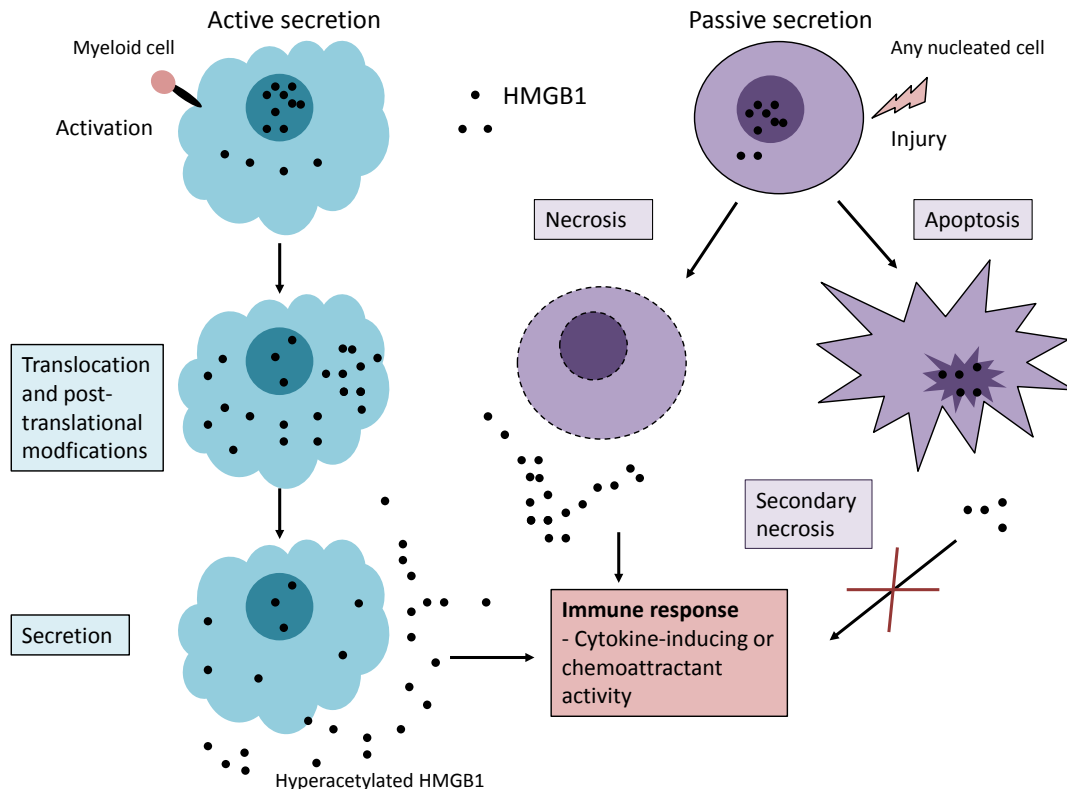


Figure 3: Extracellular release of HMGB1. HMGB1 can be released from cells in different ways. Active secretion is mainly studied in myeloid cells activated by receptor-ligation and involves post-translational modifications and subsequent release. During pyroptosis (sometimes denoted inflammatory cell death or 'programmed necrosis'), HMGB1 is released in response to inflammasome- and caspase-1 activation. HMGB1 release during necrosis is passive and occurs instantly when the plasma membrane breaks. During apoptosis, HMGB1 is initially retained in the nucleus but can be released during secondary necrosis if the apoptotic cell is not properly removed by a phagocytic cell. The actively released HMGB1 is hyper-acetylated in the NLS-regions, in contrast to passively released HMGB1. HMGB1 released from activated cells and from necrotic cells can induce immune responses by cytokine-inducing or chemoattractant activity.

2.4 HMGB1 RECEPTORS

One distinction from canonical proinflammatory cytokines (e.g. TNF and IL-1) is that HMGB1 elicits cellular and biological inflammatory responses by signal transduction through receptors that were previously identified for interaction with foreign molecules. Unlike TNF and IL-1, whose cognate plasma membrane receptor families are clearly defined, HMGB1 interacts with several seemingly unrelated receptors that had been previously identified for their capacity to transduce activation signals from exogenous (TLR2, TLR4, and TLR9) and endogenous (RAGE) ligands. The first receptor implicated as a binding partner for

HMGB1 is the receptor for advanced glycation end products (RAGE), a transmembrane, cell surface, multiligand member of the immunoglobulin superfamily. Since then HMGB1 has been demonstrated to interact and signal through several additional receptors including at least TLR2, TLR4, TLR9 and CD24/Siglec.

RAGE is a 35 kDa transmembrane receptor with N-glycosylations belonging to a superfamily of immunoglobulins. It was first determined to bind AGEs (advanced glycated end products) but has since then been described to interact with many different molecules including HMGB1, β -amyloid protein, S100 proteins, C3a-anaphylatoxin and unmethylated cysteine-guanine-rich DNA (hCpGA) (reviewed in [61]). RAGE was first described as a receptor for HMGB1 in 1995 [62] and the binding site has later been mapped to amino acids 150-183 [63]. The binding of HMGB1 to RAGE promotes chemotaxis and stimulation of cell growth, differentiation of immune cells, the migration of immune cells and smooth muscle cells and up-regulation of cell surface receptors [53,64-66]. However, it has recently been suggested that the signalling of HMGB1 via RAGE mainly induces production of CXCL12, a chemokine that synergistically promotes chemotaxis together with HMGB1 [67,68]. The reports regarding whether HMGB1-RAGE interaction induce cytokine production diverge [20,69,70], possibly due to different origins and redox states of the used HMGB1 (as will be discussed further in section 2.5.3).

Toll-like receptors were first described to bind PAMPs and to activate the innate immune response against pathogens. During recent years they have also been shown to bind endogenous DAMPs (discussed in section 1.1.1) which activate immune responses. TLR2 binds many different ligands derived from bacteria such as Lipoteichoic acid (reviewed in [61]). It has also been reported that HMGB1 binds to TLR2 and induces NF- κ B activation [19], although a detailed molecular understanding of this interaction is still missing.

The prototypic HMGB1 receptor is the TLR4-MD2 complex, that was initially discovered as the LPS-receptor and then also as a receptor for certain other molecules e.g. heat shock proteins, some S100 proteins and fibrinogen from host cells (reviewed in [61]). The recombinant HMGB1-TLR4 interaction has been demonstrated by FRET (fluorescence resonance energy transfer), immunoprecipitation and functionally by induction of NF- κ B activation [18,19].

The interaction between endogenous HMGB1 and TLR4 has been shown in synovial fibroblasts by PLA (proximity ligation assay). The binding of HMGB1 to TLR4 promotes cytokine production. By stimulating TLR4-deficient macrophages it was confirmed that TLR4 is required for cytokine production. This study also reveals that the cysteine in position 106 is critical for the binding and signaling through TLR4 [20]. HMGB1 binds to the MD-2 molecule in the TLR4 receptor complex with the same avidity as LPS [Yang *et al.* unpublished data].

Another TLR receptor suggested for HMGB1 signaling is TLR9. TLR9 binds HMGB1 and is important in the stimulation with HMGB1-DNA complexes as shown by immunoprecipitation and confocal microscopy [71,72]. Again, it is presently unknown how HMGB1 interacts with the TLR9 receptor complex.

There is also a receptor described for HMGB1 that induces down-regulation of the immune response. The receptor CD24-Siglec-10 complex protects against drug-induced liver injury by selectively dampening the HMGB1-induced, but not pathogen-induced TLR4 immune responses [73].

2.5 FUNCTIONS OF EXTRACELLULAR HMGB1

Extracellular HMGB1 was initially described by Heikki Rauvala and colleagues as a protein that interacts with plasminogen and tissue type plasminogen activator (tPA) and enhances plasmin generation at the cell surface. At that time HMGB1 was termed *amphoterin* and was known to mediate neurite outgrowth [74]. Since then numerous functions of HMGB1 in the extracellular space have been described and range from inducing a strong inflammatory response to involvement in healing and tissue regeneration.

2.5.1 Pro-inflammatory role of HMGB1

The first study proposing a pro-inflammatory role of HMGB1 was published in 1999 by Kevin Tracey and colleagues. They found that mice subjected to gram-negative bacterial sepsis died up to five days after attainment of the peak levels of TNF and IL-1 β , the then suggested pathogenic mediators in lethal endotoxemia and gram-negative sepsis. To identify potential “late” mediators, macrophages were stimulated with LPS in cell culture and extracellular release of HMGB1 into the cell culture media was subsequently discovered. They also measured HMGB1 levels in sera from mice during endotoxemia and determined that it peaked 16-32

hours after initiation of disease. Survival in this model was markedly improved by injecting anti-HMGB1 antibodies up to 24 hours after the initiation of the disease, making HMGB1 a completely unique target and providing a wider therapeutic window than any other studied pro-inflammatory mediator [48].

Since that discovery, it has been shown that HMGB1 is not only a product of activated macrophages but is also a potent inducer of a number of cytokines and chemokines. Human monocytes stimulated with purified recombinant HMGB1 can release TNF, IL-1 α , IL-1 β , IL-1RA, IL-6, IL-8, macrophage inflammatory protein (MIP)-1 α , and MIP-1 β [75]. However, for several years there have been contradictory results from different groups regarding the cytokine-inducing capacity of HMGB1 [paper II, [72,76-79]. The reasons for these divergent results are due to the fact that experiments have been performed with batches of HMGB1 with uncontrolled redox states. We now know that post-translational redox modifications totally regulate HMGB1 functions in a mode to be outlined later on in this text.

2.5.2 HMGB1 in co-operation with other molecules

Another pro-inflammatory function of HMGB1 is to form complexes with other molecules and to thus enhance the immune response in a strong, synergistic manner. This mechanism may be a way of accelerating events during innate immune reactions. Stimulation with HMGB1 in complex with low amounts of the exogenous TLR ligands LPS, CpG-ODN, Pam₃CSK₄ or Lipoteichoic acid (LTA), respectively, results in strongly enhanced cytokine production compared to stimulation with these molecules alone [Paper II, [71,72,76,79-81]. HMGB1 can also interact with the endogenous proteins IL-1 α , IL-1 β and CXCL12 and enhance their functions paper II [78,82,83].

These complexes signal through the receptor of the ligand that is complexed to HMGB1. In cell cultures of macrophages deficient for TLR2 and TLR4 it was demonstrated that HMGB1-LPS complexes used TLR4 and that HMGB1-Pam₃CSK₄ complexes used TLR2 for signaling [84]. HMGB1-IL-1 α and HMGB1-IL-1 β complexes use the IL-1 type 1 receptor since blockade of the cell activation was possible with IL-1 receptor antagonist (IL-1RA) [paper II, [78]. The complex between HMGB1 and the chemokine CXCL12 signals via CXCR4, the reciprocal receptor for CXCL12 [67] and the previously known HMGB1 receptors TLR4 and

RAGE does not seem to play a role in this indirect cytokine-stimulating mechanism.

HMGB1 that leaks out from apoptotic cells are attached to nucleosomes. Such complexes have immunostimulatory effects and signal through TLR2 [85]. However, even though HMGB1 binds to many molecules it does not bind and enhance the stimulation of TNF, RANKL, IL-18 or Poly(I:C), suggesting a selective mechanism [76].

In contrast to bacterial DNA, mammalian DNA is normally not immunogenic. It can acquire immune stimulatory properties when associated with HMGB1 and other proteins that promote receptor interactions, thereby breaking immune tolerance [86].

2.5.3 The effect of post-translational modifications of HMGB1 function

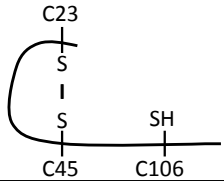
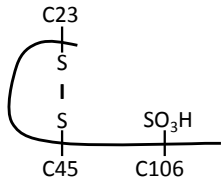
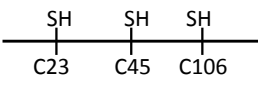
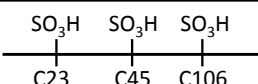
It was recently reported that the redox state of HMGB1 determines its pro-inflammatory capacity. HMGB1 has three redox-sensitive cysteines and the redox state of each of these regulates the function of extracellular HMGB1. The different redox forms of HMGB1 are summarised in table 1. If the cysteines in position 23 and 45 form a disulfide bridge and the cysteine in position 106 is in thiol form HMGB1 acts as a pro-inflammatory mediator that signals via TLR4-MD2 [20,87]. All three cysteines in reduced form render HMGB1 chemoattractant properties and terminal oxidation of all cysteines to sulfonate terminates both activities. During an inflammatory reaction the cytokine-inducing HMGB1 (with disulfide bond and reduced C106) was most abundant in serum in a mouse model of drug-induced liver injury. However, during the resolving phase of inflammation and hepatic regeneration, the non-inflammation inducing form was the most abundant. This indicates that dynamic redox changes during different stages of inflammation tightly regulate the function of HMGB1 [68,87].

The formation of the disulfide bridge between thiols is caused by reactive oxygen species (ROS) production that occurs during inflammation [88,89] and the disulfide bridge is important in the folding and stability of proteins in the extracellular milieu [90]. The ligation of TLRs during pyroptotic cell death leads to release of the cytokine-inducing form of HMGB1 whereas non-ligated cells release the chemotactic variant. This indicates that it is not the pyroptosis *per se* that regulates the redox status of HMGB1 but rather the ROS production associated

with the priming event [Nyström et al, EMBO J, in press]. Mitochondrial ROS production can be induced by extracellular TLR priming of inflammasomes [91], and this might enable the disulfide bridge formation in HMGB1 released from primed pyroptotic cells. More extensive ROS production during apoptosis can inactivate HMGB1 by terminally oxidizing its cysteines to sulfonates [60,68].

The effect of other post-translational modifications including acetylation, phosphorylation and methylation on HMGB1 function is so far unknown. However, levels of hyperacetylated HMGB1 in serum were only elevated in patients who died or required a liver transplant, while total HMGB1 serum levels and other biomarkers were less selectively predictive [92].

Table 1: Redox state of HMGB1

Schematic molecular overview	Released during			Cytokine-inducing activity	Chemo-attractant activity
	Apoptosis	Necrosis	Pyroptosis		
 <p>C23 S S C45 SH C106</p>		X	X (LPS primed)	Yes	No
 <p>C23 S S C45 SO₃H C106</p>			X (LPS primed)	No	No
 <p>SH SH SH C23 C45 C106</p>		X	X	No	Yes
 <p>SO₃H SO₃H SO₃H C23 C45 C106</p>	X			No	No

2.5.4 Regenerative role of HMGB1

Tissue repair is an important outcome of the inflammation triggered by tissue and organ damage, both in the presence and absence of invading pathogens, and aims to restore tissue homeostasis (discussed in section 1.3). It is therefore not surprising that HMGB1, as an inflammatory mediator and danger signal, not only has a role during the pro-inflammatory phase of inflammation but also during the healing process to repair the damaged tissue. It has recently been

demonstrated that the redox modifications of HMGB1 mediate its different functions during inflammation and its resolution (discussed in previous section).

The first role of HMGB1 regarding regeneration processes was its involvement in neurite outgrowth in the developing nerve system [93]. Since then, the beneficial role of HMGB1 has been studied in the context of many disease models. In an *in vitro* model of skin healing HMGB1 induces cell migration and cell proliferation of 3T3 embryonic mouse skin fibroblasts in a RAGE-dependent manner [94]. Administration of HMGB1, with all cysteines in a reduced, 'all-thiol' form, to the skin wound of diabetic mice with impaired wound repair accelerates the wound healing process, but had no effect in healthy mice. However, HMGB1 inhibitors delayed skin repair in normal mice, but not in diabetic mice. These data suggest that the low levels of HMGB1 in diabetic patients and mice affect their decreased ability to heal [95].

Vessel-associated stem cells (mesoangioblasts), that are known to migrate to and participate in the generation of damage muscle tissue, migrate towards HMGB1 in an all-thiol form both *in vitro* and when injected in healthy muscle. HMGB1 has therefore been suggested to play a role in muscle tissue regeneration. This activity was not dependent on RAGE signaling [96]. HMGB1 is released during hypoxia from human dermal microvascular endothelial cells and addition of HMGB1 to ischemic muscle improved limb perfusion and increased endothelial density following ischemic injury [97]. Stressed muscle fibers induced an antioxidant response to ROS production that generated a reduced extracellular microenvironment at the site of injury. This promoted the activity of HMGB1 which otherwise became inactive when oxidized (discussed in previous section) and preserved the HMGB1 muscle regenerative properties [98]. Injection of HMGB1 in 'all-thiol' form induced myocardial regeneration after myocardial infarction [99]. The beneficial effects were mediated by enhanced angiogenesis, restored cardiac function and improved survival after myocardial infarction *in vivo* in HMGB1 transgenic mice [100].

2.6 PATHOLOGICAL ROLE OF HMGB1 IN DISEASES

The pathogenic role of HMGB1 has been described in many human diseases and animal disease models. In this section I have chosen to describe the role of HMGB1 in bacterial sepsis and endotoxemia as examples of infectious inflammation and in rheumatic diseases as examples of sterile inflammation.

2.6.1 Sepsis and endotoxemia

Sepsis is a systemic inflammation with more than 30% lethality that is triggered by an infection [101]. Sepsis was the first disease model in which the pathological role of HMGB1 was demonstrated (discussed in section 2.5.1). Although endotoxins stimulate the release of IL-1 β and TNF, blockade of these have yielded limited effect in clinical trials of sepsis. Release of HMGB1 has a delayed kinetic and was therefore possible to block with specific antibodies in experimental sepsis, even if the treatment was initiated 24h after the mice were subjected to caecal ligation and puncture (CLP) surgery [48,102].

The successful blockade of HMGB1 at highly clinically relevant time points in experimental models makes HMGB1 a very attractive therapeutic target molecule for studies of treatment in clinical sepsis. The relevance of HMGB1 in septic patients was shown by higher serum levels in patients that survived compared to non-infected controls, and even higher levels in patients who did not survive [48], but has not been confirmed in other studies [103]. Moreover, the presence of autoantibodies to HMGB1 in sepsis was associated with a favorable outcome in critically ill patients [104].

In sepsis, 25% of the survivors develop cognitive impairment and HMGB1 has been indicated to be involved. Survivors of the experimental sepsis model, CLP, have higher serum HMGB1 levels for a long period subsequent to the acute disease as compared to controls, and administration of anti-HMGB1 antibodies during the post-sepsis period significantly improved memory impairments and brain pathology [105].

2.6.2 Rheumatic diseases

HMGB1 has been defined as a key mediator in arthritis. A possible involvement is described in figure 4. HMGB1 is over-expressed in the synovial tissue and increased levels are recorded in synovial fluids (SF) of RA patients

[106-108] and of JIA patients [paper IV]. In adults HMGB1 levels are several-fold higher in SF from rheumatoid arthritis patients than in SF from osteoarthritis patients, while the plasma HMGB1 levels were comparable in both groups [107]. There are also reports demonstrating significantly increased serum HMGB1 concentrations in RA patients as compared to healthy controls [109]. Destructive arthritis can also be induced by intra-articular injection of recombinant HMGB1 into mouse joints [110]. Autoantibodies against HMGB1 have been reported in RA [111,112] and in JIA [113,114], but it is presently unknown whether they exert any functions.

Another autoimmune disease in which HMGB1 has been implicated to be involved is Systemic lupus erythematosus (SLE). One important feature of SLE is increased apoptotic cell death and a reduced ability to degrade and eliminate apoptotic debris. Another important pathogenetic feature of SLE is the production of anti-nuclear antibodies that will form immune complexes with components from dead cells. HMGB1 can form complexes with nucleosomes and induce autoantibody response to histones and DNA, while nucleosomes alone cannot [85]. The levels of HMGB1 in SLE serum are increased compared to healthy controls, correlate with disease activity [115-117] and correlate inversely to levels of complement C3 and C4 [118]. HMGB1 ELISA assays generally provide false negative results in SLE due to the masking of HMGB1 with autoantibodies and serum proteins [117,119]. The presence of anti-HMGB1 antibodies in SLE has been reported by several groups [111,112,120] and correlates with SLE disease activity index [117].

HMGB1 is also involved in heterogeneous rheumatic muscle diseases including idiopathic inflammatory myopathies (IIMs) that are characterised by muscle weakness, inflammatory cell infiltrates and MHC class I expression on muscle fibers. HMGB1 expression is increased in the invading inflammatory infiltrates in IIM patients but this is not evident in healthy controls. Treatment with prednisone decreases the extracellular HMGB1 expression in muscle biopsies [121]. HMGB1 induces increased MHC class I expression and muscle fatigue in cultured mouse muscle fibers [122,123].

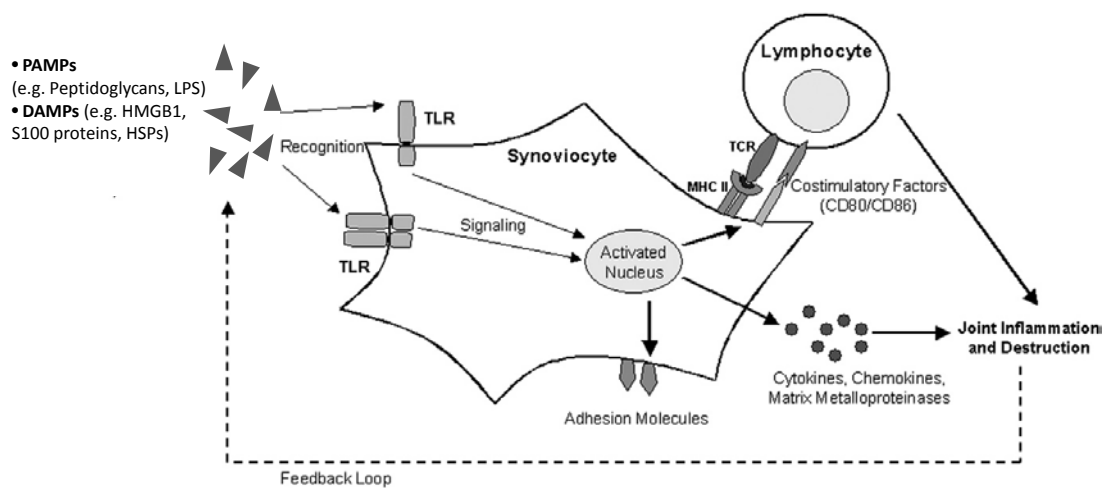


Figure 4: Possible involvement of HMGB1 and other danger signals in the pathogenesis of rheumatic joint diseases. Synovial fibroblasts and macrophages are activated by DAMPs or PAMPs and release proinflammatory cytokines, chemokines, and tissue destructive enzymes. In a hypothetical feedback loop, DAMPs generated during joint inflammation and tissue destruction may result in chronic stimulation of synovial cells. Adapted from Brentano et al. *Cell Immunol.* 2005

2.7 THERAPEUTIC BLOCKADE OF HMGB1

Extracellular HMGB1 and its function can be blocked in different ways, such as through nuclear sequestering, specific antibodies or receptor blockade. Several pharmaceutical agents have been demonstrated to inhibit the release of HMGB1. Anti-rheumatic drugs such as dexamethasone, gold sodium thiomalate and chloroquine decrease the secretion of HMGB1 in vitro [paper III,[124]. HMGB1 expression in tissue can also be down-regulated by corticosteroids administered intra-articularly or systemically in patients with chronic arthritis or myositis [106]. The anti-cancer drug oxaliplatin sequesters HMGB1 in the nucleus and oxaliplatin treatment of collagen-induced arthritis (CIA) in mice ameliorates disease, although this effect was transient [125]. Ethyl pyruvate is another agent that inhibits HMGB1 secretion from human macrophages and attenuates inflammation in endotoxemia and sepsis models [126]. Ethyl pyruvate also attenuates spinal cord ischemic injury and reduces the levels of HMGB1 in serum and spinal cord tissue [127]. Glycyrrhizin, a natural anti-inflammatory drug in clinical use that is derived from the liquorice plant, binds to both HMGB1 DNA binding boxes and inhibits the chemoattractant activities of HMGB1 through blocking the formation of HMGB1-CXCL12 complexes [67,128].

During the new era of biological therapeutics, monoclonal antibodies and recombinant proteins have been used successfully for treatment of inflammatory

diseases for more than a decade. This will be discussed more in section 3.3.4. Antibodies that block extracellular HMGB1 have proven successful in many experimental disease models (reviewed in [129]), but no HMGB1-specific therapy has yet reached clinical trials. Neutralizing polyclonal antibodies are beneficial in many disease models. In CIA the anti-HMGB1 polyclonal antibodies significantly reduce the arthritis score, disease-induced weight loss and histological severity of arthritis and tissue destruction in both mice and rats [130]. In addition to arthritis, polyclonal anti-HMGB1 antibodies are effective in disease models of pancreatitis [131], islet transplants in diabetes [132], sepsis [48], endotoxemia [133], hemorrhagic shock [134,135] and ischemic stroke [136]. However, the disease models with positive effect of monoclonal HMGB1 antibodies are restricted to arthritis [paper IV], sepsis [102], drug-induced liver injury [73], stroke [137] and traumatic brain injury [138].

The soluble form of the HMGB1 receptor RAGE can bind HMGB1 and can thereby act as a decoy receptor [62]. A recombinant peptide constituting the A-box domain of HMGB1 has been successfully used for treatment in many disease models and was from the beginning anticipated to be a receptor antagonist for HMGB1. Among disease models in which A box has been clinically efficacious are arthritis [130,139], sepsis [133], pancreatitis [140,141], ischemia-reperfusion injury [142] and epilepsy [143]. However, the mechanism underlying the beneficial properties of the recombinant A box are still unknown and the blockade of any known HMGB1 receptor has not been demonstrated. Nonetheless, it has recently been demonstrated that recombinant A box protein inhibits the chemoattractant activity of HMGB1 but not the cytokine-inducing ability, indicating that its therapeutic potential in experimental models are assigned to the decreased recruitment of inflammatory cells in the injured tissue [68].

3 RHEUMATIC JOINT DISEASES

Rheumatic joint diseases are a family of diseases characterised by chronic inflammation causing swelling and pain in one or more joints. Each disease presents with a unique cluster of typical symptoms and classifications.

3.1 JUVENILE IDIOPATHIC ARTHRITIS

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory disorder in children typified by diverse articular and extraarticular manifestations of unknown etiology. The prevalence is difficult to estimate because of variations in diagnostic criteria, low disease frequency and small study numbers. Epidemiological studies report prevalence rates between 7 and 400 per 100 000 children and the annual incidence is between 0.8 and 22.6 per 100 000 children [144].

3.1.1 Classification and etiological features

JIA is not one disease but rather a heterogeneous group that can be divided into seven subgroups according to the International League of Associations for Rheumatology (ILAR) [145]. Common for all subgroups is onset before 16 years of age and persistent arthritis for at least 6 weeks, and each category has its own exclusion criteria. The exclusion of differential diagnoses is important, especially arthritis caused by infection, neoplasm or other autoimmune diseases. The features of the seven subgroups are summarised in table 2. Oligoarthritis is the most prevalent (~40-50%) and is characterised by a maximum of four joints being affected during the first 6 months of disease. This subgroup is further divided into *persistent* or *extended* depending on the additional number of joints affected at a later stage. Patients with persistent oligoarthritis often enter remission and patients with extended oligoarthritis have a higher risk of developing a more chronic disease course. The second most common subgroup is polyarthritis (~30%) with more than four joints being affected during the first 6 months of disease. Rheumatoid factor (RF) positive polyarthritis, with a disease onset in late childhood or early adolescence, is more likely to be erosive and to share many clinical and immunogenic characteristics with adult RA. Systemic arthritis with systemic manifestations and fever is less common (~10%) [144,145,146].

Patients with systemic JIA have an increased risk of developing MAS (macrophage activation syndrome), a severe and potentially life-threatening complication. The incidence is 7-13% in systemic JIA and the mortality is about 20-30%. Although the cause of MAS is unknown, activation of macrophages and T-cells leading to an overwhelming inflammatory response with massive cytokine overproduction has been implicated in this syndrome [147].

JIA is a multifactorial autoimmune disease with both environmental and genetic factors involved. Family studies indicate that the susceptibility of JIA is inherited since siblings have a 15-30 times higher risk of developing JIA than is the general population [148,149]. Several studies indicate that there are associations between some JIA subgroups and HLA genes. However, there is no association with the subgroup systemic JIA lack and HLA genes. This subgroup is instead associated with polymorphisms of IL-6 and MIF (macrophage inhibitory factor).

Environmental factors have also been suggested to be involved in the disease onset of JIA. The association of viral and bacterial infections with JIA onset has been proposed but results are inconclusive. Maternal smoking during pregnancy was associated with a higher risk of polyarthritis in the child (reviewed in [150]).

Table 2: Classification of JIA

JIA subtype	% of total JIA	Onset age, sex ratio	Joint involvement	Other features
Oligoarthritis	40-50 %	Early childhood, F>M	≤ 4 joints affected Large joints: knees, ankles, wrist Persistent disease: never >4 joints Extended disease: >4 joints after first 6 mo of disease	60-80% ANA positive 30% uveitis
Polyarthritis (RF-negative)	20-25%	2 peaks: 2-4y and 6-12y, F>M	>5 joints, symmetric	25% ANA positive 15% uveitis
Polyarthritis (RF-positive)	5%	Late childhood/early adolescence, F>M	>5 joints, small and large joints, symmetric	25% ANA positive <1% uveitis Fatigue
Systemic arthritis	5-10%	Throughout childhood, F=M	Poly or oligoarticular	Daily (quotidian) fever for ≥2 weeks Skin rash Lymphadenopathy Hepatosplenomegaly Serositis <1% uveitis
Enthesitis-related arthritis	5-10%	Late childhood/Adolescence M>F	Weight-bearing joint especially hip and intertarsal joints	Enthesitis HLA-B27-positive Axial involvement (including sacroiliitis) ~7% uveitis
Psoriatic arthritis	5-10%	2 peaks: 2–4 y and 9–11 y F>M	Asymmetric or symmetric small or large joints	Nail pits, onycholysis Dactylitis Psoriasis 10 % uveitis
Undifferentiated arthritis	10%			Does not fulfill criteria for any above category or fulfills criteria for >1 category

Abbreviations: JIA = Juvenile idiopathic arthritis, F = Female, M = Male, ANA = Antinuclear antibody, RF = Rheumatoid factor

3.1.2 Pathogenesis and joint inflammation

JIA is a very heterogeneous and multi-factorial autoimmune disease and the pathogenesis and disease progression varies between subgroups and individuals. The pathogenesis of oligo/polyarthritis is distinct from systemic JIA. Oligo/polyarthritis is more a lymphocyte-mediated antigen-driven autoimmune disease and systemic JIA is an autoinflammatory disease with loss of control of phagocytes and release of proinflammatory mediators [150]. However, the processes mediating joint damage appear to be common for all JIA subtypes. The increased vascularisation and endothelial activation promote infiltration of inflammatory cells and activation of osteoclasts and proteases, especially matrix metalloproteinases (MMPs). In addition to cartilage and bone destruction, the tissue repair is reduced due to increased apoptosis of osteoblasts (reviewed in [151]).

The association of HLA alleles with poly and oligoarthritis suggests a role for adaptive immunity and especially T cells in the pathogenesis. Autoreactive T cells with specificity for the joint-related proteins aggrecan and fibrillin are evident in the circulation of JIA patients but not in healthy controls [152]. Several of the danger signals HSPs are also autoantigens in JIA (reviewed in [151]). Regulatory T cells are suggested to limit the disease progression in persistent oligoarthritis since a higher number of CD4⁺CD25^{hi} T_{REG} cells are observed in these patients compared to in patients with extended oligoarthritis, polyarthritis and systemic JIA [153,154].

The role of autoantibodies including antinuclear antibodies (ANA), RF and antibodies to citrullinated protein antigen (ACPA) on the disease development has not been fully elucidated. The RF⁺/ACPA⁺ subset of patients with polyarthritis suffers from a significant joint damage [151]. It is also proposed that the ANA⁺ patients form a clinical homogenous group regardless of JIA subgroup [155]. ANA positivity, together with early disease onset, is also associated with an increased risk of developing uveitis [156].

Proinflammatory cytokines are involved in patients with oligo and polyarticular JIA. Serum levels of IL-6 and TNF are elevated compared to controls, although even higher levels in synovial fluid indicate a local production [151].

Anti-TNF therapy is beneficial in patients with extended oligoarthritis and polyarthritis, indicating a key role of TNF in these subsets [157].

Overproduction of IL-6 is associated with systemic JIA and these patients do respond very well to anti-IL-6 therapy [158]. The IL-1 levels are not increased but they have a clear IL-1 signature since incubation of healthy mononuclear cells with plasma from these patients leads to upregulation of innate immune genes including IL-1 [159].

Common for several subgroups of JIA is the crucial involvement of immune activation induced by DAMPs in JIA pathogenesis. Serum levels of S100 proteins (also known as myeloid-related proteins (MRPs)) (discussed in section 1.1.1), secreted from activated neutrophils and monocytes, are increased in JIA. MRP8/14 serum levels correlate with disease activity in JIA and are suggested as a biomarker to monitor treatment response [160]. S100A12 are also increased in serum and correlate to degree of inflammation and disease activity in JIA [161].

3.2 RHEUMATOID ARTHRITIS

RA is a chronic, disabling disease characterised by synovitis, destruction of bone and cartilage and loss of joint function. Approximately 0.5-1% of the adult population are affected worldwide, and 20–50 cases per 100 000 occur annually [162].

3.2.1 Classification and etiological features

Diagnostic criteria were developed 1987 by The American College of Rheumatology (ACR) [163] (table 3). Four of the criteria must be fulfilled and must persist for at least six weeks. Recently, these criteria were updated in order to identify patients in early stage of disease previously missed by the former criteria [164].

The aetiopathogenesis of RA is complex and incompletely understood although both genes and environment are involved. This has been demonstrated in twin studies where smoking was studied. In 12 of 13 pairs of monozygotic twins, the smoker was the one who developed RA and the non-smoker was healthy [165]. Several large studies have also demonstrated that smoking is an environmental risk factor for developing RA [166,167]. Moderate consumption of alcohol can reduce the risk for RA since there was a decreased risk for developing RA among

women who drank >4 glasses of alcohol (1 glass = 15 g of ethanol) per week compared with women who drank <1 glass per week or who never drank alcohol [168,169]. It has been suggested that sex hormones are involved in RA since the incidence is higher in women than in men and the peak incidence in women coincides with menopause [170]. Treatment with estrogens suppresses CIA in mice [171] and has ameliorating effects on inflammation in women with RA [172].

Autoantibodies including RF and ACPA are sometimes present before the clinical onset of disease. The division of RA patients into two subsets depending on the presence or absence of ACPAs has been valuable since these groups differ regarding causes and severity [173].

Several genetic loci are associated with increased risk of developing RA and HLA DR is the most established. Most HLA DR alleles that are associated with RA had a common amino acid sequence motif, named the shared epitope, in the β chain of the HLA DR molecule. This indicates that the MHC class II activation of T cell and B cell is important in the RA pathogenesis. Another genetic risk factor described for RA is PTPN22 (protein tyrosine phosphatase, non-receptor type 22). Both HLA BRB1 shared epitope and PTPN22 risk alleles are only associated with the ACPA-positive subset of RA (reviewed in [174]).

Table 3.

RA classification criteria. ACR 1987

1. Morning stiffness
2. Arthritis of 3 or more joint areas
3. Arthritis of hand joints
4. Symmetric arthritis
5. Rheumatoid nodules
6. Serum rheumatoid factor
7. Radiographic changes

3.2.2 Pathogenesis and joint inflammation

In the inflamed joint there are several inflammatory cascades that trigger different parts of the immune system. The inflammatory activity is destructive for the synovial tissue if not properly regulated. The synovial membrane that lines the joints normally consists of 1-2 cell layers but during joint inflammation it expands to being 15 cell layers depth or more (synovial hyperplasia). Inflammatory cells including macrophages, mast cells, T cells, NK cells and plasma cells also infiltrate the synovium, while the concomitant excess of synovial fluid (causing swelling of the joint) is dominated by neutrophils. The inflamed synovium invades adjacent cartilage and promotes articular degradation. The unusual pathophysiological environment inside the joint, with hypoxia and variable biomechanical stress, also induces resident cells to release inflammatory mediators and danger signals, further enhancing the inflammation. Endogenous PRR ligands such as heat shock proteins, fibrinogen, hyaluronan and HMGB1 are commonly found in inflamed joint [107,108,175] (also discussed in section 2.6.2). Macrophages as well as synovial fibroblasts that are activated by their PRR that recognizes DAMPs and PAMPs are an important source of cytokines. The imbalances in regulation of cytokines and inflammatory mediators are involved in the pathogenesis [176]. Proinflammatory cytokines such as TNF, IL-1 and IL-17 can stimulate fibroblasts and macrophages to release MMPs. Especially MMP-1 and MMP-3 have been reported to be important in RA since they are able to degrade all vital proteins in the extracellular matrix of cartilage [174]. Furthermore, RANKL expression can be induced by TNF, IL-1, IL-6 and IL-17. Stimulation of RANK by RANKL on monocytic osteoclast precursors mediates the differentiation to osteoclasts with subsequent destruction of bone [177].

3.3 ANTI-RHEUMATIC THERAPIES

There are different therapeutic strategies to control the symptoms of synovial inflammation although no treatment today can completely cure the disease. Many of the conventional anti-rheumatics were developed for other diseases and were later empirically shown to also have effects in arthritis, but their mechanisms of action are still not completely understood. The newer biological drugs have been developed to target specific molecular mechanisms

concluded from research. To study the effects of anti-rheumatic drugs gives us a better understanding of the pathogenesis in arthritis but is also important for development of new therapeutics.

3.3.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs reduce pain, inflammation and fever by blocking of cyclooxygenases (COX) 1 and COX-2 with subsequent reduction of proinflammatory prostaglandins. Prostaglandins are involved in inflammation by mediating edema, vasodilation and pain. However, many body maintenance functions are associated with COX and prostaglandins, including kidney function, platelet aggregation and protection of the mucosa from acid damage. The blockade of COX-1 can therefore cause gastrointestinal toxicity as a side-effect [178]. In contrast, selective COX-2 inhibitors are associated with an increased risk for cardiovascular complications [179]. NSAIDs are nevertheless commonly used for treatment of JIA and RA.

3.3.2 Glucocorticoids

Glucocorticoids are potent anti-inflammatory drugs widely used to dampen inflammation in a broad range of inflammatory diseases and conditions in addition to arthritis. Patients with arthritis have been treated for decades with either systemically or with local intra-articular injections of glucocorticoids with an excellent effect on synovial inflammation. However, the side-effects of glucocorticoid treatment such as osteoporosis and insulin resistance have limited their usefulness. The general anti-inflammatory effects of glucocorticoids are many, including inhibition of inflammatory proteins and induction of anti-inflammatory proteins and can be mediated both through genomic and non-genomic mechanisms [180]. Locally, intra-articular injections of glucocorticoids decreases the production of pro-inflammatory mediators and adhesion molecules including TNF, IL-1 β , HMGB1, ICAM-1 and VEGF [106] and infiltration of T cells [181].

3.3.3 Disease-modifying anti-rheumatic drugs (DMARDs)

DMARDs reduce the progression of the disease and prevent or reduce joint damage. Methotrexate (MTX) is often the first-line choice in treatment of both JIA and RA. It was developed as a cancer treatment to inhibit cell proliferation by antagonizing folate and interfering with DNA synthesis. However, its anti-proliferative effects cannot fully explain the anti-inflammatory properties of low-dose treatment in arthritis. Its metabolites, MTX polyglutamates, inhibit the enzyme AICAR (5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide) transformylase which increases the release of the potent anti-inflammatory mediator adenosine [182]. The mechanism of action is not completely understood but adenosine can inhibit cytokine transcription and suppresses the inflammatory function of neutrophils, monocytes/macrophages, dendritic cells and lymphocytes, important in the joint inflammation [183]. The effect of MTX is reduced in patients receiving adenosine receptor antagonist, indicating the involvement of adenosine [184]. MTX can cause hematologic and mucosal side-effects, but these are related to folate antagonism and can be reduced by folic acid supplementation [182].

Hydroxychloroquine has been used as an anti-malarial agent for hundreds of years but has more recently also been used to treat rheumatic diseases. Chloroquine has immunomodulatory effects but the mechanism is still unclear. It is a weak base and can easily enter into cells and affect lysosomes and other cytoplasmic vesicles, thereby altering protein processing. This effect may be responsible for a number of mechanisms of action including inhibited cytokine production from macrophages, especially IL-1 and IL-6, inhibition of MMPs and antigen presentation [185] [182]. More recently, hydroxychloroquine has also been demonstrated to inhibit the function of TLR3, TLR7 and TLR9. The sensing of TLRs by self-DNA, -RNA and immune complexes is important in the pathogenesis of RA and SLE. The inhibition of TLR function by hydroxychloroquine may therefore be a part of its mechanism of action in these two diseases [182].

Gold sodium thiomalate (GST, Myocrisin) has been used as anti-rheumatic drug for a long time but its molecular mechanism of action is still not fully understood. It is known to modulate phagocytic cells [186]. It has also been demonstrated that gold compounds inhibit COX-2 with subsequent inhibition of

PGE₂ production and also MMP-3 and IL-6 expression in articular cartilage from RA patients [187].

Sulfasalazine and Leflunomide are other DMARDs that sometimes are used for treatment of rheumatic diseases.

3.3.4 Biologics

Biologics are purified, modified and/or reconstructed proteins and are used to modify the immune response by inhibition of specific key molecular targets. The first available biologics for use in rheumatic diseases, the TNF inhibitor etanercept (Enbrel), was approved in 1998. Etanercept is a human IgG-TNF receptor fusion protein. Several other TNF inhibitors are now approved for treatment of rheumatic diseases including infliximab (Remicade), adalimumab (Humira) certolizumab (Cimzia) and golimumab (Simponi) which are all anti-TNF monoclonal antibodies. Etanercept and adalimumab are approved for use in JIA and all five are approved for use in RA.

Abatacept (Orencia) is a CTLA4-Ig fusion protein that blocks the second signal of T cell activation by antigen-presenting B cells which suppress cytokine release.

Rituximab (Rituxan) is a chimeric monoclonal antibody that blocks the CD20 surface protein on mature B cells, resulting in depletion of B cells. This affects both the B cells antibody production and interaction with T cells and is more effective in ACPA⁺ and RF⁺ patients. The depletion persists for up to 12 months.

IL-1 β inhibitors have a dramatic effect in autoinflammatory syndromes in which IL-1 β plays a key role, including gout and familial Mediterranean fever. However, the efficacy in RA has proven inferior to that of anti-TNF. Anakinra (Kineret) is an IL-1 receptor antagonist that is sometimes used as a treatment of RA and JIA.

Tocilizumab (RoActemra) is a humanized monoclonal antibody that blocks soluble and membrane-bound IL-6. It is used in RA patients that do not respond to TNF blockade but has also shown good effect in systemic JIA [182].

4 AIMS OF THE THESIS

HMGB1 has been defined as a key mediator in arthritis although its pathogenic role in arthritis is not fully understood. Although HMGB1 blockade has proven efficacious in many disease models in addition to arthritis, no anti-HMGB1 therapeutic has yet been tested in clinical trials. The general objective of my thesis work was therefore to study the role of HMGB1 in arthritis, in humans as well as in animal models, and to assess its potential as a therapeutic target.

The specific aims of the thesis were to:

- Determine the expression of extracellular HMGB1 in inflamed joints of juvenile idiopathic arthritis (JIA) patients with active disease and its correlation to other inflammatory mediators
- Study the stimulatory effect of HMGB1 alone and in complex with other inflammatory mediators on synovial fibroblast from patients with arthritis in an *in vitro* system
- Evaluate the effects of well-known anti-rheumatic drugs on HMGB1 secretion from human monocytes
- Investigate the therapeutic potential of a monoclonal anti-HMGB1 antibody in two different animal models of arthritis

5 METHODOLOGICAL CONSIDERATIONS

In this section some of the methods used in my research are discussed. Details are described in the separate papers.

5.1 THE BIOBANK AND SAMPLE HANDLING

In order to improve the knowledge of the pathogenesis in JIA and its different disease subgroups we established a biobank called “Juvenile Arthritis BioBank Astrid Lindgren Children’s Hospital” (JABBA) for samples from JIA patients. Collection of samples started in 2010 and this far we have collected plasma, synovial fluid, peripheral blood mononuclear cells (PBMCs), DNA, urine samples and a few synovial biopsies from more than 250 patients connected to a patient register.

The overall aim with the JABBA biobank is to provide a unique opportunity to study different aspects of JIA pathogenesis. However, we have special interest in HMGB1 as an inflammatory mediator and a biomarker in JIA and when quantifying levels of HMGB1 in clinical samples the sample handling is crucial to avoid incorrect results. Increasing levels of HMGB1 over time in plasma and serum samples from healthy volunteers due to *ex vivo* cell death is a common cause of falsely high HMGB1 recordings [Sundberg, unpublished data, 188]. Before we started to collect clinical samples for inclusion in the JABBA biobank we carefully investigated how long a blood sample could be stored at room temperature before centrifugation and without spontaneous HMGB1 release occurring. We could determine that no changes in HMGB1 levels could be detected after 4 hours, and this timepoint was hence set to the maximum allowed time span between sampling and centrifugation of all samples.

5.2 DETECTION AND QUANTIFICATION OF HMGB1

5.2.1 Detection of HMGB1 in biological samples

Quantification of HMGB1 in biological samples is of great importance in the evaluation of its role in the pathogenesis of multiple diseases. In the past no commercial method for HMGB1 detection was available and different methods were used by different research groups. A few years ago a new **HMGB1 ELISA** was introduced onto the market [189] and is now widely used in the field of HMGB1

research. A commercially available method makes the quantification of HMGB1 levels in recent studies more comparable and I have used this method in **paper I**.

However, even now when a commercially available assay exists, the detection of HMGB1 in biological samples is complicated and it is worth pointing out some difficulties in its measurement. One obstacle when measuring HMGB1 in clinical samples is that the biological ability of HMGB1 to bind factors can complicate the detection [119,190], possibly by masking epitopes used for antibody binding in ELISA. In patients with high levels of autoantibodies e.g. SLE, the detection of HMGB1 with ELISA is difficult since the anti-HMGB1 antibodies block HMGB1 [117]. The **Western blot** technique dissociates such immune complexes and may therefore be a better method for HMGB1 detection in these patients. However, if samples are ultrafiltered to remove proteins above 100kDa the immune complexes will be excluded and significant amounts of HMGB1 might be removed before the actual measurement. In our cohort of JIA patients the levels of anti-HMGB1 antibodies are very low [Schierbeck *et al.*, unpublished data] and should not affect HMGB1 detection using ELISA.

An alternative method for HMGB1 detection, not used in my studies, is a non-antibody- based method. It is based on the property of HMGB1 to bind to radiolabeled hemicatenated DNA loops with high affinity and then analyzed by electrophoresis in non-denaturing polyacrylamide gels using the band-shift assay method. Purification of HMGB1 with perchloric acid further increased the sensitivity of the method [191]. However, this time-consuming method is not widely used.

5.2.2 Detection of HMGB1 in *in vitro* cell cultures

In **paper III** I have used a sensitive **ELISPOT** method previously developed in our group for detection of HMGB1 [192]. Cells are added directly to the antibody-coated wells and following incubation the number of secreting cells is detected since one spot on the well membrane corresponds to one secreting cell. This method is very sensitive and a low number of cells are needed, but it is not possible to detect the total level of secreted protein. Cells that are cultured at high cell density can be stressed because of the high consumption of nutrients in the medium and HMGB1 will be passively released. The ELISPOT therefore enables

HMGB1 detection from physiologically cultured cells and minimizes the background secretion.

The cell morphology and translocation of HMGB1 from the nucleus to the cytoplasm can be visualised by **immunocytochemistry** (used in **paper III**). This enables studies of the localization of HMGB1 in the cell in response to stimulation or inhibition of HMGB1 release. However, it is difficult to determine if HMGB1 is extracellularly released or only translocated to the cytoplasm.

5.2.3 Characterisation of post-translational modifications of HMGB1

All methods described above detect the total level of HMGB1 and do not distinguish between different isoforms of HMGB1 (discussed in section 2.5.3).

Liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) can be used to further characterise different HMGB1 isoforms. With this method it is possible to both detect the redox state of HMGB1 and to quantify the level of hyperacetylated HMGB1 associated with active secretion [193]. This method is time-consuming and therefore not applicable for screening of a large number of samples.

5.3 PREPARATION OF HMGB1

To study the role of HMGB1 in complex with other molecules (used in **paper II**) we used recombinant rat HMGB1 expressed in *E. coli* and containing a calmodulin-binding peptide tag (for sequence see [48]).

The protein was produced in the presence of dithiothreitol (DTT), which reduces three the cysteines in HMGB1 to thiols and makes it chemotactically active but not cytokine-inducing (discussed in section 2.5.3, summarised in table x) [68]. It is therefore very important to consider the production protocol and redox status of HMGB1 used in different publications.

In general, when studying the immune activation and TLR involvement induced by recombinant proteins expressed in bacteria it is important to consider that they can be contaminated with LPS, bacterial DNA or other microbial products, yielding false positive results. Our protein was highly purified and endotoxin-free as determined by Limulus assay and DNA-free as determined by gel electrophoresis.

5.4 DETECTION OF SOLUBLE PROTEINS

The detection of soluble proteins in synovial fluid can be more complicated than detection in serum/plasma and cell supernatants since the methods are not validated for such samples and the samples contain many large-sized charged molecules. There are many different methods for measurement of soluble proteins in biological samples and supernatants from cell cultures. ELISA (used in **paper I** and **paper II**) is the most widely used and has even shown high reproducibility between dilutions in synovial fluid [Schierbeck, unpublished data]. The limitations are that larger sample volumes (compared to bead-based methods) are sometimes required. Other bead-based immunoassays such as CBA (cytometric bead array, BD Bioscience) (used in **paper I** and **paper II**) and BioPlex (BioRad) are based on the same principles as ELISA but have the great advantage that numerous proteins can be simultaneously measured with high sensitivity in a small sample volume. This is favorable when working with valuable clinical samples (as in paper I) and is also time-saving as compared to performing several ELISAs. The ELISPOT method (described in the previous section) was also used in **paper II** and **paper III** for detection of TNF release from cell cultures.

5.5 EXPERIMENTAL ARTHRITIS MODELS

Experimental animal models of arthritis are important tools for the development and evaluation of new therapies and for revelation of immunological mechanisms involved in arthritis pathogenesis. There are several animal models, both inducible and spontaneous, with similarities and differences compared as to human disease [194]. The choice of a relevant animal model depends on the aim and research question in the current study. The heterogeneity in human diseases indicates that it is favourable to use several models with distinct pathogenesis.

5.5.1 Arthritis models in this thesis

In **paper IV** I have used two different experimental arthritis models: CIA and a spontaneous arthritis model in mice deficient for DNase II and IFNRI, respectively. CIA is the most commonly used model of human RA and is induced by injection of collagen type II either with Freund's complete adjuvant (inactivated mycobacteria suspended in mineral oil) or Freund's incomplete

adjuvant (mineral oil alone) [195]. CIA shares many clinical, histological and immunological features with the human disease. These similarities include symmetric joint involvement, synovitis with cartilage and bone erosions and also presence of RF [196,197]. As in the case of RA, the susceptibility of CIA has been associated with the mouse homolog to HLA class II alleles [198]. A difference between CIA and human disease is that mice with CIA develop anti-collagen antibodies to a greater extent compared to what is detected in RA patients [196]. Another difference to RA is the transient joint pathology in CIA.

More recently, a new mouse model of arthritis was described in which mice double- deficient for DNase II and IFN-IR (interferon type I receptor) spontaneously develop destructive chronic polyarthritis [199]. DNase II deficiency prevents macrophages from digesting DNA from engulfed apoptotic cells and expelled erythroid cell nuclei. Due to the unexplained constitutive production of IFN- β , DNase II^{-/-} mice die as embryos but can be rescued by a deficiency of the IFN-IR gene [200]. Mice double deficient for DNase II and IFN-IR spontaneously develop a clinical disease at the age of around 6 weeks. The disease has many similarities with human RA e.g. high serum levels of ACPA, RF and MMP-3. In contrast to human RA this model is not T cell-dependent since prevention of lymphocyte development in these mice by deletion of recombination activation gene (Rag) accelerates the arthritis progression. Since macrophages are unable to digest DNA, they become activated and produce cytokines that are involved in the disease pathogenesis [199]. We chose these two models because of their similarities with human disease and for the involvement of HMGB1 in the pathogenesis that has previously been shown [130,139].

5.6 IN VITRO AND IN VIVO MODELS

In vitro and experimental *in vivo* models are important tools for studying mediators of disease pathogenesis and general mechanisms. However, there are different advantages and disadvantages with these systems and it is important to consider the differences compared to human disease when interpreting the results.

In vitro culturing of cells probably influences differentiation and/or activation of cells in general and the *in vivo* conditions are difficult to mimic. However, such a system is useful for study of simple reactions of cause-and-effect.

The use of cell lines can be criticized since they are transformed and may not correspond to normal cells. We have therefore chosen to use cultures of primary cells; in paper II we used human synovial fibroblasts never cultured for more than 8 passages and in paper III we used freshly isolated human monocytes. The isolation step of the latter via positive selection of CD14⁺ monocytes may affect the cells. However, the low secretion of HMGB1 detected from unstimulated cells indicates that the isolation step has a minor impact on these cells since HMGB1 is easily released from stressed cells.

Even though cell culture conditions are optimized to mimic the physiological condition with 37°C and 5% CO₂, the O₂ level (21%) does not correspond to the level in the joint. The supply of oxygen to the inflamed joint is decreased, leading to profound local hypoxia with oxygen levels of 2-4% in RA joints compare to 9-12% in healthy joints [201]. Despite this, the synovial fibroblasts in an arthritic joint differ considerably from those in a healthy joint regarding receptor expression and immune response [202], and studying these *in vitro* provides knowledge regarding the milieu in the arthritic joint.

In vivo models of experimental diseases in animals not only provide a tool for pre-clinical testing of novel therapeutic targets but also enable the study of immunological mechanisms in the pathogenesis. The heterogeneity of genes and environmental factors are difficult to consider in studies of human diseases. These variations are eliminated when investigating mechanisms in a disease models since mice are genetically identical and exposed to the same environmental factors.

Many therapies have shown dramatic effects in experimental models but no or little effect in clinical trials [203]. There are differences in metabolism in different species that may explain the discrepancies. In paper IV we chose to test our therapy in two different arthritis models with completely different disease causes, but both resembling aspects of human RA. This strengthens our hypothesis that HMGB1 blockade may be beneficial in human disease.

6 RESULTS AND DISCUSSION

6.1 HMGB1 EXPRESSION IS INCREASED IN INFLAMED JOINTS OF PATIENTS WITH JIA (PAPER I)

HMGB1 is highly expressed extracellularly in synovial fluid and synovial tissue from patients with RA [106-108], but there are currently no studies defining the role of HMGB1 in JIA. Overall, JIA is not as thoroughly studied as RA and conclusions based on RA studies are sometimes translated to JIA. However, although RA and JIA share clinical features there are significant differences. To improve the knowledge regarding the pathogenesis of JIA and its different disease subgroups we established a biobank of samples from JIA patients. In 2010 we started to collect plasma, synovial fluid, peripheral blood for DNA extraction, urine and a few synovial biopsies from JIA patients registered at the pediatric rheumatology unit at Astrid Lindgren Children's Hospital.

Since our group has a special interest in HMGB1, the aim of the first study (paper I) was to investigate whether HMGB1 might be an inflammatory mediator involved in the pathogenesis of JIA. We set out to measure HMGB1 levels in plasma and synovial fluid and to correlate the recorded levels to other measured inflammatory mediators and clinical parameters.

We analysed levels of HMGB1, sRAGE and MMP-3 by ELISA in plasma and levels of HMGB1, sRAGE, MMP-3 MRP8/14, S100A12, MCP-1, IP-10, RANTES, IL-1 β , IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN- α , IFN- γ and TNF using ELISA or CBA (cytometric bead array) in synovial fluid from 23 JIA patients. We could determine that HMGB1 levels were significantly higher in synovial fluid as compared to plasma. Conversely, levels of the HMGB1 decoy receptor sRAGE were significantly lower in synovial fluid compared to plasma. S100 proteins form a group of proteins also classified as danger signals and the variants MRP8/14 and S100 A12 can be used as serum biomarkers for disease activity and treatment response in JIA [160,161,204]. We detected high levels of the studied S100 members in synovial fluid, but a high variability between patients was also recorded. The level of S100 proteins correlated to IL-6 and IL-8 levels, but not to HMGB1 levels in synovial fluid. MMPs are known to contribute to bone and cartilage destruction and MMP-3 has been suggested as a specific marker for disease activity in JIA [205]. The levels of MMP-3 were significantly higher in synovial fluid as compared

to plasma. When correlating measured mediators to clinical parameters we could demonstrate that synovial fluid levels of HMGB1 correlate with age at disease onset, but not with disease duration. HMGB1 levels were higher in patients with an early disease onset. HMGB1 was present in synovial fluid from patients with active disease independent of disease duration. In contrast, levels of S100 proteins and IL-8 correlate with disease duration and were highest at the beginning of the disease.

In summary, this is the first study describing the presence of HMGB1 in synovial fluid from JIA patients and this may indicate a role for HMGB1 in the pathogenesis. The variation of HMGB1 and other inflammatory mediators such as S100 proteins and IL-8 indicates differences in inflammatory phenotype of the patients. The different mediators may also be of varying importance during the inflammatory phases of the disease. The lack of correlation between HMGB1 and the other measured parameters implies that HMGB1 could be released from different cell types or during different phases of inflammation than other mediators.

My study suggests HMGB1 as a mediator in the pathogenesis of JIA and warrants further investigations of its role as a potential biomarker or as a therapeutic target. The high levels of HMGB1 in patients with an early onset indicate a distinct phenotype with high persisting levels during the progression of JIA. Early disease onset is one of the predictors for persistent disease and joint erosions [206] even though many JIA patients recover completely from their disease. The development of uveitis in girls with JIA is the most common and most significant extra-articular manifestation of JIA and is also associated with early disease onset [156]. Further investigation is necessary to determine if this group of patients is different from other JIA patients regarding inflammatory markers.

The new knowledge regarding the redox regulation of HMGB1 function and the ability to analyse different isoforms of HMGB1 will give us a deeper understanding in the role of HMGB1 in the inflammatory processes in JIA and other diseases.

6.2 HMGB1 IN COMPLEX WITH ENDOGENOUS AND EXOGENOUS DANGER MOLECULES ENHANCES CYTOKINE AND MMP RELEASE FROM SYNOVIAL FIBROBLASTS (PAPER II)

The cytokine-inducing capacity of HMGB1 initially described [75] has been questioned by several research groups over the years. To date, we know that the redox state of HMGB1 determines the inflammatory properties of the protein (discussed in section 2.5.3) and that previous work with poorly defined HMGB1, from a redox point of view, explains the difficulties for certain laboratories to reproduce the HMGB1 cytokine-inducing effects. We also know that HMGB1 in synergy with low amounts of exogenous or endogenous inflammatory mediators strongly enhances cytokine production as compared to stimulation with these mediators alone [71,72,76,78-80].

The aim of this study was to evaluate the potential of HMGB1 to interact with other molecules to synergistically enhance cytokine release from synovial fibroblasts isolated from rheumatoid arthritis (RA) and osteoarthritis (OA) patients and also to study reciprocal receptors mediating this effect. This *in vitro* model explains a role for HMGB1 to potentiate inflammation in the arthritic joint, a location where HMGB1 is highly expressed [107,108]. In order to study the potential of HMGB1 to enhance cytokine release in cell cultures we selected rHMGB1 batches that did not induce cytokine production on their own. This *E. coli*-expressed HMGB1 contains a calmodulin-binding tag and is highly purified and endotoxin-free. We chose to study complexes formed by HMGB1 and LPS, IL-1 α or IL-1 β , respectively, since these ligands may be present in the arthritic joint [207-211]. The synergistic effect of HMGB1 complexes was only detected if the complex was preformed overnight, but not when the separate molecules were added directly to the cell culture [76].

We demonstrated that HMGB1 together with suboptimal doses of LPS, IL-1 α or IL-1 β , respectively, induce an augmented release of TNF, IL-6 and IL-8 from both OA and RA synovial fibroblasts. HMGB1/IL-1 β complexes also induced enhanced MMP-3 secretion. To elucidate the receptors involved in this cytokine-enhancing property of HMGB1 complexes we used detoxified LPS as a receptor antagonist for TLR4 and an IL-1 receptor antagonist (IL-1RA also known as Anakinra®). We showed that HMGB1/LPS complexes signals are mediated

through TLR4 and HMGB1 in complex with IL-1 α or IL-1 β signals through the IL-1 type I receptor.

In summary, we have demonstrated that HMGB1 forms complexes with LPS, IL-1 α or IL-1 β , respectively, and thereby induces enhanced cytokine production from synovial fibroblast by ligation of the receptor of the ligand partner of the complex. Since all these mediators are present in the inflamed joints of arthritic patients, my results demonstrate a mechanism through which HMGB1 may aggravate joint inflammation by increased cytokine production and cartilage destruction via MMP-3 production.

6.3 INHIBITORY POTENTIAL OF ANTI-RHEUMATIC DRUGS ON THE RELEASE OF HMGB1 FROM HUMAN PRIMARY MONOCYTES (PAPER III)

The highly beneficial effect of HMGB1 blockade was first demonstrated in 1999 by Kevin Tracey and colleagues, anti-HMGB1 antibodies being administered in an animal model of gram-negative sepsis [48]. Moreover, the therapeutic effects were reproduced when HMGB1 antagonists were added in a delayed fashion up to 24 hours after the initial initiation of peritonitis. This is of particular clinical interest since no other immunomodulatory compounds can be successfully administered at this late stage of experimental sepsis.

However, no specific anti-HMGB1 therapy yet exists for use in human diseases. I demonstrated inhibitory effects on HMGB1 release of some well-known anti-rheumatic drugs were demonstrated in pilot studies in the mouse monocytic cell line RAW 264.7 when HMGB1 levels were analysed by western blotting [Schierbeck *et al.*, unpublished data]. Since the results were interesting I wanted to confirm the data using primary human monocytes freshly isolated from healthy blood donors. The aim of this study was therefore to evaluate the effect of selected anti-rheumatic drugs on HMGB1 release from human monocytes compared with the effect on TNF secretion.

Measurement of HMGB1 levels in supernatants with western blot analysis requires large amounts of cells that were unfeasible when using primary blood monocytes. I therefore further refined the ELISPOT technique previously developed by our group [192] for enabling detection of HMGB1 release from

primary human monocytes. The ELISPOT is a sensitive method for quantification of the number of HMGB1 secreting cells and can be performed with a low number of cells.

In this study I demonstrated that pharmacologically relevant doses of dexamethasone, chloroquine and gold sodium thiomalate (GST) all inhibit the extracellular release of HMGB1 from LPS- and IFN γ - stimulated monocytes, respectively. Other studied agents including cortisone, MTX, colchicine, etanercept and anakinra had no effect on HMGB1 release. Dexamethasone and chloroquine also inhibited TNF release but the effect of GST was exclusively on HMGB1 release. Etanercept, a soluble TNF receptor, binds TNF and blocks its detection as expected. The absence of cortisone-mediated inhibition was expected since primary monocytes lack the 11 β -hydroxysteroid dehydrogenase type 1 enzyme required for conversion of cortisone to cortisol, the functional end product of this potent endogenous anti-inflammatory system [212]. The disease-modifying actions of MTX, the most commonly used anti-rheumatic drug, are suggested to be mediated by metabolites including MTX polyglutamates that increases the release of the potent anti-inflammatory mediator adenosine [182]. MTX has a latent period of weeks before the effects are appreciated in patients with RA, suggested to be due to the need of metabolism of MTX [213]. This may explain the lack of efficiency of MTX in the short term *in vitro* system used in this study. The effect of MTX on HMGB1 release may instead be studied *ex vivo* in primary monocytes from patients treated with MTX.

We have previously reported that glucocorticoids down-regulate HMGB1 and TNF expression in synovial tissue from patients with chronic arthritis or myositis [106,122]. In this study, I demonstrated using immunocytochemistry that the mechanism by which dexamethasone inhibits HMGB1 release is through inhibition of its translocation from nucleus to the cytoplasm. It has previously been reported that GST inhibits HMGB1 release in the same manner [124]. Chloroquine also induces apoptosis, which can explain a part of its inhibition of HMGB1 release, as HMGB1 is tightly sequestered to chromatin during apoptosis [58]. Thus to summarise we have shown that dexamethasone, chloroquine and GST can inhibit HMGB1 release from human monocytes, which may be a part of their mechanism of action.

6.4 THERAPEUTIC BLOCKADE USING A HMGB1-SPECIFIC MONOCLONAL ANTIBODY IS BENEFICIAL IN TWO DIFFERENT MODELS OF ARTHRITIS (PAPER IV)

Experimental disease models provide an important tool during development and evaluation of new therapeutics. In this study we chose to use two different experimental arthritis models for evaluating the therapeutic potential of a monoclonal antibody directed against HMGB1. Firstly, CIA is the most commonly used arthritis model for evaluating new therapies and shares many features with human RA (discussed in section 5.5.1). It is induced by subcutaneous injection of collagen type II into the base of the tail of DBA/1 mice and a booster injection at day 28. In this model HMGB1 has previously been shown to be a mediator of the pathogenesis since administration of polyclonal anti-HMGB1 antibodies ameliorates disease [130]. The second is a spontaneous arthritis model. This spontaneous arthritis model, in DNase II ^{-/-} x IFN-IR ^{-/-} mice, was first described by Nagata and colleagues in 2006 [199] and has a very high resemblance to human RA (discussed in section 5.5.1). Following import of these mice into the animal facilities at the Karolinska University Hospital we studied the involvement of HMGB1 in this model. A clear correlation between severity of disease and extracellular HMGB1 levels was demonstrated. HMGB1 appeared in serum two weeks before disease onset, peaked at onset of disease and then decreased during established arthritis. Autoantibodies against HMGB1 were also present before, during and after onset of arthritis [139].

In the previous study (paper III), I showed a therapeutic strategy to inhibit HMGB1 secretion by nuclear sequestration in monocytes treated with anti-rheumatic drugs, but specific HMGB1 blockade is still important since some of the tested drugs have adverse effects. Blockade of nuclear sequestration of HMGB1 will probably only inhibit the active, but not the passive release of HMGB1. There will thus still be considerable amounts of HMGB1 in the extracellular space due to release from injured and dying cells, especially in diseases with a destructive pathogenesis. An antibody that neutralizes extracellular HMGB1 may therefore be a more favourable alternative. The effect of polyclonal antibodies has previously shown but only an antibody of monoclonal origin can be considered for clinical trials.

The aim of this project was to evaluate the effects of a monoclonal anti-HMGB1 antibody (2G7, mouse IgG2B) in these two arthritis models. We showed that the anti-HMGB1 antibody significantly decreased the clinical arthritis scores in the DNase II^{-/-} x IFN-IR^{-/-} mice when treatment was initiated before clinical onset of disease and continued every second day for five weeks. Histological parameters studied in joints included inflammatory cell infiltration, bone erosion and depletion of cartilage matrix. The antibody also significantly decreased the clinical arthritis score in the CIA model. The treatment was initiated when the mice had reached a clinical arthritis score ≥ 2 and mice were treated once a day for seven days. This model has a more aggressive and faster disease course compared to the spontaneous model. Despite this difference the 2G7 mAb demonstrated a good effect when treatment was initiated after disease onset. Fewer animals in the 2G7-treated group reached maximal disease severity (score 9) compared to in the control group.

Both these models have previously been shown to be HMGB1-dependent although they have different characteristics. The CIA model has a fast and aggressive disease course and was therefore treated once a day for only one week. The spontaneous arthritis model is less aggressive and was therefore treated for five weeks but with two-day intervals. The treatment was initiated before disease onset since very high levels of HMGB1 are already present before clinical onset.

This monoclonal antibody has beneficial effects in experimental sepsis even when the antibody was administered 24h after disease initiation. This is of particular clinical interest, since no other immunomodulatory therapeutics can be successfully administered at this relevant time point. Sepsis is a severe infection-triggered systemic inflammation with more than 30% lethality and 25% of the survivors develop cognitive impairment and new therapeutics for this disease is therefore in acute need.

In summary, I have demonstrated that a monoclonal anti-HMGB1 antibody reduces clinical arthritis in two different arthritis models. Polyclonal anti-HMGB1 antibodies have been efficient in many experimental disease models [129], but a monoclonal antibody is more relevant for further development of a humanized antibody that could be used for clinical trials.

7 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

During my thesis work I have studied the role of extracellular HMGB1 in arthritis, both in *ex vivo* samples from patients with JIA and *in vitro* models of human synovial fibroblasts from RA and OA patients, and finally HMGB1-targeted therapeutic intervention based on anti-HMGB1 mAb in two experimental arthritis models in mice. I have demonstrated the following:

- HMGB1 is overexpressed in inflamed joints of patients with JIA
- One mechanism by which HMGB1 can mediate inflammation is by forming complexes with other endogenous or exogenous inflammatory mediators, thereby enhancing their inflammatory actions.
- The proinflammatory activity of HMGB1 can be successfully targeted either by blocking extracellular release using certain anti-rheumatic drugs already clinically approved, or by neutralizing extracellular HMGB1 by administration of HMGB1-specific monoclonal antibodies, thereby ameliorating experimental arthritis

Arthritis in adults as well as in children is associated with chronic fatigue and chronic pain that restricts physical activities and quality of life. There is also a risk for permanent structural damage in the joints which can lead to permanent disability. Chronic inflammation in children generally confers systemic growth disruption. Existing therapies provide good chances for preserving joint function and improving quality of life, yet many patients are not helped enough. It is therefore important to identify and define new therapeutic strategies to improve treatment of chronic arthritis and other inflammatory diseases.

Disease progression varies between subgroups and individuals and children with JIA, and unlike adults with RA, they have a significant chance to recover with minimal or no residual state of the disease [214]. A challenge for the treating clinician is thus to detect and treat overt inflammation with accompanying tissue destruction while at the same time avoid overtreatment with associated potential side-effects for a growing child. The knowledge of immunological mechanisms

involved in disease progression and the immunological differences between JIA subgroups is not complete. Hence in order to define biomarkers for inflammation and joint destruction which would enable more precise surveillance of disease progression, there is a need to better evaluate the inflammatory phenotype of JIA patients.

HMGB1 is a danger signal that drives the inflammation in arthritis and also in many other diseases and conditions. Its inflammatory importance has been demonstrated by the ameliorating effect of HMGB1 blockade in experimental arthritis and in many other different inflammatory diseases models. Arthritis can be induced by intra-articular injections of recombinant HMGB1, indicating an important role of HMGB1 in the induction of the disease. The extracellular levels of HMGB1 have also been determined to be increased in many other inflammatory diseases, supporting a general role of HMGB1 in the pathogenesis of inflammatory diseases (reviewed in [129]). Although studies of the presence of HMGB1 in RA have been performed, the role of HMGB1 in JIA had not yet been investigated when we initiated study I.

The presence of HMGB1 in JIA synovial fluid indicates a role of HMGB1 in the pathogenesis of JIA. This study is descriptive and forms the basis for future studies of the role of HMGB1 in JIA. There are many questions that remain to be answered. The correlation between HMGB1 and age at onset indicates a certain role of HMGB1 in patients with an early onset. However, whether these patients have a different phenotype needs further investigation. Larger patient cohorts will be needed in future studies in order to evaluate the biological role of HMGB1 in subgroups of patients. The biobank sample collection from JIA patients is ongoing, which gives us a future opportunity to study JIA pathogenesis issues from different perspectives.

The recent discoveries of how post-translational modifications of HMGB1 affects the function of the protein will be very helpful for future studies of the contribution of HMGB1 in arthritis. Analysis of HMGB1 isoforms (redox, acetylation, methylation, phosphorylation) in biological samples from patients will without doubt provide a deeper understanding of the HMGB1-mediated regulation in the pathogenesis of JIA.

The quantification of hyperacetylated HMGB1 is a very useful biomarker for patients with acute drug-induced liver injury following acetaminophen overdose [92]. Hyperacetylated HMGB1 serum levels were only elevated in patients who died or required a liver transplant, while total HMGB1 serum levels were less predictive. These results indicate that hyperacetylated HMGB1 levels may represent a novel, predictive biomarker in severe systemic inflammation. The activation of cells induces hyperacetylation of the two NLS needed for subsequent HMGB1 release during programmed inflammatory cell death (pyroptosis), a caspase-1 dependent process [28]. In contrast, HMGB1 leaking out from necrotic cells is not hyper-acetylated, which makes acetylated HMGB1 a marker for actively secreted HMGB1. The hyperacetylation of HMGB1 therefore gives a clue as to the origin of the extracellular HMGB1 in biological samples. Cysteine-specific redox modifications of HMGB1 regulate its inflammatory function (discussed in section 2.5.3) during inflammation and its resolution [68,87]. This determines whether HMGB1 will have migratory, cytokine-inducing properties or no detectable inflammatory function and changes in the redox environment determine these isoforms. Today, these modified HMGB1 isoforms are analysed by liquid chromatography followed by tandem massspectrometry analysis [193], which is a very time-consuming and costly approach for studying large patient cohorts. In an ongoing study in collaboration with Daniel Antoine in Liverpool, we are analysing isoforms and acetylation patterns of HMGB1 in synovial fluids from JIA patients. Ultimately, multiple ELISA systems for quantification of different isoforms of HMGB1 will be required to run biological samples in more a high-throughput manner.

The results of my thesis also demonstrate collaboration between HMGB1 and other endogenous and exogenous danger signals, generating a synergistic effect on the production of proinflammatory cytokines and MMP in synovial fibroblasts from OA and RA patients. This reveals an additional important role of the increased extracellular HMGB1 levels in the inflamed joints of RA and JIA. The ability of HMGB1 to form functional complexes with LPS, IL-1 α or IL-1 β is not dependent on the redox state of HMGB1. In a previous study by our group we used four different batches of endotoxin-free HMGB1, two produced in *E. coli*, one produced in a eukaryotic baculovirus system, and native HMGB1 purified from

thymic tissue. We now know that these batches had different redox states, but all synergistically enhanced IL-6 production in cultured human PBMCs [76]. HMGB1 in synovial fluids from patients with JIA also expresses different redox states [Peter Lundbäck, personal communication]. This will restrict the capacity of HMGB1 to directly interact with reciprocal receptors, but will still enable synergistic complex formation with LPS, IL-1 α and IL-1 β , factors that may co-exist in the inflamed joint. However, there are as yet no published reports about the identification of HMGB1-partner molecule complexes detected in *in vivo* samples. The 'high mobility' feature of HMGB1 in the nucleus, where it associates with DNA or nuclear proteins for milliseconds and then dissociates, may possibly complicate the recognition of HMGB1-protein complexes *in vivo*.

The beneficial effects of HMGB1 blockade demonstrate the pathogenic role of HMGB1 in many diseases. However, so far no HMGB1-specific blockade has reached clinical trials. I have demonstrated that the anti-rheumatic drugs dexamethasone, GST and chloroquine can all inhibit the release of HMGB1 *in vitro*. Treatment based on these compounds can be associated with severe side-effects at high doses or after long-term administration. Glucocorticoids are often administered locally via intra-articular injections to prevent systemic side-effects but are also given orally. Taken together, the results of my study show that some anti-rheumatic drugs inhibit HMGB1 release, indicating that this might be a part of their anti-inflammatory modes of action. To efficiently neutralize excessive HMGB1 levels we still need more specific therapeutic tools in the future.

The HMGB1-specific antibodies used for therapeutic blockade in preclinical studies have almost exclusively been of polyclonal origin and useful monoclonal antibodies have only been reported in a few studies (reviewed in [129]). Despite earlier extensive evaluation by our group of numerous HMGB1-specific mAbs in experimental arthritis, none of them had the property to ameliorate the disease [Therese Östberg, personal communication]. The attribute of HMGB1 to form complexes with other factors, as described in my thesis, may complicate the binding of neutralizing antibodies. The ability of HMGB1 to form complexes *in vivo* with factors in serum and plasma and interference with ELISA detection systems has been demonstrated [117,119]. One pitfall during anti-HMGB1 mAb production is that HMGB1 may leak out from stressed or dying hybridoma cells and can then

block the secreted HMGB1-specific antibodies already present in the cultures. Thus controlling the culturing conditions is very important to produce active anti-HMGB1 mAb.

The identification of a functional monoclonal anti-HMGB1 antibody, presented in this thesis, is thus highly interesting with obvious clinical therapeutic potential. The mouse 2G7 mAb has recently been converted to a humanized version in our laboratory [Helena Harris, personal communication].

However, it is also justified to have concerns regarding future therapeutic strategy based on HMGB1 blockade since certain HMGB1 isoforms may also exert important roles in regeneration and tissue repair. These aspects need further studies. It may be possible to circumvent this by developing HMGB1-specific antibodies or other antagonists that specifically block the TLR4/MD2-dependent cytokine-inducing HMGB1 isoform, or the CXCR4-dependent chemotactic activity by HMGB1, but not possible HMGB1 isoforms that may be needed for healing processes. The vital nuclear functions of HMGB1 regulating gene transcription, chromatin replication and DNA repair are not expected to be affected by extracellular HMGB1-specific antibodies. Therapeutic strategies based on manipulating intracellular HMGB1 translocations could be potentially dangerous, since a lot still remains to be elucidated about the intracellular roles of HMGB1.

Finally, my thesis work has aided in further defining a pathogenic role of HMGB1 in arthritis, suggesting a mechanism whereby HMGB1 may indirectly contribute to the arthritic inflammatory processes in addition to its endogenous inflammatory roles, and I have defined clinically used anti-rheumatic drugs with the capacity to reduce extracellular HMGB1 release. Furthermore, I have described a monoclonal antibody targeting HMGB1 with the capacity to ameliorate experimental arthritis. These results have strengthened the evidence that HMGB1 is a relevant therapeutic target in arthritis, and suggests a means of inhibiting HMGB1 release during arthritis that deserves further developmental work. My work has also initiated further studies of HMGB1 as a prognostic biomarker in arthritis.

It is my hope that my results will lead to improvement for patients with arthritis as well as other inflammatory conditions. Today, it is difficult to predict

the disease progression in a newly diagnosed patient. It is particularly important in children with JIA, since some patients recover with no residual state of the disease while other patients develop a life-long destructive disease. The therapy can also have potential side-effects for a growing child and it is important to avoid over-treatment. Further studies of HMGB1 levels and characterisation of HMGB1 isoforms present during JIA will reveal whether such measurements can be used as biomarkers to subdivide patients according to inflammatory mechanisms active in the disease, and thereby predict disease progression and treatment response. Our studies in JIA will also have bearings for other inflammatory diseases.

I also hope that HMGB1-based therapy will be used in the clinic to treat arthritis and other inflammatory diseases e.g. sepsis. My study has contributed to defining a monoclonal antibody effective in experimental models of both arthritis and of sepsis, which could be further developed for clinical studies.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

När vår kropp skadas, antingen på grund av en infektion eller på grund av trauma så startar en inflammationsreaktion. Den har till uppgift att dels försvara oss mot infektionen men också att läka den skada som uppkommit. En inflammation som inte avtar är i sig självt mycket skadlig för vävnaden och kronisk ledinflammation (reumatism) eller andra autoimmuna sjukdomar såsom multipel skleros (MS), diabetes eller Crohns sjukdom kan utvecklas.

I min avhandling har jag studerat ett kroppseget protein kallat HMGB1 som finns i alla celler. När en cell skadas, dör eller aktiveras av inflammation så utsöndras HMGB1. Väl utanför fungerar HMGB1 som en farosignal för att signalera till andra cellerna att en vävnadsskada har skett. Av okänd anledning, kan det vid autoimmuna sjukdomar bli en ond cirkel eftersom farosignalerna orsakar inflammation och vävnadsskada vilket leder till att nya farosignaler utsöndras ur celler. På det här viset tros HMGB1 driva inflammationen i autoimmuna sjukdomar.

Flera studier har visat att HMGB1 är involverad i sjukdomsförloppet i kronisk ledinflammation. Förhöjda nivåer av HMGB1 har påvisats i den inflammerade leden hos vuxna patienter med reumatism, dock har inte HMGB1s roll i kronisk ledinflammation hos barn (juvenil artrit) studerats. Tillsammans med diabetes typ 1 är juvenil artrit en av de vanligaste autoimmuna sjukdomarna hos barn och leder till trötthet, ledsmärta och begränsningar i det vardagliga livet. Det föreligger också en risk för permanenta skador i lederna som kan leda till invalidisering och tillväxthämningar eftersom de behandlingsmetoder som finns idag inte fungerar tillfredställande på alla patienter.

Syftet med min avhandling var att studera HMGB1s roll i juvenil artrit och att utreda mekanismer som kan ligga bakom proteinets skadliga aktivitet i ledinflammation samt möjligheten att med behandling blockera HMGB1.

I min avhandling har det för första gången visats att HMGB1 finns i förhöjda nivåer i den inflammerade leden hos patienter med juvenil artrit. Jag har visat att HMGB1 kan fungera som en förstärkare och sammarbeta med andra inflammatoriska molekyler och därigenom öka den inflammatoriska och vävnadsnedbrytande förmågan hos specifika ledceller.

Jag har vidare visat att HMGB1s aktivitet kan hämmas genom olika behandlingsstrategier. Utsöndringen av HMGB1 från celler kan blockeras med välkända läkemedel och HMGB1 som redan är utanför cellerna kan blockeras med en specialkonstruerad antikropp som binder specifikt till HMGB1. Jag har visat att behandling med denna antikropp lindrar sjukdomen hos försöksdjur med ledinflammation vilket är ett första steg i utvecklingen av nya behandlingsmetoder.

Sammanfattningsvis har mitt avhandlingsarbete bidragit med ytterligare bevis för att HMGB1 är involverad i ledinflammation samt visat en möjlig förstärkningsmekanism som kan orsaka detta. Ytterligare har jag visat att blockering av HMGB1s aktivitet är en möjlig ny behandlingsmetod vid ledinflammation. Jag hoppas att mina resultat ska bana väg för utveckling av nya medicinska terapier och komma till nytta för patienter med kronisk ledinflammation och andra autoimmuna sjukdomar.

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