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DANGER WITHIN

-HMGB1 AS A MEDIATOR OF ARTHRITIS

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Connecting new knowledge in the head of a young boy and in the world of science.
To my family
ABSTRACT

Chronic arthritis occurs in childhood as juvenile idiopathic arthritis and in adults most commonly as rheumatoid arthritis. The clinical expression of these diseases differ and it is presently unknown to what extent they share pathogenic mechanisms. Optimal modes of therapy in adults generally coincide with those in children. Patients with chronic arthritis do not only suffer from fatigue and recurrent joint inflammation but also the risk of irreversible joint destruction, functional disability and even early death. Anti-inflammatory, immunomodulatory compounds and physical therapy provide the best chances of preserving joint function and improving quality of life in these patients. However, many patients do not respond satisfactorily to present therapies, indicating a need for further understanding of disease mechanisms providing tools to target novel mediators of disease. One such pro-inflammatory molecule of interest is the protein high mobility group box chromosomal protein 1 (HMGB1).

Aim: The purpose of my thesis was to evaluate a role of HMGB1 in chronic joint inflammation and the possibility of using HMGB1 as a target molecule for therapy.

Results: HMGB1 was demonstrated to be prominently expressed as an extracellular and cytoplasmic protein in synovial tissue in preclinical models of arthritis. This distribution differed distinctly from that in articular tissue in control animals, where the HMGB1 localization was mainly intranuclear. Aberrant, extranuclear HMGB1 expression in synovitis and elevated synovial fluid HMGB1 levels were also verified in samples from patients with rheumatoid arthritis. Longitudinal studies of pro-inflammatory mediators during the course of collagen type II-induced arthritis in rats indicated that aberrant HMGB1 expression preceded clinical symptoms and coincided with onset of TNF and IL-1β production. Maximal HMGB1 protein and mRNA synthesis occurred in areas of cartilage and bone destruction. The functional importance of increased HMGB1 expression was evaluated in other studies of collagen-induced arthritis using HMGB1-specific therapeutic intervention. Polyclonal anti-HMGB1 antibodies as well as truncated HMGB1, acting as a receptor antagonist, ameliorated established arthritis and reduced the histological severity of the synovitis including IL-1β expression. Synovial biopsy specimens were taken arthroscopically before and during therapy with TNF blocking treatment in nine patients with rheumatoid arthritis in order to investigate a relationship between HMGB1 and the central mediator of arthritis TNF. HMGB1 expression was not influenced by the anti-TNF therapy, suggesting that the two studied molecules perform independently or that HMGB1 acts up-stream of TNF in the pro-inflammatory cascade. Since chronic arthritis often depends on activation of T lymphocytes in an adaptive immune response I also investigated whether HMGB1 may influence T cell performance. HMGB1 acted as a proliferative signal for cultured purified human CD4+ or CD8+ T cells when the cells were stimulated in a suboptimal way, but had no detectable effect on resting T cells.

Conclusion: The successful clinical outcome observed in preclinical models of arthritis with HMGB1-specific treatment is encouraging. Future studies are warranted to elucidate the most effective strategies for counteracting extracellular HMGB1-induced inflammation and determine the nature of side effects that may develop using HMGB1 as a target molecule in chronic inflammatory conditions.
Kronisk ledinflammation förekommer hos såväl barn som vuxna med varierande intensitet och duration. De vanligaste formerna av reumatisk sjukdom hos barn är juvenil idiopatisk artrit och hos vuxna reumatoid artrit. Båda dessa tillstånd medför återkommande lidande i form av trötthet, ledvärk och smärtor som begränsar dagliga aktiviteter. Även risk för permanenta, invalidiserande ledskador föreligger liksom risk för tillväxtstörningar hos barnen. Ny kunskap om kroppsegna signalämnen som driver på inflammationen har under de senaste åren lett till en glädjande utveckling av nya mer effektiva läkemedel riktade mot vissa inflammationsdrivande molekyler som TNF eller IL-1. Ett annat sådant signalprotein som kan öka inflammation är high mobility group chromosomal box protein 1 (HMGB1). HMGB1 finns i cellkärnorna i kroppens allra celler och har där många väl studerade viktiga DNA-relaterade funktioner. Man har ganska nyligen förstått att HMGB1 dessutom kan exporteras ut ur celler och att proteinet oväntat nog kan påverka och aktivera immunsystemet. HMGB1 fungerar då delvis som en alarmsignal.

Målsättningen med mitt arbete har varit att undersöka om HMGB1 bidrar till kronisk ledinflammation och om specifik neutralisering av överskott av HMGB1 skulle kunna erbjuda nya behandlingsmöjligheter mot kronisk artrit. Jag gjorde detta genom att studera effekter av HMGB1 på odlade vita blodkroppar från friska personer samt förekomst och lokalisation av HMGB1 i ledhinna erhållna från patienter med reumatoid artrit. I experimentella djurmodeller för kronisk artrit har jag studerat tidsamband mellan sjuklig HMGB1 förekomst i ledhinnor och kliniska tecken på artrit. Dessutom har jag behandlat etablerad experimentell artrit med HMGB1-neutraliserande terapi.

Resultaten visar att nivåerna av extracellulärt HMGB1 i leder ökar tidigt under utvecklingen av experimentell artritsjukdom och att nivåerna jämfört med friska är starkt ökade hos både förödeldjur och människor med kronisk ledinflammation. Intressant är också att produktionen av HMGB1 är som störst i områden av leden där brosk- och benskador är mest uttalade. Vid blockering av HMGB1 hos förödeldjur med ledinflammation noteras en påtaglig minskning av ledsvullnad och andra inflammationstecken. Däremot förändras inte nivåerna av HMGB1 hos patienter med reumatoid artrit vid behandling med TNF blockerande läkemedel, trots att patienterna förbättrades i sin sjukdom. En förklaring till detta kan vara att HMGB1 driver inflammation oberoende av TNF och därför behöver blockeras samtidigt för maximal dämpning av inflammationen. HMGB1 visar sig också kunna stimulera det, för reumatiska sjukdomar viktiga, förvärvade immunförsvar, som har immunologiskt minne med potential att permanenta en oönskad inflammation.

Sammantaget ger mina resultat belägg för att HMGB1 tidigt och påtagligt bidrar till kronisk ledinflammation med förmåga att aktivera det viktiga förvärvade immunförsvar och att modern TNF blockerande terapi inte kan nedreglera HMGB1 i lederna. Detta och den goda behandlingseffekten med HMGB1-blockerande behandling ger hopp om framtida effektiva läkemedel riktade mot HMGB1 med kapacitet att minska besvär och ledskador hos barn och vuxna med kroniska ledinflammationer.
LIST OF PUBLICATIONS

I. High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis.
*Arthritis Rheum* 2002;46:2598-603

II. Morphological characterization of intra-articular HMGB1 expression during the course of collagen-induced arthritis
*Arthritis Res Ther* 2007;9:R35

III. Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity
*Arthritis Rheum* 2003;48:2052-8

IV. Systemic TNF blockade does not modulate synovial expression of the pro-inflammatory mediator HMGB1 in rheumatoid arthritis patients - a prospective clinical study
Sundberg E, Grundman C, Af Klint E, Lindberg J, Ernestam S, Ulfgren A K, Harris H E, Andersson U.
*Arthritis Res Ther* 2008;10:R33

V. High mobility group box chromosomal protein 1 acts as a proliferation signal for activated T lymphocytes
Sundberg E, Fasth A, Palmblad K, Erlandsson Harris H, Andersson U.
*In press, Immunobiology, 2008*
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<tr>
<td>ACPA</td>
<td>Anti-citrullinated protein antibodies</td>
</tr>
<tr>
<td>ACR</td>
<td>American college of rheumatology</td>
</tr>
<tr>
<td>ANA</td>
<td>Anti-nuclear antibodies</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>CIA</td>
<td>Collagen – induced arthritis</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>DAS</td>
<td>Disease activity score</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno sorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>GST</td>
<td>Gold sodium thiomalate</td>
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<tr>
<td>HMGB1</td>
<td>High mobility group box chromosomal protein 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JCA</td>
<td>Juvenile chronic arthritis</td>
</tr>
<tr>
<td>JIA</td>
<td>Juvenile idiopathic arthritis</td>
</tr>
<tr>
<td>JRA</td>
<td>Juvenile rheumatoid arthritis</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa B ligand</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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HMGB1 AS A MEDIATOR OF ARTHRITIS

INTRODUCTION

Acute inflammation is a physiological response to any kind of tissue injury. It is an integrated part of tissue healing, remodeling and is of great importance for the clearance of foreign bodies, infectious agents and tumor cells. In that sense inflammation is a good and beneficial process. On the other hand dysregulated inflammation, that is exaggerated, insufficient or inappropriate, may cause disease and mortality. Chronic inflammation always represents a physiological failure and is a highly unwanted outcome that may mediate for example tissue destruction due to the powerful tools of the immune system and associated tissue factors. For children and adults with this kind of repeated, potentially destructive inflammation affecting the joints the consequences among others are; swollen painful joints, growth deviations, fatigue and joint destruction. Some have fever, rash, internal organ failure, functional disabilities and even suffer early death, not to forget endless years of psychosocial aspects of growing up with or living with a chronic and potentially destructive disease. Described above is a panorama of what is usually referred to as rheumatic disease. Joint inflammation, arthritis, is the hallmark of the most common forms of rheumatic diseases, in children being juvenile idiopathic arthritis (JIA) and in adults rheumatoid arthritis (RA).

Historically chronic deformative joint disease is mentioned in classical literature from Hippocrates and others. Despite studies of Medieval human skeletons with signs of arthritis solid proof concerning the existence of RA in ancient times is in fact rare[1]. Some even believe that the disease evolved due to a changing microbe milieu after European contacts with the New World in the 1400s. The French physician Ballonius working in Paris in the 1500s is by many regarded as the father of rheumatology since he made distinctions between different types of inflammatory joint diseases [2]. Doubtlessly, it took much effort to distinguish RA as a separate disease entity. Differences between RA and gout was described in the middle of the 1600s but was not well accepted until in the 1800s. Separating RA from acute rheumatism later named rheumatic fever was even more difficult. Parallel with the evolving field of microbiology at the end of the 1800s, RA and rheumatic fever were still considered as variants of a common disease, likely caused by bacteria. A bacterial association with rheumatic fever was demonstrated during the first years of the 1900s and the relation to group A hemolytic streptococci was established in the 1930s [3]. With the breakthrough of sulfanilamide and later penicillin it was easier in the 1940s to establish that RA was not caused by streptococci and potentially causative agents are still sought in the 21st century [4, 5].

In view of this historical perspective the distinction of juvenile arthritis from adult RA in the mid to late 1800s was not such a late event. First to report differences between child and adult rheumatic disease was Jean-Martin Charcot, later well known neurologist, in his thesis work from 1853. The classic forms of juvenile arthritis was well formulated in London by George Fredric Still, one of the fathers of modern pediatrics, who also in an early publication 1896 gave name to a subgroup of juvenile arthritis patients [6].
The pathogenesis of chronic arthritis is still not clearly defined. There are genetic, endocrine, environmental factors of importance and most likely infectious factors as well. For obvious reasons more is known concerning the molecular hallmarks of ongoing arthritis than the specific factors that initiate inflammation and later on abate it. Ongoing joint inflammation is characterized by increased activities both in the innate and adaptive parts of the immune system as well as in cells of the tissues in affected organs.

Innate immunity has its most important functions to early recognize and engulf the foreign microbes and particles, killing them off when possible and presenting their antigens to the adaptive immune system for later recognition. Simplified, innate immunity consists of various kinds of phagocytes, natural killer (NK)-cells and complement, molecules that rapidly stick to foreign invaders. Adaptive immunity with its lymphocytes, T-cells and B-cells, has the ability to make antibodies, to orchestrate and fine tune the specificity of the immune response, and equally important to keep memory of foreign invaders over time. Cells of the immune system communicate with each other and with other tissues either by means of secreted signal molecules like cytokines or by cell to cell contact via receptors and even by direct delivery of molecules from one cell to another.

High Mobility Group Box Chromosomal protein 1 (HMGB1) is present in the nucleus of all cells, can also be secreted extracellularly from several innate immune cells and is also released in tissues with dying cells. HMGB1 can activate immune cells resulting in a proinflammatory response. This renders HMGB1 interesting in one of the most common forms of longstanding inflammatory disease, that of chronic arthritis.

**Rheumatic joint disease**

*Juvenile Idiopathic Arthritis*

Juvenile Idiopathic Arthritis is one of the most prevalent acquired autoimmune childhood diseases with a yearly incidence of 11-15/100,000 children in the Scandinavian countries [7, 8]. JIA is a heterogeneous group that can be divided into seven subgroups according to the revised International league of associations for rheumatology (ILAR) criteria adapted in Edmonton 2001 [9]. Oligoarthritis with a maximum of 4 joints affected, often larger joints like knee or ankle, is the most common entity in early childhood. It has the greatest association with positive anti-nuclear antibodies (ANA) and the highest risk for uveitis. The polyarticular rheumatoid factor (RF) negative form of JIA occurs in childhood or adolescence and is characterized by a symmetrical arthritis including smaller joints in hands and feet as well as fatigue. The polyarticular RF positive type is quite similar to the RF negative type but more aggressive with higher risk of persistent functional impairment. Psoriatic arthritis, uncommon in younger children, presents with arthritis of larger and/or smaller joints and often times with involvement of the spine. This diagnosis requires psoriatic skin manifestation of the patient or a close relative. Entesitis-related arthritis also develops in later childhood or adolescence and as the name implies also includes enthesitis apart from arthritis in the extremities, spine and sacroiliac joints. Systemic juvenile arthritis is characterized by longstanding remittent fever, arthritis, exanthema, lymphadenopathy, hepatosplenomegaly, myocarditis and serositis, a
clinical entity with an intensive inflammatory reaction that markedly differs from the other JIA subtypes. This systemic inflammatory entity was historically referred to as Still’s disease. The seventh and last group according to ILAR criteria is the group of unspecified arthritis either filling the criteria for two subtypes or not filling criteria for any of the mentioned groups though still presenting arthritis for more than 6 weeks, the definition of a chronic arthritis according to ILAR.

It should be noted that other present classification systems of juvenile chronic arthritis exist. Juvenile Rheumatoid arthritis (JRA) according to American College of Rheumatology (ACR) and Juvenile Chronic Arthritis (JCA) according to European League Against Rheumatism (EULAR) define the subgroups differently, of utmost importance when comparing study results and of some importance in the daily clinical work [8].

Chronic arthritis is also an important clinical feature in other systemic autoimmune childhood diseases including systemic lupus erythematosus (SLE) and dermatomyositis. Arthritis also occurs in vasculitides and in several autoinflammatory diseases of childhood.

**Rheumatoid Arthritis**

Rheumatoid Arthritis with a prevalence of 0.5-1.0 % and an incidence of 20-30/100 000 in the western world is one of the most common chronic inflammatory diseases.

Diagnosis, like for children, requires symptoms for at least 6 weeks. The seven criteria for RA according to ACR[10] are displayed in table 1.

Like in children and adolescence, chronic arthritis in adults occur in several other systemic rheumatic diseases.

<table>
<thead>
<tr>
<th>RA classification criteria. ACR 1987.</th>
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<tr>
<td><strong>Morning stiffness</strong></td>
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<tr>
<td><strong>Arthritis of 3 or more joint areas</strong></td>
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<tr>
<td><strong>Arthritis of hand joints</strong></td>
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<tr>
<td><strong>Symmetric arthritis</strong></td>
</tr>
<tr>
<td><strong>Rheumatoid nodules</strong></td>
</tr>
<tr>
<td><strong>Serum rheumatoid factor</strong></td>
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<td><strong>Radiographic changes</strong></td>
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Table 1. RA classification criteria. At least 4 out of the 7 criteria must be fulfilled for diagnosis.
Therapy

For treatment of chronic arthritis NSAIDs may prove effective but regularly more potent treatment is warranted. Corticosteroids are used for intraarticular injections or as per oral administration. Side effects of systemic corticosteroids limit their use and the addition of disease modifying anti-rheumatic drugs (DMARDs) is often necessary. Methotrexate is the most widely used DMARD but the group also includes sulphalazine, chloroquine and cyclosporine. Formerly gold salts were frequently used. During the last decade a new group of targeted therapies, so called biological, has emerged. Cytokine blockers targeting TNF (infliximab, etanercept, adalimumab), IL-1 (anakinra) and IL-6 (tocilizumab) pathways have provided rheumatologists and patients with invaluable tools for reducing joint destruction and increasing quality of life [11-14]. Antibodies for B-cell depletion (rituximab) or T-cell activation blockade (abatacept) have also proven effective. With the rapidly evolving knowledge of the pathogenesis of inflammation several new drugs are on trial for future treatment of chronic arthritis.

Host defense

Evolution of the immune system

Man has the unfortunate ability to kill each other as well as animals and plants for better or worse. Voluntary killing of threatening microorganisms is harder due to their limited size and great numbers. For that reason man, as other species, has evolved an in greater part autonomous system for host defense of pathogenic microorganisms. This immune system has developed parallel and in harmony with the evolution of our present race homo sapiens sapiens.

Early unicellular organisms developed means of protection against other microorganisms that have been inherited all the way to humans. Strategies like cellular self killing, so called programmed cell death or apoptosis, represents one mechanism of preventing an attacking microorganism to nourish and spread. The creation of antibacterial peptides was also an evolutionary early method of host defense. Specific receptors like lectin, recognizing subsets of pathogenic microorganisms, is another feature that emerged already in unicellular organisms.

Later during evolution in multicellular organisms some 500 million years ago broader receptor repertoire evolved recognizing several different types of hostile invaders. Of these pattern recognition receptors (PRRs), toll like receptors (TLRs) often serve as model receptors [15]. TLRs have recently come to much attention not only in research concerning host defense but also in autoimmunity. Due to the need of disposal of damaged or malfunctioning cells in multicellular organisms, specialized eater cells, phagocytes developed. Being a body of many cells it was advantageous not only to recognize most foreign intruders but also to tag your own and sound cells to avoid an immune attack against your self. Thus molecular self-tagging evolved in parallel to specialized immune cells with the potential of targeted cell killing. With multicellular organisms the complement system is still crucial for host defense and with implication in autoimmunity development.
Adaptive immunity is a relatively late invention emerging at first with yawed vertebrates some 450 million years ago [16]. Adaptive immunity means that we after birth acquire an ability to detect and remember new foreign antigens. This is an advantage for a species like ours, depending on slow evolutionary changes of host defense genes compared to many microorganisms. The fact that we also want to inhabit most biotopes on earth and maybe inhabit other planets also makes our adaptive immunity a necessity. One unfortunate draw back is the risk that activated immune cells of the adaptive immune system start recognizing the own body as foreign and hostile thus starting the development of autoimmunity.

We must realize that the evolution of host defense in man is parallel and closely linked to the evolution of pathogenic factors and resistance mechanisms in the microorganisms that we live in close contact with. In the present evolutionary moment man is actually doing quite well with its upgraded immune system in spite of repeated reports of multi-resistant bacteria.

As a last general remark concerning the evolution of the immune system it should be noted that it has evolved not only due to a constant and changing microbial surrounding pressure but also as a consequence of the phenotypic development of man. Thus several entities of the immune system are equally important for the homeostasis and developmental features of our bodies.

Cytokines

Cytokines are very potent signal protein molecules that mediate autocrine, paracrine or to some extent endocrine signaling. Immune cells as well as cells outside the immune system may produce cytokines and are dependent on cytokine signaling. Just counting the cytokine subgroup of interleukins will provide us with over 30 members. Cytokines can have different, sometimes seemingly opposite effects, depending on type of cell interaction and co-existing signaling molecules. Cytokines exert their effects by binding to reciprocal specific cell surface receptors. The ligand-receptor interaction will start an intracellular signal cascade that will eventually initialize nuclear transcription activity. Cytokines will control numerous biological events like cell survival or cell death, proliferation, differentiation, pro- or anti-inflammatory activities, type of immune response and much more. Generally Interleukin (IL)-1, IL-6, IL-12, TNF and IFN-γ are proinflammatory. Typical examples of anti-inflammatory cytokines are IL-10 and under certain circumstances Transforming growth factor (TGF)-β. Interferons type I, (IFN)-α/β, upregulate major histocompatibility complex (MHC) I and is of great importance in anti-viral defense. Chemokines are cytokine related signal molecules that induce migration of cells.

RAGE and other receptors

Important immune-regulating receptors on immune cells and other cells are those reciprocal to cytokines and chemokines mentioned above. Others are cell type-specific like B-cell and T-cell receptors, unique due to their immense ability to discriminate myriads of antigens. Present on immune cells and mesenchymal and ectodermal cells are PRRs that recognize potentially dangerous microbes by conserved pathogen structures, the so-called pathogen-associated molecular patterns (PAMPs). PRRs are also receptors for certain conserved endogenous molecular structures, so called damage associated molecular patterns (DAMPs), which signal tissue damage. PRR signaling
activates several inflammatory responses including complement activation, opsonization and proinflammatory cytokine production. The best characterized PRRs are toll-like receptors (TLRs). As of today, thirteen mammalian TLRs have been described recognizing a wide variety of antigens from viruses, parasites and bacteria as well as endogenous DAMPs.

HMGB1 is suggested to signal through TLR2 and TLR4, β2-integrin which is also a PRR and the receptor for advanced glycation end products (RAGE) [17-20]. RAGE is a multi-ligand receptor of the immunoglobulin superfamily of importance in homeostasis, development and inflammation and is expressed in RA synovia [21]. RAGE ligands other than HMGB1 include advanced glycation end products (AGEs), some S100 proteins and β-amyloids[22]. RAGE can be expressed by most cell types and may be up-regulated in many tissues including a variety of tumors[23-25]. Ligation of RAGE activates the CDC42/Rac1 signaling pathway generating cytoskeletal changes. Also the MAPK pathway is activated resulting in activation of NFκB, involved in up-regulation of many proinflammatory cytokines and adhesion molecules[19, 26]. Interestingly HMGB1 seems to induce an unexceptionally long standing activation of NFκB partly through a positive loop since NFκB activation increases expression of RAGE [27]. Intracellular signaling due to TLR2 or TLR4 ligation overlaps to a great extent, utilizing the MyD88 pathway to generate activation of NFκB that will propagate transcriptional activity leading to pro-inflammatory and anti-apoptotic activities [28, 29].

Also receptors formerly thought to exist only in the nervous system have been identified on immune cells. The knowledge of links between the nervous system and the immune system increases rapidly. One direct neuro-immunologic route of interaction is the cholinergic inflammatory pathway designating acetylcholine signaling via the vagus nerve directed towards acetylcholine receptors on macrophages or postganglionic autonomic nerve fibers as a master sensor over immune responses acting with short notice [30, 31]. This system also mediates sensory input, alerting the central nervous system (CNS) of peripheral ongoing inflammation via cytokine, IL-1 and TNF, signaling via vagal afferent fibers. Also adrenergic receptors on immune cells modulate immune responses [32]. It has also been demonstrated that transcutaneous vagus nerve stimulation reduces serum HMGB1 levels and improves survival in murine sepsis [33].

**Innate immunity**

Innate immunity is inherited in our germline genes and is the result of billions of years of evolution. A foreign intruder has to evade many obstacles to be able to cause infection. First are physiological barriers consisting of the mechanical shield in the skin and other epithelial surfaces. In addition to this mechanical obstacle there are chemical barriers including low pH, enzymes and antibacterial peptides. Commensal non-pathogenic microbes provide another problem to concur. Overcoming this, an invading pathogen will face the rest of the innate immune system; principally consisting of effector molecules and immune cells with different, partly overlapping functions.

**Cellular components**

Dendritic cells (DCs) are both resident and mobile cells, which are the most efficient antigen-presenting cells for naïve T cells and thus of crucial importance to initiate
adaptive immunity. Dendritic cells engulf and digest microorganisms in peripheral tissues and migrate to regional lymph nodes to present foreign antigens to T-cells. Another important innate immune cell is the monocyte, more numerous than dendritic cells, circulating in the blood and after entering tissues differentiating into macrophages. Macrophage, meaning big eater, has a profound ability to engulf and degrade tissue debris and microorganisms by killing them via reactive oxygen species (ROS), lysozymes and pH shift followed by further enzymatic degradation. They also share the competence for antigen presentation with dendritic cells. In addition there are different forms of granulocytes, the most abundant being the neutrophil. These cells are very active phagocytes, but unlike macrophages they die within hours after performing their activity. NK-cells, elusive in their origin, can functionally be regarded as part of innate immunity or as a bridge between innate and adaptive immunity [34]. NK-cells use MHC class I, in man HLA-A, B, C, as a marker of self and will recognize lack of MHCI as a signal of missing self activating the NK cell to initiate killing of the non-self cell either by transfer of cytotoxic substances like perforin, granzyme or nitric oxide (NO) or to initiate inflammation by the production of cytokines [35, 36].

**Humoral factors**

The complement system includes about 20 proteins mainly synthesized in the liver. Activation of this system leads to a series of events including proteolytic cleavage of complement factors most importantly C3 and C5. This serves as initiation of a membrane attack complex (MAC) on the cell membrane of the intruder. A MAC consists of several aggregated complement factors that together make a hole in the cell membrane which will kill the target cell. Cleavage products of C3 and C5 increase permeability of blood vessels and are chemotactic factors for phagocytes, also activating their phagocytic capability. Other complement factors attach to apoptotic cells and induce enhanced phagocytosis. Known direct effects bridging complement system to adaptive immunity is the fact that cleavage products of C3 can act as a proliferation signal for B-cells and also affect T-cell maturation[37, 38]. Acute phase proteins are released from the liver as a consequence of cytokine stimulation, most notably by IL-1, IL-6 and TNF. Acute phase proteins like C-reactive protein (CRP) and mannan-binding lectin (MBL) induce opsonization and complement activation.

**Adaptive Immunity**

Adaptive immunity is acquired during life and its stored memory of antigen encounters is not inherited. This type of immunity is exclusive for vertebrates and has evolved during the last 450 million years. The main cellular constituents of adaptive immunity are B and T lymphocytes. Related to T-cells are natural killer T (NKT)-cells capable of significant cytokin production but lacking the receptor variability that is a hallmark for adaptive immunity.

**B-cells**

B-cells are lymphocytes with membrane-bound antibodies as surface receptors. These B-cell receptors (BCR) can be of different Ig classes on one and the same B-cell but share the exact same antigen specificity. B-cells are activated by presenting antigen
engulfed by the BCR and presented on MHC II to a CD4⁺ T-cell recognizing the same epitope. The T-cell will in this case induce a receptor-mediated activation signal. Activation results in up regulation of surface molecules, proliferation and a gene recombination process leading to the generation of new B-cells with higher affinity for the specific antigen as well as antibody receptor isotype or subclass switching. Activation can also take place independent of a T-cell by certain antigens that activate several different clones of B-cells with limited specificity for the antigen. Also cross-linking of repetitive sequences like polysaccharides can serve as activation signal. Some B-cells transform into a plasma cell giving up their ability to alter subclass and antigen specificity. Instead the plasma cell carries efficient protein machinery capable of large scale antibody production of one certain type of antibody. Circulating antibodies can contribute to immunity in three principal ways: Antigen-neutralization, opsonization or cell killing.

T-cells

T-cells are lymphocytes characterized by a hypervariable T-cell receptor (TCR). T-cells come in two major forms, CD4⁺ T-cell also named T helper cells (Th) and CD8⁺ cells or so-called cytotoxic cells. CD4⁺ T-cells have many immunoregulatory functions of which several are mediated through secretion of signal molecules, so-called cytokines. These cells are activated by presentation of a short piece of foreign peptide, antigen, by an antigen-presenting cell (APC) like macrophages, B-cells, but most efficiently by dendritic cells. The APC presents the peptide on a major MHCII molecule together with a second signal via a co-stimulatory receptor interaction between the APC and the T-cell. Dependent of the milieu in which the CD4⁺ T-cell is activated it will mature into separate effector subsets. At least 4 subsets are characterized. Stimulation to a Th1 subset is mediated in part by the presence of IL-12. Th1 subsets producing INF-γ is important in defense against intracellular pathogens. Th2 generation mediating help for antibody production is stimulated by the presence of IL-4. Th2 cells also have impact on immunity towards parasitic worms and are implicated in the development of asthma and allergy. More recent studies have defined regulatory T-cells (Treg) as a subset likely to develop under the influence of TGF-β. These cells are of importance due to their immunosuppressive capabilities. On the contrary, if the CD4⁺ T-cell is exposed not just to TGF-β but in addition to IL-6 and IL-23, Th cells secreting IL-17, Th17 cells, will differentiate [39]. Th17 cells have drawn much attention lately, not only because of important effects in defense against extracellular bacteria, but also due to association with autoimmunity. CD8⁺ T-cells are activated in a similar way as CD4⁺ T-cell but the antigenic peptide is here presented by an APC on a MHC I. CD8⁺ T-cells are also highly dependent on a sufficient and well-balanced cytokine milieu for proliferation and maturation into effector cells. These will in turn kill infected or transformed cells that present the foreign antigen on MHC I. The process of killing mimics the NK cell mode of action with transfer of cytotoxic granulae but also by inducing apoptosis by activating death receptors, Fas, on the target cell. Killing of virally infected or tumor transformed cells are assignments suitable for CD8⁺ T-cells.

There is an additional subset of T-cells called γδ-T-cells mainly present in mucous membranes, where they exert cytotoxic activity and produce cytokines. The TCR repertoire of these cells is limited compared to conventional αβ-T cells.
Pathogenesis of RA and JIA

As noted before, chronic arthritis is common in a wide variety of chronic inflammatory diseases usually referred to as rheumatic diseases. Rheumatic diseases could in theory be autoimmune, autoinflammatory or infectious. Starting with infections, it is possible that one or several of our rheumatic diseases are caused by a microorganism, but it has been exceedingly difficult to verify any today known infectious agent as sufficiently prevalent in any rheumatic subgroup, making this assumption still hard to prove [5, 40-42]. Autoinflammatory diseases have persistent inflammation but no adaptive immune response to an endogenous antigen as part of its etiology. Autoinflammation is rather a malfunctioning innate immune response. Typical autoinflammatory diseases are familial Mediterranean fever (FMF) Muckle-Wells Syndrome (MWS) and Neonatal Onset Multi-system Inflammatory disease (NOMID) also called CINCA. The latter two diseases are caused by mutations in the CIAS1/NALP3 gene affecting the cryopyrin-dependent inflammasome which is of importance for IL-1β processing and secretion [43, 44]. Innate autoinflammation can also be a consequence of polymorphisms in genes of the complement system [45]. Finally an autoimmune disease implies a disease process with adaptive features directed to endogenous antigens. Graves’s disease with antibodies directed to the receptor for Thyroid-stimulating hormone is a typical autoimmune disease. Many of our chronic inflammatory joint diseases including RA and several subtypes of JIA display autoimmune features. Endogenous etiologic antigens remain elusive despite several candidate molecules. It should again be emphasized that the pathogenesis of JIA and RA are multifactorial which is true not only with regard to environmental factors like smoking and hormonal gender-related factors but likely as well with regard to different types of triggering inflammatory responses. Possibly a genetic susceptibility of autoimmune responses can be coupled with a genetic autoinflammatory predisposition with infections as trigger of the final loss of tolerance resulting in chronic arthritis.

Tolerance

Adaptive immunity has an enormous antigen-specific repertoire due to recombination of T-cell and B-cell receptors. This ability harbors a risk of adaptive immune response toward self antigens resulting in loss of self tolerance. Central tolerance-preserving mechanisms in bone marrow for B-cells and in thymus for T-cells results in depletion of autoreactive lymphocytes. In the thymus T-cells that bind too firmly to presented MHC complexes are deleted, so called negative selection. Peripheral tolerance in tissues is regulated by lack of sufficient local costimulatory signaling directed towards the T-cell. This in turn will evoke apoptosis, programmed cell death or anergy in autoreactive T-cells [46, 47].

Genetic and etiologic factors

Human leukocyte antigens have been linked to RA since decades [48]. HLA-DRB1 gene locus in particular correlates highly to RA susceptibility, genetically both linked to and associated with the disease. Also non-HLA genetic factors contribute to RA susceptibility. Single nucleotide polymorphism (SNP) of tyrosine phosphatase non-receptor 22 (PTPN22), a protein with essential importance for TCR signal transduction,
has been associated with RA recently and may serve as an example [49, 50]. Genetic variations altogether is calculated to account for around 50% of disease susceptibility in RA.

In JIA the polyarticular RF positive subtype, that mostly resembles adult RA, is associated with the same HLA-DRB1 allele as RA. The other JIA subtypes associated with other alleles within the DRB1 region [51]. The HLA-B27 genotype is associated with the enthesitis-related arthritis JIA subtype. The presence of several non-linked alleles within the DRB1 region further increases the risk of certain JIA subtypes. Alleles in the HLA-DQ region are also associated with JIA but close linkage to the DR region makes it hard to distinguish the disease susceptibility contribution. Also in JIA non-HLA gene association, like PTPN22 and others, has been confirmed but odds ratios are low making relative contribution to disease unsure[52]. Notably, the multitude of JIA subtypes interfere with the genetic dissection of associated genes. Focusing for instance on systemic JIA associations with SNPs in the promoter region of IL-6, not present in other JIA subtypes, has been demonstrated [53].

The gender difference female:male in RA being 3:1 indicate hormonal factors as an important contribution to disease pathogenesis. Disease flares postpartum and decreasing ratio in older patients underline this [54-58]. However, gender differences in JIA being at least 3:2 implies other sex chromosome-related differences.

Smoking is the strongest environmental independent risk factor for RA [59, 60]. Recently smoking in combination with RA associated HLA-DRB1 shared epitopes (SE) is demonstrated to be an exceedingly high risk combination between genes and environment with and odds ratio of 7.5 or more for RA [61].

Epigenetics is a new field that also may be important for development of RA or JIA. It refers to non germ line inheritable regulatory processes of DNA transcription due to DNA methylation or histone modifications. Of importance for inflammatory processes epigenetics has so far not conclusively been linked to RA pathogenesis despite findings like DNA hypomethylation in leukocytes from RA patients [62-65].

**Joint synovia and arthritis**

Our joints consist of evolutionary tailored bone surfaces covered with cartilage, lubricated by synovial fluid, encompassed by the joint capsule which on the interior is covered by the synovial membrane. This membrane is thin, only one to two cell layers normally, consists of synovial fibroblasts and synovial macrophages and is the source of synovial fluid.

This balanced and peaceful setting comes to an end with the initiation of chronic arthritis. Propagation of arthritis is characterized by synovial hypertrophy with increased proliferation of synovial cells and extracellular matrix. Further activation of innate as well as adaptive immune responses with influx of immune cells contributes to the formation of synovitis. Complement products are increased in the synovial fluid with activation of both the alternative and classical pathways generating chemotactic signals for immune effector cells [66-68]. Complement receptors are up-regulated further indicating complement being important [69]. Immune complexes are also formed together with antibodies in the joints and can be detected in the circulation. A classical antibody in RA and some JIA polyarthritis patients is RF an autoantibody reacting with the Fc portion of IgG subtype antibodies and first described by C Warden in 1909. Though highly prevalent in RA and a risk factor for a more aggressive disease
in both RA and JIA, RF has a low specificity. Another autoantibody in RA reacts with type II collagen, which is abundant in cartilage, but has also low specificity. Anti-citrullinated proteins antibodies (ACPAs) have recently come to much attention due to a high sensitivity of more than 95% for RA and occur in serum of patients years before onset of clinical disease [70-72]. Together this points to a possible pathogenic effect of these antibodies. In JIA ACPAs are rare but can be detected in a subgroup of RF positive polyarthritis patients. Presence of ACPAs indicates a development of a more destructive arthritis in adults as well as in pediatric patients.

Influx of immune cells to the synovial membrane is enhanced by release of chemokines from synovial fibroblasts, synovial immune cells and by complement degradation products. Neutrophils are recruited in great amounts to the synovial fluid, whereas monocytes differentiating to macrophages are recruited to the synovia. Present in the synovia are also B-cells and T-cells, both of the CD4⁺ and the CD8⁺ subtype. Other less numerous immune cells in the inflamed synovia include plasma cells, NK, NKT and mast cells.

![Schematic illustration of a normal joint (left) and a joint with inflammation (right).](image)

**Figure 1.** Schematic illustration of a normal joint (left) and a joint with inflammation (right).

Activation of synovial cells, macrophages, T-cells and to some extent B-cells generates local release of cytokines with migrating, proliferative, differentiating and proinflammatory consequences. Tissue degrading molecules like matrix metalloproteinases (MMPs) are also released. All together this results in proliferation of the synovia and initiation of cartilage damage and bone resorption through osteoclast.
activation. Synovial fibroblasts and macrophages are important contributors of TNF, IL-1 and receptor activator of nuclear factor κB ligand (RANKL) driving osteoclast differentiation with subsequent bone resorption. Moreover, macrophages release a whole array of additional important arthritis mediators, including IL-18. Main T-cell cytokines implied as most important for pathogenesis are IFN-γ, TNF, RANKL and more recently and of special interest IL-17. IL-17 induces maturation and activation of synovial fibroblasts, neutrophils and monocytes with resultant release of chemokines, cytokines and increased MMP and prostaglandin production [73, 74]. IL-17 also enhances maturation of DCs. Other cytokines released by several cell types linked to arthritis pathogenesis are IL-6, IL-15, macrophage migration inhibitory factor (MIF) and B-cells proliferating signals APRIL and BAFF. The inflammatory end result and hallmark of advanced RA is the synovial transformation to an invasive pannus with cartilage- and bone-destructive features. Down regulatory mechanisms of inflammation include IL-10 and TGFβ production by regulatory T-cells and soluble receptors neutralizing proinflammatory cytokines.

Thus chronic arthritis is the result of a combination of genetic and environmental factors acting together. The potential destructive arthritis involves not only a close interplay between innate and adaptive immune responses but an equally close interplay between the local joint tissues and the immune system.

HMGB1

General remarks

HMGB1 is a 25 kD protein. It was discovered in the early 1970s and was mostly studied for almost 30 years as a crucial intranuclear protein. The protein binds strongly to cruciform or bent DNA and is involved in nuclear functions regulating transcription, DNA repair, recombination and chromatin fiber assembly. During the last decade it has been intensely studied as an extracellular molecule involved in processes that mediates neuronal differentiation, stem cell recruitment and innate immunity.

HMGB1 structurally consists of 214 amino acids and is named due to its ability to migrate quickly in the electric field of a gel electrophoresis [75, 76]. HMGB1 is uniquely well preserved among species indicating crucial functions for life throughout evolution[77]. Identity between man and other mammals is 99%[78]. HMGB1 shares about 80% homology with HMGB2 and HMGB3, the other two members of the HMGB family. HMGB proteins are present even in the smallest multicellular animals again pointing to these proteins to be evolutionary very important [79]. The N-terminal and central part of the molecule is strongly positively charged and the long repeated acidic sequence of the C-terminal is strongly negative. The mid main part of the molecule consists of two DNA-binding structures called the A and B box with ability to bind to the minor groove of DNA regardless of nucleotide sequence.

HMGB1 is abundantly expressed in the nucleus as a chromatin-associated protein, only histones outnumber HMGB1. It is also present in the cytoplasm and was first detected expressed on cell surfaces by Heikki Rauvala in Helsinki in his studies of neurite outgrowth in the embryonic brain and was later also discovered as a protein on the
surface of activated platelets[80-83]. The presence in circulation was not evident until Kevin J Tracey and coworkers in New York detected HMGB1 in sepsis patients and demonstrated the release of HMGB1 by activated macrophages [84]. This finding published in 1999 was of course central for the concept of HMGB1 as a possible biologic factor outside the cell. No effort has later been spared to elucidate the properties of HMGB1 shuttling out of the cells and of its biologic actions.

Extracellular HMGB1 release has been studied from the angle of active as well as passive release. A mechanism of active release from monocytes by a non-classical secretory lysosome pathway was demonstrated in 2002[85]. Hypermethylation, acetylation and phosphorylation weaken the DNA binding capacity of HMGB1 and prevents the protein to reenter the cell nucleus resulting in a translocation of HMGB1 to the cytoplasm where its is accumulated in secretory lysosomes for subsequent extracellular secretion [86-88]. Release of HMGB1 is demonstrated in a wide range of myelopoetic and other cell types including; macrophages, monocytes, dendritic cells, NK-cells, osteoblasts, osteoclasts, hepatocytes, enterocytes and pituicytes [84, 89-94]. Active release from endothelial cells and T-cells is also reported [95]. Stimuli inducing active HMGB1 release include exogenous signals like TLR2, TLR3 and TLR4 ligands including LPS, as well as endogenous proinflammatory signals like IFN-γ, INF-α/β, nitric oxide (NO), IL-1 and TNF [19, 84, 89, 93, 96-98]. In addition, it has recently been demonstrated that live cells stressed by oxygen deprivation will release HMGB1 by as yet undefined mechanisms [99].

Passive release of HMGB1 from cells undergoing necrosis is a distinct and confirmed finding. Apoptosis was initially reported to prevent extracellular HMGB1 release, but several reports have indicated that secondary necrosis of apoptotic bodies may lead to passive leakage of HMGB1 [100-106]. In one study the HMGB1 immunogenicity was reduced by oxidation of ROS during apoptosis as opposed to during necrosis[107]. Even though biologically active in greater amounts after necrosis and lesser amounts after apoptosis, the relative functional contribution of these two routes of passive release is presently not known.

**Biologic effects**

Inside the cell HMGB1 has functions crucial for life. It binds to DNA and has the ability to modify chromatin structure thereby regulating transcription [108]. HMGB1 interaction with transcription factors by protein-protein interaction is another route for transcription regulation [109]. The importance as an intranuclear regulator is underlined by fatal neonatal hypoglycemia in HMGB1 knockout mice and by marked phenotypic
changes including severe growth retardation, blindness, lack of subcutaneous fat and additional features [110].

Biological effects of released HMGB1 in the immune system include chemotaxis of certain stem cells and myeloid cells, induction of proinflammatory cytokine and MMP production in monocytes/macrophages. It is also a maturation factor for dendritic cells and facilitates their homing to lymph nodes [29, 84, 103, 111-120]. Chemotaxis and activation of neutrophils is also evident[112, 121]. T-cells differentiate towards a Th1 phenotype in response to HMGB1[24, 25]. HMGB1 acts as an adjuvant in B-cells to enhance antigen specific IgG production and is crucial for activation of chromatin reactive B-cells[103, 122]. Of particular interest to the pathogenesis of chronic arthritis, HMGB1 induces osteoclast differentiation with subsequent TNF release and enhance proinflammatory cytokine- and MMP production in synovial fibroblasts [123] (data not shown). Endothelial cells are activated due to HMGB1 stimulation with subsequent proinflammatory cytokine release and upregulation of adhesion molecules [124, 125].

As a chemotactic factor promoting proliferation and differentiation of stem cells and smooth muscle cells also pro-angiogenic for endothelium it displays the fundamental functions for tissue repair[100, 126-128]. Apart from physiological regeneration of tissue damage the latter properties also have implications in tumors in which HMGB1 are strongly overexpressed and enhances invasiveness in parallel with MMP formation [26, 129, 130]. In the nervous system HMGB1 is also involved in tissue repair as well as inflammation. Neurons exhibit HMGB1 on the outside of cell membrane in neurites promoting their outgrowth[17]. Astrocytes, as in the case of macrophages, respond to HMGB1 with increased chemotactic, proinflammatory and MMP activity[131, 132]. Intriguingly HMGB1 also displays capabilities similar to antibacterial peptides, being not only chemotactic and immune cell activating but also bactericidal[133]. This implicates possible protective effects on epithelial surfaces or in the tissues. Being an evolutionary ancient molecule HMGB1 may predate not only all of what is referred to as adaptive immunity but also parts of innate immunity as an important first line defense against microbial attacks. It is possible that HMGB1 as an abundant and bactericidal molecule still exerts an important selective pressure on several important pathogenic bacterial strain maybe even those likely to cause septic arthritis.

Interestingly, in the same year as HMGB1 was discovered in the blood of patients with sepsis, Polly Matzinger et al showed that not only exogenous but also endogenous signals could activate DCs and thus provoke an immune response [134, 135].

Molecules with such a feature, notifying the immune system of tissue damage, is now regarded as endogenous danger signals and often referred to as damage-associated molecular patterns (DAMPs). The number of detected DAMPs has grown steadily and the concept of “danger within” is now accepted in immunology. The burning medical issue is in what way we can benefit by modifying DAMP signaling within.

HMGB1 signaling and blocking agents

Extracellular HMGB1 was first described as a pro-inflammatory mediator. Early efforts to investigate HMGB1 signaling pointed to RAGE and TLR 2 and 4 as the important receptors for protein ligation [17-19]. Later also β2-integrin (Mac-1) has been proposed as an HMGB1 receptor [20]. Intracellular signaling followed by HMGB1 ligation to
RAGE includes activation of mitogen-activated protein kinase (MAPK) resulting in nuclear translocation of nuclear factor kappa B (NF-kB). In view of its proinflammatory properties the addition of NF-kB activation early implied HMGB1 as a cytokine-like protein. In time the knowledge of HMGB1 signalling has become more complex. It is now evident that most of the cytokine-stimulating capacity is lost if HMGB1 preparations are highly purified giving the possibility that HMGB1 signals in concert with one or several other molecules[136, 137]. Indeed HMGB1 potentiates proinflammatory responses when binding to TLR ligands or IL-β [119, 138, 139] (data unpublished). On the contrary, other effector functions of HMGB1 activation like cell migration and neurite outgrowth may stay intact even with highly purified preparations without any need for co-factors.

Figure 3. The role of HMGB1 in immune responses associated with chronic arthritis.

Mechanisms of counteracting the pro-inflammatory effects of HMGB1-complexes have been sought for possible treatment of inflammatory conditions like sepsis and autoimmune diseases. So far two principally different ways have been tried; that of blocking extracellular HMGB1 and that of preventing secretion of the protein. Extracellular neutralization of HMGB1 is possible with specific antibodies directed towards the protein. These kind of studies have been successful in animal models of sepsis and chronic arthritis as well as in ischemic, partly HMGB1 mediated CNS injuries[114, 140-142]. Neutralizing HMGB1 is also possible by adding its main receptor as a decoy soluble RAGE (sRAGE) to bind and deactivate HMGB1. Also this approach is beneficial in animal models of chronic arthritis and sepsis. Studies of mutant HMGB1 led to the knowledge that the pro-inflammatory effects of HMGB1 are
mediated by the B-box. The A-box on the contrary inhibits B-box receptor ligation and can therefore for practical reasons be regarded as antagonist of HMGB1 pro-inflammatory action. A-box will in fact also ameliorate models of chronic arthritis, sepsis and pneumonia [112, 116, 140]. HMGB1 can also be bound and neutralized by the N-terminal lectin-like domain of thrombomodulin, an endothelial anticoagulant cofactor. This recombinant lectin-like domain also suppresses arthritis in several models [143].

Preventing release of HMGB1 from macrophages is demonstrated with ethylpyruvate, alpha7nAChR ligation, stearoyl lysophosphatidylcholine, oxaliplatin, gold sodium thiomalate and ghrelin [144-149]. More specific blocking of HMGB1 translocation from nucleus to the cytoplasm is demonstrated for oxaliplatin, gold sodium thiomalate and ghrelin. Of these substances, gold salts are unique as a well established treatment of RA. Ghrelin, a small polypeptide with growth-related and anti-inflammatory properties, are of special interest since it is an endogenous product. Ligation of alpha7nAChR by nicotine is also an endogenous route for HMGB1 sequestration and pharmacologically tailored specific ligation of alpha7nAChR may well be a suitable target for anti-inflammatory therapy [150].
AIM OF THE STUDY

The aim of my thesis has been to clarify whether HMGB1 has a pathogenic role in chronic arthritis making it a valid target for therapeutic intervention. At the starting point of this thesis work nothing was known about HMGB1 as a mediator of arthritis or other autoimmune diseases. For that reason the early studies focused on defining the presence of HMGB1 in arthritic joints later addressing the questions of HMGB1 as a therapeutic target and the possible relation to adaptive immune responses.

Specific aims were as follows

- To investigate the presence of aberrant extranuclear HMGB1 in joints with chronic arthritis.
- To document the temporal and spatial expression of HMGB1 during the course of developing experimental arthritis.
- To evaluate if extracellular HMGB1 blocking therapy is beneficial in experimental arthritis.
- To evaluate the mutual HMGB1 and TNF dependence in chronic arthritis.
- To investigate whether HMGB1 may have a direct effect on adaptive immunity.
METHODS

In these papers samples from adult patients as well as from healthy individuals and from experimental models of arthritis are collected and analyzed. We used Western-blot, ELISA, immunohistochemistry and immunocytochemistry for protein detection and in situ-hybridization and RT-PCR for mRNA detection. As an assay of cell proliferation, incorporation of radioactive thymidine was used. Clinical outcome for RA patients was evaluated by DAS 28 and ACR and in the experimental models weight and arthritis index for swollen paws were used for assessment.

All studies were approved by Ethical committee North, Stockholm or the Local Ethical committee Karolinska University Hospital, Solna, Stockholm.

Patients

For technical, ethical and epidemiologic reasons synovial biopsies are more difficult to obtain from patients with JIA. Due to this, studied patients in this thesis are all adults. In paper I synovial biopsies where collected from 3 RA patients with active disease fulfilling the ACR criteria and 1 patient with osteoarthritis (OA). Biopsies were collected during joint replacement surgery. RA patients, of which one was female and two were males, ranged in age from 40 to 80 year with disease duration of 8 weeks to 8 years. The OA patient was a male of 60 years with 8 years of disease. All had ongoing NSAID medication, but one RA patient also had methotrexate. One of the other RA patients had sulfasalazine in addition to NSAID. Surgery was performed at the orthopedic department, Karolinska University Hospital, Solna, Stockholm.

In paper IV patients were recruited at the Rheumatology unit, Karolinska, Solna, Stockholm. Included patients had active RA, fulfilled the ACR criteria and were provided infliximab infusions as TNF-blocking therapy due to therapeutic failure and all gave their informed consent. The included 9 patients, 7 females and 2 males, ranged from 25-69 years of age with disease duration ranging from 0.6 to 18 years. All patients were treated with stable doses of methotrexate; four had prednisone in addition and one also cyclosporine as a third medication before and during the study period. Synovial biopsies were obtained by arthroscopic technique 1 to 21 days before start of infliximab therapy and for each patient follow up biopsies were collected from the same area in the joints 8-10 (median 9) weeks later. Median disease activity as measured by DAS 28 was 5.95 before infliximab start and decreased significantly to 4.41 at the end of the study period.

Blood donation

For studies of leukocytes from healthy individuals in paper V one healthy donor was recruited at the rheumatology research lab, CMM, Karolinska Institutet and buffy coats where purchased from the Karolinska University Hospital blood center. The advantage of fresh blood from a donor is the good viability of cells and the diminished risk of unwanted cell activation. Buffy coats on the other hand give a greater number of leukocytes permitting large scale experiments but may depending on the timing of the experiment provide less viable cells or unwanted cell activation. A buffy coat contains principally the cells left after a blood donation when most of erythrocytes and serum are removed (ie white blood cells and platelets) in the process of making blood products for therapeutic use. For practical reasons, buffy coats used in paper V rested at room temperature 16-18 hours prior to the start of experiments.
From fresh blood or from buffy coats, peripheral blood mononuclear cells (PBMCs) were extracted by use of density gradient centrifugation with Ficoll-Paque™. To obtain pure cultures of CD4⁺ and CD8⁺ cells, PBMCs were stained with fluorochrome-conjugated antibodies directed against the T-cell surface markers CD3, CD4 and CD8 at 4°C for 20 min. Separation of cells were then performed by a MoFlo high speed cell sorter. The two cell populations collected were cells double positive for CD3 and CD4, referred to as a CD4⁺ population, and cells double positive for CD3 and CD8, referred to as a CD8⁺ population. The purity of sorted cells was assessed by FACS and ranged between 95-98%, most contaminating cells being NK cells and a few B-cells.

**Experimental models of arthritis**

Experimental arthritis can be provoked by several different means. Mostly used species are inbred strains from mice or rats with a great variation of arthritis susceptibility dependent on strain, reflecting the MHC-association described for RA. Some strains even develop arthritis spontaneously over time. The most common arthritis models in rodents are collagen-induced arthritis (CIA) and adjuvant arthritis (AA). These model diseases are induced by subcutaneous injections of collagen type II emulsified in mineral oil or heat-killed mycobacteria dispersed in mineral oil, respectively. Both models share several features with RA and have been used in multiple studies regarding pathogenesis as well as anti-arthritic therapy.

In this thesis we used female Lewis rats for AA in paper I for the initial descriptive analysis of HMGB1 distribution in arthritic joints. Rats were immunized by an injection at the base of the tail with heat-killed *Mycobacterium Tuberculosis* dispersed in mineral oil. Clinical onset of arthritis was around day 11. In paper II and paper III we used male DBA/1 mice and male DA rats for the induction of CIA. These models were suitable for kinetic studies of HMGB1 expression and treatment efficacy due to its reasonably good resemblance to human chronic arthritis and its stable longstanding arthritis phenotype. In addition, the CIA model is well established at our research facility providing consistent handling and results.

All animals were kept at the animal unit at Karolinska Hospital, Stockholm, with light and darkness cycles of 12 hours. They were fed standard rodent chow and water was provided ad libitum. Health status was monitored according to the guidelines of Swedish Veterinary Board (SVA) and animals were reported free of screened pathogens. Mice were injected on day 0 at the base of the tail with a mixture of heat-killed, freeze-dried *Mycobacterium tuberculosis*, bovine collagen type II and Freund’s incomplete adjuvant, a mineral oil-based adjuvant solution. Day 21 mice were boostered with a repeated injection of bovine collagen type II emulsified in mineral oil, resulting in arthritis onset around day 28. In rats a single injection at the base of the tail was performed on day 0 with a mixture of bovine collagen type II and mineral oil and arthritis onset was around day 12. In paper I and III rodents were sacrificed and perfused with fixating formaldehyde solution. Paws were dissected and decalcified for 3 weeks in 4% EDTA solution followed by two days in 20% sucrose phosphate buffer. In paper II, due to methodological improvement of the tissue handling procedure, rats were sacrificed and perfused with 4% formaldehyde with the addition of 0.2 % picric acid, paws dissected and left in solution of the same fixative over night. Specimens were then washed in PBS twice daily for 3-4 days until clear of picric acid. Subsequent decalcification was performed in 4% EDTA with 0.2 M sodium cacodylate for approximately 4 weeks followed by 8 days in 20% sucrose. Synovial tissue sections (7-8 µm) were then cut with a cryostat and mounted on microscope slides for later immunohistochemical evaluation.
Methods of evaluation

Clinical evaluation of arthritis

Mice and rats were closely monitored by evaluation of general well-being and weight. Rodent paws were observed daily for erythema and swelling in paper II and paper III and scoring of joints was performed as follows. In mice each paw was evaluated using a scale ranging from 0–3 in which 0 = no signs of arthritis, 1 = one type of joint affected, 2 = two types of joints affected, and 3 = the entire paw affected, giving a maximum score of 12. In rats each paw was evaluated using a scale ranging from 0–4 in which 0 = no signs of arthritis, 1 = one type of joint affected, 2 = two types of joints affected, 3 = three types of joints affected and 4 = three types of joints affected and maximal erythema and swelling, giving a total maximum score of 16.

Patients in paper IV were evaluated clinically by DAS 28 and ACR criteria.

mRNA detection

Detection of mRNA in paper II was accomplished by mRNA in situ hybridization in which tissue sections from joints with experimental arthritis were exposed to a 32P-dATP labelled 50 base pair HMGB1 oligonucleotide probe. After subsequent hybridization of the radioactive probe to tissue-produced HMGB1 mRNA, tissues were washed. Tissue slides were then dipped in photoradiographic emulsion, left for exposure, developed and counterstained with haematoxylin. For each tissue section, the number of labelled cells was evaluated by an observer blinded to the identity of the samples.

Tissue samples from biopsies in paper IV were small and suited better for mRNA detection by RT-PCR. Following extraction of mRNA, cDNA was prepared. Amplification of cDNA with RT-PCR was carried out using 20 base pair forward and reverse HMGB1 primers and a fluorescent detection primer. β-actin was used as housekeeping reference gene for evaluation of HMGB1 mRNA expression.

Protein detection

Protein detection of HMGB1 in synovial fluid in paper I was performed by Western blot and ELISA. Western blotting with specific anti-HMGB1 polyclonal antibodies followed protein separation on an SDS-PAGE gel and semi-quantitative protein levels were calibrated against a rHMGB1 dilution curve in a gel image analysis system. TNF protein detection from synovial fluid was performed with commercial ELISA-kit. Tissue sections in paper I–IV were investigated by immunohistochemistry for synovial protein expression. As primary antibodies for HMGB1 (conc. 0.5-1.0 µg/ml) and TNF (2 µg/ml), peptide affinity-purified polyclonal rabbit antibodies were used and for IL-1β detection (2 µg/ml) a polyclonal affinity-purified goat antibody was used. Incubation of secondary biotinylated antibodies directed to the Fc-part of the primary antibodies was followed by incubation with avidine-horseradish peroxidase conjugates and visualization was performed with diaminobenzidine as substrate. Species-specific irrelevant primary antibodies were used as negative controls. The specificities of extracellular and intracellular cytokine immunoreactivities were verified by their complete inhibition in blocking experiments with preabsorption of the cytokine-specific antibody with recombinant cytokine prior to staining. For HMGB1 one additional
polyclonal and one additional monoclonal primary anti-HMGB1 antibody verified the staining patterns.

**Proliferation assay**

Purified CD4⁺ and CD8⁺ T-cells were cultured in medium with 5% human sera in 96-well plates. Plate-bound anti-CD3 at concentrations ranging from 0-1.0 µg/ml served as activation signal for the T-cell populations and rHMGB1 in concentrations ranging from 0-4 µg/ml was added to the cultures from start. At 48 h [³H] thymidine was added and 16 h later cells were harvested and radioactive labeling was measured by a β-counter to estimate the proliferation of cells.

**RESULTS**

**HMGB1 is aberrantly expressed in chronic arthritis.**

Since HMGB1 earlier had been demonstrated to possess proinflammatory properties we decided to investigate if the protein was expressed in an aberrant manner in chronic arthritis. In paper I ankle joints from rats with peak intensity of experimental adjuvant arthritis were examined. Tissue sections were analyzed by immunohistochemical staining for HMGB1. In healthy rats the HMGB1 expression was predominantly intranuclear in the synovial membrane as well as in many chondrocytes in the cartilage. In contrast, tissue sections from rats with arthritis demonstrated cytoplasmic staining for HMGB1 in macrophage-like mononuclear cells in the inflamed synovial membrane. A cytoplasmic staining pattern was also evident in the same type of macrophage-like cells in the synovial fluid whereas adjacent polymorphonuclear cells stained negative. We also examined biopsies from 3 RA patients and 1 OA patient with the same technique. Results demonstrate occasional cytoplasmic staining in synovial membrane from the single OA patient but a markedly increased cytoplasmic pattern in RA synovial membranes. Synovial fluid was also collected from 15 additional RA patients and levels of HMGB1 were measured by Western blotting. TNF levels in the samples were measured by ELISA. In 14 out of 15 patients HMGB1 levels were elevated, ranging from 1.8 to 10.4 µg/ml. TNF in 12 out of 15 patients were detected in the range of 8 to 1,608 pg/ml. There was no correlation between HMGB1 and TNF levels. Altogether, the results indicate an aberrant and high expression of HMGB1 in the joints of patients with RA and in experimental arthritis as compared to controls and raises the question of HMGB1 as a mediator of chronic arthritis.

**HMGB1 is an early mediator of experimental arthritis**

With the knowledge of aberrant HMGB1 expression in some forms of chronic arthritis we hypothesized that HMGB1 may be an effector molecule in the development of chronic arthritis. We therefore, in paper II, investigated the temporal and spatial expression of HMGB1 at the early onset of arthritis in the experimental rat CIA model. HMGB1 expression was related to the expression of two known mediators of arthritis, TNF and IL-1β, and all three were detected by immunohistochemistry. For HMGB1 mRNA detection by in situ hybridization was also performed.
We found HMGB1 to be expressed in most cells with an intranuclear pattern before disease onset. 10 days after immunization, close to but predating clinical arthritis onset, a slight aberrant HMGB1 expression was detected in the lining and sublining layer of the synovia. At clinical onset day 15 this aberrant expression was more enhanced and increased expression of TNF and IL-1 was also evident in the synovia. Peak expression of HMGB1, TNF and IL-1 was detected from day 21 and onwards, corresponding to the most pronounced disease activity. Of the three investigated proteins TNF was most abundantly expressed at the later time points. Aberrant protein expression in the synovial vessels was first detected for TNF at clinical onset day 15. From day 21 and onwards TNF expression in vessels was extensive and accompanied by a moderate aberrant expression of HMGB1 and IL-1β. In cartilage constitutive extranuclear expression of HMGB1 and IL-1β was evident from start and increased from day 21. TNF was not expressed in the cartilage. Once joint destruction had started, all three investigated proteins were highly expressed in the destructive zone. The expression of HMGB1 mRNA was evident in both healthy and inflamed synovia but a marked upregulation was noted in the area most adjacent tissue destruction. Our results demonstrate aberrant expression of HMGB1 as early as or earlier than TNF and IL-1β expression at the very onset of chronic arthritis. Furthermore, HMGB1 protein synthesis is upregulated only in areas adjacent to tissue destruction. Altogether this indicates that HMGB1 is translocated from nuclei and that expression increases in chronic arthritis strengthening the hypothesis that HMGB1 contributes to the disease process.

HMGB1 blocking therapy ameliorates experimental arthritis

With data indicating HMGB1 as a mediator of arthritis we wanted to investigate the possibility that blocking of HMGB1’s extracellular effects could ameliorate chronic arthritis. In paper III we treated mice with CIA by intraperitoneal injections of either a neutralizing polyclonal rabbit anti-HMGB1 antibody or recombinant glutathione S-transferase (GST)-tagged A-box. As controls an irrelevant rabbit antibody or a GST-tag were used. Also rats with CIA were treated with A-box and GST-tag as control. We could demonstrate that anti-HMGB1 antibodies ameliorated clinical disease as measured by mean arthritis index which was 2.4±1.8 for the treatment group and 5.7±1.8 for the control group at the end of the study, 8 days after start of treatment. Significant reduction in the number of affected paws was also evident as well as decreased weight loss in the treatment group. For A-box treated mice mean arthritis index was reduced to 3.2±1.8 compared to 5.4±1.7 in the control group. As with polyclonal antibody treatment, A-box treatment significantly reduced the number of affected paws and also reduced weight loss. Histopathological evaluation of paws demonstrated a significant reduction in synovial proliferation and tissue destruction in mice treated with anti-HMGB1 antibodies or with A-box. Also the synovial expression of IL-1β was reduced in both treatment groups compared to the control groups as detected by immunohistochemistry.

In rat CIA A-box treatment significantly reduced mean arthritis index to 5.6±2.2 compared to 9.5±1.6 in control treated rats. Weight reduction and the number of affected paws were not significantly reduced.

The results in this study demonstrate that HMGB1 is important for the pathogenesis of chronic arthritis in two experimental models. Furthermore, HMGB1 blocking therapy
significantly ameliorates disease severity including tissue destruction. The expression of IL-1β is reduced as a consequence of HMGB1 blocking therapy. Altogether the results propose HMGB1 as a novel target molecule for treatment of chronic arthritis.

**HMGB1 is a TNF independent mediator of arthritis**

Since TNF is an important mediator of arthritis and our earlier findings had proposed HMGB1 as a mediator of arthritis we decided to evaluate the mutual HMGB1 and TNF dependence in chronic arthritis. In *paper IV* we studied synovial biopsies from 9 patients with active RA before and after established TNF-blockade with infliximab. Patients were subjected to an arthroscopy-guided biopsy sampling before and 9 (8-10) weeks after start with infusions of 3 mg/kg infliximab. Treatment resulted in an expected reduction of symptoms and a significant decrease in disease parameters as measured by ACR and DAS 28, the latter decreasing from a mean index 5.95 to 4.41. Expression of HMGB1 was evaluated by immunohistochemistry and aberrant expression was determined in defined areas of the synovia. Cellular infiltrates, lining layer and endothelium were evaluated separately. In addition, the overall aberrant HMGB1 expression was evaluated. In 6 out of the 9 patient biopsies HMGB1 mRNA levels were evaluated by RT-PCR.

Our results demonstrate that the aberrant protein expression of HMGB1 was decreased in five patients, remained unchanged in one patient and increased in 3 patients. Overall HMGB1 expression remained unchanged in all defined areas of the synovia for the group. There was no correlation between clinical response and the direction of change of HMGB1 expression in individual patients. Similarly, levels of HMGB1 mRNA were not altered as a consequence of infliximab treatment. Altogether the results indicate that TNF is not the main inducer of HMGB1 in chronic arthritis. Since HMGB1 may represent a TNF independent mediator of arthritis the possible success of future HMGB1-directed treatments is supported.

**HMGB1 modulates adaptive immune response**

Support for HMGB1 as a mediator of chronic arthritis and the evidence of adaptive immune features in most forms of chronic arthritides generated the hypothesis that HMGB1 in some context affects adaptive immunity. In *paper V* I decided to investigate whether or not HMGB1 may have a direct effect on T-cell proliferation. For T-cell purification PBMCs were obtained at seven occasions from one healthy donor and an additional five buffy coats were used for the purpose of evaluating inter individual variations of HMGB1-induced T-cell responses. Pure cultures of CD4+ and CD8+ T-cells were generated by Ficoll separation followed by immunofluorescent staining and FACS sorting. Cells were cultured in the presence of plate-bound anti-CD3 antibodies simulating T-cell activation through the CD3 complex. CD3 antibody coating concentrations ranged from 0 to 2.5 µg/ml. E-coli derived rHMGB1 was added to cultures in concentrations ranging from 0 to 4 µg/ml. Proliferation was assessed by [3H]thymidine incorporation. Results demonstrate that HMGB1 does not enhance proliferation of naïve CD4+ or CD8+ T-cells without anti-CD3 stimulation. Neither does HMGB1 act as a proliferation signal on any of the T-cell populations subjected to marked activation by higher anti-CD3 concentrations. On the contrary, HMGB1
significantly increased proliferation in both CD4+ and CD8+ cell populations when anti-CD3 stimulation was suboptimal. Our results indicate that HMGB1 acts as a proliferation signal for activated T-cells, thus having the ability to modulate adaptive immune responses.

**DISCUSSION**

HMGB1 is an evolutionary ancient protein with many divergent functions of outmost importance. As a nuclear protein it is crucial for proper activity of many transcription factors and for correct, dynamic structure of the chromatin. Extracellular HMGB1 further regulates mesenchymal cell migration and proliferation. This is fundamental for tissue regeneration after injury as well as for expansion of growing tissues. HMGB1 has been demonstrated as an essential molecular signal for wound healing in the skin and in the myocardium after ischemic infarction partly by activation of residing stem cells[128, 151]. Neurons also use HMGB1 in order to build neuronal networks. HMGB1 expression in the cytoplasm and on the extracellular surface at the leading edges of neurites growing out from the neuron is markedly increased and this outgrowth is regulated by HMGB1 ligation to RAGE[17, 130].

In the field of pathological tissue growth, tumours expanding beyond tissue boundaries also depend on HMGB1 for optimal invasiveness [26]. The tissues mentioned above may not necessarily serve as models for what actually occurs in the joint, but indicates important features regarding tissue growth that can be enhanced by HMGB1. Observations more relevant to joint tissue remodelling is that therapeutic intervention targeting HMGB1 ameliorates preclinical models of arthritis. The beneficial therapy results of anti-HMGB1 treatment and sRAGE therapy of CIA included less tissue damage, supporting the notion that HMGB1 is part of the mechanism involved in articular destruction[140, 152]. Furthermore, HMGB1 activates plasminogen a protein crucial for arthritis development in the CIA model and it also has the capacity to induce synovitis with pannus formation when injected into mice joints [82, 153, 154]. HMGB1 is crucial for proper bone formation during embryogenesis by enhancing expansion of osteoblasts, osteoclasts and blood vessels into the cartilage during endochondral ossification[155]. The fact that we could also demonstrate a local increase in HMGB1 mRNA only in areas adjacent to cartilage and bone destruction warrants further investigation of the role of HMGB1 in synovial transformation to a pannus[156]. Whether or not HMGB1 mediates development of pannus formation by activation of synovial fibroblasts or residing synovial stem cells has not yet been investigated thoroughly[157].

The finding of excess extranuclear HMGB1 in the joints of RA patients and in the CIA model of arthritis has recently been highlighted in a study of hypoxia in the equivalent settings of arthritides. Hypoxia can readily be demonstrated in RA and CIA synovitis. Interestingly HMGB1 expression correlated with the degree of local hypoxia whereas hypoxia was not demonstrated to correlate to TNF and IL-6 expression[99]. Furthermore, hypoxia *in vitro* mediated release of HMGB1 in a dose-dependent manner in the investigated cell lines of synovial, chondrocyte and macrophage origin. The results imply that HMGB1 secretion under hypoxia was not a passive release from injured cells, but rather preceded hypoxia-induced cell damage. Altogether this points
to hypoxia as an important mediator of the abundant HMGB1 expression in RA arthritis. Therapeutic reduction of hypoxia with hyperbaric oxygen may thus possibly attenuate inflammation and tissue destruction by decreasing the level of extracellular HMGB1 as well as by oxidizing the protein reducing its pro-inflammatory capacity.

The pro-inflammatory effects of HMGB1 have been discussed for almost 10 years. Apart from implications in acute infections and in tumour formation HMGB1 has also been associated with aberrant expression patterns in several chronic inflammatory diseases. Atherosclerosis, inflammatory bowel disease, diabetes and autoimmune hepatitis are all possibly HMGB1-dependent diseases of which a model of autoimmune hepatitis as well as colitis can be ameliorated by blocking HMGB1. [158-164]. Chronic arthritides, myositis, Sjögren syndrome and SLE are disease entities in the autoimmune area associated with HMGB1 and its connection with arthritis is the entity most thoroughly characterized so far [122, 165-167].

My thesis work includes the first demonstration of high aberrant HMGB1 expression in RA and experimental arthritis which has later been confirmed by other investigators [168-170]. Moreover, the results from paper II in my thesis indicates that HMGB1 expression is an early synovial manifestation in CIA, suggests a pathogenic role rather than an epiphenomenon late during the course of the disease [156]. The coinciding expression of the arthritis-promoting cytokines TNF and IL-1β, raises the question whether synovial HMGB1 increase is caused by any of these two cytokines. I doubt that TNF is the main inducer of HMGB1 in arthritis since TNF seems to be a weak stimulus for HMGB1 release in cultured macrophages [93]. HMGB1 on the other hand is a rather potent stimulus for TNF production in macrophages and DCs and also regulates TNF transcription in osteoclasts, [93, 111, 114, 123]. Persistent expression of HMGB1 in synovitis from RA patients during TNF blockade with infliximab was demonstrated in paper IV[171]. This result combined with the fact that HMGB1 blocking therapy in other disease models has the ability to down-regulate TNF, rather suggest HMGB1 as an inducer of TNF than the opposite[116, 142]. It is also a possibility that TNF and HMGB1 act independently of each other, since intra-articular HMGB1 injections in TNF gene-deficient mice generate arthritis like in wild type control mice [172]. In contrast, IL-1 type I receptor deficient mice did not develop arthritis after intra-articular HMGB1 injection, highlighting a need for IL-1/HMGB1 collaboration to establish arthritis[153]. The recent reports, confirmed in our laboratory, that HMGB1 needs to bind to certain endogenous (such as IL-1β) or exogenous inflammatory mediators for mediating proinflammatory activity supports the notion that HMGB1 mediates arthritis in synergy with IL-1β[119] [122] [138, 139]. During my thesis work, my collaborators and I have neutralized extracellular HMGB1 effects with A-box or with anti-HMG1 polyclonal antibodies in rodent CIA models which markedly ameliorated disease and IL-1β expression in synovial. This, again, underlines the importance and close interplay between these two mediators of arthritis[140]. This early treatment study, emphasizing the importance of HMGB1 in the pathogenesis, has later been followed by others confirming that blocking HMGB1 ameliorates disease in experimental models of arthritis. This has been done by means of blocking extracellular HMGB1 with sRAGE, thrombomodulin or antibodies of other origin than ours and by preventing release of HMGB1[99, 143, 147, 149, 152, 173].
An adaptive immune response is present in most patients with chronic arthritis. Recent findings indicate HMGB1 as capable of supporting adaptive immune responses. Endogenous DC-derived HMGB1 acted as a mediator to promote Th1 responses and RAGE expressed on T-cells is important for antigen-specific T-cell expansion [24, 25]. In T-cell dependent models of acute allograft rejection HMGB1 was essential indicated by increased graft survival after HMGB1 blocking therapy with A-box or sRAGE[174, 175]. My investigation of T-cell proliferation as a consequence of HMGB1 stimulation supports these data, demonstrating HMGB1 as a proliferation signal for anti-CD3 activated CD4+ and CD8+ T-cells[176]. My source of HMGB1 differs from the earlier mentioned study, since I used bacterially derived recombinant protein for stimulation rather than endogenous DC-derived protein. Consistent results with different sources of HMGB1 strengthen validity of these T-cell activating effects. However, in the recent light of HMGB1 functioning as a booster of other proinflammatory mediators there remains a possibility that Dumitriu and co-workers actually stimulated T-cells with HMGB1 complexed to IL-1β and I stimulated T-cells with HMGB1 complexed to a bacterially derived compound since my rHMGB1 preparations were endotoxin low but not endotoxin free. I speculate that bacterially derived rHMGB1 in my experiments might be complexed with LPS or unmethylated CpG DNA. Receptors for these bacterial compounds, TLR4 and TLR9 respectively, are expressed on T-cells and can co-activate at least CD4+ T-cells stimulated though the CD3 complex [177]. In analogy HMGB1 by ligation of RAGE on B-cells has the ability to enhance TLR9 [122]. This scenario could implicate a novel possible role for HMGB1 in adaptive immune response not only as a booster of endogenous adaptive signalling but also as a result of exogenous challenge.

My major drive to pursue the biology of HMGB1 in this PhD project has been and still is a wish to improve therapy of chronic inflammatory diseases. I believe that the results of the studies included in my thesis and the results of several other preclinical studies support the idea that HMGB1 indeed is a very interesting novel target molecule for therapy. However, the potential risk of targeting HMGB1 has not been addressed and remains to be elucidated. A number of studies demonstrating important physiological tasks for extracellular HMGB1 have been published during the period I have spent with my PhD project. The idea of therapeutic targeting of HMGB1 in dysregulated inflammation is not to eliminate/neutralize all extracellular HMGB1 molecules, but to reduce exaggerated levels. The reasoning is very analogous to that of the anti-TNF concept, which theoretically also could be dangerous. The fact that extracellular HMGB1 levels in general are much higher than those of TNF could possibly complicate the anti-HMGB1 approach from a practical point of view. However, we do not presently know anything about how much of the total extracellular pool of HMGB1 that is biologically active. It is also an unresolved issue whether a strategy based on interference with already released HMGB1 or therapeutic intervention preventing HMGB1 release is to be preferred. Many exciting aspects need to be sorted out in the near future.
CONCLUSION

HMGB1 is an ancient molecule that during the last decade has come in focus due to the emerging comprehension for its regenerative, tissue remodelling and proinflammatory properties. Much is still to learn about the biologic functions of this protein in health and disease. This work has contributed to the present knowledge of HMGB1 as a mediator of chronic arthritis. The strong and early expression of extracellular HMGB1 together with good response to HMGB1 blocking therapy in experimental models highlights the general importance of this protein in chronic arthritis. Persistent HMGB1 expression in RA synovia during TNF blocking treatment supports the interpretation that HMGB1 blocking therapy could be beneficial for patients with JIA or RA. The fact that synovial HMGB1 protein synthesis is up-regulated mostly in areas adjacent to joint destruction warrants further evaluation of its role in synovial invasiveness. The role of HMGB1 in adaptive immune responses has only very recently been addressed but points to regulating properties important in autoimmunity. Since beneficial biological effects could be mediated by a future HMGB1-directed therapy suitable routes for HMGB1 inhibition should be evaluated. It is my belief that HMGB1 blocking therapy may provide a powerful contribution to treatment of systemic inflammatory conditions.
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REFERENCES


