From the Department of Medicine, Rheumatology Unit,
Karolinska Institutet, Stockholm, Sweden

HMGB1 in inflammation - secretion and function

Heidi Wähämaa

Karolinska Institutet

Stockholm 2008

Published by Karolinska Institutet. Printed on recycled paper by Larserics Digital Print AB, Sweden

ISBN 978-91-7409-251-6

With love to my family

ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by progressive joint destruction. The inflammatory and joint destructive processes in RA are mediated by resident synovial cells and cells recruited from the blood stream and bone marrow. A variety of cytokines, chemokines and proteases contribute to the cartilage and bone destruction.

High mobility group box chromosomal protein 1 (HMGB1) was discovered over three decades ago as a transcription-regulating protein. In addition to its nuclear role, HMGB1 expression was detected at the leading edges in motile cells and its active secretion from immune cells was demonstrated. An excessive HMGB1 expression has been recorded in arthritic joints and in synovial fluid from RA patients and HMGB1-blocking therapies have been demonstrated to attenuate the disease course in murine arthritis models, suggesting that HMGB1 is a key player in arthritis.

The focus of this thesis work has been to further the understanding of HMGB1 as an inflammatory mediator and its role in arthritis. More specifically, I have studied the induction of HMGB1 secretion from a variety of inflammatory cells, how HMGB1 blockade affects the proinflammatory cytokine pattern in cell cultures and how HMGB1-targeting therapy affects the disease development in collagen-induced arthritis (CIA). Finally, I have also studied the inflammation-inducing capacity of HMGB1 alone and in complex with other proinflammatory molecules.

In order to quantify HMGB1 secretion from different cell types an HMGB1-specific ELIspot method was developed. We could demonstrate that HMGB1 was secreted from macrophage/monocytic cells during inflammatory conditions and that the secretion could be inhibited by gold salts and oxaliplatin treatment as detected by ELIspot. We could demonstrate that oxaliplatin-treatment attenuated disease development in murine CIA. A rebound effect with severe and aggressive disease course was demonstrated after one week of treatment which correlated with an excessive extranuclear HMGB1 pattern in the affected joints, indicating an HMGB1-mediated joint inflammation.

Furthermore, we have demonstrated *in vitro* that the proinflammatory activity of HMGB1 is dependent on complex formation between HMGB1 and other inflammation-promoting molecules, such as IL-1β, LPS and CpG-DNA. Studies using synovial fibroblasts obtained from arthritis patients demonstrated enhanced induction of TNF, IL-6 and IL-8 production and an enchased production of matrix metalloproteinase-1 and -3 when stimulated with HMGB1 in complex with IL-1β or LPS as compared to either substance alone. Thus, these results suggest that HMGB1 in complex with IL-1β or LPS can mediate both inflammation and destruction in RA.

In conclusion, the studies presented in this thesis strengthen the view of HMGB1 as an inflammation- and destruction-promoting molecule. I have demonstrated HMGB1 secretion from cell types present in the arthritic joint, defined two therapies in clinical use which have the potential to block HMGB1 secretion and verified the anti-rheumatic effect of one of these therapies in murine CIA. By *in vitro* studies, I have extended the knowledge of the proinflammatory features of HMGB1 and demonstrated both a proinflammatory and prodestructive effect of HMGB1 on synovial fibroblasts. Taken together, the studies in this thesis suggest that HMGB1 is one of the key mediators of arthritic inflammation and joint destruction.

LIST OF PUBLICATIONS

I. WÄHÄMAA H, Vallerskog T, Qin S, Lunderius C, LaRosa G, Andersson U, Erlandsson Harris H
HMGB1-secreting capacity of multiple cell lineages revealed by a novel HMGB1 ELISPOT assay
Journal of Leukococyte Biology 2007 Jan;81(1):129-36.

II. Zetterström CK, Jiang W, WÄHÄMAA H, Östberg T, Aveberger AC, Schierbeck H, Lotze MT, Andersson U, Pisetsky DS, Erlandsson Harris H
Pivotal Advance: Inhibition of HMGB1 nuclear translocation as a mechanism for the anti-rheumatic effects of gold sodium thiomalate
Journal of Leukococyte Biology 2008 Jan;83(1):31-8

III. Östberg T, WÄHÄMAA H, Palmblad K, Ito N, Stridh P, Shoshan M, Lotze MT, Erlandsson Harris H, Andersson U
Oxaliplatin Retains HMGB1 Intranuclearly and Ameliorates Collagen Type II-induced Arthritis
Arthritis Research & Therapy 2008;10(1):R1

IV. Hreggvidsdottir H, Östberg T, WÄHÄMAA H, Schierbeck H, Aveberger AC, Klevenvall L, Palmblad K, Ottosson L, Andersson U, Erlandsson Harris H
The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation
Submitted for publication

V. WÄHÄMAA H, Schierbeck H, Hreggvidsdottir H, Aveberger AC, Andersson U, Erlandsson Harris H
HMGB1 in complex with IL-1β promotes an inflammatory phenotype in synovial fibroblasts
Manuscript

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACPA	anti-citrullinated protein antibodies
ACR	the American College of Rheumatology
AP-1	activator protein-1
CIA	collagen-induced arthritis
DAF	decay-accelerating factor
DAMPs	damage associated molecular patterns
DC	dendritic cells
DMARDs	disease-modifying anti-rheumatic drugs
EC	endothelial cells
ELIspot	enzyme-linked immunospot
EPC	endothelial progenitor cells
FLS	fibroblast-like synoviocytes
HLA	human leukocyte antigen
ICAM-1	intracellular adhesion molecule 1
IFN	interferon
IL	interleukin
MC	mast cells
MMP	matrix metalloproteinase
MVB	multivesicular bodies
MyD88	myeloid differentiation primary response protein 88
NK cells	natural killer cells
NF-κB	nuclear factor κB
PAMPs	pathogen associated molecular patterns
PDGF	platelet derived growth factor
PgE_2	prostaglandin E_2
PRRs	pattern recognition receptors
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end products
RANKL	receptor activator of the nuclear factor κB ligand
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RF	rheumatoid factor
SCID	severe combined immunodeficiency
SE	shared epitope
SP	substance P
TGF-β	transforming growth factor-β
TIRs	Toll/IL-1 receptors
TLR	toll-like receptor
TIRAP	TIR-domain containing adaptor protein
TNF	tumour necrosis factor
Treg	T regulatory cells
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

RHEUMATOID ARTHRITIS

Inflammation is a complex defence mechanism that aims to control and resolve infection, toxic stress or tissue damage, and which protects the integrity of human body. Chronic inflammation is a pathological condition which is characterised by a continuous inflammatory response, which is self-perpetuating, causing tissue damage and impairing the function of the affected organ.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by swelling and pain in multiple joints, often initially occurring in the joints of the hands, wrists and feet [1]. Extra-articular manifestations such as vasculitis, respiratory and cardiac dysfunctions occur in patients with more severe disease [2]. The prevalence is about 0.5-1%, with an annual incidence of 25/100 000 persons in Western countries [3], preferentially affecting women after menopause [4].

Etiology

A detailed epidemiological population-based study determined that the incidence of RA continues to increase with age. The yearly incidence of RA around the age of 30 is about 10 per 100 000 individuals and this is about 10-fold higher at the age of 60 years [4]. Age is thus considered to be one of the strongest risk factors for RA.

Although the etiology of RA is not known there is strong evidence that both genes and environmental factors play roles in its development. The genetic influence has been demonstrated by studies showing a greater concordance in monozygotic (12-15%) as compared to dizygotic twins (3-4%) [5]. The most important genetic risk factor for RA is linked to the human leukocyte antigen (HLA) class II genes. HLA contains three regions, DP, DQ and DR, and the association is particularly strong for HLA DRB1 alleles that code for a similar amino acid sequence termed shared epitope (SE). The five peptides at positions 70-74 are located in the third hypervariable region within the peptide binding groove of HLA [6, 7], suggesting an involvement in peptide presentation to T cells [8]. It has been suggested that the SE determines the severity rather than susceptibility of RA [9, 10] and recently it was demonstrated that SE was only associated with individuals displaying antibodies against citrullinated peptides (anti-CCP) (discussed in more details in the B cell chapter) [11]. Interestingly, other

genes associated with RA displayed a correlation with occurrence of anti-CCP antibodies, implying different etiologies and pathogeneses in RA patients respectively seropositive or seronegative to anti-CCP antibodies [11-14].

Hormonal influences also contribute in development and progression of RA. As the disease has a three-fold predominance in women [8] and the incidence of disease increases through the fifth and sixth decades of life, this suggests that alternation in sex hormone levels plays a role in RA. It has been proposed that androgen protects younger men against development of RA and loss of estrogen at menopause elevates the risk for RA in women. Additionally, oral contraceptives confer as reduced risk of developing RA [15]. A decreased disease activity during pregnancy with disease flare during breastfeeding [16, 17] further indicate that the hormonal balance is central to the development of RA.

Environmental factors are estimated to contribute to at least one-third of the risk for developing RA [18]. Smoking it self is a risk factor [19, 20] but the risk is further increased in patients with SE and anti-CCP antibodies [21] thus indicating an interaction between environmental and genetic factors. No specific viral or bacterial infection has consistently been proven to precede RA development, but Epstein-Barr virus (EBV) is commonly detectable in synovial membranes of RA patients but not in osteoarthritis patients [22] and about one-third of RA patients display occurrence of cytomegalovirus (CMV) in the synovial membrane [23]. Furthermore, the presence of retroviral particles in synovial fluid [24] and bacterial compounds such as LPS and bacterial DNA in the synovial membrane in RA patients [25-27] supports the idea of a viral and/or bacterial contribution to RA. Other environmental factors that have been suggested to increase the risk of RA development include exposure to silica [28] and mineral oils [29]. Dietary factors have also been suggested to be important in RA outcome [30, 31] while contradictory statements concerning obesity and RA development have been reported [32, 33]. Interestingly, alcohol consumption has a protective effect [34] and even protects mice from arthritis [35].

Clinical manifestations in RA

The symptoms of RA vary between patients, but general disease features include fatigue, lack of appetite, low-grade fever, muscle and joint aches accompanied by stiffness. Muscle and joint stiffness are usually most notable in the morning and after

periods of inactivity. During disease flares joints become swollen, painful and tender, caused by local inflammation in the synovia with an excessive production of synovial fluid and an influx of inflammatory cells into the joint.

Multiple joints are usually inflamed in RA in a symmetrical fashion beginning with the small joints of the hand and feet. Although RA is acute or subacute in about 25% of patients its onset can be palindromic or monoarticular, or it can begin with extra-articular synovitis (tendosynovitis, bursitis) or with polymyalgia-like symptoms such as fever, fatigue and weight loss [36].

Since RA is clinically a heterogenous disease, with no single diagnostic or pathogonomic symptom or laboratory analysis, the diagnosis of RA is facilitated by use of The American College of Rheumatology (ACR) classification criteria from 1987 [37] (Table 1). Patients fulfilling at least four of seven of these criteria are classified as having RA and criteria 1 through 4 must have been present for at least 6 weeks.

Table 1. The 1987 ACR classification criteria for RA

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around joints lasting at least one hour a day.
2. Arthritis of three or more joint areas	At least three joint areas simultaneously with soft tissue swelling or fluid. 14 possible areas: left and right proximal interphalangeal (PIP), metacarpo-phalangeal (MCP); wrist, elbow, knee, ankle, and metatarsophalangeal (MTP) joints.
3. Arthritis of the hand joints	At least one swollen area in the wrist, MCP or PIP joint.
4. Symmetry of arthritis	Simultaneous involvement of the same joint areas (defined in 2.) on both sides of the body PIP, MCP, or MTP joints are acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules over bony prominences, extensor surfaces, or juxtaarticular regions.
6. Rheumatoid factor	Detected by a method positive in less than 5% of normal controls.
7. Radiographic changes	Typical for rheumatoid arthritis on posteroanterior hand and wrist radiographs. Must include erosions or unequivocal bony decalcification adjacent to the involved joints.

However, the ACR 1987 classification criteria are not best adapted to diagnose early RA since some of the criteria are not fulfilled during the first year after onset. A recent study indicates that the sensitivity and specificity of the ACR 1987 criteria in early RA is low and should not be used as a diagnostic tool in early RA, suggesting a revision of the classification criterias [38]. The Leiden group recently developed new diagnostic criteria set for early arthritis with the ability to discriminate, at the first visit, between self-limiting, persistent non-erosive and persistent erosive arthritis. This set consists of 7 criteria: symptom duration at first visit, morning stiffness of at least 1 hour, arthritis in ≥3 joints, bilateral compression pain in the metatarsal phalangeal joints, IgM-RF positivity, anti- CCP positivity, and erosions on radiographs of the hands and feet [39, 40].

Joint anatomy

A normal synovial joint consists of opposing bones with surfaces of unmineralised cartilage. Between the bone and the surface cartilage there is a mineralised cartilage layer. The joint is surrounded by a capsule covered with a thin layer of synovial tissue.

Normal healthy synovial tissue consists of two anatomically distinct compartments: a lining layer and a sublining layer. The lining layer, which is one-to-three cell layers thick, is in direct contact with the intra-articular space. It is a loosely organised, avascular tissue not supported by a basement membrane. Macrophage-like synovicytes (type A) and fibroblast-like synovicyte (type B) are the predominant cell types in healthy synovial membrane (reviewed in [41]).

The sublining layer is even more loosely organised, comprising a loose fibro-adipose tissue, interspersed with cells and blood and lymph vessles and nerves [42]. Two-thirds of native synovicytes are B-type fibroblast-like synovicytes (FLS), which in contrast to type A cells are $CD68^-$. FLS appear to belong to specialised fibroblast populations. DAF and VCAM-1 are expressed by activated FLS while other fibroblasts, including skin fibroblasts, do not express these markers [41]. Currently the best markers to identify FLS are vimentin, prolyl-5-hydroxylase and Thy-1 [43]. FLS play critical roles in normal joint homeostasis by synthesising and secreting hyaluronan, proteoglycans and a balanced amount of cytokines and MMPs [44].

PATHOGENESIS IN RHEUMATOID ARTHRITIS

Synovial pathophysiology in RA is a complex and synergistic interplay between different cell populations within the joint characterised by chronic inflammation and progressive joint destruction. Each cell type within the synovium contributes significantly to the initiation and perpetuation of RA. The resident cells within the synovia are the adipocytes, nerve cells, macrophage-like synovicytes, fibroblasts-like synovicytes and endothelial cells. These cells are described first, as they build up the "host-tissue" of RA inflammation. A schematic picture of synovial inflammation and pannus formation is presented in figure 1.

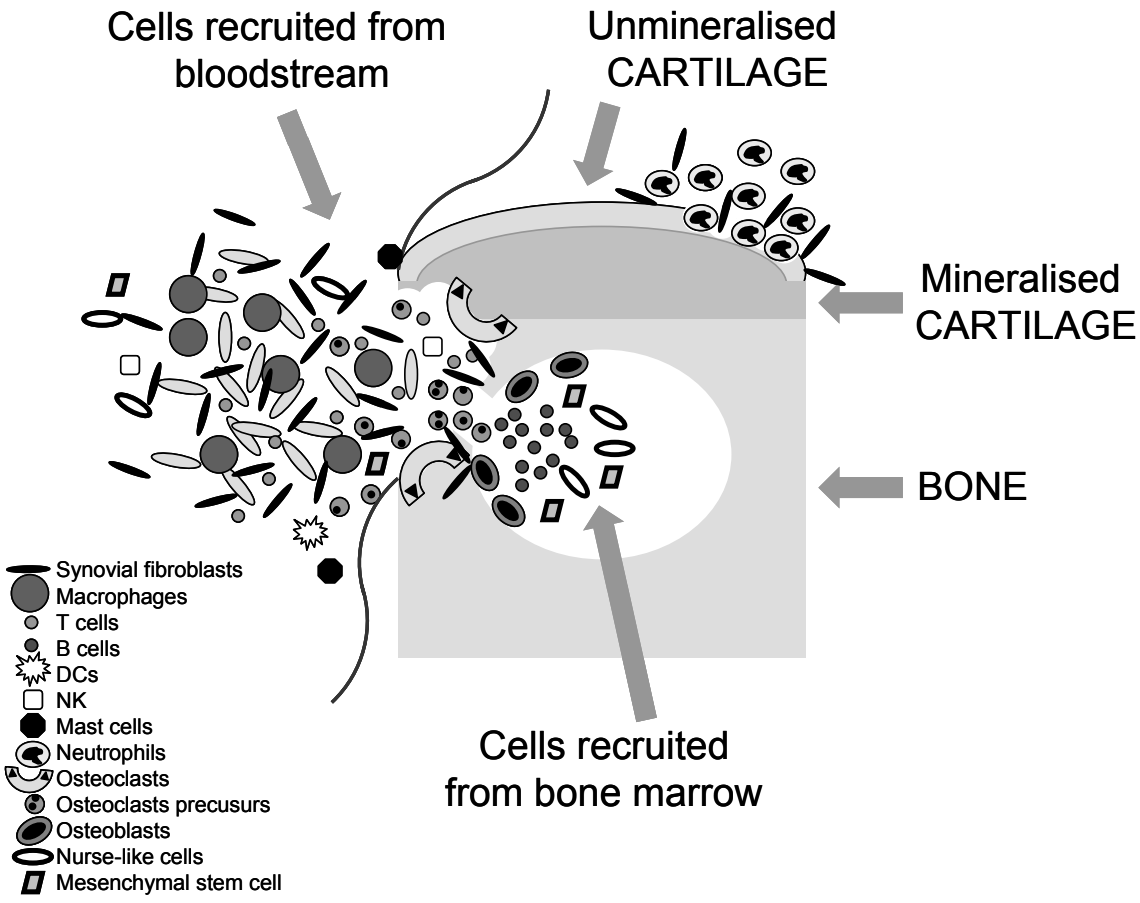

Figure 1. Hyperproliferating pannus tissue, with influx of inflammatory cells from bloodstream and bone marrow.

Adipocytes

Adipocytes have the general ability to synthesise and release proinflammatory cytokines, complement factors, growth factors and adhesion molecules [45] thus having a capacity to promote inflammation. However, the biological function of articular and synovial adipose tissue in RA is not well characterised, and conflicting data exists regarding the inflammatory and anti-inflammatory function of adipocytes in RA [46, 47]. It has been demonstrated that cytokines can regulate FLS differentiation to adipocyte-like cells [48] and further that adipocytes can dedifferentiate into fibroblast-like cells in a reversible manner [49]. Thus the phenotype of adipocytes might vary depending on the stage of the pathological process (metabolic or inflammatory) or the differentiation/maturation stage.

Nerve fibres

The innervation of RA synovia is altered, with loss of sympathetic nerve fibres [50, 51] Instead, substance-P (SP) produced by sensory nerve fibres contributes to the severity of experimental arthritis [52] with activation of synovial fibroblasts [53] and enhanced proinflammatory cytokine secretion from different cell types [54-56]. A predominance of SP positive sensory nerve fibres in RA patients compared to OA patients further supports the importance of SP in the inflammatory process in RA [57].

Macrophage-like synoviocytes

Macrophage-like synoviocytes or type A synoviocytes are considered to be tissue resident macrophages even though they are derived from blood-borne mononuclear cells. Type A cells have a round morphology and are located in the synovial lining layer with about half of the cell bodies projected over the surface line of the synovial membrane. These cells play an important role in maintaining normal joint homeostasis by absorbing and degrading extracellular constituents, cell debris and microorganisms in the synovial fluid and intimal matrix. Type A synoviocytes have the ability to phagocytose foreign substances that enters into the joint cavity, demonstrated with latex particles, iron dextran and colloidal gold particles (reviewed in [58]). As an antigen-presenting cell, type A synoviocytes express MHCII and have a broad spectrum of lysosomal enzymes facilitating the degradation of ingested material [59]. Latex

particles ingested by type A cells are gathered at the synovium cartilage junction which is highly vascularised, suggesting that intra-articular particles are drained via blood circulation. However, it has also been suggested that type A cells could remove the ingested material to lymphatic channels of the sublining layer (reviewed in [58]).

Fibroblasts-like synoviocytes

The most common cell type at the sites of bone and cartilage invasion is synovial fibroblasts that play crucial roles in both joint damage and the propagation of inflammation [41, 60, 61] FLS differ considerably from synovial fibroblasts from healthy joints. FLS in RA have been demonstrated to accumulate a number of mutations similar to those evident in malignancy [62] but these appear to be a consequence of the inflammatory environment rather than a primary cause of RA. However, once established these mutations facilitate FLS invasiveness, proliferation and resistance to apoptosis.

The specific features of RA FLS are now described in more detail, namely their: *invasiveness, hyperplasia* and their *inflammation*-promoting characters.

Invasiveness

The primary mechanism by which FLS erode cartilage appears to be via the synthesis and secretion of matrix metalloproteinases (MMPs), including MMP 1, 3, 8, 9, 10 and 13 (reviewed in [41]). MMP1 (collagenase-I) has the capacity to digest collagen type II which is an abundant cartilage compound and MMP3 (stromelysin-I) degrades matrix proteins [63]. Levels of MMP1 and MMP3 are elevated in synovial fluid and serum of RA patients [64-66] and are suggested to be useful predictive markers for an erosive disease [64-66]. In cell culture FLS produce MMP1 and MMP3 in response to the proinflammatory cytokines IL-1β and TNF, TLR ligands, direct cell-cell contact with T cells and under hypoxic condition [67-70]. These are all features characterising the arthritic joint. In addition to protease-mediated cartilage destruction, *in vitro* studies have revealed that FLS have the capacity to ingest cartilage by phagocytosis [71]. Studies of joints have demonstrated mineralised cartilage is rapidly resorbed by osteoclasts, whereas unmineralised surface cartilage remains intact for a longer time. Unmineralised cartilage degradation is mediated by FLS, neutrophils and chondrocytes themselves [72].

FLSs are also important key players in bone destruction. A key factor for osteoclastogenesis is the production of the receptor activator of the nuclear factor κB ligand (RANKL), which is produced and expressed on the surface of FLS following stimulation with proinflammatory cytokines and TLR ligands [73-75]. Activation of resident osteoclast progenitors in the bone or recruited macrophages [76] or DC [77] are able to mature to osteoclasts through RANKL-mediated activation. Additionally, FLS have been demonstrated to directly invade bone and to mediate osteoclast-independent bone resorption [78].

The invasive phenotype of FLS is suggested to be induced through expression of cellular proto-oncogenes, a feature occurring abundantly at the sites of invasion into cartilage and bone [79] although distinct from inflammation [80]. The inflammation-independent activation of synovial fibroblasts was confirmed by studies performed in the severe combined immunodeficiency (SCID) mouse. RA synovial fibroblasts were co-implanted with cartilage explants from the same donor into SCID mice, and even after 60 days the implanted fibroblasts retained their activated appearance and grew into the cartilage. This feature was not detected in synovial fibroblasts obtained from osteoarthritis or healthy individuals [81] further underlining the activated phenotype of RA synovial fibroblasts.

Synovial hyperplasia

Another hallmark of RA is synovial hyperplasia. The lining layers expand from 1-2 cell layers to being 15 cell layers depth or even more. Despite that the sublining layer becomes infiltrated with a variety of inflammatory cells, the FLS population in this region is also expanded [82]. In addition to the inflamed environment in whic several growth factors from adjacent cells promote FLS proliferation, at least three different mechanisms in FLSs themselves can contribute to increased FLS populations in RA, namely: hyperproliferation, decreased apoptosis and decreased senescence (reviewed in [41]).

Hyperproliferation of FLS can be driven by both over-expression of proteins and mutations in genes that regulate proliferation. Indeed, both pathways seem to be affected in RA. Excessive expression of growth factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) is evident in FLS [83] as is increased expression of Erk that regulates mitosis in FLS [84].

Decreased apoptosis: In the absence of apoptosis cells survive and accumulate abnormally. Death signals that promote apoptosis include oxidants that damage mitochondria as well as specific pro-death ligands promoting cell death through specific receptors. Human studies indicate dysregulated CD95-receptor mediated apoptosis in FLS [85] and a high expression of Bcl-2 which suppress apoptosis. Heterogeneous mutations of the tumour suppressor gene *p53* have been documented in RA FLS, permitting cell division in the presence of DNA damage [62, 86]. Mutations in *p53* are suggested to be a consequence of oxidative and inflammatory stress rather than being the primary etiology of RA [87].

Decreased senescence: Senescence denotes the process by which aging cells enter a permanently non-proliferative state. The mitotic life of a cell is principally determined by telomeres, the repeating DNA sequences at the ends of chromosomes. With each cell division, telomere number is usually decreased while in stem cells, cancer cells and RA FLS the enzyme telomerase replaces lost telomeres and permits unlimited mitosis [88, 89].

Inflammation

Another important feature of RA FLS is their capacity to produce and secrete a wide range of proinflammatory mediators including cytokines, growth factors and lipidic mediators of inflammation.

Chemokines and their receptors are crucial elements in the migration of leukocytes during inflammation. FLS can produce chemokines constitutively due to hypoxia [90] or following stimulation by proinflammatory cytokines, microparticles [91] or TLR ligands [92] An early activation and chemokine production of FLS by exogenous and endogenous TLR ligands might be a crucial step in the initiation of the chronic inflammatory process in RA. TLR pathways can be activated by exogenous stimuli such as bacteria and viruses that are evident in RA joints [26, 27, 93] and endogenous stimuli such as mRNA and fibrin [94, 95].

Endothelial cells

Under normal circumstances the adult vasculature is mostly quiescent, and angiogenesis does not take place except during wound healing and the female

reproductive cycle. Dysregulated angiogenesis and vasculogenesis contribute to RA pathology. Angiogenesis describes the formation of new vessels by activating pre-existing mature endothelial cells (EC) [96] and vasculogenesis describes the *de novo* formation of blood vessles by recruiting circulating $CD34^+$ endothelial progenitor cells (EPCs) to ischemic tissues [97].

Vascular endothelial growth factor (VEGF) is the key regulator of both angiogenesis and vasculogenesis. The expression of VEGF is induced under hypoxic conditions in the RA joint and induces EC proliferation, migration and survival [98, 99] thereby promoting EPC recruitment to the sites of vascular injury [100].

Endothelial activation during inflammation involves upregulation of vascular cell adhesion molecule-1 (VCAM-1) that together with increased angiogenesis and increased vascular permeability of endothelial cells facilitates the influx of inflammatory cells from the bloodstream into the joint [41, 101].

Cells infiltrating the sublining layer

The specific features of the different cell populations recruited to the arthritic joint are further described cell-by-cell.

Nurse-like cells and mesenchymal stem cells

A specific cell population distinct from fibroblasts, called *nurse-like cells* [102] is present in the synovial tissue and bone marrow. These fibroblastic bone marrow stromal cells migrate into the joint cavity through bone canals and proliferate in the synovial tissue [103]. Nurse-like cells have the capacity to adhere and allow lymphocytes to crawl beneath them, a process termed *pseudoemperipolesis* (adhesion and holding beneath). These cells interact with lymphocytes and monocytes, promoting cytokine production, cell proliferation, IgG production [104, 105] and osteoclast maturation from monocytes [106]. Multipotent mesenchymal stem cells from bone marrow are yet another cell population present in the arthritic synovia, with the multipotency to develop into cartilage, bone, fat and muscle cells [107] and reviewed in [102].

T cells

Substantial evidence for a T cell influence in RA pathogenesis include the association of HLA-DR1 and HLA-DR4 alleles, the presence of activated T helper cells within inflamed RA synovium, the ability to actively transfer RA to immunodeficient mice using T cells from human RA synovium and the anti-arthritic effects of T cell-directed therapies (reviewed in [108].

Of the infiltrating cells, 30-50% are T cells, predominantly of the $CD4^+$ subtype [109]. The vast majority of T cell clones from the human synovial membrane and synovial fluid represent Th1 cells producing IFNγ and IL-2. Activated $CD4^+$ T cells are also present in the peripheral blood and the frequencies of IFNγ producing cells in patients with new-onset synovitis correlate well with disease activity, emphasizing the role of Th1 cells in the initiation of disease (reviewed in [108]).

Pregnancy improves the symptoms of RA in about 75% of women, with significant resolution of inflammation. Interestingly, during pregnancy a marked decrease in Th1-mediated immunity has been determined and recently, a placental-derived protein (placental protein 14) was determined to inhibit Th1 immune responses and to synergise with IL-4 promoting Th2 immunity [110]. This further strengthens the evidence of a Th1-mediated immune response in RA.

It has become apparent that in RA the $CD4^+$ T cell subsets with regulatory capacity are functionally impaired, thus allowing Th1-driven immunity to progress into chronic inflammation. A T cell subset with regulatory capacity is the $CD4^+CD25^+$ T regulatory cells (Tregs). Between 5% and 15% of $CD4^+$ T cells in the peripheral blood of healthy individuals are Treg cells, which have the capacity to inhibit activation-induced proliferation of autologous Th1 cells (reviewed [108]). Despite the high levels of Tregs in the synovial fluid of RA patients, they are ineffective/defective in their capacity to control inflammatory responses. A recent study suggested that TNF inhibits the suppressive activity of Treg cells, as treatment with anti-TNF antibody could restore the suppressive function of Tregs [111, 112].

A second $CD4^+$ T cell population with the potential to counteract T cell-driven inflammation in RA is the Th2 cell population. Th2 cells produce IL-4 and can prevent the generation of Th1 and Th17 cells, and downmodulate their effector functions [113]. However, the majority of RA patients already have an impaired Th2 cell differentiation at the initial phase of disease and it is demonstrated that reduced Th2 cell generation is

associated with persistently aggressive and erosive disease (reviewed in [114]). Furthermore, an association between RA severity and impaired IL-4R signalling has been demonstrated [115].

T cells are also important contributors to the pathogenesis of bone erosion in RA through an antigen-independent process. $CD4^+$ Th17 cells are distinct from Th1, Th2 and Treg cells and are present in the synovium and circulation of RA patients. Recent studies have identified Th17 cells as an exclusive osteoclastogenic T cell subset among the $CD4^+$ T cell lineage [116, 117] and IL-17 mRNA levels in synovial membranes have been demonstrated to be predictive of joint damage in RA [118]. RANKL expression by Th17 cells is thus the key link between immune activation and bone loss.

$CD8^+$ T cells produce high levels of TNF and IFNγ, thereby contributing to RA pathogenesis. Interestingly, subgroups of $CD8^+$ T cells recognising Cytomegalovirus, Epstein-Barr virus and influenza virus, respectively, are enriched in the synovial fluid compared to in peripheral blood, reflecting the enrichment of memory T cells specific for foreign antigens [119]. $CD8^+$ T cells are also required for the formation of germinal–like centre structures in RA synovia [120, 121] and these structures facilitate the interaction between T and B cells, as well as between T cells, macrophages and fibroblasts [122]. Direct cell-cell interactions of FLS with T cells were demonstrated to prevent programmed cell death of T cells [123, 124] and induced IL-6, MMPs and PGE2 production in fibroblasts. T cell contact with macrophages induces IL-1, TNF and IL-6 production [69, 125-127], demonstrating that T cells are able to drive chronic inflammation through an antigen-independent mechanism.

B cells

Successful treatment of RA through B cell depletion provides evidence for the relevance of B cells in RA pathogenesis [128-130]. B cells are apart from being Ig producing cells, very efficient antigen presenting cells and contribute to T cell activation through expression of costimulatory molecules. B cells both respond to and produce chemokines and cytokines, thereby promoting synovial inflammation [131]. Direct cell-cell interactions of B cells with FLS were demonstrated to prevent programmed cell death of B cells [123, 124] and stimulation of FLS with TNF and IFNγ induce production of B cell activation and survival factor (BAFF) [132] thus increasing the longevity of plasma cells.

Several different autoantibodies have been detected in RA patients but only rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) have sufficient sensitivity and specificity to be used in clinical practice (reviewed in [133]).

RF is an autoantibody directed against the Fc portion of IgG and can be detected in 75-80% of patients suffering from RA [134]. Combined elevation of IgM-RF and IgA-RF appears to be more specific for RA than IgM-RF alone [135] and high titer IgA-RF is associated with more rapid disease progression, worse outcome and extra-articular manifestations [136].

Although circulating RF has been considered as an important marker for RA, autoantibodies directed against citrullinated peptides have recently been identified in RA patients termed as *anti-citrullinated protein antibodies* (ACPA). Citrullination is a post-translational modification of arginine catalyzed by the enzyme family peptidylarginine deaminase (PAD) [137]. Citrullinated proteins are detectable in the arthritic synovium in humans [138] and in animal models of arthritis [139].

ACPA provide greater diagnostic specificity than RF and have high predictive value for RA several years before disease onset [140] with erosive disease being more likely to develop in $ACPA^+$ positive patients compared to in $ACPA^-$ patients [141]. ACPA producing B cells have been observed in synovial tissue and the abundance of ACPA is higher in synovial fluid and synovial tissue than in sera from RA patients [142, 143] suggesting a local antibody production against citrullinated antigen within the joint. This could indeed be the case, since germinal center-like structures are formed within the synovial tissue [144] and hypermutation and terminal B cell differentiation is suggested to occur within these germinal centre-like structures [145].

Macrophages

Macrophages are important key players in the pathogenesis of rheumatoid synovitis. Macrophages present in the inflamed synovium have two origins: the resident synovial lining layer type A synovicytes, and sublining macrophages which have migrated from blood circulation as monocytes and become tissue macrophages [146]. The number of type A synovicytes is higher in the inflamed synovial lining layer than in healthy conditions [147] and the number of macrophages in the pannus tissue correlates well with both radiological joint damage [148] and with joint pain and inflammation [149]. Macrophages, together with fibroblasts and endothelial cells, thus promote cartilage

destruction [150, 151] and through stimulation by RANKL and M-CSF monocytes are able to differentiate to osteocasts [152] promoting bone destruction.

In RA tissue macrophages have been demonstrated to be resistant to apoptosis [153] contributing to the persistent release of growth factors, cytokines, chemokines [154] and MMPs-1, -2 and -9 (reviewed [155]). Growth factors released by tissue macrophages include platelet derived growth factor (PDGF) [156] basic and acidic fibroblast growth factor (bFGF, aFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β) [154].

TNF, IL-1β, IL-6, IL-12, IL-15 and IL-18 are some of the proinflammatory cytokines produced by tissue macrophages [154]. Interestingly, IL-10 produced by monocytes is no longer considered to be an anti-inflammatory cytokine in RA [157] since IL-10 was determined to induce TNF receptor expression on monocytes [158]) and an increased expression of interferon-γ-inducible genes [157]. Among chemokines, IL-8 produced by macrophages functions as a recruiting factor for neutrophils [154] and fractalkine, while promoting angiogenesis, also acts as a chemotactic agent for monocytes and lymphocytes [159].

Neutrophils

Neutrophils are the most abundant cell population in the synovial fluid but are sparsely distributed in the synovial membrane.

A variety of chemokines produced by FLS, among others IL-8, MCP-1 and RANTES, attract neutrophils to the inflamed RA joint (reviewed in [160]) and locally produced complement components [161] followed by complement activation generates anafylatoxin C5a which also functions as chemoattractant for neutrophils [162]. C5a further induces the expresson of FcRγIII on neutrophils [162] and facilitates Fc-receptor mediated phagocytosis of immune complexes in the synovial fluid. Phagocytosis further triggers the release of hydrolytic enzymes, production of reactive oxygen species [163] and production of IL-1α and IL-1β thereby promoting local joint inflammation and tissue degradation, and as such are important mediators of surface cartilage degradation (reviewed in [164]). During neutrophil activation and degranulation nociceptin is released, which is a neuropeptide associated with pain [165] thus accounting for joint pain in RA.

NK cells

NK cells are distributed throughout lymphoid and non-lymphoid tissues. NK cells are a minor cell population of human peripheral blood, conmprising 2%-18% of the total lymphocyte count (reviewed in [166]). NK cells mediate clearance of antibody-coated cells, mediate early protection against viruses and kill cancer cells (reviewed in [167]).

In humans NK cells are divided into $CD56^{dim}$ NK cells which express perforin and produce IFNγ, and $CD56^{bright}$ NK cells which lack perforin and produce cytokines upon stimulation. NK cells are able to induce maturation and activation of DCs, macrophages and T cells but are also able to kill immature cells DCs, activated $CD4^+$ T cells, hyperactivated macrophages and cells not expressing MHC class I molecules (reviewed in [166]).

Since NK cells are able to skew the immune system towards a Th1 or Th2 response depending on the cytokine milieu and tissue localisation these cells can both promote or protect from autoimmunity [167]. The function of NK cells in RA is not fully elucidated but $CD56^{bright}$ NK cells accumulate in both lymph nodes [168] and in chronically inflamed RA joints [169]. $CD56^{bright}$ NK cells are also detected in synovial fluid from RA patients and they spontaneously produce IL-10 and TGFβ [170]. It has been demonstrated that NK cells have the ability to induce TNF production from monocytes [171] and can trigger $CD14^+$ monocytes to differentiate into DCs [172].

Dendritic cell

Dendritic cells (DC) play a pivotal role in T cell immunity and tolerance due to their ability to stimulate naïve T cells and to direct effector cell function. Human DCs are divided into two different subsets: myeloid- (mDC) or plasmacytoid dendritic cells (pDC) and both mDCs and pDCs are present in the synovial tissue in RA patients.

Myeloid DCs are found in peripheral blood, synovial fluid and in synovial tissue. In the synovial tissue they are localised in the vicinity of T cells, where they express IL-12 and IL-23, important cytokines for the induction/expansion of Th1 and Th17 T cell subsets, respectively. TLR ligands induce chemotaxis and production of various chemokines from mDCs, in particular IL-8. Peripheral blood $CD14^+$ monocytes are able to differentiate into a specific type of myeloid DCs, designated as $CD1a^+$ mDC (mo-DC). In chronically inflamed tissues or in lymph nodes NK cells trigger the differentiation of mo-DC into potent Th1-promoting cells. These cells are abundant in

the RA synovium and are primarily located in the perivascular areas and lymphoid-like structures (reviewed in [173]).

Plasmacytoid DCs are found with reduced numbers in peripheral blood in RA patients compared with healthy donors while a higher concentration of pDC was is reported in synovial fluid and synovial tissue in RA patients compared in osteoarthritis patients [174] . Importantly, pDCs induce both T cell-dependent and –independent B cell differentiation into antibody-producing plasma cells. Interestingly, pDC numbers are especially increased in ACPA positive RA patients (reviewed in [173]).

Mast cells

Mast cells (MCs) are multifunctional cells containing a vast numbers of preformed granules with proinflammatory and fibrogenic mediators. An increased number of MCs occurs in the synovial tissues of RA patients, especially at the sites of cartilage destruction [175] where the activation and degranulation of MCs are also detected [176]. Upon activation MCs are able to produce or release the preformed granules containing cytokines, chemokines and proteolytic enzymes. MCs are also a major source of vasoactive and chemotactic factors, facilitating the recruitment of other inflammatory cells into the joint (reviewed in [131]). Interestingly, a MC-membrane stabilising agent was shown to suppress arthritis progression in a collagen-induced arthritis model (reviewed in [177]).

ANTI-RHEUMATIC THERAPIES

In the normal healthy joints there is a balance between pro- and anti-inflammatory cytokines, whereas in the arthritic joint an imbalance between these mediators causes the persistence of inflammation and joint destruction [178]. Therapy in RA aims to suppress chronic inflammation and structural protection of cartilage and bone. The available therapies for RA are directed against individual proinflammatory cytokines or against individual effector cells, including therapies inhibiting the Th1/Th17 cytokine-driven disease through favouring Th2 differentiation.

Cytokine-inhibiting therapy

TNF

TNF is a major mediator of structural damage in RA by inducing osteoclast formation. This is accomplished by: *i)* inducing the expression of RANKL on mesenchymal cells and lymphocytes; and *ii)* direct engagment of TNF receptor type I on the surface of osteoclast precursors. TNF has also a primary role in RA pathogenesis by inducing production of adhesion molecules facilitating the influx of osteoclast precursors (monocytes) and other inflammatory cells [179].

TNF additionally induces the production of MMPs, IL-1 and other proinflammatory cytokines, thereby sustaining both inflammation and in turn promoting cartilage and bone destruction (reviewed in [180]. TNF stimulates macrophages to produce reactive oxygen [181, 182] that induce other synovial cells to peoduce more TNF [183] creating an intristic loop for sustained TNF production.

Available targeted therapies are: **Infliximab (Remicade®)**; a humanised chimeric monoclonal antibody which binds to both soluble and membrane bound TNF. **Adalimumab (Humira®)**; a human monoclonal antibody (produced by phage display) that binds TNF. **Etarnecept (Enbrel®)**; a soluble TNF receptor with a human IgG Fc that binds to TNF and lymphotoxin.

IL-1

Several reports demonstrate that IL-1 (in particular IL-1β) is a key mediator of joint damage. Osteoclast formation is dependent on IL-1 since the abolition of IL-1 in TNF-transgenic mice completely protected these animals from cartilage breakdown, and reduced bone destruction considerably despite ongoing synovitis [184]. An earlier study also demonstrated that therapy with IL-1RA or induced IL-R1 abolition completely rescued TNF-initiated bone loss [185]. In addition, mice that are IL-1RA-deficient spontaneously develop destructive polyarthritis [186]. Furthermore, high IL-1 levels are correlated with joint inflammation and are responsible for the articular destruction in RA by inhibiting proteoglycan synthesis [187] and by stimulating the release of MMP1 and MMP3 [188].

Available targeted therapy: **Anakinra (Kineret®)**; a soluble IL-1 receptor antagonist. Novartis has an anti-IL-1β mAb in clinical trials. This antibody works well in IL-1β caused disorders like Muckle-Wells and CINCA syndromes.

IL-6

IL-6 is the major inducer of CRP synthesis in the liver and mediates systemic inflammation [179]. IL-6 is also one of the cytokines that induces RANKL expression on mesenchymal cells and T cells, which in turn can activate osteclastogenesis and bone degradation [189]. A recent study demonstrated that blockade of IL-6 by neutralising antibodies against the IL-6 receptor effectively blocked the inflammatory process in RA [190].

Available targeted therapy: **Tocilizumab**: an antibody directed against the IL-6 receptor.

Effector cell-directed therapy

B cell depletion

B cell depletion appears to inhibit inflammatory responses by: i) decreasing the amount antigen presenting cells; ii) impairing the formation of immune complexes; and iii) blockade of proinflammatory cytokine production. Activated B cells express RANKL and support osteoclastogenesis; thus B cell depletion could affect the structural damage in RA.

Available targeted therapy: **Rituximab**; $CD20^+$ B cell depleting antibody, detects B cells in the periphery but does not deplete plasma cells.

Costimulation blockade

Endogenous CTLA4 expressed on Treg cells (and activated T cells) binds with high affinity to CD80 and CD86 expressed on the surface of antigen presenting cells, inhibiting the costmulatory signal for T cells. Thus recombinant soluble CTLA4 has anti-inflammatory properties by inhibiting T cell activation [191] but also by targeting CD80/CD86-bearing monocytes and thereby inhibiting the differentiation of monocytes into osteoclasts [192].

Available targeted therapy: **Abatacept**; recombinant CTLA4 with IgG Fc.

Modulation of Th1 toward Th2 balance

Disease-modifying anti rheumatic drugs (DMARDs)

The majority of T cell clones from the human synovial membrane and synovial fluid represent $CD4^+$ Th1 cells producing IFNγ and IL-2. (reviewed in [108]).

It has become apparent that in RA T cell subsets with regulatory capacity, such as Th2 cells and $CD4^+CD25^+$ Treg cells, are functionally impaired, thus allowing the progression of Th1- mediated inflammation (reviewed in [108, 114])

The concept of modulating the Th1/Th2 balance has been successful in animal models of arthritis [193, 194]. Several recent studies have indicated that several DMARDs and glucocorticoids appear to be able to modulate the Th1/Th2 balance.

Available targeted therapies are: **Methotrexate** [195], **Leflunomide** [196], **Sulfasalazine** [197], **Cyclosporine** [198] and **Glucocorticoids** [199, 200].

ANIMAL MODELS OF ARTHRITIS

Experimental arthritis models are appropriate tools for the study of a complex disease with interactions between genetic and environmental factors. Different animal strains have specific genetic backgrounds, where every strain may resemble one subgroup of RA patients. This offers the possibility to study how different disease inducers influence the disease course and to evaluate the effects of different anti-rheumatic therapies. The advantage of using animal models includes the possibility to study the entire disease course, from the induction phase to the progressive erosive disease.

Collagen-induced arthritis

In this thesis work, I have used Collagen-induced arthritis (CIA) in mice as a model of human arthritis. CIA is the most commonly used experimental model of arthritis and has been used since the late seventies [201]. Injection into the tail base of heterologous type II collagen (CII) suspended in mineral oil together with inactivated mycobacteria (complete Freund´s adjuvant, CFA) induces a severe destructive arthritis in DBA/1 mice [202, 203]. The susceptibility to CIA is related to MHC class II alleles. Mouse strains bearing MHC I-A^q, I-A^r or I-A^b develop arthritis following challenge with CII [204]. In CIA both cellular and humoral immune responses drive the inflammation, resulting in cartilage and bone erosion. CIA has many similarities to human RA being chronic, symmetric, affecting peripheral joints and inclusion of formation of synovial pannus tissue [203, 205].

Although an adaptive immune response to CII is pivotal for the development of disease, macrophages and their products (ie TNF, IL-1, IL-6) play a major role in the disease pathogenesis [186, 206-208]. Blockade of these cytokines ameliorate, as in RA, the disease symptoms [209-211]. The differences between mouse CIA and RA are: the abscence of lymphoid aggregate-formation, that no difference in sex predisposition exists and that no RF can be demonstrated in mouse CIA [203, 205].

HMGB1

HMGB1 as a nuclear protein

The human HMGB1 gene is located on chromosome 13q12 [212] and encodes a 215 amino acid polypeptide. HMGB1 is highly conserved between species with 99% identity between rodent, bovine and human proteins [213]. HMGB1 has a tripartite domain organisation: the A- and B- HMGB boxes, each around 75 amino acids in length, are connected by a short linker and an acidic tail formed by 30 consecutive glutamate and aspartate residues. The A-box consists of 3 α-helices folded in an L-shape and the B-box is also folded in an L-shape similar to the A-box [214-219]. The acidic tail modulates interaction with DNA and its helix-distorting ability [220-222] which modulates the interaction with nucleosomes and chromatin remodelling machineries [223, 224]. The acidic tail also modulates the acetylation of HMGB1 mediated by histone acetyltransferases [225] (reviewed in [215]).

HMGB1 is an abundant and highly conserved non-histone chromosomal protein that binds to the minor groove of DNA without sequence specificity. It associates with high affinity to DNA with highly bent structures, such as four-way junctions and cisplatin-modified DNA [226]. HMGB1 has a functional importance as a regulator of transcription, either by remodelling chromatin and nucleosome structure or direct interaction with transcription factors such as steroid hormone receptors, p53 and NF-kB [227]. Furthermore, HMGB1 knockout mice die shortly after birth due to hypoglycemia and exhibit a defect in the transcriptional function of the glucocorticoid receptor [228]. HMGB1 is expressed in all nucleated cells but the expression level of HMGB1 is much higher in transformed cell types compared to in non-transformed cells [229-232].

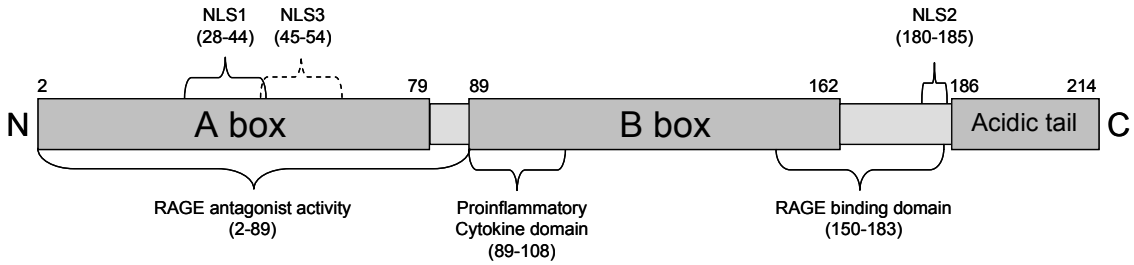

Figure 2. Structure of HMGB1

Extracellular functions of HMGB1

The cytoplasmic occurrence of HMGB1 was already demonstrated in 1979 [233] and HMGB1 expression at leading edges in spreading and motile cells was demonstrated in 1987 [229, 234]. HMGB1 binding to plasminogen and plasminogen activator [235] was demonstrated to play a crucial role during cell invasion and tissue remodelling by activating MMP2 and MMP9 that degrade the extracellular matrix [236, 237]. HMGB1 was also found in the cytoplasm of resting platelets and to be expressed on platelet surface upon activation [238].

In 1999 HMGB1 was discovered as an extracellular mediator of inflammation released by macrophages [239]. The cytokine activity of HMGB1 is ascribed to the B-box structure while the A-box behaves as a competitive inhibitor of HMGB1 [240-243]. The structure and the functional domains of HMGB1 are further described in Fig 2. During the last decade HMGB1 has been demonstrated to be secreted from various types of cells, promoting both inflammation and tissue regeneration [244] and to possess bactericidal effects [245].

Secretion of HMGB1

Active HMGB1 secretion

HMGB1 is an extremely mobile nuclear protein which rests at a specific DNA site for only fractions of a second [246] and in most cells HMGB1 shuttles between the nucleus and cytoplasm. The nuclear import is an active process while the cytoplasmic translocation may occur via passive diffusion or by CRM1-mediated active export. Activation of monocytes with proinflammatory agents induces hyperacetylation of HMGB1which inhibits the active transport of HMGB1 to the nucleus, resulting in cytosolic accumulation [247]. In myeloid cells HMGB1 is then accumulated in secretory lysosomes followed by lysophosphaditylcholine (LPC)-mediated exocytosis [248]. In addition, the ATP binding cassette transporter 1 inhibits both HMGB1 and IL-1β secretion from monocytes and macrophages [249, 250] and recently a specific ABC transporter (multi-drug resistant protein 1, MRP-1) was suggested to mediate active HMGB1 secretion from macrophages [251]. The mechanism by which cell types lacking the secretory lysosomal pathway, such as smooth muscle cells and endothelial cells [252, 253] secrete HMGB1 is not yet elucidated.

Passive HMGB1 release

The other setting in which HMGB1 release occurs is during cell death. Since HMGB1 is loosely bound to chromatin it may leave the nucleus when membrane integrity is lost. Induction of necrotic cell death through physical or metabolic injury leads to HMGB1 release [246]. Late apoptotic cells undergoing secondary necrosis also release HMGB1 [254-257]. One recent report suggests that hypoxia may even induce active HMGB1 release, that precedes hypoxia-induced apoptosis or necrosis [257].

Exosomal HMGB1 release

Exosomes are small, 30-100 nm membrane vesicles believed to originate from late endosomal compartments called multivesicular bodies (MVBs). MVBs are involved in transporting proteins for degradation in lysosomes. Intraluminal vesicles are formed by inward budding of the endosomal membrane of MVB and may be released when MVB fuse with the plasma membrane, these intra luminal vesicles are then called exosomes [258, 259]. HMGB1 has been detected in exosomal compartments and in exosome-depleted supernatants of Caco-2 cells suggesting that HMGB1 was released by several routes from these cells [374].

Modifications of HMGB1

HMGB1 is further directed to diverse post-translational modifications. ADP ribosylation of HMGB1 regulates gene transcription and is a cellular response to DNA damage [260, 261]. HMGB1-ADP ribosylation can be induced by alkylating agents and was shown to induce HMGB1 relocalisation from the nucleus to cytoplasm and subsequent release during necrotic cell death [262].

During apoptosis caspases 3 and 7 mediate oxidation of HMGB1 in HeLa cells [263]. Activation of RAW 264.7 cells induced phosphorylation of HMGB1 and inhibited the nuclear transport of HMGB1 by reduced binding to nuclear cargo carrier protein KAP-α1 [264]. Furthermore, mono-methylated HMGB1 was detected in the cytoplasm but not in the nucleus of neutrophils [265].

Depending on the activation status of cells at the time point of passive HMGB1 liberation, it is therefore likely that all these different HMGB1 isoforms may occur extracellularly. HMGB1 can also be cleaved by Thrombin-thrombomodulin complexes

[266] and extracellular HMGB1 can further make complexes with IgG [267, 268], LPS, CpG [269-271, *paper IV*] and IL-1β [272, *papers IV,V*]. All these modifications of HMGB1, (both post-translational and the binding to other molecules), influence the mode of receptor binding, the choice of receptor/receptors and the subsequent signalling cascade (Figure 3).

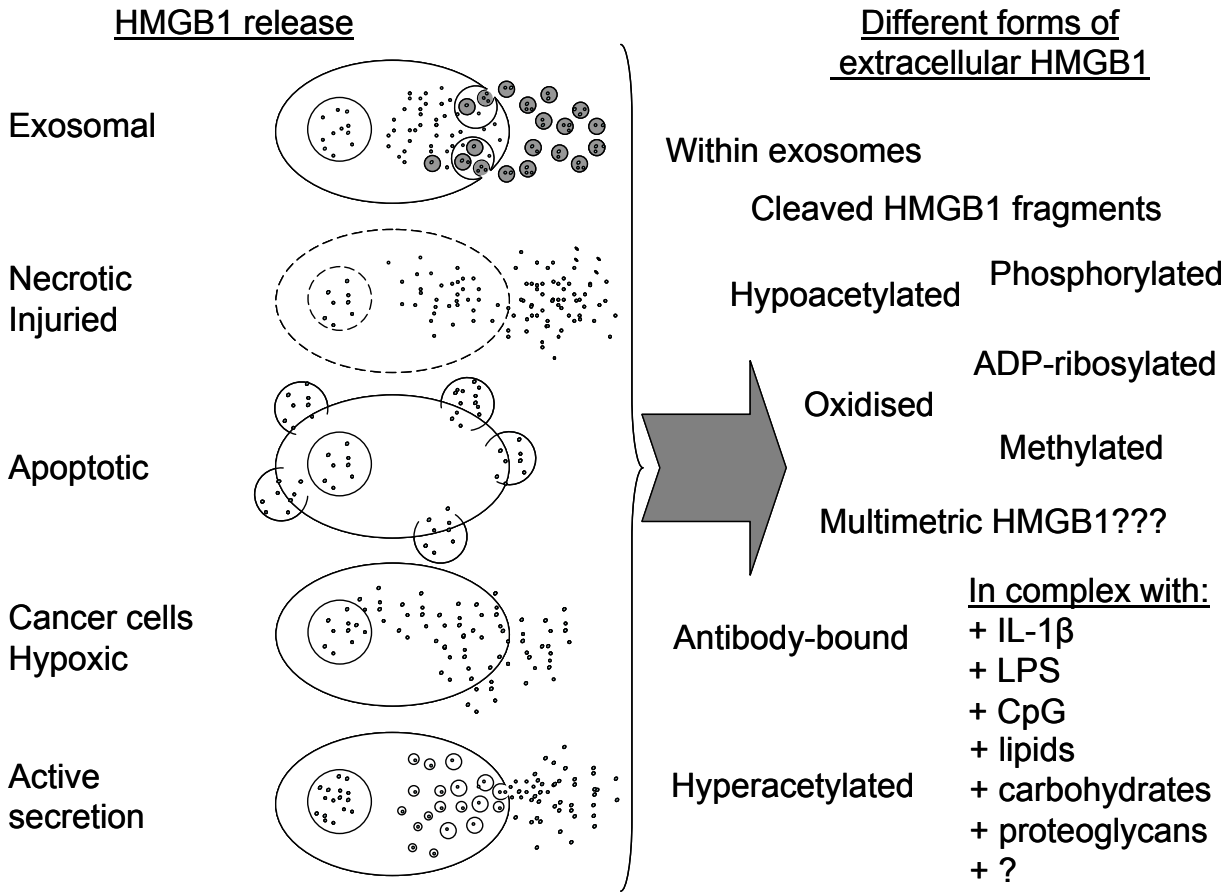

Figure3. SUMMARY: HMGB1 release and different forms of extracellular HMGB1

HMGB1 receptors

HMGB1 has been demonstrated to interact with a wide range of proteins including proteoglycans [273, 274] lipids and carbohydrates [275] transcriptional factors [227, 276-278] and viral proteins [279, 280]. Using a phage display approach HMGB1 was demonstrated to bind to more than 12 different peptide sequences, some being strictly associated with the A-box or B-box, respectively, while several were associated with both A- and B-box structures [281]. The heterogeneity of peptide and protein binding to HMGB1 thus implies interaction with several extracellular receptors. The suggested receptors for HMGB1 are summarised in Table 2 and the following receptors are discussed more detailed in next section: Receptor for advanced glycation end products (RAGE), TLR2, TLR4 and TLR9.

Receptor for advanced glycation end products (RAGE)

RAGE belongs to the immunoglobulin superfamily of cell-surface molecules and is composed of an extracellular region containing of one V-type and two C-type immunoglobulin domains [282, 283] followed by a hydrophobic transmembrane-spanning domain and a highly charged cytoplasmic domain which is essential for RAGE signalling [284, 285]. RAGE has several isoforms that code for both transmembrane and soluble proteins (sRAGE) [286, 287]. sRAGE has been suggested to function as a decoy receptor since higher plasma levels of sRAGE are associated with a reduced risk for arthritis and many other inflammatory diseases (reviwed in [288]).

HMGB1interaction with membrane-bound RAGE was originally described to promote neuronal outgrowth [229, 289, 290]. The A- and B-boxes can independently promote cell migration in rat smooth muscle cells via a RAGE-dependent manner [291] while the neuronal outgrowth is promoted by the C-terminal motif [217]. It is therefore likely that additional molecules are involved in the regulation of cell migration. It was recently demonstrated that both RAGE and Mac-1 β2 integrin interaction was required in HMGB1-mediated neutrophil recruitment in an *in vivo* model of peritonitis [292].

RAGE is also described to be the major receptor for HMGB1-induced cytokine production and cell migration in macrophages [250, 293]. However, the cytokine-inducing capacity of HMGB1 is ascribed to the B-box domain [240, 263, 294-296] whereas the interaction with RAGE is located to amino acids 150-183, just before the

C-terminal acidic tail [217] implying an interaction between HMGB1/RAGE and additional receptor/receptors. Full-length RAGE has two potential N-linked glycosylation sites which occur in the ligand-binding V-domain [283] and the subsequent glycocylation of RAGE was reported to enhance the binding to HMGB1 [297].

Interestingly, S100, which is a ligand for RAGE, has been detected as a tetrameric state in the human brain. Ostendorp and colleagues proposed that these S100 complexes could trigger multimersation of RAGE and initiation of signal transduction [298, 299]. Since HMGB1 has a charged dipolar structure it has a strong tendency to form polymeric structures, which was first described for the protein isolated from brain tissues [234]. There are no studies addressing whether multimeric HMGB1 is needed for RAGE signalling and/or if a multimerisation of RAGE is needed for HMGB1-mediated signal transduction. Conversely, it has been demonstrated that RAGE/TLR9 associates in HMGB1/CpG-mediated signalling in pDCs [271].

Toll-like receptors

The primary response to pathogens in the innate immune system is triggered by Pattern Recognition Receptors (PRRs) that bind Pathogen Associated Molecular Patterns (PAMPs). Toll-like receptors (TLRs) are well-characterised signal generating receptors among PPRs and recognise a vast number of complex PAMPs [300] and Disease Associated Molecular Patterns (DAMPs), thereby initiating key inflammatory responses [301]. All known TLRs (presently 14) known in mammals are type I integral membrane glycoproteins containing an extracellular domain with leucine-rich repeats responsible for ligand recognition [302]. Most of TLRs are cell-surface receptors but TLR3, TLR7, TLR8 and TLR9 reside in intracellular organelles [303]. TLRs act as homo- or heterodimers or together with other PPRs recognising diverse bacterial and viral ligands [304] and even host DNA [305, 306]. The cytoplasmic domains of TLRs and IL-1-receptors are homologous, thus called Toll/IL-1 receptors (TIRs) [302]. TIR is required for signalling pathways activating the transcription factors nuclear factor κB (NFκB), activator protein-1 (AP-1), which is common to all TLRs, and interferon regulatory factor 3 (IRF) and IRF7. NFκB and AP-1 leads to proinflammatory cytokine production while IRF 3 and IRF7 promote the production of IFNβ and IFNα, -type I interferons.

TLR2 and TLR4

Both TLR2 and TLR4 are membrane-bound receptors. Among a vast number of ligands, TLR2 recognises lipoteichoic acid from gram-positive bacteria and TLR4 binds LPS from gram-negative bacteria (reviewed in [307]). While both TLR2 and TLR 4 are membrane-bound receptors they can be expressed as soluble proteins. Soluble TLR2 is produced through a post-translational modification and is detectable in human plasma and milk, while soluble TLR4 in mice is a result of alternative splicing (reviewed in [300]).

More recently a direct interaction of HMGB1 with TLR2 and TLR4 was determined using fluorescence resonance analysis and immunoprecipitation [308] and HMGB1-mediated TNF release from macrophages obtained from $TLR2^{-/-}$ and $TLR4^{-/-}$ mice was determined to be compromised in comparison to wild-type mice [309].

HMGB1-mediated NFκB activation via TLR2 is dependent on Myeloid differentiation primary response protein 88 (MyD88) and TIR-domain containing adaptor protein (TIRAP) binding to TIR. Furthermore, expression of dominant-negative forms of IL-1R associated kinase 1 (IRAK1), IRAK2, IRAK4, TGF-β activated kinase 1 (TAK1), Tak 1 binding protein 2 (TAB2), TNF receptor-associated factor 6 (TRAF6) or p38 inhibited HMGB1-mediated NFκB activity in macrophages. The signalling cascades initiated by TLR2 and TLR4 exhibit significant overlap but HMGB1-mediated TLR4 signalling seem to be less dependent on TAK1 and TAB2 pathways [310].

A partial overlap exists between RAGE and TLR2 and TLR4 signalling pathways, as depicted in Fig 4 [311, 312]. This sharing of signalling components makes genetic studies and heterologous expression systems difficult to interpret, and has led to contradictory conclusions regarding the roles of TLR2, TLR4 and RAGE, respectively, in HMGB1 signal transduction [293, 310]. Furthermore, the signalling cascades and substrate specificities are also modulated by TLR homo- and hetero-oligmerisation. Thus the binding of HMGB1 to homo- or heterodimeric TLRs remains to be determined. Furthermore, the fact that HMGB1 is very prone to form complexes with several TLR ligands such as LPS and endogenous molecules such as IL-1β complicates the issue of defining HMGB1-specific receptors.

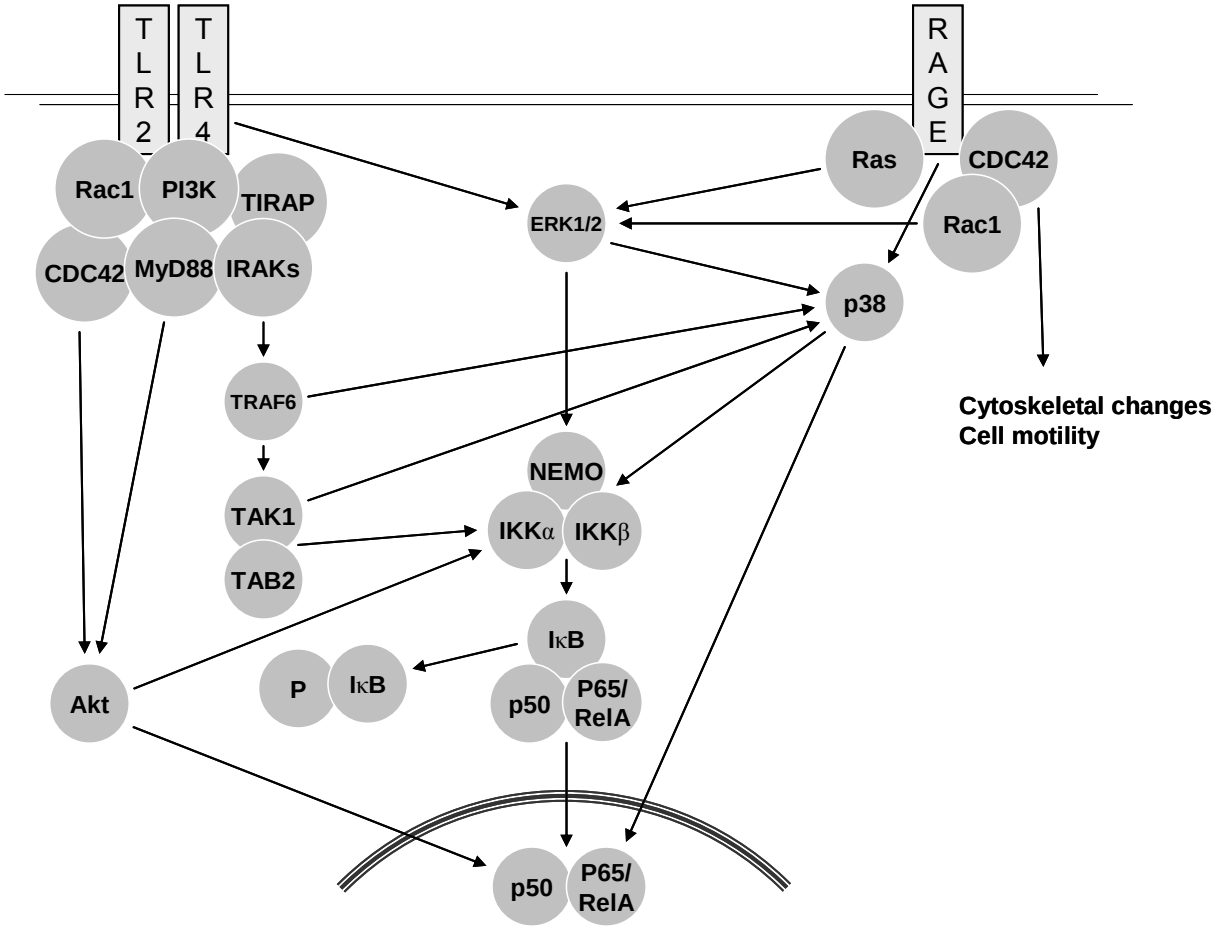

Figure 4. Convergence of TLR2 ,4 and RAGE signalling.
Picture modified from van Beijnum et al. (ref.311)

Apetoh and colleagues illustrated the importance of adequate interaction between HMGB1 released from dying tumour cells and TLR4 expressed on DCs. The cross-presentation of tumour antigens and the promotion of tumour-specific cytotoxic T-cell responses required both HMGB1 and TLR4. Mutation in TLR4 (Asp299Asp to Asp299Gly) decreased its binding to HMGB1 and individuals bearing this mutation failed to cross-present antigens to CTLs from dying melanoma cells. Furthermore, knockdown of HMGB1 with siRNA or treatment with neutralising antibody directed against HMGB1 blunted antigen presentation of tumour antigens and inhibited T-cell priming. However, addition of recombinant HMGB1 could not restore the effects of HMGB1 released from Doxorubicin-treated tumour cells, indicating a requirement of tumour-specific HMGB1 [313, 314] (reviewed in [315]).

Interestingly, the way of dying and the resulting post-translational modifications of HMGB1 seem to be important for HMGB1-mediated DC responses. Kazama and colleagues demonstrated that oxidation of Cysteine 106 alone was sufficient to block the immunogenic activity of HMGB1. Thus DCs are activated by HMGB1 released from necrotic cells which is not oxidised while oxidised HMGB1 released from apoptotic cells induces tolerogenic signals in DCs. Oxidation of HMGB1 was reported to be mediated by caspase 3- and 7-induced ROS activation [263, 316].

ADP-ribosylation of HMGB1 occurs in cells treated with alkylating agents. How this affects the cross-presentation of tumour antigens is as far as I know not yet studied. It is interesting that cell supernatants from cells deficient in the enzyme (PPAR-1) responsible for polyadenylation fail to induce inflammation [262].

TLR9

TLR9 is primarily confined to cells of the immune system and is highly expressed in plasmacytoid DCs but not in conventional DCs [317]. It is initially localised in the endoplasmic reticulum, but redistributes to early endosomes upon stimulation with CpG-DNA [318]. TLR9 recognises synthetic CpG oligonucleotides and unmethylated CpG motifs in bacterial and viral DNA [317] but also host DNA and RNA [305, 319].

While the exact mechanism leading to TLR9 activation by CpG-DNA remains unclear, HMGB1 was recently determined to be an important regulator in this process. It was demonstrated that HMGB1 interacts with TLR9 in the endoplasmic reticulum Golgi intermediate compartment (ERGIC) in quiescent macrophages and accelerates the redistribution of TLR9 to early endosomes in response to CpG-ODN stimulation. Thus immune cells lacking HMGB1 demonstrated a delayed redistribution of TLR9 to early endosomes and an impaired cytokine response to CpG-ODN. Interestingly, the decreased response to CpG-ODN in HMGB1-defective cells could be restored by addition of extracellular HMGB1, suggesting a common feedback loop in which innate immune cells secrete HMGB1 and HMGB1 sensitises immune cells to CpG-DNA through interaction with TLR9 and RAGE [270, 271].

Table 2. HMGB-1 receptors and signaling

Receptor	Comment
RAGE [217, 289, 293]	Interactions with HMGB1 result in MAP kinase activation, enhanced tumor growth, metastases, and release of MMPs. RAGE-HMGB1 also interact with the Rho family of GTPases, Cdc42 and Rac to regulate cell motility.
TLR2/TLR4 [308]	Interaction with HMGB1 results in activation of macrophages and maximal stimulation of NFκB activity in macrophages, along with induction of neovascularization in periods of innate immune system activation.
TLR9 [270, 271]	Interaction with HMGB1 within the ERGIC in quiescent macrophages and accelerates the redistribution of TLR9 to early endosomes upon CpG activation.
Syndecan [274]	HMGB1 links syndecan to the extra cellular matrix participates in cell adhesion and migration of simple epithelial cells, particularly in early cell spreading.
Phosphacan/protein-Tyr Phosphatase γ/β [273]	This receptor participates in proteoglycan-mediated regulation of cell adhesion, neurite growth and cell migration during central nervous system development. It links phosphacan in the extracellular matrix or the transmembrane phosphatase on adjacent cells, to cell surface glycoproteins such as contactin to which phosphacan alone binds only minimally.
Plasminogen [235]	This activates plasmin via formation of ternary complexes, also binds tissue-type plasminogen activator.
TREM -1 [320, 321]	TREM is expressed broadly on myeloid cells. Interaction with HMGB1 amplifies the immune response on THP-cells.

Modified from JE Ellerman 2007, Clin Cancer Research

HMGB1 expression in the arthritic joint

High levels of HMGB1 are present in serum and synovial fluid in rheumatoid arthritis patients [257, 322] with a higher concentration in synovial fluid from RA patients compared with osteoarthritis patients [323]. The hyperplastic synovial tissue in RA patients demonstrates a significant increase in extracellular HMGB1 expression [257, 324].

Tissue hypoxia occurs in the inflamed RA joints due to accelerated oxygen consumption in accumulated inflammatory cells and due to the hyperproliferation of synovial cells. Hypoxia has been considered to have a pathological role in arthritis development through induction of cytokines, matrix-degrading enzymes and angiogenic factors [325]. Thus the increased number of blood vessels detected within the inflamed synovial tissue is suggested to be a consequence of the hypoxic environment. Recently, hypoxia was demonstrated to induce HMGB1 release in cell-based *in vitro* experiments and HMGB1 detected in synovial fluid collected from patients suffering from RA correlated with lactic acid concentrations, which is a tissue hypoxia marker [257]. Thus HMGB1 released during hypoxia is yet a source of extracellular HMGB1 within RA tissue.

Putative role of HMGB1 in RA

In the collagen-induced arthritis model both the protein and mRNA levels of HMGB1 were elevated at the areas of cartilage bone destruction [326].

Interestingly, FLS in the arthritic joint express RAGE [323, 327, 328] and binding of HMGB1 induces invasive feature of FLS. HMGB1-mediated invasiveness could be completely inhibited by antibody treatment targeting RAGE, suggesting that RAGE-targeted therapy could inhibit the cartilage and bone invasion by FLS [329]. In our studies we have demonstrated that HMGB1 in complex with trace amounts of LPS or IL-1β induced TNF, IL-6 and IL-8 production from FLSs and enhanced their MMP1 and MMP3 production, thus increasing the inflammatory and destructive phenotype of FLS (*Paper V*).

HMGB1 promotes vasculogenesis by inducing chemotactic migration of adult human peripheral blood-derived endothelial progenitor cells (EPC), an important mechanism in rescue of ischemic tissues. The enhanced adhesion of EPCs at a site of ischemia and active angiogenesis is mediated by HMGB1/RAGE-induced β-integrin upregulation on EPCs [330].

The initiation of angiogenesis, the growth of new blood vessels from pre-existing ones, begins with the degradation of the basement membrane by activated endothelial cells. These proliferate and migrate, leading to the formation of solid sprouts to the stroma. Macrophages produce a number of potent angiogenetic cytokines and growth factors, e.g. TNF, IL-8 and VGEF. Recently, HMGB1 has been demonstrated to promote

angiogenesis in a dose-dependent manner in an *in vitro* angiogenesis assay [331]. Blocking of RAGE inhibited HMGB1-induced neovascularisation *in vivo* and endothelial cell proliferation and membrane ruffling *in vitro,* demonstrating HMGB1 and RAGE dependency during blood vessel formation and in wound healing [332].

Interestingly, a high HMGB1 expression pattern was demonstrated in the endothelial cells of RA synovial tissue samples despite local glucocorticoid treatment [324], and an active HMGB1 release has been demonstrated from activated endothelial cells [252, 333] and from smooth muscle cells lining the arteries [252] suggesting that local HMGB1 production could promote angiogenesis in RA.

An activated endothelium is required for the transmigration and accumulation of immune cells to the site of inflammation. Recent publications demonstrate that HMGB1 possesses the capacity to both activate the endothelial cells by upregulating the adhesion molecules ICAM-1, VCAM-1 and E-selectin, and through attracting inflammatory cells to the site of inflammation by inducing IL-8 and MCP-1 production [242, 246, 295, 334]. Treutiger and colleagues demonstrated that neutrophil adhesion to activated endothelial cells was partly mediated by RAGE. Furthermore, it was recently demonstrated that both RAGE and Mac-1 β2 integrin interaction was required in HMGB1-mediated neutrophil recruitment in an *in vivo* model of peritonitis and for increased adhesion to ICAM-1 *in vitro* [292, 335]. Since Mac-1 expression is only restricted to neutrophils and macrophages it is plausible that recruitment of other proinflammatory cells requires interaction with other molecules together with RAGE. Additionally, HMGB1 activates neutrophils [336].

When NK cells are in physical contact with immature DCs they start to produce IL-18 that stimulates NK cells to produce HMGB1 [337]. Extracellular HMGB1 in turn has been demonstrated to promote DC migration [338] and maturation [339, 340]. HMGB1 is further secreted from stimulated DCs, creating an intrinsic loop of inflammation-promoting events. HMGB1 secreted from DCs is crucial for T cell proliferation, survival and polarisation to a Th1 phenotype, and this is mediated by HMGB1/RAGE interaction [340, 341]. Among T cells, Th1 cells are the most prominent cell population in the inflamed RA synovial tissue [108, 109] mediating inflammation and bone destruction.

Phagocytosing macrophages release HMGB1 [342] and extracellular HMGB1 can further induce monocyte migration [250] and enhance the expression and secretion of

other proinflammatory cytokines such as TNF, IL-1β, IL-6 and IL-8 [343, *paper V*]. All these cytokines occur in the arthritic joint, promoting inflammation and bone destruction. Bone destruction in RA is mainly mediated by osteoclasts and HMGB1 and RAGE are mediators of osteoclastogenesis [344]. HMGB1 is also released by osteoclast precursor cells, osteoclasts and osteoblasts [345]. It is also demonstrated that HMGB1 promotes chemotaxis and osteoblast differentiation from mesenchymal stem cells [346] indicating a bone anabolic activity of HMGB1.

Taken together, HMGB1 is an abundant molecule in the arthritic joint and different cell populations, namely endothelial cells, neutrophils, NK-cells, DCs, T-cells, monocytes and macrophages, are potent sources of secreted HMGB1. Extracellular HMGB1 is a potent amplifier of local inflammatory responses by enhancing the release of cytokines, chemokines and by promoting osteoclastogenesis and pannus invasion into the cartilage and bone. Modulating HMGB1 activity may provide a new strategy to block the intrinsic loop of inflammation and joint destruction.

HMGB1 targeting therapies

The importance of HMGB1 in arthritis has been further demonstrated by use of animal models, in which the blocking of HMGB1 activity using different strategies has had beneficial effects on arthritis development. To inhibit HMGB1-mediated activities the strategies which have been used include: *i)* targeting of extracellular HMGB1; *ii)* receptor blocking (may be different for inflammation and invasiveness); and *iii)* inhibition of HMGB1 secretion.

Targeting extracellular HMGB1

HMGB1 neutralising polyclonal antibody and the antagonistic A-box residue ameliorated the disease course of collagen-induced arthritis in both the mouse and rat. The mean arthritis score, disease-induced weight loss and histological severity of arthritis as well as IL-1β expression in the synovial tissue were significantly reduced in the treated animals [257, 347].

Thrombomodulin is an endogenous endothelial anticoagulant that binds to HMGB1 and prevents the extracellular proinflammatory activities of HMGB1 [348]. Treatment with truncated forms of thrombomodulin was a successful therapy in several forms of experimental arthritis [349].

Receptor blocking

Administration of sRAGE suppressed clinical and histological signs of arthritis and decreased the RAGE expression, TNF, IL-6, MMP-3, -9 and -13 levels in DBA/1 mice joints, limiting both inflammation and bone and cartilage destruction [350].

Inhibition of HMGB1 secretion

Ghrelin (GHR) is a 28-aa polypeptide mainly produced by stomach cells and is involved in the control of growth hormone secretion and adiposity [351]. Collagen-induced arthritis treated with GHR attenuated the arthritis severity, with decreased HMGB1 levels in serum compared with untreated animals. Translocation of HMGB1 from the nucleus to the cytoplasm in LPS-stimulated macrophages was inhibited by GHR treatment, indicating a decreased secretion of HMGB1 [352].

Oxaliplatin a commonly used anti-cancer drug creating platinated DNA adducts where HMGB1 is sequestered [353]. Systemic treatment with oxaliplatin ameliorated collagen type-II induced arthritis in mice and blocked the nuclear translocation of HMGB1. However, the treatment effect was not persistent and a rebound effect was determined with an increased disease induction and severe synovial inflammation. This rebound effect correlated with an increased extracellular HMGB1 expression (*Paper III*).

METHODS USED IN THE PAPERS

Cell cultures

C1MC/C57.1	*(I)*
HCT 116	*(I)*
HMC-1.2	*(I)*
PBMC	*(IV)*
RAW264.7	*(I, II, III)*
Synovial fibroblasts	*(V)*
THP-1	*(II)*

Detection of cytokines and MMPs

CBA	*(V)*
ELISA	*(II, IV)*
TNF ELIspot	*(II, IV)*
Western blotting	*(II, IV)*

Detection of HMGB1

ELIspot	*(I, II, III)*
Immunohistochemistry	*(III)*
Immunocytochemistry	*(I, II, III)*
Western blotting	*(III, IV)*

Induction and evaluation of arthritis

CIA	*(III)*

RESULTS & DISCUSSION

Development of an HMGB1-specific ELIspot assay and determination of HMGB1 secreting capacities of several cell lines.

(*Paper I*)

In the first study an HMGB1-specific ELIspot method for both adherent and non-adherent cells was developed. Several different antibodies were screened in order to elucidate the optimal combination of antibodies to detect HMGB1 secretion from cells. The capture antibody in our HMGB1 ELIspot is a non-commercial mouse monoclonal antibody, 2G7 CTI (Boston, MA, USA) that recognises the amino acid sequence 53-63 within the A-box region of the HMGB1 molecule. The detection antibody is a commercial affinity-purified rabbit polyclonal antibody PharMingen (San Diego, CA, USA) detecting amino acids 166-181 at the linker region between the B-box structure and the acidic tail of HMGB1. The specificity of the ELIspot assay was confirmed by replacing the capture or detection antibodies with isotype- or irrelevant control antibodies, or by omitting the detection antibody. No spots were detected with these control antibodies from either stimulated or unstimulated RAW246.7 cells.

The kinetic pattern of HMGB1 release could be determined by discriminating between low- and high- intensity spots. Low intensity spots reflect the initial phase of HMGB1 secretion and high intensity spots signify high output HMGB1 secretion. Stimulation with IFNγ alone induced a fast HMGB1 release from RAW264.7 cells detected as the highest amount of low intensity spots after 24h of stimulation. Conversely, TNF was a poor inducer of HMGB1 secretion. Stimulation with LPS alone or with IFNγ+LPS generated a granular HMGB1 pattern after 24h of stimulation as detected by immunocytochemistry, indicating that HMGB1 was secreted via secretory lysosomal pathway from the RAW264.7 cells. However, in IFNγ stimulated cells this pattern occurred after 48h of stimulation, demonstrating that HMGB1 secretion was not always preceded by HMGB1 granule formation, suggesting additional pathways for HMGB1 secretion.

HMGB1 is known to be highly expressed in malignant cells [229, 354]. In my study I could demonstrate that not all transformed cell lines tested were able to actively secrete HMGB1. The human colon cancer cell line HCT 116 had a high spontaneous secretion of HMGB1, while the mast cell lines C57 and HMC-1.2 did not even release HMGB1 following stimulation. HMGB1 release was further detected from water-disintegrated cells (resembling necrotic cells), but was not detected from apoptotic cells (induced by p53 activator PRIMA-1).

Although we did not detect HMGB1 release from apoptotic or all transformed cell lines tested, we should consider the possibility that it might depend on conformational differences or post-translational modifications of HMGB1 [260-265]. For instance, PRIMA-1 induces apoptosis by activating p53 which in turn activates the caspase proteinases which induce oxidation of HMGB1 [263]. It is possible that oxidised HMGB1 is not detected by the antibody pair used in our ELIspot assay. It is therefore crucial to evaluate the specificity of HMGB1 antibodies that are used in different bioassays.

HMGB1 secreted by the exosomal pathway would neither be detected by ELIspot or ELISA, since HMGB1 is then encircled by membrane, pinpointing the necessity to combine different techniques when studying HMGB1 secretion.

Inhibition of HMGB1 secretion by inducing nuclear retention of HMGB1

(*Paper II & III*)

In the second and third studies we investigated the capacity of gold sodium thiomalate and oxaliplatin to inhibit HMGB1 secretion.

The first clinical trial with gold compounds was led by a French physician, Forestier in 1929. Thirty years later (1960) gold therapy was demonstrated to be clinically efficient in a controlled study (reviewed in [355]). The intramuscular gold treatment in subgroups of patients with RA reduced both disease activity [356] and cartilage destruction [357]. Although the clinical efficacy of gold salts is well established in RA the mechanism of action is not fully understood. The so far described anti-

inflammatory effects of gold salts include suppression of the proinflammatory mediators IL-1β, NO and PGE2 [358] and inhibition of NF-κB activity [359].

After absorption the gold complex dissociates rapidly in blood plasma, generating gold-albumin complexes and the thiomalate moiety is converted to a free thiolate form. After repeated administration gold is concentrated in the kidneys, liver, spleen and synovial tissue. It is taken up by macrophages and the gold is almost exclusively deposited in lysosomes in macrophages (reviewed in [355]).

I wanted to elucidate whether Myocrisin (GST), the most commonly used gold compound, could inhibit HMGB1 secretion from human THP-1 monocytes and murine RAW264.7 macrophage cell lines. We started to investigate whether IFNγ+LPS- or p(I:C)-induced-HMGB1 secretion could respectively be inhibited by GST treatment.

THP-1 and RAW264.7 cells pretreated with GST and followed by IFNγ+LPS stimulation displayed a dose-dependent decrease in HMGB1 secretion determined by both ELIspot and Western blotting analyses. Intracellular HMGB1 staining of IFNγ+LPS stimulated RAW264.7 cells displayed a cytoplasmic HMGB1 pattern compared with GST-treated cells, which display a more nuclear HMGB1 staining indicating a nuclear retention and a decreased HMGB1 secretion. Similarly, GST-mediated inhibition of HMGB1 secretion was determined by p(I:C)- or LPS-stimulated RAW264.7 cells (determined by Western blot). In agreement with previous data [358], TNF production was not affected by GST treatment.

NO and type I IFNs have been reported to be downstream key mediators of HMGB1 release [254]. NO production is triggered by LPS, p(I:C), IFNγ and TNF stimulation and IFNβ production is induced by LPS and p(I:C). We therefore further investigated whether GST also influenced the production of these key mediators and if GST could inhibit HMGB1 secretion induced by NO and IFNβ. Indeed, RAW264.7 cells pretreated with GST followed by stimulation with either LPS or p(I:C) demonstrated decreased IFNβ and NO levels as determined by ELISA and the Greiss method, respectively. Furthermore, the IFNβ and NO donor NOC-15-induced HMGB1 secretion was attenuated by GST-treatment as determined by Western blotting, demonstrating that GST-treatment affects LPS-, p(I:C)-, IFNβ- and NO-induced HMGB1 release.

We also verified that the GST-mediated inhibition of HMGB1 was related to the gold component itself rather than to the thiomalate moiety in the GST, since the thiomalate

moiety itsef did not induce HMGB1 attenuating effects. We could also demonstrate that $AuCl_3$, another gold compound, attenuated both HMGB1 and NO secretion from RAW264.7 cells, which further strengthens the view that gold compounds in general function as inhibitors of HMGB1 secretion.

We did not elucidate whether the phagocytosis of gold particles *per se* inhibited HMGB1 release from monocytes/macrophages. There is one report concerning increased HMGB1 release during phagocytosis of apoptotic material [342] thus describing a distinct event from gold particle phagocytosis.

Paper III

Oxaliplatin and other platinated anti-tumour compounds generate DNA adducts, leading to nuclear sequestration of HMGB1 [360]. In the third study we aimed to verify the oxaliplatin sequestering capacity of HMGB1 both in *in vitro* and *in vivo* models and to study whether that influenced the course of collagen type II-induced arthritis.

In vitro studies using RAW264.7 cells stimulated with IFNγ+LPS demonstrated a nuclear retention of HMGB1 when co-cultured with oxaliplatin. In addition, the secretion of HMGB1 was inhibited by oxaliplatin treatment as determined by ELIspot. Conversely, TNF production was not affected by oxaliplatin treatment. Cultures with lymph node cells challenged with ovalbumin displayed an inhibited proliferative activity in the presence of oxaliplatin, an important finding since CIA is a T cell-dependent arthritis model.

DBA/1 mice were challenged with bovine collagen type II and treated with one intraperitoneal injection of oxaliplatin at the expected onset of CIA. Administration of oxaliplatin in early arthritis delayed the disease onset and ameliorated the clinical signs of arthritis. In an attempt to prolong the positive effect of oxaliplatin treatment the study was repeated and the animals were treated with an additional injection of oxaliplatin. The additional dose of oxaliplatin prolonged the period of reduced arthritis but did not affect the arthritis incidence. In both settings an aggressive disease flare was observed one week after the last dose of oxaliplatin was given. Analysis of articular tissue demonstrated a nuclear HMGB1 staining pattern which correlated well with the low clinical arthritis score, while an excessive cytoplasmic and extracellular HMGB1 pattern correlated with the time point of disease flare.

Apetoh and colleagues reported that HMGB1 is released from oxaliplatin-treated cells undergoing apoptosis, thereby confirming our results [313, 314]. They further described that cross-presentation of tumour antigens and the promotion of tumour-specific cytotoxic T-cell responses required tumour-derived HMGB1 to bind to TLR4 on DCs. It is currently not known if disease flares in oxaliplatin treated CIA mice are dependent on such DC-mediated immune responses. Alkylating agents have been demonstrated to induce ADP-ribosylation of HMGB1 [262]. Oxaliplatin belongs to this group of drugs and it is therefore plausible that the released HMGB1 is ADP-ribosylated. Whether ADP-ribosylated HMGB1 is more immunogenic than unmodified HMGB1 or if ADP-ribosylation is required for TLR4 binding is yet to be determined.

It is also possible that the rebound effect with aggressive arthritis in oxaliplatin-treated CIA mice could depend on HMGB1-mediated chemoattraction of proinflammatory cells.

Studies of the cytokine inducing capacity of HMGB1 alone or in complex with inflammation promoting molecules

(*Paper IV &V*)

Highly purified HMGB1 batches have been demonstrated to possess low or no cytokine-inducing activity [275, 361]. In these last two studies I wanted to further study the cytokine-inducing capacity of highly purified HMGB1. I studied HMGB1 alone or in complex with IL-1β, LPS, Pam3CSK4, CpG-ODN, TNF, RANKL, p(I:C) or IL-18, respectively.

In study IV we used peripheral monocytes (PBMCs) to investigate the proinflammatory capacity of HMGB1. Native HMGB1 purified from calf thymus or recombinant HMGB1 purified from viral or bacterial sources were used. None of these HMGB1 batches induced IL-6 production from freshly isolated PBMCs. In contrast, IL-6 production was synergistically enhanced when PBMCs were stimulated with HMGB1/LPS complexes. These HMGB1/LPS complexes contained suboptimal concentration of LPS, which alone did not induce IL-6 production. HMGB1 together with Pam3CSK4 or CpG-ODN also displayed synergistic effects on IL-6 production in

PBMCs, even though the effect was not as pronounced as was the HMGB1/LPS complex-induced IL-6 production. Monocytes express high levels of TLR1/TLR2 that mediates Pam3CSK4 signalling while the TLR9 expression is very low on monocytes. It has been reported that the pDC-containing monocyte population is sensitive to CpG-ODN-mediated stimulation while pure monocyte populations do not respond to stimulation with CpG-ODN. The authors further suggested that monocyte activation within human PBMCs is due to secondary effects of CpG-ODN-mediated signalling via pDCs [362]. It is therefore not surprising that the CpG-ODN was a weak inducer of IL-6 production from our PBMC cultures.

HMGB1 together with TNF, RANKL, p(I:C) or IL-18 failed to induce IL-6 production from PBMCs. A recent publication reported that HMGB1 had only a weak association to TNF, while HMGB1 was shown to bind avidly to IL-1β [272]. In our study we did not investigate if these latter molecules failed to form complexes with HMGB1, but we clearly demonstrated that HMGB1 acts selectively together with certain molecules, and that the synergistic activity of HMGB1 is not only dependent on non-specific interaction with each and every protein.

Paper V

In study V we investigated the proinflammatory effect of HMGB1 alone or in complex with IL-1β or LPS on synovial fibroblasts (RASF) from RA and osteoarthritic (OASF) patients. Cells were stimulated for 9h and the number of TNF producing cells was determined by ELIspot. After 24 hours of stimulation the supernatants were collected and IL-1β, IL-10, IL-6 and IL-8 levels were determined using a CBA assay and MMP-1 and MMP-3 levels were analysed by ELISA. HMGB1 alone did not induce cytokine or MMP production. In contrast, stimulation with HMGB1 in complex with IL-1β or LPS had a synergistic effect on TNF, IL-6 and IL-8 production, respectively, and enhanced the MMP-1 and MMP-3 production as compared with stimulation by IL-β or LPS alone. This is the first report to demonstrate the effects of HMGB1-complex-mediated activation of synovial fibroblasts.

In my study, I could not determine any difference between the HMGB1/IL-1β-induced cytokine and MMP production between RA and OA fibroblasts. Interestingly, high levels of IL-1β are evident in RA synovial tissues and to a lesser extent in OA patients. The quantities of HMGB1 in the synovial fluid from OA patients are much lower

compared to the levels in RA patients [323]. It is therefore possible that the complex formation between HMGB1 and IL-1β occurs more frequently in the arthritic joint, leading to a more pronounced inflammation with an increased bone and cartilage destruction in RA patients compared to in OA patients.

CONCLUDING REMARKS & FUTURE PERSPECTIVES

The scientific messages and innovations in this thesis work can be summarised as follows:

- An HMGB1-specific ELIspot assay was developed that enabled assessments of active and passive HMGB1 release from both adherent and non-adherent cells.
- Gold salt was determined to reduce HMGB1, IFNβ and NO secretion from macrophages, while it did not influence their TNF secretion.
- Oxaliplatin ameliorated experimental arthritis by inducing nuclear retention of HMGB1. The observed disease rebound effect correlated well with increased amounts of extracellular HMGB1, indicating the importance of HMGB1 as a pro-inflammatory mediator in arthritis.
- The inflammatory and destructive activity of HMGB1 was demonstrated to be mediated by complex formation of HMGB1 with other inflammation-inducing molecules.

Rheumatoid arthritis is a chronic systemic inflammatory disease encompassing severe inflammation and destruction of synovial joints. An increasing body of evidence suggests that HMGB1 has a central role in RA pathogenesis since increased levels of HMGB1 are evident in the synovial fluid and an extracellular HMGB1 pattern can be demonstrated in the synovial tissues of RA patients. HMGB1 is released by a variety of cells in the inflamed RA joint and reportedly promotes a vast number of inflammation-promoting features such as angiogenesis, endothelial activation, inflammatory cell recruitment and tissue destruction [363].

Changes that occur in the synovial lining layer during RA development resemble the peritoneal lining during chronic ambulatory dialysis in which epithelial cells become hyperplastic and exhibit a transformed mesenchymal (myofibroblast) phenotype. These changes are induced in a process called ‘epithelial-to-mesenchymal transition’ (EMT) [364]. In a recent study, Steenvoorden and colleagues demonstrated that the myofibroblast marker α-sma was expressed in RA FLS but not in healthy subjects, indicating a transformed EMT phenotype in RA FLS. Interestingly, α-sma expression

in heathy FLS could be induced by stimulation with synovial fluid obtained from RA patients [365]. TGF-β is the most well known inducer of EMT, but it can also be induced by the RAGE ligands S100 calgranulins and AGEs [240]. Increased levels of TGF-β, AGEs and S100 calgranulins are detected in synovial fluid in RA patients [366, 367]. Several S100 proteins and AGEs are ligands to RAGE. Together with increased expression of RAGE in FLS the induction of the EMT phenotype may be promoted by RAGE/RAGE-L interaction in RA. Since it has been reported that HMGB1 competes with AGEs for binding to the RAGE ectodomain [284] it would be interesting to investigate if HMGB1 could induce EMT in healthy synovial fibroblasts and thereby reveal if HMGB1 may be an important molecule already at the early phase of RA.

Pullerits and colleagues demonstrated that intra-articular HMGB1 injections did not induce arthritis in IL-1R gene deficient mice [243] demonstrating co-operation between HMGB1 and IL-1β. My results presented in this thesis demonstrate a distinct co-operation between HMGB1 in complex with IL-1β, generating a synergistic effect on proinflammatory cytokine and MMP production in synovial fibroblasts (*paper V*). Such complex formation between HMGB1 and IL-1β may occur *in vivo* since both HMGB1 and IL-1β are highly expressed within the arthritic joint [323].

Interestingly, several reports demonstrate that IL-1β is able to drive both bone and cartilage destruction independently of TNF [184]. TNF gene deficient animals displayed a similar degree of joint inflammation as the corresponding control TNF wildtype animals when challenged with intra-articular HMGB1 [368] thus demonstrating that HMGB1-mediated joint inflammation is not dependent on TNF induction. Furthermore, a recent study from Sundberg and colleagues demonstrated that the extracellular HMGB1 expression pattern did not decrease in the synovial tissues following anti-TNF treatment [369]. All these results thogether indicate that TNF and HMGB1 may act independently of each other and that HMGB1-mediated joint inflammation and destructive features of synovial fibroblasts may dependent on IL-1R-mediated signalling.

Taken together, these results suggest new approaches to treat patients who do not respond to anti-TNF therapy.

It will be a future challenge to develop drugs which specifically target the disease-promoting HMGB1 molecule since an increasing body of evidence suggests that pure HMGB1 promotes tissue regeneration and cell migration [275] whereas in complex with inflammation-promoting agents such as CpG, IL-1β and LPS it promotes inflammation and tissue destruction [269-272, *papers IV,V*].

It is also apparent that HMGB1 also undergoes diverse post-translational modifications [260-263, 265]. It can be cleaved by Thrombin-thrombomodulin complexes [266] and is also able to make complexes with IgG [267, 268]. These modifications and molecular interactions of HMGB1 most likely influence the wide receptor binding capacity and diverse functions of HMGB1.

A recent report illustrated the importance of HMGB1 in the error-free repair of DNA damage. The lack of HMGB1 led to increased mutagenesis and reduced cell survival in mammalian cells following exposure to DNA-damaging agents [373] thus emphasising the importance of targeting only the disease-promoting form of HMGB1.

Protein transduction domains which promote cargo delivery cross cell and organell membranes are typically rich in basic amino acids (lysine, arginine and histidine) [370]. HMGB1 has an exceptional dipolar structure with a highly basic amino-terminal structure, consisting of 184 amino acids there on an average every fourth amino acid is a lysine [229]. Thus, the structure of HMGB1 might influence the capacity of HMGB1 to enter cells through non-receptor mediated mechanism. Since HMGB1 has been used as a nonviral gene delivery agent [371, 372] it is a possibility that therapies directed against HMGB1 could be delivered into cells in a cargo dependent manner.

One potential approach to identify HMGB1 suppressive agents is to study the HMGB1-suppressing capacity of already known drugs that have been shown to be safe in clinical use. In this thesis two well-known compounds were studied regarding their capacities to inhibit HMGB1 secretion. Both oxaliplatin and gold salt induced nuclear HMGB1 retention and decreased HMGB1 release. From a clinical point of view regarding arthritis it was interesting to study gold salts which are used as efficacious anti-rheumatic drugs in a subgroup of RA patients. Gold salt treatment of macrophages displayed a suppressive effect on HMGB1 release but did not suppress their TNF secretion. Thus patients with HMGB1- (and IL-β)-specific cytokine profiles might in particular benefit from therapy based on gold salt treatment.

SVENSK SAMMANFATTNING

Reumatoid artrit eller som sjukdomen i allmänhet kallas: reumatism, är en inflammatorisk sjukdom som främst drabbar lederna med påföljande vävnadsskada och invaliditet. Forskarna och läkarna är överens om att den ihållande inflammationen drivs av olika proteiner bland andra TNF och IL-1β. Terapier riktade mot dessa proteiner hjälper många patienter, men det finns fortfarande en stor grupp av reumatiker som har en aktiv sjukdom med pågående inflammation som ger upphov till vävnadsskador i både ledbrosket och i benet.

Färska studier visar att det finns ytterligare andra proteiner som kan vara viktiga i den reumatiska inflammationen. HMGB1 är ett protein som har påvisats i stora mängder i den artritiska leden samt i ledvätskan hos reumatiker. Djurstudier har visat att behandlingar riktade till att hämma HMGB1 minskar ledinflammationen och vävnadsskadorna hos försöksdjuren.

Syftet med mina studier har varit att vidare studera betydelsen av HMGB1 i reumatism. Vi utvecklade en känslig metod för att på cellnivå kunna mäta utsöndringen av HMGB1 från olika celletyper. Genom att studera inflammatoriska celler och deras förmåga att aktivt utsöndra HMGB1 har vi kunnat med hjälp av läkemedel påverka utsöndringsgraden av HMGB1. Vi har kunnat visa att en hämning av HMGB1-utsöndring resulterar i ett minskat inflammatoriskt svar från celler och i en minskning av ben- och brosknedbrytning. Vi har vidare kunnat visa att HMGB1 samarbetar med andra inflammatoriska proteiner och därmed intensifierar inflammationen och den vävnadsskadande förmågan hos ledspecifika celler.

Våra studier har därmed bekräftat att HMGB1 är en central molekyl i den reumatiska inflammationen och därmed en potentiell målmolekyl för kommande terapier.

Vår förhoppning är att våra studier ska leda till, att fler patienter med svår reumatism ska kunna få en mer effektiv behandling.

ACKNOWLEDGEMENTS

Eftersom alla mina engelska ord har tagit slut, kommer den här sektionen att skrivas på svenska. Jag vill uttrycka mitt hjärtligaste tack till alla, som på ett eller annat sätt har stöttat mig på vägen, och hjälpt mig med att nå mitt mål. Ett extra stort tack vill jag rikta till:

Helena Erlandsson Harris, som har varit min huvudhandledare under mina år som doktorand. Du har alltid ställt upp, både som en vetenskaplig mentor och som en medmänniska. Du har varit lyhörd, hjälpsam och uppmuntrande. Jag älskar våra hemliga projekt!!! Jag är ytterst tacksam för att jag fick vara din kyckling och att du lärde mig att hitta de vetenskapliga vetekornen bland agnarna.

Ulf Andersson, min bihandledare, som med skräckblandad förtjusning? har funnits vid min sida. Tack för allt stöd, alla vetenskapliga diskussioner och alla uppmuntrande mail :). Du har lärt mig att fokusera och därmed bli en mer effektiv forskare!

Lars Klareskog, tack för att du med ditt brinnande intresse för vetenskapen bidrar till den kreativa forskningsmiljön. Du är generös och har alltid tid med uppmuntrande ord.

Tomas Olsson, som hjälpte mig att hitta ett bra doktorandprojekt hos Helena.

Bob Harris, som med kort varsel och på sin lediga tid granskade mitt avhandlingsarbete! Jag vill också tacka dig för att du alltid ställer upp och att du bidrar med god stämning på labbet.

HMGB1-gruppen. Hur ska jag kunna tacka er alla? Alla ni har betytt otroligt mycket för mig! Jag börjar i den ordningen jag träffade er. Lotta, du har varit en helt fantastisk arbetskamrat! Tack för alla sena kvällar vid mikroskopet och alla hysteriska skratt och stöttande kramar när experimenten inte fungerade. Tack för att du trodde på mig!
Riikka Kokkola (Söderling), tack för din vänskap när jag kom till labbet, nu kommer Turpos bok! Karin, tack för alla glada stunder på labbet. Jag vill speciellt tacka dig för din omtänksamhet under mitt avhandlingsarbete, -tack för att du hjälpte mig med layouten trots att du fick jobba fram till småtimmarna!
Therese, tack för alla gånger du har fixat flygbiljetter och andra blanketter till mig! Tack för att du har guidat mig i Stockholm, Island och i New York! Erik, vår filosof som får mig på gott humör! Du håller alltid vad du lovar... 21:e är ett exempel på det! Cecilia, tack för ett roligt samarbete med guld-peket! Du är en frisk och glad fläkt! Du måste komma tillbaka och lösa IL-1R och HMGB1-sambandet? Tomas (lurifax), tack för alla luffarschackturneringar, -man ska aldrig ge upp!!! Tack Fredrik för alla försök att hitta HMGB1 i serum... jag har lite prover kvar i en handske i frysen... när kommer du igen? Lasse, min hemliga beundrare, tack för all hjälp med datorn och alla goda hembakade pajer, du är så bra! Hulda, min lott-kompis! Tack för att du alltid finns till hands, du kan konsten att lugna ner mig när jag är stressad. Hanna, vad skulle jag ha gjort utan dig? Tack för all ”Slabb-hjälp” och att du har tagit ansvar för gastuberna!!! Och till sist Lena, jag är så glad att du har börjat hos oss, nu är det ännu roligare på labbet! Du fixar allt: från frisörtid till att uppehålla paketbud med din kvinnliga charm! Vad hade kalendern varit utan dig?

Tack Ewa W och Karin L för er vänlighet, för alla vetenskapliga diskussioner och all hjälp på labbet. Rüdiger för ditt glada och impulsiva sätt som alltid får mig på ett glatt humör!

ALLA medförfattare och alla ni som jag har samarbetat med. Tusen TACK!!!

Jag vill tacka ALLA på reumatologens- och ALLA på neurologensforskningslab. Ett specellt stort tack till Emeli, min statistik-guru och Sevim min datorhjälpreda under åren!

Ett extra tack till alla laboratorieassistenter, ni har delat med er av ert kunnande och tid och i vissa situationer har ni betytt ALLT för mig! I bokstavsordning: Britt för alla roliga E-mail, kramar och alla djupa samtal i soprummet, Eva J för allt du gör för labbet och alla uppmuntrande ord och ditt tålamod när jag för 50-elfte gången frågar var apoteksbeställningslistan ligger..., Eva L som alltid tar sig tid att hitta antikroppar, telefonnummer eller hemliga recept, Gull-Britt för alla spännande och roliga historier under kafferasterna, Marianne Engström som med stor entusiasm driver på vårt ”hemliga sidoprojekt”, du är en kunskapsgruva, och Åse, tack för shoppingturerna och alla berättelser om livet, blommor och onda magar, du har alltid tid när jag har behövt ha någon att prata med!
Camilla Berg, Gunnel Bremerfeldt och Susanne Karlfeldt med er hjälp blir allting så mycket enklare! Hur ska jag någonsin kunna tacka er...TACK!!!

Rudolf och Dagmar för all datorhjälp (inga frågor är dumma) och Katarina för alla roliga morgonstunder och för snabb hjälp med instrumenten när det behövdes!

Mina Uppsala-kollegor; Kristina Nilsson Ekdahl och Bo Nilsson, tack för att ni gav mig möjligheten att göra mitt examensarbete hos er och Kristina för att du tände min låga för immunologi! Tack ALLA på komplementlabbet, jag har saknat er och jag SKA höra av mig!

Therese V, för din vänskap, stöd och lottintresse! Du är en labb-klippa! och jag lovar att höra av mig nu när disputations-cirkusen är över!!! Kram på dig over there!

Monica, Madeleine och Karin, tack för att ni har tvingat mig att göra saker utanför labbet! Jag SKA fixa en fest hemma hos mig! Och Persbrant, vi måste dejta igen!

Jag vill tacka alla lärare och handledare på Kalmar Högskola som lärde mig vikten av kritiskt tänkande och gav mig grunderna för att kunna skriva en läslig labrapport! Tack också till alla klasskamrater för allt kul under studietiden! Bozena, du har varit en fantastisk vän genom alla åren! Tack för ditt stöd och uppmuntran, alla borde ha en vän som du! Tack till din underbara familj, Sam, Vincent och Tristan.

Tack till ALLA vännerna utanför labbet, som har fått mig att tänka på andra saker än HmbGett, även ni som jag sällan hör av mig till. Ett specellt tack till mina nya vänner i Uppsala och Tobo. Ett tack till mina trogna tåg-kompisar, Rita, Bengt & Anders och Janne, jag har lärt mig en hel del om gener, fåglar, patentansökningar och växrter.

Kiitokset kaikille sukulaisille, ystäville ja kumminkaimoille siellä Suomessa ja Johannalle siellä Englannissa!

Kiitokset äiti ja isä kaikesta! Olette aina tukeneet ja yrittäneet ymmärtää. Kiitokset ihanista kesistä mökillä ne ovat olleet henkireikiäni. Kiitokset Jukka-veljelleni, Marionella- siskolleni sekä Johanille iloisista hetkistä. Kiitokset Ailalle ja Hansille kaikista rohkaisevista sanoista ja ihanista illoista. Kiitokset Simolle, Jaanalle ja Veralle hauskoista tuokioista luonanne.

Till sist, till min underbara lilla familj. Tack Iris för din kärlek! Alla små lappar med hjärtan och med ”Du-är-bäst-mamma”-meddelanden. Tack för KÄMPE-kaninerna, dödskalle-amuletten och alla ”en-liiiten-stund-till-kramar”, du har gett mig kraft och styrka att orka vidare! Toni, min älskade man och vän. Vad vore jag utan dig? Tack för allt stöd, kärlek, all omtanke och alla kramar under den här tiden. Ni två betyder ALLT för mig! Jag älskar er!

REFERENCES

1. Firestein, G.S., *Evolving concepts of rheumatoid arthritis.* Nature, 2003. **423**(6937): p. 356-361.
2. Rose, N.R. and I.R. McKay, eds. *The autoimmune diseases*. 3rd ed. 1998, Academic press.
3. Cooper, G.S. and B.C. Stroehla, *The epidemiology of autoimmune diseases.* Autoimmun Rev, 2003. **2**(3): p. 119-25.
4. Doran, M.F., et al., *Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period.* Arthritis Rheum, 2002. **46**(3): p. 625-31.
5. Silman, A.J., et al., *Twin concordance rates for rheumatoid arthritis: results from a nationwide study.* Br J Rheumatol, 1993. **32**(10): p. 903-907.
6. Wordsworth, B.P., et al., *HLA-DR4 subtype frequencies in rheumatoid arthritis indicate that DRB1 is the major susceptibility locus within the HLA class II region.* Proc Natl Acad Sci U S A, 1989. **86**(24): p. 10049-53.
7. Thomson, W., et al., *Quantifying the exact role of HLA-DRB1 alleles in susceptibility to inflammatory polyarthritis: results from a large, population-based study.* Arthritis Rheum, 1999. **42**(4): p. 757-62.
8. Gregersen, P.K., J. Silver, and R.J. Winchester, *The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis.* Arthritis Rheum, 1987. **30**(11): p. 1205-13.
9. Gorman, J.D. and L.A. Criswell, *The shared epitope and severity of rheumatoid arthritis.* Rheum Dis Clin North Am, 2002. **28**(1): p. 59-78.
10. Khani-Hanjani, A., et al., *Expression of QK/QR/RRRAA or DERAA motifs at the third hypervariable region of HLA-DRB1 and disease severity in rheumatoid arthritis.* J Rheumatol, 2002. **29**(7): p. 1358-65.
11. Kallberg, H., et al., *Gene-gene and gene-environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis.* Am J Hum Genet, 2007. **80**(5): p. 867-75.
12. Lorentzen, J.C., et al., *Association of arthritis with a gene complex encoding C-type lectin-like receptors.* Arthritis Rheum, 2007. **56**(8): p. 2620-32.
13. Sigurdsson, S., et al., *Association of a haplotype in the promoter region of the interferon regulatory factor 5 gene with rheumatoid arthritis.* Arthritis Rheum, 2007. **56**(7): p. 2202-10.
14. Verpoort, K.N., et al., *Association of HLA-DR3 with anti-cyclic citrullinated peptide antibody-negative rheumatoid arthritis.* Arthritis Rheum, 2005. **52**(10): p. 3058-62.
15. Silman, A.J. and J.E. Pearson, *Epidemiology and genetics of rheumatoid arthritis.* Arthritis Res, 2002. **4 Suppl 3**: p. S265-72.
16. Brennan, P. and A. Silman, *Breast-feeding and the onset of rheumatoid arthritis.* Arthritis Rheum, 1994. **37**(6): p. 808-13.
17. Silman, A.J., *Parity status and the development of rheumatoid arthritis.* Am J Reprod Immunol, 1992. **28**(3-4): p. 228-30.
18. MacGregor, A.J., et al., *Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins.* Arthritis Rheum, 2000. **43**(1): p. 30-7.
19. Silman, A.J., J. Newman, and A.J. MacGregor, *Cigarette smoking increases the risk of rheumatoid arthritis. Results from a nationwide study of disease-discordant twins.* Arthritis Rheum, 1996. **39**(5): p. 732-5.

20. Stolt, P., et al., *Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases.* Ann Rheum Dis, 2003. **62**(9): p. 835-41.
21. Klareskog, L., et al., *A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination.* Arthritis Rheum, 2006. **54**(1): p. 38-46.
22. Takeda, T., et al., *Lytic Epstein-Barr virus infection in the synovial tissue of patients with rheumatoid arthritis.* Arthritis Rheum, 2000. **43**(6): p. 1218-25.
23. Mehraein, Y., et al., *Latent Epstein-Barr virus (EBV) infection and cytomegalovirus (CMV) infection in synovial tissue of autoimmune chronic arthritis determined by RNA- and DNA-in situ hybridization.* Mod Pathol, 2004. **17**(7): p. 781-9.
24. Stransky, G., et al., *Virus-like particles in synovial fluids from patients with rheumatoid arthritis.* Br J Rheumatol, 1993. **32**(12): p. 1044-8.
25. Sacre, S.M., et al., *Could toll-like receptors provide a missing link in chronic inflammation in rheumatoid arthritis? Lessons from a study on human rheumatoid tissue.* Ann Rheum Dis, 2007. **66 Suppl 3**: p. iii81-6.
26. Siala, M., et al., *Analysis of bacterial DNA in synovial tissue of Tunisian patients with reactive and undifferentiated arthritis by broad-range PCR, cloning and sequencing.* Arthritis Res Ther, 2008. **10**(2): p. R40.
27. Kempsell, K.E., et al., *Reverse transcriptase-PCR analysis of bacterial rRNA for detection and characterization of bacterial species in arthritis synovial tissue.* Infect Immun, 2000. **68**(10): p. 6012-26.
28. Stolt, P., et al., *Silica exposure is associated with increased risk of developing rheumatoid arthritis: results from the Swedish EIRA study.* Ann Rheum Dis, 2005. **64**(4): p. 582-6.
29. Klareskog, L., et al., *Genes and environment in arthritis: can RA be prevented?* Arthritis Res, 2002. **4 Suppl 3**: p. S31-6.
30. Linos, A., et al., *Dietary factors in relation to rheumatoid arthritis: a role for olive oil and cooked vegetables?* Am J Clin Nutr, 1999. **70**(6): p. 1077-82.
31. Hafstrom, I., et al., *A vegan diet free of gluten improves the signs and symptoms of rheumatoid arthritis: the effects on arthritis correlate with a reduction in antibodies to food antigens.* Rheumatology (Oxford), 2001. **40**(10): p. 1175-9.
32. Symmons, D.P., et al., *Blood transfusion, smoking, and obesity as risk factors for the development of rheumatoid arthritis: results from a primary care-based incident case-control study in Norfolk, England.* Arthritis Rheum, 1997. **40**(11): p. 1955-61.
33. Escalante, A., R.W. Haas, and I. del Rincon, *Paradoxical effect of body mass index on survival in rheumatoid arthritis: role of comorbidity and systemic inflammation.* Arch Intern Med, 2005. **165**(14): p. 1624-9.
34. Kallberg, H., et al., *Alcohol consumption is associated with decreased risk of rheumatoid arthritis; Results from two Scandinavian case-control studies.* Ann Rheum Dis, 2008.
35. Jonsson, I.M., et al., *Ethanol prevents development of destructive arthritis.* Proc Natl Acad Sci U S A, 2007. **104**(1): p. 258-63.
36. Klareskog, L., T. Saxne, and Y. Enman, *Reumatologi*. 1st ed. 2005, Lund: Studentlitteratur.
37. Arnett, F.C., et al., *The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.* Arthritis Rheum, 1988. **31**(3): p. 315-24.
38. Banal, F., et al., *Sensitivity and specificity of the American College of Rheumatology 1987 criteria for the diagnosis of rheumatoid arthritis according to disease duration: a systematic literature review and meta-analysis.* Ann Rheum Dis, 2008.

39. Visser, H., et al., *How to diagnose rheumatoid arthritis early: a prediction model for persistent (erosive) arthritis.* Arthritis Rheum, 2002. **46**(2): p. 357-65.
40. van der Helm-van Mil, A.H., et al., *A prediction rule for disease outcome in patients with recent-onset undifferentiated arthritis: how to guide individual treatment decisions.* Arthritis Rheum, 2007. **56**(2): p. 433-40.
41. Mor, A., S.B. Abramson, and M.H. Pillinger, *The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction.* Clin Immunol, 2005. **115**(2): p. 118-28.
42. Bick, E.M., *Surgical pathology of synovial tissue.* The Journal of Bone and Joint Surgery, 1930(12): p. 33-44.
43. Muller-Ladner, U., et al., *Cells of the synovium in rheumatoid arthritis. Synovial fibroblasts.* Arthritis Res Ther, 2007. **9**(6): p. 223.
44. Knedla, A., E. Neumann, and U. Muller-Ladner, *Developments in the synovial biology field 2006.* Arthritis Res Ther, 2007. **9**(2): p. 209.
45. Coppack, S.W., *Pro-inflammatory cytokines and adipose tissue.* Proc Nutr Soc, 2001. **60**(3): p. 349-56.
46. Ehling, A., et al., *The potential of adiponectin in driving arthritis.* J Immunol, 2006. **176**(7): p. 4468-78.
47. Dayer, J.M., et al., *Adipose tissue has anti-inflammatory properties: focus on IL-1 receptor antagonist (IL-1Ra).* Ann N Y Acad Sci, 2006. **1069**: p. 444-53.
48. Yamasaki, S., et al., *Cytokines regulate fibroblast-like synovial cell differentiation to adipocyte-like cells.* Rheumatology (Oxford), 2004. **43**(4): p. 448-52.
49. Tagami, M., et al., *Genetic and ultrastructural demonstration of strong reversibility in human mesenchymal stem cell.* Cell Tissue Res, 2003. **312**(1): p. 31-40.
50. Miller, L.E., et al., *The loss of sympathetic nerve fibers in the synovial tissue of patients with rheumatoid arthritis is accompanied by increased norepinephrine release from synovial macrophages.* Faseb J, 2000. **14**(13): p. 2097-107.
51. Weidler, C., et al., *Low density of sympathetic nerve fibres and increased density of brain derived neurotrophic factor positive cells in RA synovium.* Ann Rheum Dis, 2005. **64**(1): p. 13-20.
52. Levine, J.D., et al., *Intraneuronal substance P contributes to the severity of experimental arthritis.* Science, 1984. **226**(4674): p. 547-9.
53. Lotz, M., D.A. Carson, and J.H. Vaughan, *Substance P activation of rheumatoid synoviocytes: neural pathway in pathogenesis of arthritis.* Science, 1987. **235**(4791): p. 893-5.
54. Wagner, F., et al., *Substance P enhances interferon-gamma production by human peripheral blood mononuclear cells.* Regul Pept, 1987. **19**(5-6): p. 355-64.
55. Ansel, J.C., et al., *Substance P selectively activates TNF-alpha gene expression in murine mast cells.* J Immunol, 1993. **150**(10): p. 4478-85.
56. Wiedermann, C.J., et al., *In vitro human polymorphonuclear leukocyte chemokinesis and human monocyte chemotaxis are different activities of aminoterminal and carboxyterminal substance P.* Naunyn Schmiedebergs Arch Pharmacol, 1989. **340**(2): p. 185-90.
57. Dirmeier, M., et al., *Lower density of synovial nerve fibres positive for calcitonin gene-related peptide relative to substance P in rheumatoid arthritis but not in osteoarthritis.* Rheumatology (Oxford), 2008. **47**(1): p. 36-40.
58. Iwanaga, T., et al., *Morphology and functional roles of synoviocytes in the joint.* Arch Histol Cytol, 2000. **63**(1): p. 17-31.
59. Moller Graabaeck, P., *Fine structure of the lysosomes in the two types of synoviocytes of normal rat synovial membrane.* Cell and Tissue research, 1985. **239**: p. 293-298.

60. Pap, T., et al., *Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis.* Arthritis Res, 2000. **2**(5): p. 361-7.
61. Kontoyiannis, D. and G. Kollias, *Fibroblast biology. Synovial fibroblasts in rheumatoid arthritis: leading role or chorus line?* Arthritis Res, 2000. **2**(5): p. 342-3.
62. Reme, T., et al., *Mutations of the p53 tumour suppressor gene in erosive rheumatoid synovial tissue.* Clin Exp Immunol, 1998. **111**(2): p. 353-8.
63. Saffarian, S., et al., *Interstitial collagenase is a Brownian ratchet driven by proteolysis of collagen.* Science, 2004. **306**(5693): p. 108-11.
64. Green, M.J., et al., *Serum MMP-3 and MMP-1 and progression of joint damage in early rheumatoid arthritis.* Rheumatology (Oxford), 2003. **42**(1): p. 83-8.
65. Kobayashi, A., et al., *Serum levels of matrix metalloproteinase 3 (stromelysin 1) for monitoring synovitis in rheumatoid arthritis.* Arch Pathol Lab Med, 2007. **131**(4): p. 563-70.
66. Yamanaka, H., et al., *Serum matrix metalloproteinase 3 as a predictor of the degree of joint destruction during the six months after measurement, in patients with early rheumatoid arthritis.* Arthritis Rheum, 2000. **43**(4): p. 852-8.
67. Dayer, J.M. and D. Burger, *Interleukin-1, tumor necrosis factor and their specific inhibitors.* Eur Cytokine Netw, 1994. **5**(6): p. 563-71.
68. Kyburz, D., et al., *Bacterial peptidoglycans but not CpG oligodeoxynucleotides activate synovial fibroblasts by toll-like receptor signaling.* Arthritis Rheum, 2003. **48**(3): p. 642-50.
69. Burger, D., et al., *Imbalance between interstitial collagenase and tissue inhibitor of metalloproteinases 1 in synoviocytes and fibroblasts upon direct contact with stimulated T lymphocytes: involvement of membrane-associated cytokines.* Arthritis Rheum, 1998. **41**(10): p. 1748-59.
70. Cha, H.S., et al., *Influence of hypoxia on the expression of matrix metalloproteinase-1, -3 and tissue inhibitor of metalloproteinase-1 in rheumatoid synovial fibroblasts.* Clin Exp Rheumatol, 2003. **21**(5): p. 593-8.
71. Hou, W.S., et al., *Cathepsin k is a critical protease in synovial fibroblast-mediated collagen degradation.* Am J Pathol, 2001. **159**(6): p. 2167-77.
72. Pap, T., et al., *Invasiveness of synovial fibroblasts is regulated by p53 in the SCID mouse in vivo model of cartilage invasion.* Arthritis Rheum, 2001. **44**(3): p. 676-81.
73. Shigeyama, Y., et al., *Expression of osteoclast differentiation factor in rheumatoid arthritis.* Arthritis Rheum, 2000. **43**(11): p. 2523-30.
74. Gravallese, E.M., et al., *Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor.* Arthritis Rheum, 2000. **43**(2): p. 250-258.
75. Kim, K.W., et al., *Human rheumatoid synovial fibroblasts promote osteoclastogenic activity by activating RANKL via TLR-2 and TLR-4 activation.* Immunol Lett, 2007. **110**(1): p. 54-64.
76. Jones, D.H., Y.Y. Kong, and J.M. Penninger, *Role of RANKL and RANK in bone loss and arthritis.* Ann Rheum Dis, 2002. **61 Suppl 2**: p. ii32-9.
77. Rivollier, A., et al., *Immature dendritic cell transdifferentiation into osteoclasts: a novel pathway sustained by the rheumatoid arthritis microenvironment.* Blood, 2004. **104**(13): p. 4029-37.
78. Pap, T., et al., *Osteoclast-independent bone resorption by fibroblast-like cells.* Arthritis Res Ther, 2003. **5**(3): p. R163-73.
79. Trabandt, A., et al., *Expression of the collagenolytic and Ras-induced cysteine proteinase cathepsin L and proliferation-associated oncogenes in synovial cells of MRL/I mice and patients with rheumatoid arthritis.* Matrix, 1990. **10**(6): p. 349-61.

80. Gay, S., R.E. Gay, and W.J. Koopman, *Molecular and cellular mechanisms of joint destruction in rheumatoid arthritis: two cellular mechanisms explain joint destruction?* Ann Rheum Dis, 1993. **52 Suppl 1**: p. S39-47.
81. Muller-Ladner, U., et al., *Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice.* Am J Pathol, 1996. **149**(5): p. 1607-15.
82. Qu, Z., et al., *Local proliferation of fibroblast-like synoviocytes contributes to synovial hyperplasia. Results of proliferating cell nuclear antigen/cyclin, c-myc, and nucleolar organizer region staining.* Arthritis Rheum, 1994. **37**(2): p. 212-20.
83. Melnyk, V.O., et al., *Synoviocytes synthesize, bind, and respond to basic fibroblast growth factor.* Arthritis Rheum, 1990. **33**(4): p. 493-500.
84. Schett, G., et al., *Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis.* Arthritis Rheum, 2000. **43**(11): p. 2501-12.
85. Okura, T., et al., *Protection against Fas/APO-1- and tumor necrosis factor-mediated cell death by a novel protein, sentrin.* J Immunol, 1996. **157**(10): p. 4277-81.
86. Firestein, G.S., et al., *Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium.* Proc Natl Acad Sci U S A, 1997. **94**(20): p. 10895-900.
87. Tak, P.P., et al., *Apoptosis and p53 expression in rat adjuvant arthritis.* Arthritis Research, 2000. **2**: p. 229-235.
88. Yudoh, K., et al., *Different mechanisms of synovial hyperplasia in rheumatoid arthritis and pigmented villonodular synovitis: the role of telomerase activity in synovial proliferation.* Arthritis Rheum, 1999. **42**(4): p. 669-77.
89. Tsumuki, H., et al., *Basic FGF-induced activation of telomerase in rheumatoid synoviocytes.* Rheumatol Int, 2000. **19**(4): p. 123-8.
90. Mapp, P.I., M.C. Grootveld, and D.R. Blake, *Hypoxia, oxidative stress and rheumatoid arthritis.* Br Med Bull, 1995. **51**(2): p. 419-36.
91. Distler, J.H., et al., *The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2892-7.
92. Pierer, M., et al., *Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands.* J Immunol, 2004. **172**(2): p. 1256-65.
93. Schaeverbeke, T., et al., *Mycoplasma fermentans, but not M penetrans, detected by PCR assays in synovium from patients with rheumatoid arthritis and other rheumatic disorders.* J Clin Pathol, 1996. **49**(10): p. 824-8.
94. Smiley, S.T., J.A. King, and W.W. Hancock, *Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4.* J Immunol, 2001. **167**(5): p. 2887-94.
95. Kariko, K., et al., *mRNA is an endogenous ligand for Toll-like receptor 3.* J Biol Chem, 2004. **279**(13): p. 12542-50.
96. Ingram, D.A., N.M. Caplice, and M.C. Yoder, *Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells.* Blood, 2005. **106**(5): p. 1525-31.
97. Hristov, M. and C. Weber, *Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance.* J Cell Mol Med, 2004. **8**(4): p. 498-508.
98. Ferrara, N. and T. Davis-Smyth, *The biology of vascular endothelial growth factor.* Endocr Rev, 1997. **18**(1): p. 4-25.
99. Ferrara, N., H.P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors.* Nat Med, 2003. **9**(6): p. 669-76.

100. Rafii, S., B. Heissig, and K. Hattori, *Efficient mobilization and recruitment of marrow-derived endothelial and hematopoietic stem cells by adenoviral vectors expressing angiogenic factors.* Gene Ther, 2002. **9**(10): p. 631-41.
101. Paleolog, E.M., *Angiogenesis in rheumatoid arthritis.* Arthritis Res, 2002. **4 Suppl 3**: p. S81-90.
102. Ochi, T., et al., *Mesenchymal stromal cells. Nurse-like cells reside in the synovial tissue and bone marrow in rheumatoid arthritis.* Arthritis Res Ther, 2007. **9**(1): p. 201.
103. Nakagawa, S., et al., *Bone marrow stromal cells contribute to synovial cell proliferation in rats with collagen induced arthritis.* J Rheumatol, 1996. **23**(12): p. 2098-103.
104. Takeuchi, E., et al., *Establishment and characterization of nurse cell-like stromal cell lines from synovial tissues of patients with rheumatoid arthritis.* Arthritis Rheum, 1999. **42**(2): p. 221-8.
105. Tanaka, K., et al., *Production of interleukin-6 and interleukin-8 by nurse-like cells from rheumatoid arthritis patients after stimulation with monocytes.* Mod Rheumatol, 2005. **15**(6): p. 415-22.
106. Toyosaki-Maeda, T., et al., *Differentiation of monocytes into multinucleated giant bone-resorbing cells: two-step differentiation induced by nurse-like cells and cytokines.* Arthritis Res, 2001. **3**(5): p. 306-10.
107. Jones, E.A., et al., *Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis.* Arthritis Rheum, 2004. **50**(3): p. 817-27.
108. Skapenko, A., et al., *The role of the T cell in autoimmune inflammation.* Arthritis Res Ther, 2005. **7 Suppl 2**: p. S4-14.
109. Kinne, R.W., E. Palombo-Kinne, and F. Emmrich, *T-cells in the pathogenesis of rheumatoid arthritis villains or accomplices?* Biochim Biophys Acta, 1997. **1360**(2): p. 109-41.
110. Mishan-Eisenberg, G., et al., *Differential regulation of Th1/Th2 cytokine responses by placental protein 14.* J Immunol, 2004. **173**(9): p. 5524-30.
111. Valencia, X., et al., *TNF downmodulates the function of human CD4+CD25hi T-regulatory cells.* Blood, 2006. **108**(1): p. 253-61.
112. Ehrenstein, M.R., et al., *Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy.* J Exp Med, 2004. **200**(3): p. 277-85.
113. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nat Immunol, 2005. **6**(11): p. 1123-32.
114. Cope, A.P., H. Schulze-Koops, and M. Aringer, *The central role of T cells in rheumatoid arthritis.* Clin Exp Rheumatol, 2007. **25**(5 Suppl 46): p. S4-11.
115. Prots, I., et al., *Association of the IL4R single-nucleotide polymorphism I50V with rapidly erosive rheumatoid arthritis.* Arthritis Rheum, 2006. **54**(5): p. 1491-500.
116. Chabaud, M., et al., *Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium.* Arthritis Rheum, 1999. **42**(5): p. 963-70.
117. Sato, K., et al., *Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction.* J Exp Med, 2006. **203**(12): p. 2673-82.
118. Kirkham, B.W., et al., *Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort).* Arthritis Rheum, 2006. **54**(4): p. 1122-31.
119. Fazou, C., et al., *Epitope specificity of clonally expanded populations of CD8+ T cells found within the joints of patients with inflammatory arthritis.* Arthritis Rheum, 2001. **44**(9): p. 2038-45.
120. Kang, Y.M., et al., *CD8 T cells are required for the formation of ectopic germinal centers in rheumatoid synovitis.* J Exp Med, 2002. **195**(10): p. 1325-36.

121. Wagner, U.G., et al., *The role of CD8+ CD40L+ T cells in the formation of germinal centers in rheumatoid synovitis.* J Immunol, 1998. **161**(11): p. 6390-7.
122. Takemura, S., et al., *T cell activation in rheumatoid synovium is b cell dependent.* J Immunol, 2001. **167**(8): p. 4710-4718.
123. Lindhout, E., et al., *Fibroblast-like synoviocytes from rheumatoid arthritis patients have intrinsic properties of follicular dendritic cells.* J Immunol, 1999. **162**(10): p. 5949-56.
124. Salmon, M., et al., *Inhibition of T cell apoptosis in the rheumatoid synovium.* J Clin Invest, 1997. **99**(3): p. 439-46.
125. Sebbag, M., et al., *Cytokine stimulation of T lymphocytes regulates their capacity to induce monocyte production of tumor necrosis factor-alpha, but not interleukin-10: possible relevance to pathophysiology of rheumatoid arthritis.* Eur J Immunol, 1997. **27**(3): p. 624-32.
126. McInnes, I.B., et al., *Interleukin-15 mediates T cell-dependent regulation of tumour necrosis factor-α production in rheumatoid arthritis.* Nature Med, 1997. **3**(2): p. 189-195.
127. Yamamura, Y., et al., *Effector function of resting T cells: activation of synovial fibroblasts.* J Immunol, 2001. **166**(4): p. 2270-5.
128. Edwards, J.C. and G. Cambridge, *Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes.* Rheumatology (Oxford), 2001. **40**(2): p. 205-11.
129. Andersson, A.K., C. Li, and F.M. Brennan, *Recent developments in the immunobiology of rheumatoid arthritis.* Arthritis Res Ther, 2008. **10**(2): p. 204.
130. Bugatti, S., et al., *B cells in rheumatoid arthritis.* Autoimmun Rev, 2007. **7**(2): p. 137-42.
131. Silverman, G.J. and D.A. Carson, *Roles of B cells in rheumatoid arthritis.* Arthritis Res Ther, 2003. **5 Suppl 4**: p. S1-6.
132. Ohata, J., et al., *Fibroblast-like synoviocytes of mesenchymal origin express functional B cell-activating factor of the TNF family in response to proinflammatory cytokines.* J Immunol, 2005. **174**(2): p. 864-70.
133. Klareskog, L., et al., *Antibodies to citrullinated proteins in arthritis: pathology and promise.* Curr Opin Rheumatol, 2008. **20**(3): p. 300-5.
134. Bukhari, M., et al., *Rheumatoid factor is the major predictor of increasing severity of radiographic erosions in rheumatoid arthritis: results from the Norfolk Arthritis Register Study, a large inception cohort.* Arthritis Rheum, 2002. **46**(4): p. 906-12.
135. Jonsson, T., et al., *Combined elevation of IgM and IgA rheumatoid factor has high diagnostic specificity for rheumatoid arthritis.* Rheumatol Int, 1998. **18**(3): p. 119-22.
136. Jonsson, T., et al., *Raised IgA rheumatoid factor (RF) but not IgM RF or IgG RF is associated with extra-articular manifestations in rheumatoid arthritis.* Scand J Rheumatol, 1995. **24**(6): p. 372-5.
137. Vossenaar, E.R., et al., *PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease.* Bioessays, 2003. **25**(11): p. 1106-18.
138. Masson-Bessiere, C., et al., *The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin.* J Immunol, 2001. **166**(6): p. 4177-84.
139. Lundberg, K., et al., *Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity.* Arthritis Res Ther, 2005. **7**(3): p. R458-467.
140. Rantapaa-Dahlqvist, S., et al., *Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis.* Arthritis Rheum, 2003. **48**(10): p. 2741-9.
141. Kroot, E.J., et al., *The prognostic value of anti-cyclic citrullinated peptide antibody in patients with recent-onset rheumatoid arthritis.* Arthritis Rheum, 2000. **43**(8): p. 1831-5.

142. Masson-Bessiere, C., et al., *In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgG than in synovial fluid and serum.* Clin Exp Immunol, 2000. **119**(3): p. 544-52.
143. Reparon-Schuijt, C.C., et al., *Secretion of anti-citrulline-containing peptide antibody by B lymphocytes in rheumatoid arthritis.* Arthritis Rheum, 2001. **44**(1): p. 41-7.
144. Krenn, V., et al., *Immunohistochemical analysis of proliferating and antigen-presenting cells in rheumatoid synovial tissue.* Rheumatol Int, 1996. **15**(6): p. 239-47.
145. Kim, H.J., et al., *Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis.* J Immunol, 1999. **162**(5): p. 3053-62.
146. Imhof, B.A. and M. Aurrand-Lions, *Adhesion mechanisms regulating the migration of monocytes.* Nat Rev Immunol, 2004. **4**(6): p. 432-44.
147. Athanasou, N.A., *Synovial macrophages.* Ann Rheum Dis, 1995. **54**(5): p. 392-4.
148. Mulherin, D., O. Fitzgerald, and B. Bresnihan, *Synovial tissue macrophage populations and articular damage in rheumatoid arthritis.* Arthritis Rheum, 1996. **39**(1): p. 115-24.
149. Tak, P.P., et al., *Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity.* Arthritis Rheum, 1997. **40**(2): p. 217-25.
150. Konttinen, Y.T., et al., *Increased expression of extracellular matrix metalloproteinase inducer in rheumatoid synovium.* Arthritis Rheum, 2000. **43**(2): p. 275-80.
151. Cunnane, G., et al., *Synovial tissue protease gene expression and joint erosions in early rheumatoid arthritis.* Arthritis Rheum, 2001. **44**(8): p. 1744-53.
152. Komano, Y., et al., *Identification of a human peripheral blood monocyte subset that differentiates into osteoclasts.* Arthritis Res Ther, 2006. **8**(5): p. R152.
153. Liu, H., et al., *Regulation of Mcl-1 expression in rheumatoid arthritis synovial macrophages.* Arthritis Rheum, 2006. **54**(10): p. 3174-81.
154. Szekanecz, Z., et al., *Cytokines in rheumatoid arthritis. Potential targets for pharmacological intervention.* Drugs Aging, 1998. **12**(5): p. 377-90.
155. Szekanecz, Z. and A.E. Koch, *Macrophages and their products in rheumatoid arthritis.* Curr Opin Rheumatol, 2007. **19**(3): p. 289-95.
156. Pohlers, D., et al., *Expression of platelet-derived growth factors C and D in the synovial membrane of patients with rheumatoid arthritis and osteoarthritis.* Arthritis Rheum, 2006. **54**(3): p. 788-94.
157. Antoniv, T.T. and L.B. Ivashkiv, *Dysregulation of interleukin-10-dependent gene expression in rheumatoid arthritis synovial macrophages.* Arthritis Rheum, 2006. **54**(9): p. 2711-21.
158. Takasugi, K., et al., *Induction of tumour necrosis factor receptor-expressing macrophages by interleukin-10 and macrophage colony-stimulating factor in rheumatoid arthritis.* Arthritis Res Ther, 2006. **8**(4): p. R126.
159. Ruth, J.H., et al., *Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis.* Arthritis Rheum, 2001. **44**(7): p. 1568-81.
160. Ospelt, C. and S. Gay, *The role of resident synovial cells in destructive arthritis.* Best Pract Res Clin Rheumatol, 2008. **22**(2): p. 239-52.
161. Breitner, S., et al., *Complement components C1q, C1r/C1s, and C1INH in rheumatoid arthritis. Correlation of in situ hybridization and northern blot results with function and protein concentration in synovium and primary cell cultures.* Arthritis Rheum, 1995. **38**(4): p. 492-8.
162. Shushakova, N., et al., *C5a anaphylatoxin is a major regulator of activating versus inhibitory FcgammaRs in immune complex-induced lung disease.* J Clin Invest, 2002. **110**(12): p. 1823-30.
163. Weissmann, G. and H. Korchak, *Rheumatoid arthritis. The role of neutrophil activation.* Inflammation, 1984. **8 Suppl**: p. S3-14.

164. Schett, G., *Cells of the synovium in rheumatoid arthritis. Osteoclasts.* Arthritis Res Ther, 2007. **9**(1): p. 203.
165. Fiset, M.E., et al., *Human neutrophils as a source of nociceptin: a novel link between pain and inflammation.* Biochemistry, 2003. **42**(35): p. 10498-505.
166. Vivier, E., et al., *Functions of natural killer cells.* Nat Immunol, 2008. **9**(5): p. 503-10.
167. Johansson, S., et al., *NK cells: elusive players in autoimmunity.* Trends Immunol, 2005. **26**(11): p. 613-8.
168. Cooper, M.A., et al., *Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset.* Blood, 2001. **97**(10): p. 3146-51.
169. Dalbeth, N. and M.F. Callan, *A subset of natural killer cells is greatly expanded within inflamed joints.* Arthritis Rheum, 2002. **46**(7): p. 1763-72.
170. Pridgeon, C., et al., *Natural killer cells in the synovial fluid of rheumatoid arthritis patients exhibit a CD56bright,CD94bright,CD158negative phenotype.* Rheumatology (Oxford), 2003. **42**(7): p. 870-8.
171. Dalbeth, N., et al., *CD56bright NK cells are enriched at inflammatory sites and can engage with monocytes in a reciprocal program of activation.* J Immunol, 2004. **173**(10): p. 6418-26.
172. Zhang, A.L., et al., *Natural killer cells trigger differentiation of monocytes into dendritic cells.* Blood, 2007. **110**(7): p. 2484-93.
173. Lebre, M.C. and P.P. Tak, *Dendritic cell subsets: their roles in rheumatoid arthritis.* Acta Reumatol Port, 2008. **33**(1): p. 35-45.
174. Lande, R., et al., *Characterization and recruitment of plasmacytoid dendritic cells in synovial fluid and tissue of patients with chronic inflammatory arthritis.* J Immunol, 2004. **173**(4): p. 2815-24.
175. Bromley, M., W.D. Fisher, and D.E. Woolley, *Mast cells at sites of cartilage erosion in the rheumatoid joint.* Ann Rheum Dis, 1984. **43**(1): p. 76-9.
176. Schwartz, L.B., *Mast cells: function and contents.* Curr Opin Immunol, 1994. **6**(1): p. 91-7.
177. Kobayashi, Y., et al., *Mechanism of the reduction of endothelium-dependent vasodilation in spontaneously hypertensive rats.* Jpn J Pharmacol, 1992. **58 Suppl 2**: p. 320P.
178. Miossec, P. and W. van den Berg, *Th1/Th2 cytokine balance in arthritis.* Arthritis Rheum, 1997. **40**(12): p. 2105-15.
179. Schett, G., et al., *How antirheumatic drugs protect joints from damage in rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(10): p. 2936-48.
180. Maruotti, N., et al., *Macrophages in rheumatoid arthritis.* Histol Histopathol, 2007. **22**(5): p. 581-6.
181. Miesel, R., R. Hartung, and H. Kroeger, *Priming of NADPH oxidase by tumor necrosis factor alpha in patients with inflammatory and autoimmune rheumatic diseases.* Inflammation, 1996. **20**(4): p. 427-38.
182. Sakurai, H., et al., *Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides.* J Clin Invest, 1995. **96**(5): p. 2357-63.
183. McInnes, I.B., et al., *Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients.* J Exp Med, 1996. **184**(4): p. 1519-24.
184. Zwerina, J., et al., *TNF-induced structural joint damage is mediated by IL-1.* Proc Natl Acad Sci U S A, 2007. **104**(28): p. 11742-11747.
185. Wei, S., et al., *IL-1 mediates TNF-induced osteoclastogenesis.* J Clin Invest, 2005. **115**(2): p. 282-90.
186. Horai, R., et al., *Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice.* J Exp Med, 2000. **191**(2): p. 313-320.

187. von den Hoff, H., et al., *Interleukin-1 reversibly inhibits the synthesis of biglycan and decorin in intact articular cartilage in culture.* J Rheumatol, 1995. **22**(8): p. 1520-6.
188. Arend, W.P., et al., *Interleukin-1 receptor antagonist: role in biology.* Annu Rev Immunol, 1998. **16**: p. 27-55.
189. Wong, P.K., et al., *Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis.* Arthritis Rheum, 2006. **54**(1): p. 158-68.
190. Maini, R.N., et al., *Double-blind randomized controlled clinical trial of the interleukin-6 receptor antagonist, tocilizumab, in European patients with rheumatoid arthritis who had an incomplete response to methotrexate.* Arthritis Rheum, 2006. **54**(9): p. 2817-29.
191. Kremer, J.M., et al., *Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig.* N Engl J Med, 2003. **349**(20): p. 1907-15.
192. Axmann, R., et al., *CTLA-4 directly inhibits osteoclast formation.* Ann Rheum Dis, 2008. **67**(11): p. 1603-9.
193. Joosten, L.A., et al., *Protection against cartilage and bone destruction by systemic interleukin-4 treatment in established murine type II collagen-induced arthritis.* Arthritis Res, 1999. **1**(1): p. 81-91.
194. Bessis, N., et al., *Attenuation of collagen-induced arthritis in mice by treatment with vector cells engineered to secrete interleukin-13.* Eur J Immunol, 1996. **26**(10): p. 2399-2403.
195. Constantin, A., et al., *Antiinflammatory and immunoregulatory action of methotrexate in the treatment of rheumatoid arthritis: evidence of increased interleukin-4 and interleukin-10 gene expression demonstrated in vitro by competitive reverse transcriptase-polymerase chain reaction.* Arthritis Rheum, 1998. **41**(1): p. 48-57.
196. Dimitrova, P., et al., *Restriction of de novo pyrimidine biosynthesis inhibits Th1 cell activation and promotes Th2 cell differentiation.* J Immunol, 2002. **169**(6): p. 3392-9.
197. Kang, B.Y., et al., *Sulfasalazine prevents T-helper 1 immune response by suppressing interleukin-12 production in macrophages.* Immunology, 1999. **98**(1): p. 98-103.
198. de Groot, K. and W.L. Gross, *Wegener's granulomatosis: disease course, assessment of activity and extent and treatment.* Lupus, 1998. **7**(4): p. 285-91.
199. Almawi, W.Y., O.K. Melemedjian, and M.J. Rieder, *An alternate mechanism of glucocorticoid anti-proliferative effect: promotion of a Th2 cytokine-secreting profile.* Clin Transplant, 1999. **13**(5): p. 365-74.
200. Hench, P.S., E.C. Kendall, and et al., *The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone; compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis.* Mayo Clin Proc, 1949. **24**(8): p. 181-97.
201. Trentham, D.E., A.S. Townes, and A.H. Kang, *Autoimmunity to type II collagen: an experimental model of arthritis.* J Exp Med, 1977. **146**: p. 857-868.
202. Courtenay, J.S., et al., *Immunisation against heterologous type II collagen induces arthritis in mice.* Nature, 1980. **283**(5748): p. 666-668.
203. Billingham, M.E., *Models of arthritis and the search for anti-arthritic drugs.* Pharmacol Ther, 1983. **21**(3): p. 389-428.
204. Campbell, I.K., J.A. Hamilton, and I.P. Wicks, *Collagen-induced arthritis in C57BL/6 (H-2b) mice: new insights into an important disease model of rheumatoid arthritis.* Eur J Immunol, 2000. **30**(6): p. 1568-75.
205. Erlandsson Harris, H., M. Liljeström, and L. Klareskog, *Characteristics of synovial fluid effusion in collagen-induced arthritis (CIA) in the DA rat; a comparison of histology and antibody reactivities in an experimental chronic arthritis model and rheumatoid arthritis (RA).* Clin Exp Immunol, 1997. **107**(3): p. 480-484.

206. Keffer, J., et al., *Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis.* EMBO J, 1991. **10**(13): p. 4025-4031.
207. Butler, D.M., et al., *DBA/1 mice expressing the human TNF-α transgene develop a severe, erosive arthritis: characterization of the cytokine cascade and cellular composition.* J Immunol, 1997. **159**(6): p. 2867-2876.
208. Sasai, M., et al., *Delayed onset and reduced severity of collagen-induced arthritis in interleukin-6-deficient mice.* Arthritis Rheum, 1999. **42**(8): p. 1635-43.
209. Thorbecke, G.J., et al., *Involvement of endogenous tumour necrosis factor α and transforming growth factor β during induction of collagen type II arthritis in mice.* Proc Natl Acad Sci USA, 1992. **89**: p. 7375-7379.
210. van den Berg, W.B., et al., *Amelioration of established murine collagen-induced arthritis with anti- IL-1 treatment.* Clin Exp Immunol, 1994. **95**(2): p. 237-243.
211. Takagi, N., et al., *Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis.* Arthritis Rheum, 1998. **41**(12): p. 2117-2121.
212. Ferrari, S., et al., *The active gene that encodes human high mobility group 1 protein (HMG1) contains introns and maps to chromosome 13.* Genomics, 1996. **35**(2): p. 367-71.
213. Paonessa, G., R. Frank, and R. Cortese, *Nucleotide sequence of rat liver HMG1 cDNA.* Nucleic Acids Res, 1987. **15**(21): p. 9077.
214. Bustin, M., D.A. Lehn, and D. Landsman, *Structural features of the HMG chromosomal proteins and their genes.* Biochim Biophys Acta, 1990. **1049**(3): p. 231-43.
215. Knapp, S., et al., *The long acidic tail of high mobility group box 1 (HMGB1) protein forms an extended and flexible structure that interacts with specific residues within and between the HMG boxes.* Biochemistry, 2004. **43**(38): p. 11992-7.
216. Huttunen, H.J. and H. Rauvala, *Amphoterin as an extracellular regulator of cell motility: from discovery to disease.* J Intern Med, 2004. **255**(3): p. 351-366.
217. Huttunen, H.J., et al., *Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis.* Cancer Res, 2002. **62**(16): p. 4805-4811.
218. Weir, H.M., et al., *Structure of the HMG box motif in the B-domain of HMG1.* Embo J, 1993. **12**(4): p. 1311-9.
219. Read, C.M., et al., *Solution structure of a DNA-binding domain from HMG1.* Nucleic Acids Res, 1993. **21**(15): p. 3427-36.
220. Sheflin, L.G., N.W. Fucile, and S.W. Spaulding, *The specific interactions of HMG 1 and 2 with negatively supercoiled DNA are modulated by their acidic C-terminal domains and involve cysteine residues in their HMG 1/2 boxes.* Biochemistry, 1993. **32**(13): p. 3238-48.
221. Stros, M., J. Stokrova, and J.O. Thomas, *DNA looping by the HMG-box domains of HMG1 and modulation of DNA binding by the acidic C-terminal domain.* Nucleic Acids Res, 1994. **22**(6): p. 1044-51.
222. Müller, S., et al., *The double life of HMGB1 chromatin protein: architectural factor and extracellular signal.* EMBO J, 2001. **20**(16): p. 4337-4340.
223. Bonaldi, T., et al., *The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding.* Embo J, 2002. **21**(24): p. 6865-73.
224. Travers, A.A., *Priming the nucleosome: a role for HMGB proteins?* EMBO Rep, 2003. **4**(2): p. 131-6.
225. Pasheva, E., et al., *In vitro acetylation of HMGB-1 and -2 proteins by CBP: the role of the acidic tail.* Biochemistry, 2004. **43**(10): p. 2935-40.
226. Thomas, J.O. and A.A. Travers, *HMG1 and 2, and related 'architectural' DNA-binding proteins.* Trends Biochem Sci, 2001. **26**(3): p. 167-74.

227. Agresti, A. and M.E. Bianchi, *HMGB proteins and gene expression.* Curr Opin Genet Dev, 2003. **13**(2): p. 170-178.
228. Calogero, S., et al., *The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice.* Nat Genet, 1999. **22**(3): p. 276-280.
229. Parkkinen, J., et al., *Amphoterin, the 30-kDa family of HMG1-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation.* J Biol Chem, 1993. **268**(26): p. 19726-19738.
230. Volp, K., et al., *Increased expression of high mobility group box 1 (HMGB1) is associated with an elevated level of the antiapoptotic c-IAP2 protein in human colon carcinomas.* Gut, 2006. **55**(2): p. 234-42.
231. Choi, Y.R., et al., *Overexpression of high mobility group box 1 in gastrointestinal stromal tumors with KIT mutation.* Cancer Res, 2003. **63**(9): p. 2188-93.
232. Kuniyasu, H., Y. Chihara, and H. Kondo, *Differential effects between amphoterin and advanced glycation end products on colon cancer cells.* Int J Cancer, 2003. **104**(6): p. 722-7.
233. Bustin, M. and N.K. Neihart, *Antibodies against chromosomal HMG proteins stain the cytoplasm of mammalian cells.* Cell, 1979. **16**(1): p. 181-9.
234. Rauvala, H. and R. Pihlaskari, *Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons.* J Biol Chem, 1987. **262**(34): p. 16625-35.
235. Parkkinen, J. and H. Rauvala, *Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin. Enhancement of t-PA-catalyzed plasminogen activation by amphoterin.* J Biol Chem, 1991. **266**(25): p. 16730-16735.
236. Rauvala, H., et al., *Heparin-binding proteins HB-GAM (pleiotrophin) and amphoterin in the regulation of cell motility.* Matrix Biol, 2000. **19**(5): p. 377-87.
237. Taguchi, A., et al., *Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases.* Nature, 2000. **405**: p. 354-359.
238. Rouhiainen, A., et al., *Occurrence of amphoterin (HMG1) as an endogenous protein of human platelets that is exported to the cell surface upon platelet activation.* Thromb Haemost, 2000. **84**(6): p. 1087-1094.
239. Wang, H., et al., *Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes.* Surgery, 1999. **126**(2): p. 389-392.
240. Li, J., et al., *Structural basis for the proinflammatory cytokine activity of high mobility group box 1.* Mol Med, 2003. **9**(1-2): p. 37-45.
241. Yang, H., et al., *Reversing established sepsis with antagonists of endogenous high-mobility group box 1.* Proc Natl Acad Sci U S A, 2004. **101**(1): p. 296-301.
242. Sitia, G., et al., *Treatment with HMGB1 inhibitors diminishes CTL-induced liver disease in HBV transgenic mice.* J Leukoc Biol, 2007. **81**(1): p. 100-7.
243. Pullerits, R., et al., *High Mobility Group chromosomal protein 1, a DNA-binding cytokine, induces arthritis.* Arthritis Rheum, 2003. **48**(6): p. 1693-1700.
244. Bianchi, M.E. and A.A. Manfredi, *High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity.* Immunol Rev, 2007. **220**: p. 35-46.
245. Zetterström, C.K., et al., *High mobility group box chromosomal protein 1 (HMGB1) is an antibacterial factor produced by the human adenoid.* Pediatr Res, 2002. **52**(2): p. 148-154.
246. Scaffidi, P., T. Misteli, and M.E. Bianchi, *Release of chromatin protein HMGB1 by necrotic cells triggers inflammation.* Nature, 2002. **418**(6894): p. 191-195.
247. Bonaldi, T., et al., *Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion.* Embo J, 2003. **22**(20): p. 5551-5560.
248. Gardella, S., et al., *The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway.* EMBO Rep, 2002. **13**(10): p. 995-1001.

249. Hamon, Y., et al., *Interleukin-1beta secretion is impaired by inhibitors of the Atp binding cassette transporter, ABC1.* Blood, 1997. **90**(8): p. 2911-5.
250. Rouhiainen, A., et al., *Regulation of monocyte migration by amphoterin (HMGB1).* Blood, 2004. **104**(4): p. 1174-1182.
251. Harris, H.E. and A. Raucci, *Alarmin(g) news about danger: workshop on innate danger signals and HMGB1.* EMBO Rep, 2006. **7**(8): p. 774-8.
252. Porto, A., et al., *Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein.* Faseb J, 2006. **20**(14): p. 2565-6.
253. Kawahara, K., et al., *Induction of high mobility group box 1 release from serotonin-stimulated human umbilical vein endothelial cells.* Int J Mol Med, 2008. **22**(5): p. 639-44.
254. Jiang, W. and D.S. Pisetsky, *The role of IFN-alpha and nitric oxide in the release of HMGB1 by RAW 264.7 cells stimulated with polyinosinic-polycytidylic acid or lipopolysaccharide.* J Immunol, 2006. **177**(5): p. 3337-43.
255. Bell, C.W., et al., *The extracellular release of HMGB1 during apoptotic cell death.* Am J Physiol Cell Physiol, 2006. **291**(6): p. C1318-1325.
256. Pisetsky, D.S., *The role of nuclear macromolecules in innate immunity.* Proc Am Thorac Soc, 2007. **4**(3): p. 258-262.
257. Hamada, T., et al., *Extracellular high mobility group box chromosomal protein 1 is a coupling factor for hypoxia and inflammation in arthritis.* Arthritis Rheum, 2008. **58**(9): p. 2675-85.
258. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* J Exp Med, 1996. **183**(3): p. 1161-72.
259. Pan, B.T., et al., *Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes.* J Cell Biol, 1985. **101**(3): p. 942-8.
260. Tanuma, S., L.D. Johnson, and G.S. Johnson, *ADP-ribosylation of chromosomal proteins and mouse mammary tumor virus gene expression. Glucocorticoids rapidly decrease endogenous ADP-ribosylation of nonhistone high mobility group 14 and 17 proteins.* J Biol Chem, 1983. **258**(24): p. 15371-5.
261. Tanuma, S., T. Yagi, and G.S. Johnson, *Endogenous ADP ribosylation of high mobility group proteins 1 and 2 and histone H1 following DNA damage in intact cells.* Arch Biochem Biophys, 1985. **237**(1): p. 38-42.
262. Ditsworth, D., W.X. Zong, and C.B. Thompson, *Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus.* J Biol Chem, 2007. **282**(24): p. 17845-54.
263. Kazama, H., et al., *Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein.* Immunity, 2008. **29**(1): p. 21-32.
264. Youn, J.H. and J.S. Shin, *Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion.* J Immunol, 2006. **177**(11): p. 7889-7897.
265. Ito, I., J. Fukazawa, and M. Yoshida, *Post-translational methylation of high mobility group box 1 (HMGB1) causes its cytoplasmic localization in neutrophils.* J Biol Chem, 2007. **282**(22): p. 16336-44.
266. Ito, T., et al., *Proteolytic cleavage of high mobility group box 1 protein by thrombin-thrombomodulin complexes.* Arterioscler Thromb Vasc Biol, 2008. **28**(10): p. 1825-30.
267. Urbonaviciute, V., et al., *Factors masking HMGB1 in human serum and plasma.* J Leukoc Biol, 2007. **81**(1): p. 67-74.
268. Qing, X., et al., *Pathogenic anti-DNA antibodies modulate gene expression in mesangial cells: Involvement of HMGB1 in anti-DNA antibody-induced renal injury.* Immunol Lett, 2008.

269. Youn, J.H., et al., *High mobility group box 1 protein binding to lipopolysaccharide facilitates transfer of lipopolysaccharide to CD14 and enhances lipopolysaccharide-mediated TNF-alpha production in human monocytes.* J Immunol, 2008. **180**(7): p. 5067-74.
270. Ivanov, S., et al., *A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA.* Blood, 2007. **110**(6): p. 1970-1981.
271. Tian, J., et al., *Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE.* Nat Immunol, 2007. **8**(5): p. 487-496.
272. Sha, Y., et al., *HMGB1 develops enhanced proinflammatory activity by binding to cytokines.* J Immunol, 2008. **180**(4): p. 2531-2537.
273. Milev, P., et al., *High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase-zeta/beta with tenascin-R, amphoterin, and the heparin-binding growth-associated molecule.* J Biol Chem, 1998. **273**(12): p. 6998-7005.
274. Salmivirta, M., et al., *Neurite growth-promoting protein (amphoterin, p30) binds syndecan.* Exp Cell Res, 1992. **200**(2): p. 444-51.
275. Rouhiainen, A., et al., *Analysis of proinflammatory activity of highly purified eukaryotic recombinant HMGB1 (Amphoterin).* J Leukoc Biol, 2007. **81**: p. 49-58.
276. Liu, H., et al., *High mobility group box-1 protein acts as a coactivator of nuclear factor of activated T cells-2 in promoting interleukin-2 transcription.* Int J Biochem Cell Biol, 2008.
277. Yamoah, K., et al., *High-mobility group box proteins modulate tumor necrosis factor-alpha expression in osteoclastogenesis via a novel deoxyribonucleic acid sequence.* Mol Endocrinol, 2008. **22**(5): p. 1141-1153.
278. Yu, M., et al., *Proteomic screen defines the hepatocyte nuclear factor 1alpha-binding partners and identifies HMGB1 as a new cofactor of HNF1alpha.* Nucleic Acids Res, 2008. **36**(4): p. 1209-1219.
279. Kajino, K., et al., *Recombination hot spot of hepatitis B virus genome binds to members of the HMG domain protein family and the Y box binding protein family; implication of these proteins in genomic instability.* Intervirology, 2001. **44**(5): p. 311-6.
280. Zhang, G., et al., *Borna disease virus phosphoprotein represses p53-mediated transcriptional activity by interference with HMGB1.* J Virol, 2003. **77**(22): p. 12243-51.
281. Dintilhac, A. and J. Bernues, *HMGB1 interacts with many apparently unrelated proteins by recognizing short amino acid sequences.* J Biol Chem, 2002. **277**(9): p. 7021-7028.
282. Schmidt, A.M., et al., *Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface.* J Biol Chem, 1992. **267**(21): p. 14987-97.
283. Neeper, M., et al., *Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins.* J Biol Chem, 1992. **267**(21): p. 14998-15004.
284. Hofmann, M.A., et al., *RAGE mediates a novel proinflammatory axis: A central cell surface receptor for S100/calgranulin polypeptides.* Cell, 1999. **97**: p. 889-901.
285. Huttunen, H., C. Fages, and H. Rauvala, *Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-κB require the cytoplasmatic domain of the receptor but different downstream signaling pathways.* J Biol Chem, 1999. **274**(28): p. 19919-19924.
286. Park, I.H., et al., *Expression of a novel secreted splice variant of the receptor for advanced glycation end products (RAGE) in human brain astrocytes and peripheral blood mononuclear cells.* Mol Immunol, 2004. **40**(16): p. 1203-11.
287. Yonekura, H., et al., *Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury.* Biochem J, 2003. **370**(Pt 3): p. 1097-109.

288. Geroldi, D., C. Falcone, and E. Emanuele, *Soluble receptor for advanced glycation end products: from disease marker to potential therapeutic target.* Curr Med Chem, 2006. **13**(17): p. 1971-8.
289. Merenmies, J., et al., *30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane.* J Biol Chem, 1991. **266**(25): p. 16722-9.
290. Hori, O., et al., *The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co- expression of rage and amphoterin in the developing nervous system.* J Biol Chem, 1995. **270**(43): p. 25752-25761.
291. Degryse, B., et al., *The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells.* J Cell Biol, 2001. **152**(6): p. 1197-1206.
292. Chavakis, T., et al., *The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment.* J Exp Med, 2003. **198**(10): p. 1507-1515.
293. Kokkola, R., et al., *RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages.* Scand J Immunol, 2005. **61**(1): p. 1-9.
294. Sappington, P.L., et al., *HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice.* Gastroenterol, 2002. **123**(3): p. 790-802.
295. Treutiger, C.J., et al., *High mobility group 1 B-box mediates activation of human endothelium.* J Intern Med, 2003. **254**(4): p. 375-385.
296. Yuan, Z., et al., *Construction and characterization of the HMGB1 mutant as a competitive antagonist to HMGB1 induced cytokines release.* Biochem Biophys Res Commun, 2008. **372**(4): p. 703-7.
297. Srikrishna, G., et al., *N -Glycans on the receptor for advanced glycation end products influence amphoterin binding and neurite outgrowth.* J Neurochem, 2002. **80**(6): p. 998-1008.
298. Ostendorp, T., et al., *Structural and functional insights into RAGE activation by multimeric S100B.* Embo J, 2007. **26**(16): p. 3868-78.
299. Dattilo, B.M., et al., *The extracellular region of the receptor for advanced glycation end products is composed of two independent structural units.* Biochemistry, 2007. **46**(23): p. 6957-70.
300. Arancibia, S.A., et al., *Toll-like receptors are key participants in innate immune responses.* Biol Res, 2007. **40**(2): p. 97-112.
301. Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nat Immunol, 2004. **5**(10): p. 987-95.
302. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.
303. Kaisho, T. and S. Akira, *Toll-like receptor function and signaling.* J Allergy Clin Immunol, 2006. **117**(5): p. 979-87; quiz 988.
304. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
305. Barton, G.M., J.C. Kagan, and R. Medzhitov, *Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA.* Nat Immunol, 2006. **7**(1): p. 49-56.
306. Rifkin, I.R., et al., *Toll-like receptors, endogenous ligands, and systemic autoimmune disease.* Immunol Rev, 2005. **204**: p. 27-42.

307. Lee, M.S. and Y.J. Kim, *Signaling pathways downstream of pattern-recognition receptors and their cross talk.* Annu Rev Biochem, 2007. **76**: p. 447-80.
308. Park, J.S., et al., *High mobility group box 1 protein interacts with multiple Toll-like receptors.* Am J Physiol Cell Physiol, 2006. **290**(3): p. C917-924.
309. Yu, M., et al., *HMGB1 signals through toll-like receptor (TLR) 4 and TLR2.* Shock, 2006. **26**(2): p. 174-179.
310. Park, J.S., et al., *Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein.* J Biol Chem, 2004. **279**(9): p. 7370-7377.
311. van Beijnum, J.R., W.A. Buurman, and A.W. Griffioen, *Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1).* Angiogenesis, 2008. **11**(1): p. 91-9.
312. Lee, M.S. and Y.J. Kim, *Signaling pathways downstream of pattern-recognition receptors and their cross talk.* Annu Rev Biochem, 2007. **76**: p. 447-480.
313. Apetoh, L., et al., *Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy.* Nat Med, 2007. **13**(9): p. 1050-9.
314. Apetoh, L., et al., *Molecular interactions between dying tumor cells and the innate immune system determine the efficacy of conventional anticancer therapies.* Cancer Res, 2008. **68**(11): p. 4026-30.
315. Tesniere, A., et al., *Immunogenic cancer cell death: a key-lock paradigm.* Curr Opin Immunol, 2008. **20**(5): p. 504-11.
316. Peter, M.E., *ROS eliminate danger.* Immunity, 2008. **29**(1): p. 1-2.
317. Kawai, T. and S. Akira, *Innate immune recognition of viral infection.* Nat Immunol, 2006. **7**(2): p. 131-7.
318. Ahmad-Nejad, P., et al., *Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments.* Eur J Immunol, 2002. **32**(7): p. 1958-68.
319. Kariko, K., et al., *Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA.* Immunity, 2005. **23**(2): p. 165-75.
320. Lotze, M.T. and K.J. Tracey, *High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal.* Nat Rev Immunol, 2005. **5**(4): p. 331-342.
321. El Mezayen, R., et al., *Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin.* Immunol Lett, 2007. **111**(1): p. 36-44.
322. Kokkola, R., et al., *High mobility group box chromosomal protein 1: A novel proinflammatory mediator in synovitis.* Arthritis Rheum, 2002. **46**(10): p. 2598-2603.
323. Taniguchi, N., et al., *High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine.* Arthritis Rheum, 2003. **48**(4): p. 971-981.
324. af Klint, E., et al., *Intraarticular glucocorticoid treatment reduces inflammation in synovial cell infiltrations more efficiently than in synovial blood vessels.* Arthritis Rheum, 2005. **52**(12): p. 3880-3889.
325. Brenchley, P.E., *Angiogenesis in inflammatory joint disease: a target for therapeutic intervention.* Clin Exp Immunol, 2000. **121**(3): p. 426-9.
326. Palmblad, K., et al., *Morphological characterization of intra-articular HMGB1 expression during the course of collagen-induced arthritis.* Arthritis Res Ther, 2007. **9**(2): p. R35.
327. Drinda, S., et al., *Identification of the receptor for advanced glycation end products in synovial tissue of patients with rheumatoid arthritis.* Rheumatol Int, 2005. **25**(6): p. 411-413.
328. Hou, F.F., et al., *Receptor for advanced glycation end products on human synovial fibroblasts: role in the pathogenesis of dialysis-related amyloidosis.* J Am Soc Nephrol, 2002. **13**(5): p. 1296-1306.

329. Steenvoorden, M.M., et al., *RAGE activation induces invasiveness of RA fibroblast-like synoviocytes in vitro.* Clin Exp Rheumatol, 2007. **25**(5): p. 740-2.
330. Chavakis, E., et al., *High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells.* Circ Res, 2007. **100**(2): p. 204-12.
331. Schlueter, C., et al., *Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule.* Am J Pathol, 2005. **166**(4): p. 1259-1263.
332. Mitola, S., et al., *Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine.* J Immunol, 2006. **176**(1): p. 12-15.
333. Kawahara, K., et al., *HMGB1 release in co-cultures of porcine endothelial and human T cells.* Xenotransplantation, 2007. **14**(6): p. 636-41.
334. Fiuza, C., et al., *Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells.* Blood, 2003. **101**(7): p. 2652-2660.
335. Orlova, V.V., et al., *A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin.* Embo J, 2007. **26**(4): p. 1129-39.
336. Park, J.S., et al., *Activation of gene expression in human neutrophils by high mobility group box 1 protein.* Am J Physiol Cell Physiol, 2003. **284**(4): p. C870-879.
337. Semino, C., et al., *NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1.* Blood, 2005. **106**(2): p. 609-616.
338. Yang, D., et al., *High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin.* J Leukoc Biol, 2007. **81**(1): p. 59-66.
339. Rovere-Querini, P., et al., *HMGB1 is an endogenous immune adjuvant released by necrotic cells.* EMBO Rep, 2004. **5**(8): p. 825-830.
340. Messmer, D., et al., *High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization.* J Immunol, 2004. **173**(1): p. 307-313.
341. Dumitriu, I.E., et al., *Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products.* J Immunol, 2005. **174**(12): p. 7506-7515.
342. Qin, S., et al., *Role of HMGB1 in apoptosis-mediated sepsis lethality.* J Exp Med, 2006. **203**(7): p. 1637-1642.
343. Andersson, U., et al., *HMG-1 stimulates proinflammatory cytokine synthesis in human monocytes.* J Exp Med, 2000. **192**(4): p. 565-570.
344. Zhou, Z., et al., *HMGB1 regulates RANKL-induced osteoclastogenesis in a manner dependent on RAGE.* J Bone Miner Res, 2008. **23**(7): p. 1084-96.
345. Charoonpatrapong, K., et al., *HMGB1 expression and release by bone cells.* J Cell Physiol, 2006. **207**(2): p. 480-490.
346. Meng, E., et al., *High mobility group box 1 protein inhibits the proliferation of human mesenchymal stem cells and promotes their migration and differentiation along osteoblastic pathway.* Stem Cells Dev, 2008. **17**(4): p. 805-13.
347. Kokkola, R., et al., *Successful therapy in collagen-induced arthritis in mice and rats targeting extracellular HMGB1 activity.* Arthritis Rheum, 2003. **48**: p. 2052-2058.
348. Abeyama, K., et al., *The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism.* J Clin Invest, 2005. **115**(5): p. 1267-1274.
349. Van de Wouwer, M., et al., *The lectin-like domain of thrombomodulin interferes with complement activation and protects against arthritis.* J Thromb Haemost, 2006. **4**(8): p. 1813-1824.
350. Hofmann, M.A., et al., *RAGE and arthritis: the G82S polymorphism amplifies the inflammatory response.* Genes Immun, 2002. **3**(3): p. 123-135.

351. Kojima, M. and K. Kangawa, *Ghrelin: structure and function.* Physiol Rev, 2005. **85**(2): p. 495-522.
352. Chorny, A., et al., *Ghrelin protects against experimental sepsis by inhibiting high-mobility group box 1 release and by killing bacteria.* J Immunol, 2008. **180**(12): p. 8369-8377.
353. Dong Xda, E., et al., *High mobility group box I (HMGB1) release from tumor cells after treatment: implications for development of targeted chemoimmunotherapy.* J Immunother, 2007. **30**(6): p. 596-606.
354. Cheng, B.Q., et al., *Serum high mobility group box chromosomal protein 1 is associated with clinicopathologic features in patients with hepatocellular carcinoma.* Dig Liver Dis, 2008. **40**(6): p. 446-52.
355. Aaseth, J., M. Haugen, and O. Forre, *Rheumatoid arthritis and metal compounds--perspectives on the role of oxygen radical detoxification.* Analyst, 1998. **123**(1): p. 3-6.
356. Ernestam, S., et al., *Evidence for immunostimulatory effects of intramuscular gold in patients with rheumatoid arthritis: correlation with skin reactions.* J Rheumatol, 2003. **30**(8): p. 1748-55.
357. Rau, R., et al., *Radiographic outcome after three years of patients with early erosive rheumatoid arthritis treated with intramuscular methotrexate or parenteral gold. Extension of a one-year double-blind study in 174 patients.* Rheumatology (Oxford), 2002. **41**(2): p. 196-204.
358. Yamashita, M., et al., *Inhibition by auranofin of the production of prostaglandin E2 and nitric oxide in rat peritoneal macrophages.* Eur J Pharmacol, 1999. **368**(2-3): p. 251-8.
359. Jeon, K.I., J.Y. Jeong, and D.M. Jue, *Thiol-reactive metal compounds inhibit NF-kappa B activation by blocking I kappa B kinase.* J Immunol, 2000. **164**(11): p. 5981-9.
360. Vaisman, A., et al., *Effect of DNA polymerases and high mobility group protein 1 on the carrier ligand specificity for translesion synthesis past platinum-DNA adducts.* Biochemistry, 1999. **38**(34): p. 11026-39.
361. Zimmermann, K., et al., *Native versus recombinant high-mobility group B1 proteins: functional activity in vitro.* Inflammation, 2004. **28**(4): p. 221-229.
362. Hornung, V., et al., *Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides.* J Immunol, 2002. **168**(9): p. 4531-7.
363. Pisetsky, D.S., H. Erlandsson-Harris, and U. Andersson, *High-mobility group box protein 1 (HMGB1): an alarmin mediating the pathogenesis of rheumatic disease.* Arthritis Res Ther, 2008. **10**(3): p. 209-219.
364. Yanez-Mo, M., et al., *Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells.* N Engl J Med, 2003. **348**(5): p. 403-13.
365. Steenvoorden, M.M., et al., *Fibroblast-like synoviocyte-chondrocyte interaction in cartilage degradation.* Clin Exp Rheumatol, 2007. **25**(2): p. 239-45.
366. Brennan, F.M., et al., *Detection of transforming growth factor-beta in rheumatoid arthritis synovial tissue: lack of effect on spontaneous cytokine production in joint cell cultures.* Clin Exp Immunol, 1990. **81**(2): p. 278-85.
367. Foell, D., et al., *Expression of the pro-inflammatory protein S100A12 (EN-RAGE) in rheumatoid and psoriatic arthritis.* Rheumatology (Oxford), 2003. **42**(11): p. 1383-9.
368. Pullerits, R., et al., *Induction of arthritis by high mobility group box chromosomal protein 1 is independent of tumour necrosis factor signalling.* Arthritis Res Ther, 2008. **10**(3): p. R72.
369. Sundberg, E., et al., *Systemic TNF blockade does not modulate synovial expression of the pro-inflammatory mediator HMGB1 in rheumatoid arthritis patients--a prospective clinical study.* Arthritis Res Ther, 2008. **10**(2): p. R33.

370. Rubartelli, A. and R. Sitia, *Entry of exogenous polypeptides into the nucleus of living cells: facts and speculations.* Trends Cell Biol, 1995. **5**(11): p. 409-12.
371. Bottger, M., et al., *Condensation of vector DNA by the chromosomal protein HMG1 results in efficient transfection.* Biochim Biophys Acta, 1988. **950**(2): p. 221-8.
372. Mistry, A.R., et al., *Recombinant HMG1 protein produced in Pichia pastoris: a nonviral gene delivery agent.* Biotechniques, 1997. **22**(4): p. 718-729.
373. Lange, S.S., D.L. Mitchell, and K.M. Vasquez, *High mobility group protein B1 enhances DNA repair and chromatin modification after DNA damage.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10320-5.
374. Liu, S., et al., *HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers.* Am. J. Physiol. Cell Physiol., 2006. **290**: p.990-999.

FOR YOUR NOTES