From Department of Medicine, Solna
Karolinska Institutet, Stockholm, Sweden

STUDIES OF THE IMMUNOPATHOGENESIS OF ARTHRITIS, WITH AN EMPHASIS ON THE ALARMIN HMGB1

Heshuang Qu

曲和爽

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Cover illustration: ***'The Journey of Science',*** handcrafted by **Charlotte De Vries** (2022). This work reflects a free interpretation of the paper "*Immunoprofiling of active and inactive systemic juvenile idiopathic arthritis reveals distinct biomarkers: a single center study*" by Heshuang Qu et al. It shows the journey of what science sometimes is; a winding path towards an unknown destination, aiming to find meaningful connections and patterns in the large and complex data being generated these days.

Studies of the immunopathogenesis of arthritis, with an emphasis on the alarmin HMGB1

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Heshuang Qu

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Principal Supervisor:
Professor Helena Erlandsson Harris
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Co-supervisors:
Associate Professor Cecilia Aulin
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Associate Professor Alexander Espinosa
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Professor Camilla Svensson
Karolinska Institutet
Department of Physiology and Pharmacology

Opponent:
Lillemor Berntson
Adjunct senior lecturer
Uppsala University
Department of Women's and Children's Health

Examination Board:
Associate Professor Charlotte Rolny
Karolinska Institutet
Department of Oncology-Pathology

Associate Professor Peder Olofsson
Karolinska Institutet
Department of Medicine, Solna

Professor Jan Ernerudh
Linköping University
Department of Biomedical and Clinical Sciences

Try your best, and time will tell.

POPULAR SCIENCE SUMMARY OF THE THESIS

Thinking about feeling sick – tiredness, weakness, fever, reduced appetite, headache or maybe feeling down in mind? Sometimes, sickness from flu or mild infection can be self-treated with some common medications such as Ibuprofen and Levofloxacin, and recovery will come over time. Sometimes, sickness stays or recurs over time.

Arthritis is a common condition that causes pain and inflammation in the joints. Arthritis affects people of all ages, including children. Usually, the symptoms of arthritis do not simply stay in joints; instead, other organs are also affected. For example, in some children with systemic juvenile idiopathic arthritis (JIA), the condition can damage a wide variety of body systems, including the skin, eyes, lungs, heart and blood vessels. Unfortunately, JIA is complex, and currently, there is no drug to cure JIA.

Doctors need to give accurate and efficient diagnosis before deciding on treatments. However, it is often problematic when symptoms from different causes look the same. For example, both bacterial infection and systemic JIA can lead to fever, but simply treating the fever would not be optimal in either case. Disease-specific markers, known as biomarkers, better facilitate diagnosis and even prediction of medicine treatment outcomes.

My PhD work focuses on arthritis in children called JIA, with a prevalence of up to 1/1,000 worldwide. We aimed to identify special markers from patient samples that can support a better diagnosis of JIA. Such markers could also potentially help understand the causes and underlying mechanisms of the disease, thereby benefiting novel drug development. Additionally, it has been suggested that children with JIA have higher incidences of psychiatric disorders, such as depression and anxiety. Therefore, we also tried to identify and explain the underlying mechanisms of central neuroinflammation in children with JIA.

From computer prediction to the wet lab, from cell culture to animal experiments and from bench to bedside, there are always unmet needs from patients and healthcare workers. As researchers, we work on answering the questions as correctly as possible. The findings in this thesis may help to better define and classify JIA, as well as draw people's attention to the well-being of children with JIA.

简明摘要

生病是什么感觉？会觉得疲倦、肌肉无力、发烧、食欲不振、头痛或情绪低落？大部分时候，流感或轻度感染都能自行治愈或通过服用布洛芬和扑热息痛等一些常用药物来治疗；但有时，生病的感觉会一直存在，甚至会随着时间的推移而反复发作。

类风湿性关节炎是一种常见的自身免疫性疾病，主要表现为关节疼痛，肿胀甚至形变。关节炎影响着所有年龄段的人，包括儿童。事实上，类风湿性关节炎的症状不仅仅局限于关节，其他器官也会受到影响。例如，在一些患有全身性幼年特发性关节炎 (sJIA) 的儿童中，疾病会损害多个器官，包括皮肤、眼睛、肺、心脏和血管。不幸的是，与流感相比，JIA 的起因和治疗都更复杂，现阶段并没有药物可以根治 JIA。

在确定治疗方案之前，医生需要给出准确有效的诊断。但是，当来自不同原因的症状看起来相同时，诊断就不会那么容易。例如，细菌感染和 JIA 都有发烧的症状，简单地服用退烧药可能不是最佳选择。在这种情况下，疾病的特异性标志物可以更好地辅助诊断，甚至预测药物治疗的效果。同时，儿童和青少年正处于生长和发育的关键阶段，传统的一以贯之的治疗模式很可能试错，从而使他们经受不必要的痛苦。精准医疗和个性化治疗能够减少药物带来的副作用。

我的博士课题的关注重点是幼年特发性关节炎（JIA），全球儿童发病率高达千分之一。我们的目标是定义血液和滑液中的生物标志物，以辅助更准确的诊断和治疗。这些生物标志物有助于了解疾病的成因，因此支持新的治疗靶点的发现和新药开发。此外，已经注意到患有 JIA 的儿童可能有更高的精神疾病发病率，例如他们比其他同龄孩子更多地表现出抑郁和焦虑；因此，我们还通过实验室研究试图解释 JIA 儿童精神疾病的潜在机制。

本论文中的第 I 篇文章重点讨论了 sJIA 在发病和消退阶段血液中炎症因子表达量的变化，并发现了可能将 sJIA 与其他儿童自身免疫疾病区分开来的特异性标记物 SCF。

第 II 篇文章重点关注少关节型幼年特发性关节炎（oligoarticular JIA，oligoJIA）。与 sJIA 不同， oligoJIA 患者的血液中的炎症因子表达量与健康人无异，提示在未来的研究中 oligoJIA 的关节滑液（SF）似乎具有更高的研究价值。我们还发现，与持续患病的患者相比，在患病初期的 oligoJIA 患者的关节滑液中趋化因子的表达量较高，因而趋化因子可能成为抑制 oligoJIA 病情发展的关键靶点。

第 III 和 IV 篇文章研究了一种炎症因子 HMGB1 在细胞层面的作用。HMGB1 的高表达与很多疾病相关，其中包括 JIA。在第 I 篇文章中，我们曾发现在 sJIA 的发病期，患者血液中的 HMGB1 的浓度有所升高，揭示了 HMGB1 与 sJIA 的联系。我们在第 III 和第 IV 篇文章中详细地研究了 HMGB1 对巨噬细胞分化的作用。

第 V 篇文章基于两种不同的类风湿性关节炎的小鼠模型，探讨类风湿性关节炎导致的精神疾病。我们重点研究了小鼠大脑中的海马体的变化。很多研究表明，海马体具调控记忆力，空间学习能力，焦虑和抑郁情绪的功能。我们发现，在患有类风湿性关节炎的小鼠的海马体中，神经细胞的增殖和更新有所减慢。这表明关节炎可能导致中枢神经系统的病变。

这本论文中的结果能够帮助科学家和医生从分子微观层面更好地定义和区分 JIA，也将 JIA 儿童可能存在的精神健康问题进行探讨，有助于医护人员在未来的随诊过程中更详尽地记录患者的症状，从而更全面，更系统地分析患者病情，制定个性化治疗方案.

ABSTRACT

Dysregulated inflammatory responses are characterized by the excessive release of endogenous inflammatory molecules, which initiate a chain of reactions, including immune cell infiltration, activation, polarization, necrosis, apoptosis and pyroptosis. These changes in cell distribution and status can lead to tissue damage, represented by swelling, pain, redness, heat and loss of function. Rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) are diseases involving dysregulated inflammatory responses and are the most common rheumatic diseases in adults and children, respectively. Although there have been many studies investigating the prognosis, diagnosis and treatment of RA and JIA, there are still unmet clinical needs in this regard. A better understanding of the pathogenesis of each disease is essential to address these unmet needs.

High mobility group box 1 (HMGB1), a prototypical damage-associated molecular pattern (DAMP), is expressed in all nucleated animal cells and platelets. The role of HMGB1 depends on its localization, post-translational modification and redox modification. HMGB1 is actively secreted or passively released into the extracellular region during cell activation or cell death. Extracellular HMGB1 acts as an alarmin that can initiate the immune response alone or combined with other molecules, such as nucleic acid, to participate in multiple biological processes. It has been evident that HMGB1 is involved in various inflammatory responses and autoimmunity, including RA and JIA. Increased levels of HMGB1 have been recorded in arthritic joints, and HMGB1 blockade ameliorates experimental arthritis.

The overall aim of this thesis is to investigate the pathogenesis of arthritic diseases, including RA and JIA, with a special emphasis on the involvement of HMGB1.

In **papers I and II**, we used proximity extension assay (PEA) to measure 92 inflammation-related proteins in plasma and synovial fluid (SF) from patients with systemic JIA (sJIA) and oligoarticular JIA (oligoJIA). By performing a cross-sectional comparison with age- and sex-matched healthy controls, we were able to not only confirm the previously reported sJIA biomarkers (IL6, IL18 and S100A12), but also find novel markers which could distinguish active sJIA from inactive sJIA or controls. The level of HMGB1 was significantly higher in active sJIA than inactive sJIA in both paired and cross-sectional analysis. In contrast, plasma proteomics did not distinguish patients with oligoJIA from healthy controls. This finding corresponds to the clinical definition of oligoJIA, where patients have local joint inflammation but lack significant systemic inflammation. Longitudinal analysis of twenty SF and ten plasma samples from an individual patient revealed the immunosuppression effects of methotrexate (MTX). Finally, the paired analysis of SF indicated that, compared to the persistent phase, cell chemotaxis was the main character defining the early phase of oligoJIA.

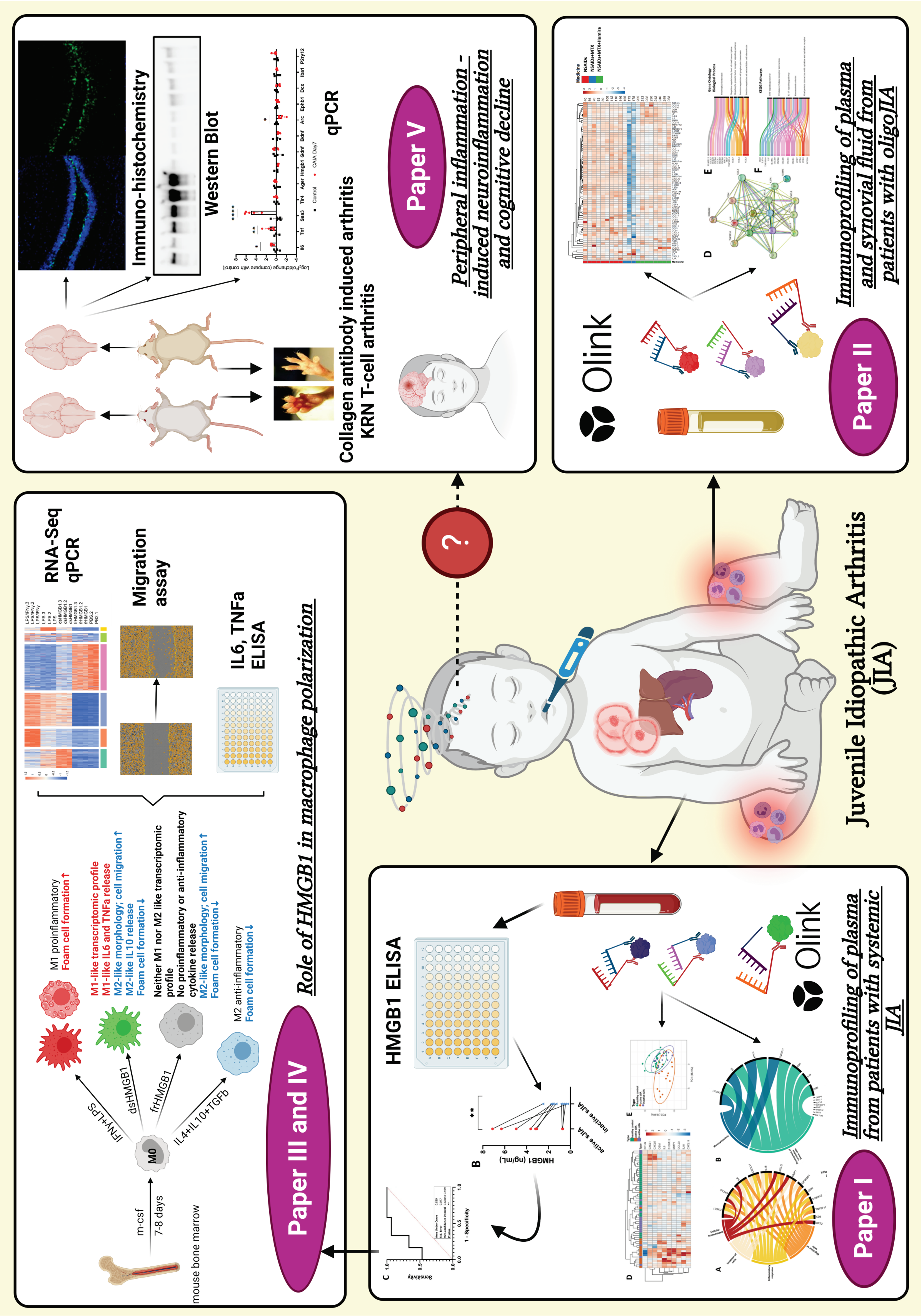
Macrophages are versatile myeloid cells that play an important role in tissue homeostasis, immune defense, and inflammatory progression and resolution. Macrophage polarization plays a role in defining the outcomes of different diseases, including arthritis. In **papers III** and **IV**, we investigated the effects of HMGB1 on the *in vitro* polarization of murine bone marrow-

derived macrophages (BMDMs). Compared to proinflammatory phenotype M1 and alternatively activated anti-inflammatory phenotype M2, disulfide HMGB1 (dsHMGB1) induced a unique macrophage phenotype that secretes proinflammatory cytokines, rather than inducing metabolic changes leading to nitric oxide production; while fully reduced HMGB1 (frHMGB1) did not trigger any significant change in cytokine release or gene expression. Both dsHMGB1 and frHMGB1 could induce cell migration. Moreover, RNA sequencing (RNA-Seq) was performed to generate overall transcriptomic profiles of HMGB1-stimulated BMDMs. The results further confirmed the initial findings that dsHMGB1 induced a distinct BMDM polarization phenotype compared to LPS/IFNγ- (M1) and LPS-induced phenotypes, while frHMGB1 failed in inducing a significant transcriptomic profile shift compared to controls.

In **paper V**, we investigated the occurrence of neuroinflammation in experimental arthritis. Two mouse arthritis models, collagen antibody-induced arthritis (CAIA) and KRN T-cell arthritis (KRN), were established, and brain tissues were analyzed, with a focus on the hippocampus area. Our data suggested that arthritis could lead to neuroinflammation, significantly upregulating proinflammatory gene expression and interfering with hippocampal neurogenesis and proliferation.

Taken together, the findings presented in this thesis contributed new knowledge about arthritis and inflammation. We discovered novel protein biomarkers for JIA, which could benefit the prognosis, diagnosis and classification of JIA. We compared HMGB1, a DAMP, with LPS, a pathogen-associated molecular pattern (PAMP), regarding their effects on macrophage polarization. We also carried out RNA-Seq analysis to obtain the transcriptomic profiles of frHMGB1 and dsHMGB1 stimulated BMDMs, which may provide good references for other researchers interested in the inflammatory properties of HMGB1 and role of HMGB1 as a mediator of arthritic inflammation. Finally, we explored the occurrence of neuroinflammation in two arthritis mouse models, revealing the potential mechanisms behind arthritis-induced cognitive disorders.

GRAPHICAL ABSTRACT


Immuno-histochemistry
Western Blot
qPCR
Collagen antibody induced arthritis
KRN T-cell arthritis
Paper V
Peripheral inflammation - induced neuroinflammation and cognitive decline
Olink
Paper II
Immunoprofiling of plasma and synovial fluid from patients with oligoJIA
?
Juvenile Idiopathic Arthritis (JIA)
RNA-Seq
qPCR
Migration assay
IL6, TNFa ELISA
mouse bone marrow
m-csf
7-8 days
M0
IFNγ+LPS
dsHMGB1
frHMGB1
IL4+IL10+TGFβ
M1 proinflammatory
Foam cell formation↑
M1-like transcriptomic profile
M1-like IL6 and TNFa release
M2-like morphology; cell migration↑
M2-like IL10 release
Foam cell formation↓
Neither M1 nor M2 like transcriptomic profile
No proinflammatory or anti-inflammatory cytokine release
M2-like morphology; cell migration↑
Foam cell formation↓
M2 anti-inflammatory
Foam cell formation↓
Paper III and IV
Role of HMGB1 in macrophage polarization
HMGB1 ELISA
HMGB1 (ng/mL)
active sJIA
inactive sJIA
**
Sensitivity
1 - Specificity
Olink
Paper I
Immunoprofiling of plasma from patients with systemic JIA

This illustration was created with BioRender.com.

LIST OF SCIENTIFIC PAPERS

I. **Qu, H.**, Sundberg, E., Aulin, C., Neog, M., Palmblad, K., Horne, A., Granath, F., Ek, A., Melén E., Olsson, M., Erlandsson Harris H.*. Immunoprofiling of active and inactive systemic juvenile idiopathic arthritis reveals distinct biomarkers: a single-center study. *Pediatr Rheumatol* 2021; 19, 173.

II. **Qu, H.**[#], Neog, M.[#], Palmblad, K., Sundberg, E., Lövquist, A., Melén E., Aulin, C., Erlandsson Harris H.*. Cross-sectional and longitudinal immunoprofiling of oligoarticular juvenile idiopathic arthritis reveals different patterns in synovial fluid and plasma specimens.
Manuscript

III. Salo, H., **Qu, H.**, Mitsiou, D., Aucott, H., Han, J., Zhang, X., Aulin, C., Erlandsson Harris, H.*. Disulfide and Fully Reduced HMGB1 Induce Different Macrophage Polarization and Migration Patterns. *Biomolecules*. 2021; 11(6):800.

IV. **Qu, H.**, Heinbäck, R., Salo, H., Ewing, E., Espinosa, A., Aulin, C., Erlandsson Harris, H.*. Transcriptomic Profiling Reveals That HMGB1 Induces Macrophage Polarization Different from Classical M1. *Biomolecules*. 2022; 12(6):779.

V. **Qu, H.***, Shen, Y.[†], Bersellini Farinotti, A.[†], Wermeling, F.[#], Svensson, C.I.[#], Erlandsson Harris, H.*. Investigating the occurrence of neuroinflammation in mice with collagen-antibody induced arthritis and KRN T-cell arthritis.
Manuscript

* Corresponding authors

[#], [†] Equally contributed

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LIST OF ABBREVIATIONS

ACD	actinomycin D
ACPA	anti-citrullinated peptide antibody
ADAM	A desintegrin and metalloprotease
ADHD	attention deficit hyperactivity disorder
AGE	advanced glycation end-products
AIA	adjuvant-induced arthritis
AID	autoinflammatory syndromes
ARC	activity-regulated cytoskeleton-associated protein
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BMDM	bone marrow-derived macrophage
CAIA	collagen antibody-induced arthritis
CASP	caspase
CBP	calmodulin binding protein
CCL	C-C motif ligand
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CLR	C-type lectin receptor
CNS	central nervous system
COX2	cyclooxygenase-2
CRP	C-reactive protein
CV	coefficient of variation
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C chemokine receptor
DAMP	damage-associated molecular pattern
DCX	doublecortin
DMARDs	disease-modifying anti-rheumatic drugs
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EphB	ephrin type-B receptor
ESR	erythrocyte sedimentation rate
FLT3LG	Fms-related tyrosine kinase 3 ligand
G6PI	glucose-6-phosphate isomerase
GC	glucocorticoid
GDNF	glial cell line-derived neurotrophic factor
GSEA	gene set enrichment analysis
HA	hyaluronan
HAse	hyaluronidase
HMGB1	high mobility group box 1
3sHMGB1	non-oxidizable chemokine-HMGB1
dsHMGB1	disulfide HMGB1
frHMGB1	fully-reduced HMGB1
oxHMGB1	sulfonyl HMGB1
HSP	heat shock proteins

ICU	intensive care unit
IFA	incomplete Freund’s adjuvant
IFN	interferon
IL	interleukin
JABBA	Juvenile Arthritis Biobank Astrid Lindgrens Barnsjukhus
JIA	juvenile idiopathic arthritis
oligoJIA	oligoarticular JIA
sJIA	systemic JIA
KITLG	ligand for the receptor-type protein-tyrosine kinase KIT
KRN	KRN T-cell arthritis
LC-MS/MS	liquid chromatography-tandem mass spectrometric analysis
LOD	limit of detection
LOX-1	low-density lipoprotein receptor-1
LPS	lipopolysaccharide
mAb	monoclonal antibodies
malPEG	methoxypolyethylene glycol-maleimide
MAS	macrophage activation syndrome
M-CSF	macrophage colony-stimulating factor
MD2	myeloid differentiation factor 2
MHC	major histocompatibility complex
MIS-C	multisystem inflammatory syndrome in children
MMP	matrix metalloproteinase
MTX	methotrexate
NEM	N-ethymaleimide
NES	nuclear export signals
NGF	nerve growth factor
NLR	NOD-like receptor
NLS	nuclear localization sequences
NO	nitric oxide
NPX	normalized protein expression
NSAID	non-steroidal anti-inflammatory drug
oxLDL	oxidized low density lipoprotein
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCA	principle component analysis
PEA	proximity extension assay
PGN	peptidoglycan
PICS	post-intensive care syndrome
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
PTM	post-translational modification
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end-products
RF	rheumatoid factor
RLR	RIG-I-like receptor
RNA-Seq	RNA sequencing

SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SF	synovial fluid
SIRT	NAD-dependent deacetylase sirtuin
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
ST1A1	sulfotransferase 1A1
SURFS	systemic undifferentiated recurring fever syndromes
TCR	T cell receptor
TGF	transforming growth factor
thiol/SH	sulfhydryl
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFSF14	TNF superfamily member 14
Trx	Thioredoxin-1
TrxR	Thioredoxin reductase
VEGF	vascular endothelial growth factor

1 INTRODUCTION

1.1 INFLAMMATION AND THE MEDIATORS OF INFLAMMATION

Inflammation is a complex biological response of an organism to stimuli, including outside invaders, such as pathogens and irritants, as well as the body's own immune system (Figure 1). Acute inflammation often causes noticeable symptoms, including fever, pain, redness and swelling, which last for several hours or days before remission. Chronic inflammation, resulting from either improper immune activation, faulty immune resolution or both, is usually accompanied by subtler and persistent symptoms, can last for months or years. Unsuccessfully eradicated acute inflammation, autoimmune disorders and long-term exposure to irritants can result in chronic inflammation.

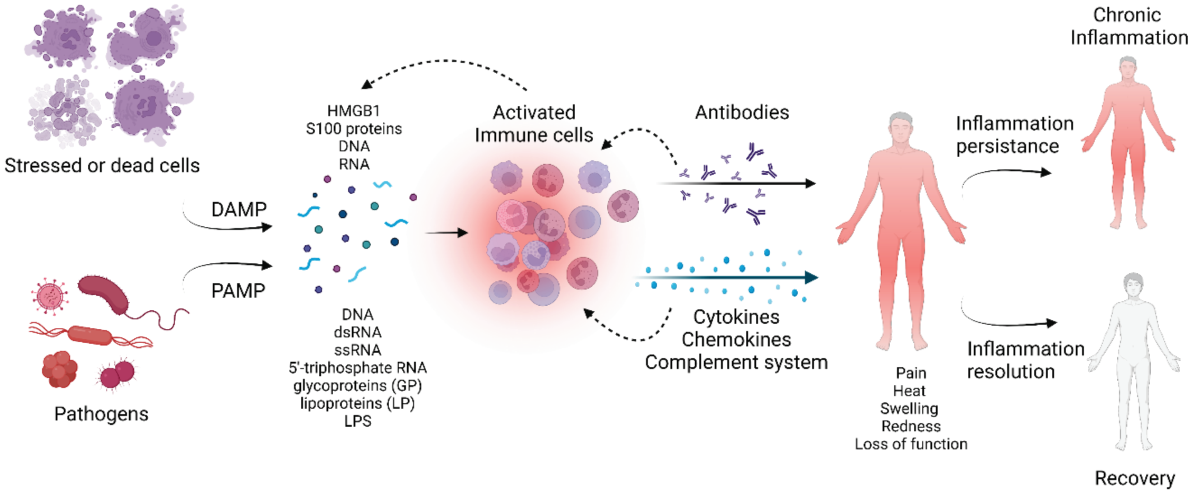

Stressed or dead cells
DAMP
PAMP
Pathogens
HMGB1
S100 proteins
DNA
RNA
DNA
dsRNA
ssRNA
5'-triphosphate RNA
glycoproteins (GP)
lipoproteins (LP)
LPS
Activated
Immune cells
Antibodies
Cytokines
Chemokines
Complement system
Pain
Heat
Swelling
Redness
Loss of function
Inflammation
persistance
Chronic
Inflammation
Inflammation
resolution
Recovery

Figure 1. Illustration of the inflammatory process. This illustration was created with BioRender.com.

Inflammation is triggered by agents of either exogenous or endogenous origin, leading to the activation of the immune system and cytokine generation (Figure 1). Triggers from endogenous origins include damage-associated molecular patterns (DAMPs), released from dead cells or secreted by activated or stressed cells [1]. In contrast, triggers with exogenous origins include pathogen-associated molecular patterns (PAMPs), which initiate infectious pathogen-induced inflammatory reactions.

PAMPs and DAMPs alert and activate cells after recognition by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) [2]. Recognition results in a cascade of events, including the activation of signaling pathways leading to the upregulation and secretion of inflammatory mediators, such as cytokines and chemokines, and ultimately the recruitment of more immune cells to the site of infection and injury.

1.1.1 Pathogen-associated molecular patterns (PAMPs)

As the name suggests, PAMPs originate from pathogens, such as bacteria and viruses. PAMPs include polysaccharides, lipoproteins, as well as DNA and RNA segments of bacterial or viral origins. One well-known PAMP is lipopolysaccharide (LPS), which is a part of the outer cell wall of gram-negative bacteria and is known to be the cause of septic shock [2]. LPS can be recognized by immune cells via TLR4 together with myeloid differentiation factor 2 (MD2) on the immune cell surface, activating MyD88-NFκB signaling pathway [3]. Caspase-4 and -11 have been shown to be cytoplasmic LPS receptors, which directly bind to LPS, resulting in inflammasome activation and pyroptosis [4].

1.1.2 Damage-associated molecular patterns (DAMPs)

The concepts of DAMPs were first proposed by Polly Matzinger in the early 1990s in the danger model of the immune system. She described that the immune system could discriminate between dangerous and safe by recognizing the signals from injured or stressed cells and tissues [5]. Nowadays, the term “DAMPs” usually describes molecules released from dead cells and trigger inflammatory and immune responses [1]. Well-known DAMPs include high mobility group box 1 (HMGB1), S100 proteins, heat-shock proteins, DNAs and RNAs.

Increased levels of DAMPs have been detected in tissues and biological fluids, such as plasma and synovial fluid (SF), derived from patients afflicted with different inflammatory diseases. HMGB1 has been found to be abundantly expressed as a nuclear, cytoplasmic, and extracellular component in synovial tissues from rheumatoid arthritis (RA) patients [6]. High levels of S100A8/A9 and S100A12 have been recorded in sera of systemic juvenile idiopathic arthritis (sJIA) patients during disease flares [7, 8]. In systemic lupus erythematosus (SLE), the serum level of HMGB1 has been found to be significantly elevated and was correlated with disease activity [9]. These associations reveal that DAMPs play roles in the pathogenesis of inflammatory diseases and may have the potential as biomarkers for disease activity or therapeutic targets.

1.2 HIGH MOBILITY GROUP BOX 1 (HMGB1)

HMGB1 was discovered and named in 1973 for its high electrophoretic mobility in polyacrylamide gel [10]. This 29 kDa ubiquitous nuclear protein consists of 215 amino acids and three parts: DNA-binding A-box and B-box, and an acidic C-tail (Figure 2A and 2B). HMGB1 is expressed in all nucleated animal cells and platelets [11]. HMGB1 is evolutionally conserved among mammalian cells, with 99% protein sequencing homology between rodents and humans [12].

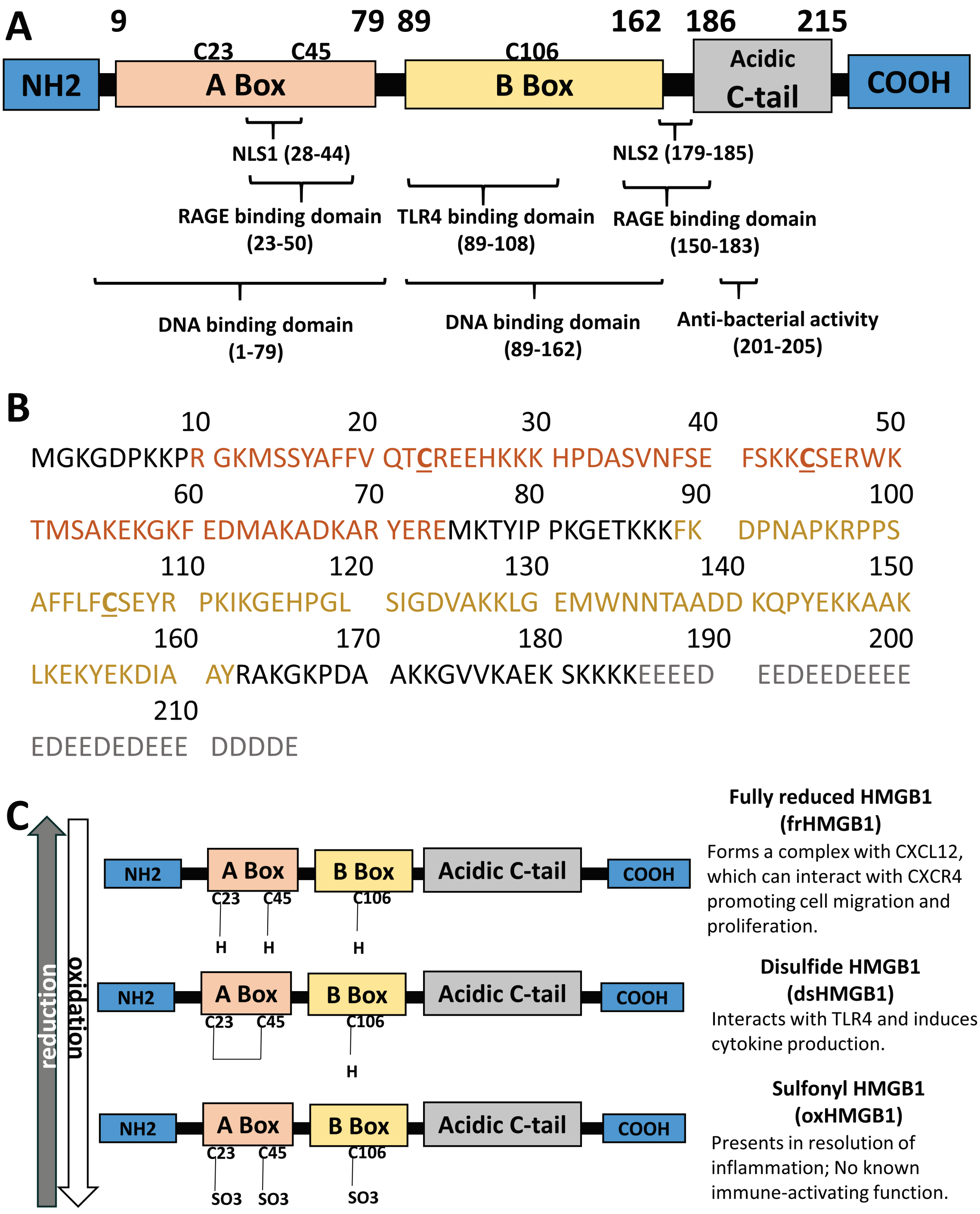

A
9
79 89
162 186 215
C23 C45
C106
NH2
A Box
B Box
Acidic C-tail
COOH
NLS1 (28-44)
NLS2 (179-185)
RAGE binding domain (23-50)
TLR4 binding domain (89-108)
RAGE binding domain (150-183)
DNA binding domain (1-79)
DNA binding domain (89-162)
Anti-bacterial activity (201-205)
B
10 20 30 40 50
MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
60 70 80 90 100
TMSAKEKGKF EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS
110 120 130 140 150
AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKAAK
160 170 180 190 200
LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK SKKKKEEEED EEDEEDEEEE
210
EDEEDEDEEE DDDDE
C
reduction
oxidation
NH2
A Box
B Box
Acidic C-tail
COOH
C23 C45 C106
H H H
C23 C45 C106
H
C23 C45 C106
SO3 SO3 SO3
Fully reduced HMGB1 (frHMGB1)
Forms a complex with CXCL12, which can interact with CXCR4 promoting cell migration and proliferation.
Disulfide HMGB1 (dsHMGB1)
Interacts with TLR4 and induces cytokine production.
Sulfonyl HMGB1 (oxHMGB1)
Presents in resolution of inflammation; No known immune-activating function.

Figure 2. HMGB1 structure and redox isoforms. (A) HMGB1 consists of three domains: DNA-binding A-box (amino acid 9-79) and B-box (amino acid 89-162), as well as an acidic C-tail (amino acid 186-21). (B) Two lysine-rich nuclear localization sequences (NLS) are located at the A-box and, the linker region between the B-box and the C-tail (see underlined, bold red characters). Three cysteines are located in amino acid position 23, 46 and 106, which redox status decide the receptor binding and biological function of HMGB1. (C) Fully-reduced HMGB1 ("C23hC45hC106h", frHMGB1), disulfide HMGB1 ("C23-C45C106h", dsHMGB1) and sulfonyl HMGB1 ("C23soC45soC106so", oxHMGB1). dsHMGB1 is the only isoform that induces proinflammatory response via binding to TLR4, resulting in cytokines release. FrHMGB1 lacks cytokine-inducing activity but induces cell migration by forming a heterodimer with CXCL12, which binds to CXCR4. oxHMGB1 presents in the resolution of inflammation, and it has no known immune-activation function. Interplay between frHMGB1 and dsHMGB1 is a reversible process dependent on the redox state of the intra- and extracellular environment, while the formation of sulfonyl HMGB1 is irreversible. This illustration was created with Microsoft PowerPoint.

HMGB1 is essential for life. Knockout of *Hmgb1* in mice resulted in a short life span of less than 24 hours, with numerous deficiencies caused by the downregulation of glucocorticoid receptors and impaired utilization of glycogen stored in the liver [13]. The functions of HMGB1 depend on its cellular location, post-translational modification (PTM), redox isoform and microenvironment.

1.2.1 HMGB1 localization

HMGB1 shuttles continuously between the nucleus and cytoplasm of resting cells, though it is mostly maintained in the nucleus during cell homeostasis [14]. Nuclear HMGB1 contributes to DNA-related events, such as DNA repair, transcription regulation and genome stability [15]. Nuclear localization signals (NLS) and nuclear export signals (NES) present in the A-box and B-box of HMGB1 regulate the location of HMGB1 (Figure 2A) [16]. Cell stress and immune responses can shift the equilibrium from nuclear towards cytoplasmic localization via increased PTMs (Figure 3A). HMGB1 phosphorylation in NLSs occurs after tumor necrosis factor (TNF) α and okadaic acid exposure, resulting in HMGB1 cytoplasmic translocation [17]. Methylation of HMGB1 in Lys42 or Lys112 also contributes to HMGB1 cytoplasmic translocation [18]. Hyper-acetylation of multiple lysines in the two NLSs prevents nuclear re-entry of HMGB1 [14, 19].

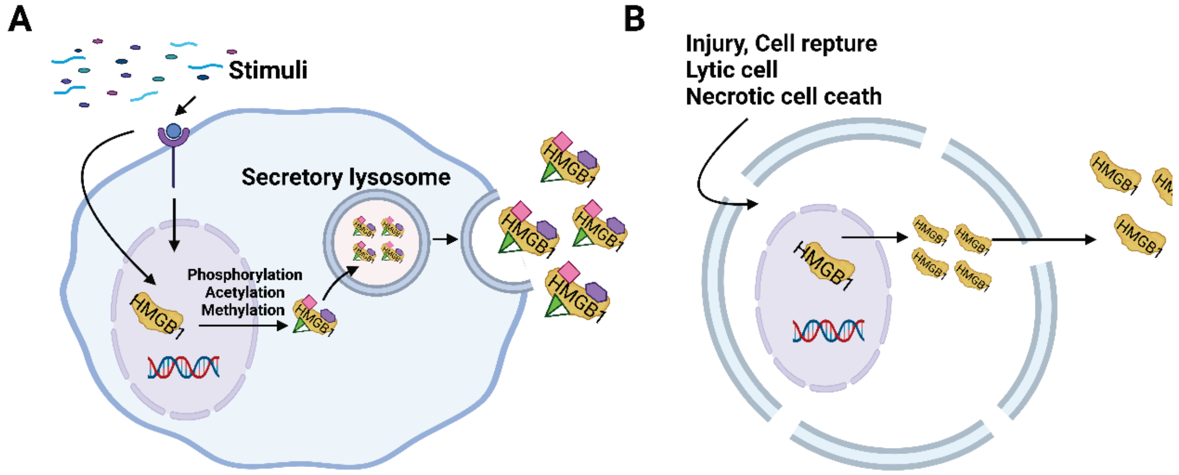


Figure 3. Translocation and release of HMGB1. There are two cellular mechanisms to release HMGB1 into the extracellular environment (active secretion and passive release). (A) Cell activation by extracellular stimuli can induce PTMs of HMGB1 through acetylation, phosphorylation, and methylation. This prevents HMGB1 from shuttling into the nucleus, resulting in accumulation in the cytoplasm. Cytoplasmic HMGB1 can then be enveloped into secretory lysosomes, which fuse with the cell membrane, and finally released into the extracellular environment. (B) HMGB1 is passively released into the extracellular space from damaged, necrotic and pyroptotic cells with leaky plasma membranes. Apoptotic cells undergoing secondary necrosis can also induce late HMGB1 release. This illustration was created with BioRender.com.

Cytosolic HMGB1 controls mitochondrial dynamics and morphology, and promotes autophagy [15, 20]. HMGB1 can be released into the extracellular space either autonomously secreted by activated immune cells, or passively by lytic cell death or sudden cell rupture, such as necrosis and pyroptosis (Figure 3B) [21, 22]. Due to the lack of a leader peptide sequence, the secretion of HMGB1 was previously claimed to be via the endo-lysosomal system rather than the well-characterized Golgi-endoplasmic reticulum pathway [23]. A recent study demonstrated that HMGB1 from bone marrow-derived macrophages (BMDMs) was only secreted under pyroptosis and indirectly dependent on gasdermin D; while HMGB1 release upon stimulation by LPS was gasdermin D-independent *in vivo* [24]. Another study proposed a model of HMGB1 secretion mediated by HSP90AA1 (Heat Shock Protein 90 Alpha Family Class A Member 1), GORASP2 (Golgi Reassembly Stacking Protein 2), the autophagy machinery, and exosomes, both *in vitro* and *in vivo* [25]. However, the exact PTMs or redox isoforms of the HMGB1 released by these distinct pathways are still unclear. It is likely that HMGB1 released passively or actively differs in PTM and may act differently in the microenvironment and on the surrounding cells.

Once secreted to the extracellular space from activated immune cells, extracellular HMGB1 acts as a prototypical alarmin, modulating the nature and magnitude of immune responses. HMGB1 conveys these roles either by directly binding to cell surface receptors, or by first forming complexes with other molecules, such as C-X-C motif chemokine ligand (CXCL) 12, LPS, DNA, interleukin (IL) 1α and IL1β, then binding to receptors as a heterocomplex [26-29]. Detection of HMGB1 by immune cells can result in proinflammatory cytokine release, apoptosis, immune cell proliferation, differentiation and chemotaxis [21]. Extracellular HMGB1 can also bind to intracellular receptors, via first uptake following binding to the receptor for advanced glycation end-products (RAGE), enabling endocytosis of HMGB1 [30]. Once inside the endosome, the amphoteric structure of HMGB1 disrupts the endosome membrane, and HMGB1 reaches the cytosol where it can potentially interact with its cytosolic sensors [30].

1.2.2 HMGB1 redox isoforms

There are three cysteines at amino acid positions 23, 45 and 106 (Figure 2A). Different extracellular functional isoforms of HMGB1 dependent on the redox status of these three conserved cysteine residues have been reported. The three different HMGB1 redox isoforms are denoted: fully-reduced HMGB1 ("C23hC45hC106h", frHMGB1), disulfide HMGB1 ("C23-C45C106h", dsHMGB1) and sulfonyl HMGB1 ("C23soC45soC106so", oxHMGB1) (Figure 2C). The interplay between frHMGB1 and dsHMGB1 is a reversible process. DsHMGB1 can be reduced to frHMGB1 by exposure to reducing reagents, for example, Dithiothreitol (DTT) *in vitro*, and frHMGB1 could be oxidized to dsHMGB1 or oxHMGB1 by air-equilibrated buffers or hydrogen peroxide *in vitro* [31, 32]. It has also been proposed that reactive oxygen species *in vivo* could oxidize HMGB1 [33]. OxHMGB1 is irreversibly transformed without any known immune-activating function [21].

The biological activity of extracellular HMGB1 depends on the redox state of the three cysteine residues. The three HMGB1 redox isoforms show distinct receptor binding capacities and trigger different cellular signaling pathways. DsHMGB1 is the only isoform known to exhibit a cytokine-inducing role. The administration of dsHMGB1 to rat brain increases the expression of major histocompatibility complex (MHC) class II and cell death in the central nervous system (CNS) [34]. FrHMGB1 induces chemotaxis of fibroblasts and monocytes into inflamed tissues, promoting wound healing and tissue repair [32, 35]. Although oxHMGB1 has no known proinflammatory function, in the highly oxidative cancer microenvironment, oxHMGB1 induces tolerogenicity of dendritic cells and benefits tumorigenesis [36].

To characterize the redox isoform of HMGB1, our lab currently uses responses of peripheral blood mononuclear cells (PBMCs) to overnight incubation with the HMGB1 protein to inform on the redox state. If proinflammatory cytokines, such as IL6 and TNFα, are measured from the cell supernatant, the HMGB1 can be regarded as dsHMGB1 [31]. However, we are not able to define the purity of the HMGB1 redox isoform, as there is no standardized cytokine concentration to evaluate whether the protein is pure dsHMGB1 or a mixture of frHMGB1 and dsHMGB1. A common strategy for deciding redox isoforms of proteins is by performing tryptic digestion and liquid chromatography-tandem mass spectrometric analysis (LC-MS/MS). However, there has been no available protocol of LC-MS/MS for HMGB1 redox isoform detection. Moreover, free thiol groups and disulfide bonds could be measured by direct colorimetric assay [37]. In addition, some chemistry compounds, including N-ethymaleimide (NEM) and methoxypolyethylene glycol-maleimide (malPEG), are reactive to the sulfhydryl (thiol/SH) group [38, 39]. In the future, combining gel and non-gel-based redox proteomics could be helpful for better characterizing the redox status of HMGB1.

1.2.3 HMGB1 receptors

Extracellular HMGB1 drives biological responses via various receptors (Figure 4). To date, 15 different receptors have been described to directly or indirectly bind HMGB1 [40]. Some receptors, for example RAGE, interact directly with HMGB1. In contrast, other receptors interact indirectly with HMGB1. For example, C-X-C chemokine receptor (CXCR) 4 binds to a heterocomplex consisting of frHMGB1 and CXCL12 [32, 35].

1.2.3.1 Toll-like Receptors (TLRs)

TLRs are evolutionarily conserved type I transmembrane proteins localized either at the plasma membrane (TLRs 1, 2, 4–6, and 10) or within the endosomal compartment (TLRs 3 and 7–9). TLRs are important components of the immune system, involved in detecting both PAMPs and DAMPs, and hence playing a core role in protecting the host against threats present in either the extracellular or intracellular environment [41].

TLR2, 3, 4, 5, 7 and 9 are known HMGB1 receptors [42]. DsHMGB1-TLR4 is the most well-studied interaction and induces proinflammatory cytokines, such as IL6, TNFα and IL1β, through the activation of MD primary response gene 88 (MyD88)-dependent NF-κβ pathway (Figure 4) [43]. It should be noted that HMGB1 could not directly bind to TLR4 independent of the HMGB1 redox isoform. Instead, HMGB1-TLR4 binding requires MD2, an extracellular

TLR4 adaptor binding specifically to dsHMGB1 [43]. In contrast, unlike dsHMGB1, frHMGB1 and oxHMGB1 have been found unable to bind to MD2 and consequently without cytokine-inducing effects [43]. Moreover, MD2 is also requisite for LPS signaling of TLR4 [3]. However, Yang et al. demonstrated that dsHMGB1 and LPS did not necessarily share the same binding epitope or structure, since the FSSE (P5779) tetramer selectively attenuated dsHMGB1–MD2–TLR4 signaling without inhibiting macrophage activation in response to LPS [44].

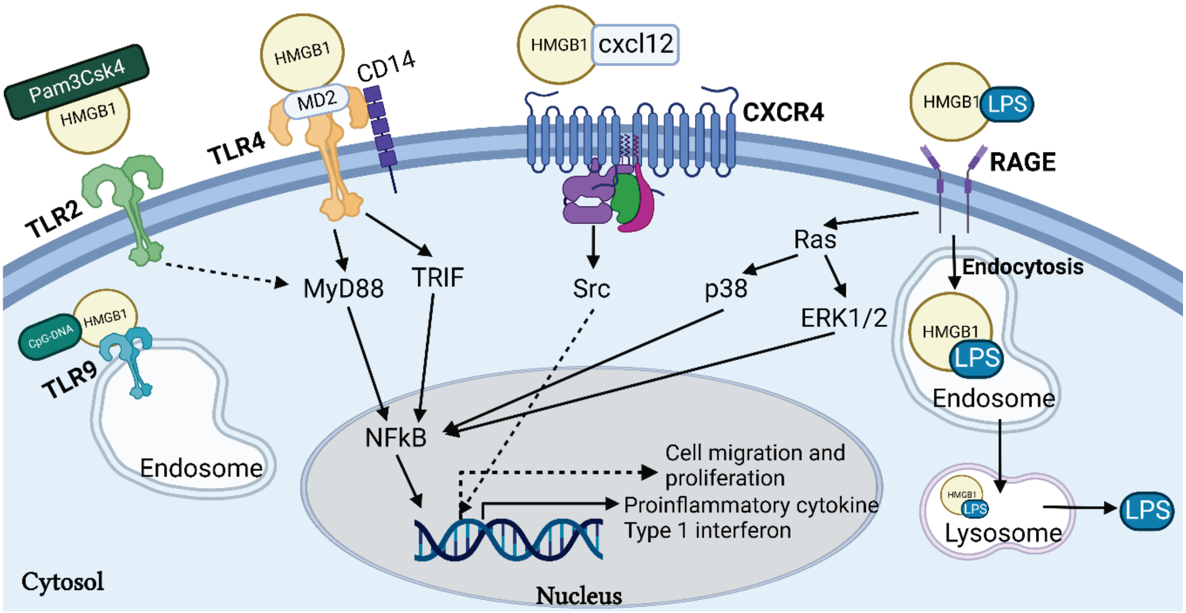

Pam3Csk4
HMGB1
TLR2
HMGB1
MD2
CD14
TLR4
HMGB1
cxcl12
CXCR4
HMGB1
LPS
RAGE
CpG-DNA
HMGB1
TLR9
Endosome
MyD88
TRIF
Src
Ras
p38
ERK1/2
Endocytosis
HMGB1
LPS
Endosome
NFkB
Cell migration and proliferation
Proinflammatory cytokine
Type 1 interferon
HMGB1
LPS
Lysosome
LPS
Cytosol
Nucleus

Figure 4. HMGB1 receptors and the corresponding downstream signaling pathways. This illustration was created with BioRender.com.

TLR2 forms heterodimers with TLR1 or TLR6. TLR2/TLR1 and TLR2/TLR6 heterodimers are known to specifically bind lipoproteins, while TLR2 homodimer has only been proposed, with no evidence proving that it triggers a signaling cascade to date. The TLR2-HMGB1 binding is indicated to regulate macrophage polarization [45, 46], articular chondrocyte homeostasis [47] and hemorrhagic stroke transformation [48]. However, the mechanism regulating the HMGB1-TLR2 axis has yet to be specified. We have previously shown that HMGB1 lacking the C-tail (Δ30), but not full-length HMGB1, binds to TLR2 [49]. However, in HEK cells transfected with TLR2, the Δ30-TLR2 axis alone did not alert any biological function but rather potentiated the inflammatory activities of the TLR2 ligand peptidoglycan (PGN) [49]. The TLR2-HMGB1 interaction is likely to be cell types and the microenvironment dependent.

1.2.3.2 Receptor of Advanced Glycation End Products (RAGE)

RAGE is a proinflammatory transmembrane receptor present in many cell types, mainly in a preformed intracellular pool that can be rapidly transported to the cell surface during cell activation [50]. RAGE is a multi-ligand receptor binding to advanced glycation end-products (AGEs), S100 proteins, HMGB1 and DNA [51]. The diversity of RAGE ligands leads to a variety of activated downstream signaling pathways, including NFκB, MAPKs, PI3K-Akt, Rho GTPases, JAK-STAT, and Src family kinases (reviewed by Sims et al. [52]).

HMGB1 has two RAGE binding domains. One is in the A-box (aa 23-50) [53] and the other in the B-box (aa 150-183) [54]. Activation of the HMGB1-RAGE axis has been widely reported in inflammatory diseases, including arthritis [55], cancer [56], diabetes [57], and multiple sclerosis [58].

The binding of HMGB1 to RAGE is reported to lead to NF-κB activation and subsequent cytokine formation [59]. In male mouse BMDMs lacking RAGE, the production of TNF, IL6 and IL1β was round to be attenuated [60]. However, our recent results showed that in female mouse BMDMs lacking RAGE, upon dsHMGB1 stimulation, IL6 and TNFα production did not differ when compared to WT BMDMs [61]. One should consider the effects of sex differences. Rudjito et al. [62] reported that dsHMGB1 induced higher levels of proinflammatory factors, including TNF, IL6 and CXCL1, in male compared to female mouse BMDMs. It is likely that dsHMGB1 triggers cytokine released via both TLR4 and RAGE, but the balance and priority of the receptor binding need further evaluation, especially considering the sex dimorphism.

The interaction between RAGE and extracellular HMGB1 enables endocytosis of HMGB1 and bound partner molecules (including DNA and LPS) (Figure 4). Once inside the endosome, the amphoteric structure of HMGB1 disrupts the endosome membrane [30]. HMGB1 and the partner molecules can thus reach the cytoplasm and induce caspase-11-dependent pyroptosis. This was demonstrated in in lethal sepsis [30]. Similarly, HMGB1 entering the cytosol potentially allows it to interact with the cytosolic sensors. Such interactions have previously been described by us [63] and others [64]. We are now performing proximity proteomics to explore how the recently demonstrated ability of extracellular HMGB1 to reach the cytosolic compartment results in activation of cytosolic signaling pathways.

1.2.3.3 C-X-C chemokine receptor type 4 (CXCR4)

CXCR4 is a G protein-coupled receptor expressed by most cells. CXCR4 activates multiple signaling pathways that orchestrate cell migration, hematopoiesis, cell homing, and retention in the bone marrow [65]. CXCL12, a ubiquitously expressed chemokine, is a ligand of CXCR4. The CXCL12-CXCR4 axis plays an important role in recruiting immune cells to the site of inflammation, inducing wound healing and tissue regeneration [32, 66]. The CXCL12-CXCR4 axis also plays a role in CNS development, regulating cerebellar granule cell development and neurogenesis [67].

FrHMGB1 forms a heterocomplex with CXCL12 and binds to CXCR4 (Figure 4), resulting in chemotaxis of fibroblasts, monocytes and HeLa cells, much stronger than CXCL12 alone [32, 68]. However, frHMGB1 cannot alone induce chemotaxis via CXCR4 [32]. The intracellular mechanism has recently been claimed to be β-arrestin2-dependent: CXCL12-HMGB1 heterocomplex maintained CXCR4 in the plasma membrane and enhanced the cell response to chemotactic factors [69]. Regarding intracellular signaling pathways, Src phosphorylation was observed two minutes after incubating human cardiac fibroblasts with frHMGB1 [32], while ERK phosphorylation was observed 10 minutes after incubating human monocytes with frHMGB1 together with CXCL12 [35]. A recent study demonstrated that the inhibition of JAK-STAT and cyclooxygenase-2 (COX2) abrogated the migratory response of human monocytes towards HMGB1-CXCL12 heterocomplex [70]. Src-MEK-ERK signaling, Src-P13K-Akt signaling and JAK-STAT signaling are related to cell proliferation and migration [66, 71]. The precise pathways activated by frHMGB1-CXCL12 have not been specified and are likely to be cell type dependent.

In contrast, dsHMGB1 or oxHMGB1 could not trigger cell migration via CXCR4 [32]. However, our recent study showed that dsHMGB1 increased the motility of mouse BMDMs in a wound-healing scratch assay, and inhibition of TLR4 diminished the migration ratio [72]. The potential explanations could be that either TLR4-NFκB signaling directly results in BMDM migration, or that the released proinflammatory cytokines and chemokines reversely affect cell motility. In addition, a mutated form of HMGB1, where serines replace all three cysteines, can directly bind to CXCR4 and is as effective as HMGB1-CXCL12 in promoting cell migration and muscle regeneration [32].

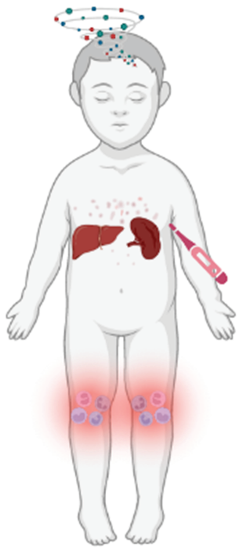
1.2.4 HMGB1 and diseases

The proinflammatory activity of HMGB1 was first discovered in studies designed to identify novel mediators of sepsis [73]. Since, multiple inflammatory diseases have been the subject of research regarding the inflammatory properties of HMGB1, including acute liver injury, arthritis, SLE, stroke and cancer. In this section, my discussion focuses on the role of HMGB1 in arthritis, including RA, JIA and neuroinflammation, as well as its potential as a therapeutic target.

1.2.4.1 HMGB1 and arthritis

RA is an autoimmune disease that not only affects joints, cartilage and bones but also causes systemic manifestations, including fever, uveitis, pleural effusions, weight loss and neurological disorders [74]. Early diagnosis of RA is based on combining symptoms with laboratory measurements, such as elevated C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPAs) [75].

JIA is a heterogeneous group of conditions characterized by arthritis of unknown origin that persists for more than six weeks in children younger than 16 years of age [76, 77]. Based on clinical presentation, JIA is classified into seven subtypes: oligoarticular JIA (oligoJIA), RF+ and RF- polyarticular JIA, systemic JIA, psoriatic JIA, enthesitis-related JIA and undifferentiated JIA [76, 77] (Figure 5).



Category		Proportion	Description
Oligoarticular JIA		27-60%	Affects **four or fewer** joints, often knees and ankles; Symptoms include pain, stiffness and swelling joints.
Polyarticular JIA	**RF+**	2–7%	Affects **five or more** joints, include knees, ankles as well as small joints of the hands. Patients have RF or anti-cyclic citrullinated peptide in the blood.
	RF-	11–30%	Affects **five or more** joints, include knees, ankles as well as small joints of the hands.
Systemic JIA		4-17%	Affects **the whole body**; Symptoms includes fever, rash, hepatosplenomegaly, lymphad-enopathy and serositis.
Psoriatic JIA		2–11%	Except from arthritis, patients also show **psoriasis.**
Enthesitis-related JIA		1–11%	Often affects legs and spine. Patients can also have inflammation at the **entheses.**
Undifferentiated JIA		11-21%	Arthritis that **does not fit** into any of the above categories **or fits into more than one** of the above categories.

Figure 5. Seven subtypes of JIA. Abbreviation: RF, rheumatoid factors. This illustration was created with BioRender.com.

SJIA accounts for 4-17% of JIA cases. SJIA is characterized by chronic arthritis accompanied by high spiking fever flares lasting for at least two weeks. Additional systemic symptoms may include rheumatic rash, hepatosplenomegaly, lymphadenopathy and serositis [77]. The pathogenesis of sJIA has been associated with dysregulation of the innate immune system, suggesting that it may instead be part of the spectrum of autoinflammatory diseases rather than autoimmune diseases [78]. No pathognomonic feature distinguishes sJIA from other conditions, but some laboratory parameters may support the diagnosis of sJIA, including elevated CRP, ESR, neutrophil and platelet counts [79]. Manifestations of sJIA, including macrophage activation syndrome (MAS), neurological complications, physical impairment and long-term damage from chronic inflammation can be severe and significantly impact patients' well-being [80].

Although the pathology of neither RA nor JIA is well understood, it is evident from experimental data that HMGB1 mediates disease progression and contributes to sustained inflammation. For example, intra-articular injection of recombinant HMGB1 into mouse knee joints resulted in arthritis with a frequency of over 80% [81]. High levels of HMGB1 have been measured in SF from inflamed joints of patients with RA and JIA [82, 83], with levels of HMGB1 much higher in SF than serum [83]. Even so, increased levels of HMGB1 in serum are associated with more destructive JIA [84] and reflect the extent of local inflammation. Additionally, our recent findings suggest that sJIA patients have higher plasma HMGB1 during their active phases than inactive phases [85], suggesting that the release of HMGB1 correlates with disease activity and inflammation.

Further evidence of the role of HMGB1 in disease progression is provided by Cecchinato et al., who demonstrated the co-localization of HMGB1, CXCL12 and Thioredoxin-1 (Trx) in the synovial membrane of patients with RA [70]. Trx and Thioredoxin reductase (TrxR) provides resistance against oxidative stress, and the concentration of Trx is positively correlated with RA disease severity [86, 87]. It has been suggested that Trx and TrxR preserved HMGB1 in the reduced form in synovium; the redox potential in the synovial microenvironment contributed to the formation of frHMGB1-CXCL12 heterocomplex, which played a role in recruiting immune cells contributing to diseases progression [70].

The role of HMGB1 has also been studied in experimental collagen-induced arthritis (CIA). Treatments with 2G7, a monoclonal antibody targeting the A-box of HMGB1 (aa 53-63), significantly ameliorated the production of inflammatory cytokines and bone destruction in CIA mice [88]. Further, targeting HMGB1 appeared to affect pain in the collagen antibody-induced arthritis (CAIA) model, with treatment using 2G7 reversing CAIA-induced mechanical hypersensitivity. Interestingly, such effect was sex-dependent, observed in only male mice and did not affect joint arthritis scores [89]. Co-localization of HMGB1 and hypoxia was observed in inflamed joints of CIA, and hypoxia increased HMGB1 release from mesenchymal cells and macrophages, suggesting that hypoxia-induced HMGB1 release may contribute to joint inflammation [90]. Meanwhile, this study demonstrated that anti-HMGB1 treatment attenuated joint inflammation [90]. Despite promising research, there has been no clinical study investigating HMGB1 neutralization in arthritis yet.

1.2.4.2 HMGB1 and neurological diseases

Systemic inflammation, characterized by increased circulating proinflammatory cytokines, has been implicated in cognitive decline and dementia. DAMPs and PAMPs in circulation may have effects not only on the local inflamed tissue but also affect CNS via blood circulation through the blood-brain barrier (BBB). Up to 70% of the patients discharged from the intensive care unit (ICU) can still suffer from a chronic functional impairment, which is called "post-intensive care syndrome (PICS)" [91]. Symptoms of PICS are not limited to bodily impairment, cognitive and psychological impairments are also included, which pose a severe problem to the affected persons and an increasingly important socio-economic problem [91]. The pathogenic mechanisms of PICS are still under research. Metabolic factors, hemodynamic factors, inflammation and toxic influences are thought to contribute to PICS [92, 93]. Interestingly, significant elevation of HMGB1 in plasma has been reported in ICU survivors at three and six months after discharge, whilst the elevation of HMGB1 was negatively correlated with rapid visual information processing in ICU survivors [94].

Although the effects on the CNS of some autoimmune or autoinflammatory diseases are still not well defined, neurological comorbidities, including cognitive impairment and depression, have been recorded in RA and SLE [95, 96]. Furthermore, RA patients showed brain metabolic changes positively associated with ESR levels and disease activity [97]. Although no severe neurological symptoms have been observed in RA, elevated choline levels are related to microglial activation and monocyte infiltration, suggesting that systemic inflammation is associated with increased brain metabolism and potential predisposition to neurological disorders [97]. In addition, hippocampal neurodegeneration has been observed in the CIA model, independent of the disease activity, indicating a potential mechanism for the memory loss and depression reported in RA [98]. Given that HMGB1 has been applied successfully as a therapeutic target in various neurodegenerative disease models, including Parkinson's disease [99] and Alzheimer's disease [100], it is likely that neurological manifestations in autoimmune diseases may be associated with HMGB1.

HMGB1 can initiate inflammation in CNS and cause brain damage by disrupting the BBB and inducing expression of the proinflammatory factor IL1β and apoptosis [34]. Serum HMGB1 was elevated in sepsis survivor mice, which showed learning and memory impairment that improved upon administration of anti-HMGB1 antibodies, possibly due to reduced spine density on dendritic processes of CA1 neurons in the hippocampus [101]. Anti-HMGB1 antibodies have also been reported to inhibit neuronal apoptosis in the hippocampus and improve sensorimotor function after traumatic brain injury [102]. In addition, Zhang et al. demonstrated the protective role of the anti-HMGB1 antibody to the BBB from ischemia-induced disruption in rats [103].

Together, these studies demonstrate the role of HMGB1 in neuroinflammation and the potential benefits of anti-HMGB1 treatment for patients with chronic inflammation and related cognitive deficits.

1.2.5 Key challenges and future perspectives in the HMGB1 research field

Neutralization of HMGB1, by anti-HMGB1 antibodies, recombinant HMGB1 A-box, and plant-derived compounds, has been demonstrated to have promising effects in various disease models [21, 104-106]. However, there is currently no proven HMGB1-targeted treatment undergoing clinical trials. One possible explanation for this could be that HMGB1 is a ubiquitous protein with diverse and complex intracellular and extracellular functions; hence targeting HMGB1 may not only relieve inflammation but also have other yet unknown side effects. The intracellular function of HMGB1 should be better understood before translating any promising pre-clinical results to clinical settings. Moreover, the time window and doses of anti-HMGB1 treatment can affect the outcome, especially for acute and fast-developing diseases, such as stroke and sepsis.

Improving *ex vivo* HMGB1 quantification is critical for expanding the knowledge of HMGB1 involvement in diseases. We, as well as others, have found that molecules forming complexes to HMGB1 in biological fluids can interfere with assays designed to detect HMGB1. For instance, though the molecular weight of HMGB1 is around 29kDa, we frequently observe a 50kDa band, independent of the reducing reagent DTT when immunoblotting using an anti-HMGB1 antibody to detect HMGB1 in plasma and SF from patients with JIA (unpublished data). In contrast, HMGB1 released from *in vitro* cell necrosis were blotted only at a size of 29kDa independent of reducing reagent DTT (unpublished data). Andersson et al. [107] reported the contradictory results of HMGB1 quantification in plasma from patients with COVID-19 via commercial enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Their results indicated that sample pre-treatment with perchloric acid before immunoblotting could dissociate molecules attached to HMGB1 and increase HMGB1 levels compared to levels recorded by commercial ELISA without pre-treatment. Willis et al. [108] also observed a high-molecular-weight HMGB1 complex in plasma and PBMCs from patients with SLE that was resistant to denaturing. They claimed that the protein-cross-linking enzyme transglutaminase-2 could catalyze the HMGB1 complex. Taken together, both sample treatment and analysis methods are important factors to consider in all *ex vivo* analysis for accurate and comparable quantifications of HMGB1.

Additionally, as discussed in section 1.2.2., no method exists to quantify HMGB1s redox isoforms or other PTMs in complex biological fluids. It is important to evaluate the redox purity of HMGB1 used in *in vitro* experiments, as well as in clinical samples. These obstacles interfere with further exploration of the fascinating biology of extracellular HMGB1.

1.3 PERIPHERAL INFLAMMATION-INDUCED NEUROINFLAMMATION

Symptoms of chronic inflammatory diseases, such as RA and JIA, are not only limited to local manifestations and peripheral inflammation, but can also involve cognitive decline and neuropsychiatric symptoms [109, 110]. Peripheral inflammation-induced neuroinflammation is attracting increasing attention, though the specific mechanisms are still under investigation.

Cognitive decline refers to the deterioration of intellectual and problem-solving abilities, associated with behavior alteration, and includes the loss of memory and executive function [111]. Up to two-thirds of RA patients were classified as cognitively impaired with a high prevalence of anxiety and depression [109, 112]. Further, RA patients significantly underperformed on verbal function, memory and attention tests compared to healthy controls [113]. Children and adolescents with JIA have been found to show a higher rate of psychoticism, anxiety, depression, withdrawal, somatic complaints, rule-breaking behaviors, and thought and social problems than the non-JIA controls [114-119]. Cognitive effects can continue into adulthood, with adults diagnosed with JIA in childhood shown to be more deficient in visuospatial function than non-JIA adults [120]. However, Berthold et al. recently reported contrary results that the risk of depression and anxiety was not increased in individuals with JIA compared to a reference group of individuals free from JIA [121].

The pathogenic mechanisms of cognitive decline in chronic inflammatory diseases are complex, as psychosocial well-being depends on multiple biological, psychological, and sociological factors (Figure 6).

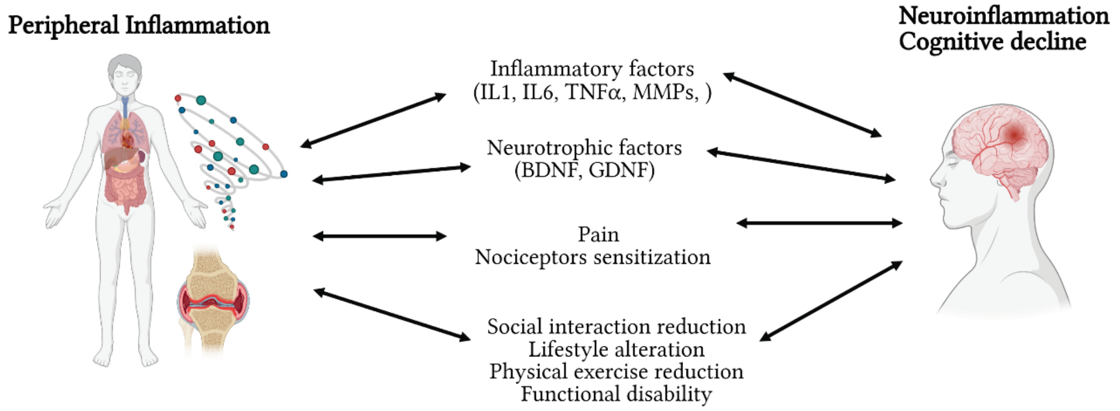

Peripheral Inflammation
Neuroinflammation
Cognitive decline
Inflammatory factors
(IL1, IL6, TNFα, MMPs,)
Neurotrophic factors
(BDNF, GDNF)
Pain
Nociceptors sensitization
Social interaction reduction
Lifestyle alteration
Physical exercise reduction
Functional disability

Figure 6. Multi-step progression of chronic peripheral inflammation to neuroinflammation and cognitive decline. This illustration was created with BioRender.com.

1.3.1 Biological factors derived from peripheral inflammation could induce neuroinflammation

1.3.1.1 Proinflammatory cytokines

The upregulation of circulating pro-inflammatory cytokines, such as IL1, IL6 and TNFα, characterizing RA and JIA are associated with poorer cognitive performance and depression in both diseases [122, 123]. Anti-TNF therapy in RA can suppress potential CNS involvement linked to BBB dysfunction [124].

Neuroinflammation, especially hippocampal inflammation, has also been observed in experimental arthritis. For example, the expression of IL1β and IL6 was upregulated in the CIA rat cortex and hippocampus [125]. Moreover, reduced integrity and enhanced permeability of brain micro-vessels of CIA rats were also observed, along with increased expression of MMP3, MMP9 and RAGE in the hippocampus, indicating BBB breakdown [125]. There are also reports of BBB impairment in CIA mouse brain [126] with a consistent increase of brain inflammatory mediators IL4, IL10, IL12 and Chemokine C-C motif ligand (CCL) 2 up to day 42 after immunization [98].

1.3.1.2 Neurotrophic factors

Two neurotrophic factors, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), are differentially expressed in peripheral blood from patients with arthritis compared to healthy controls, with RA patients having higher BDNF levels, but lower GDNF levels [122, 127].

BDNF

BDNF is involved in synaptic plasticity, neuronal differentiation and survival of neurons [128]. Klein et al. demonstrated that blood BDNF concentrations reflect brain-tissue BDNF levels in rats and pigs [129]. Erickson et al. [130] reported the relationship between age-related memory impairment, reduced hippocampus volume and decreased serum BDNF levels. There have also been studies demonstrating that reduced plasma BDNF levels are associated with depression [131-133]. Therefore, serum BDNF is regarded as a reliable marker of BDNF present in the brain, as well as of depression.

Symptoms of depression are frequent in RA. However, the elevation of plasma BDNF in RA seems to indicate a different mechanism between plasma BDNF and depression [122, 134] than those of the aforementioned studies. Indeed, in the adjuvant-induced arthritis (AIA) rat model, BDNF expression was significantly lower in both the cortex and hippocampus but higher in serum [135]. The authors proposed that impairment of both neuron and endothelial cells contributed to the decreasing BDNF levels in the AIA brain [136]. However, the reason for increasing plasma BDNF levels have not been clearly elucidated. One hypothesis is that high-grade inflammation in RA leads to peripheral expression and secretion of BDNF, as circulating BDNF might primarily be derived from leukocytes in inflammatory diseases [137]. Therefore, blood BDNF levels might not be a reliable marker of cognitive decline of arthritic diseases.

GDNF

GDNF is involved in neuronal survival and regeneration. Increasing plasma levels of GDNF have been associated with cognitive dysfunction, including attention deficit hyperactivity disorder (ADHD) and bipolar disorder [138-141]. Decreased levels of GDNF have been reported in patients with RA [122], chronic pain [142] and depression [143]. De Ceuninck et al. [144] reported GDNF being produced by chondrocytes during joint inflammation, while there have been few reports about the origin of GDNF in peripheral blood.

GDNF protein expression is enriched in the brain, kidney, urinary bladder, sexual organs (placenta and testis) and soft tissue. As BBB hyper-permeability is raised in experimental arthritis, one hypothesis could be that GDNF in the blood is derived from the brain or inflamed joint. Our recent study showed increasing *Gdnf* mRNA expression in mouse BMDMs upon exposure to LPS and Interferon (IFN) γ (unpublished data). Thus, defining the origin and induction mechanisms of GDNF is important for understanding the role of GDNF in chronic inflammation.

1.3.1.3 Neurogenesis-related proteins

Doublecortin (DCX) is an important regulator of hippocampal neurogenesis, implicated in memory function, mood and cognitive disorders [145-147]. The potential role of DCX in neuropsychiatric symptoms in arthritis appears complex as illustrated in experimental arthritis. For example, Sutthiwarotamakun et al. observed a significant reduction of DCX-positive cells in CIA DBA/1 mouse brains on day 42 following immunization, regardless of disease severity

[98]. In contrast, Wolf et al. [148] reported an increasing number of DCX-positive cells and hippocampus neurogenesis in AIA C57BL/6 mice on day 7 after induction. The contradictory results could be due to different arthritis-inducing procedures between CIA and AIA. The immune response of CIA involves both T- and B-cells, while the AIA model is Th1-cell and neutrophil dependent. Different inflammatory profiles may cause different effects on hippocampus neurogenesis.

1.3.2 The interaction between pain, inflammation and cognitive decline

Pain in chronic arthritis occurs both spontaneously and as a result of pressure on affected joints. Pain and functional limitations can cause reductions in physical exercise, social interactions, and hence the quality of life for patients, which may be reflected in mental illnesses, such as depression and anxiety [149, 150]. Conversely, patients with depressive disorders are also more prone to develop chronic pain [151]. Taken together, chronic pain and depression interact bi-directionally in the CNS.

Neuro-immune interactions modulate both peripheral and central mechanisms of pain. Sensitization of nociceptors occurs due to biochemical changes within the local synovium and SF, the dorsal root ganglion and the spinal cord. These changes include altered cytokines (for example, IL1β, IL6, IL17, TNF), chemokines (for example, CCL2), and growth factors (for example, vascular endothelial growth factor (VEGF) and β nerve growth factor (NGF)) [152]. These inflammatory mediators can negatively affect neurogenesis even though the illness is not manifested. On the other hand, proinflammatory biomarkers have been found to be higher in depressed individuals, and inflammatory challenges trigger depressive behaviors and brain activity alterations in animal models [153].

In summary, in chronic arthritis, pain and cognitive decline are highly associated. More interestingly, in recent years, anti-depressants have been used to relieve chronic pain [154], and anti-inflammatory therapies are being examined to treat bipolar disorder [155]. I would expect that in the future, more shared pathways between peripheral and CNS inflammation will be identified, which will aid in the discovery of novel treatments.

2 RESEARCH AIMS

The overall aim of this thesis was to investigate the pathogenesis of arthritic diseases, with a particular focus on sJIA and oligoJIA and with a special emphasis on the involvement of HMGB1. Furthermore, we aimed to study the molecular mechanism of macrophage polarization upon HMGB1 stimulation.

In **papers I** and **II**, we aimed to define biomarkers relevant for clinical use as well as to reveal the immune mechanisms in JIA by performing immunoprofiling of plasma and SF samples from patients with sJIA and oligoJIA,

In **papers III** and **IV**, we aimed to clarify how the interplay of HMGB1 and macrophages can contribute to inflammatory processes and the impact of HMGB1 redox isoforms on macrophage polarization. We set out to perform an in-depth analysis of BMDM phenotypes induced by different HMGB1 redox isoforms in depth using RNA sequencing (RNA-Seq) transcriptomic profiling, ELISA for induced cytokine release and *in vitro* functional assays for migration and foam cell formation.

In **paper V**, we aimed to study the connection between arthritis and neuroinflammation by investigating the occurrence of arthritis-induced neuroinflammation in two different experimental arthritis models.

3 METHODOLOGICAL CONSIDERATIONS

All methods applied have been described in the "Methods and Materials" section of each included paper or manuscript. I would like to address the considerations and discuss the advantages and limitations of the main methods herein.

3.1 PAPERS I AND II

3.1.1 Clinical samples from patients with JIA

Patient samples used during the completion of this work are from the Juvenile Arthritis Biobank Astrid Lindgrens Barnsjukhus (JABBA) biobank, which was founded in 2009. The plasma samples used in **papers I** and **II** were collected during the period of 2010 to 2018; the SF samples involved in **paper II** were collected during the period of 2011 and 2019. Researchers have claimed that proteomic biomarker discovery might be affected by storage time even at -80 °C [156, 157].

All plasma samples from patients with JIA were processed by centrifugation within 4 hours of blood draw, for 10–15 minutes at 3000–6000 g with brake and then stored at -80 °C until analysis. Healthy control samples, from a population-based cohort (Barnens miljö- och hälsoundersökning) in the Stockholm region [158], were collected during the period of May 2003 and June 2004; after centrifugation, the samples were stored for 1-8 hours at -20 °C before transferring to -80 °C until analysis. The influence of processing time on protein analysis results has been investigated. Huang et al. [159] reported that one-quarter of all proteins measured in EDTA plasma were significantly affected by centrifugation delays. Concentrations of caspase (CASP) 8, NAD-dependent deacetylase sirtuin (SIRT) 2 and sulfotransferase 1A1 (ST1A1) in plasma increased following extraction, up to 300-700% of baseline measurements within the first 24-hour delay. Nevertheless, they did not show data from any shorter delay than 24 hours. There was no report of centrifugation delay from either JABBA or the healthy control biobank, but we have reasons to be skeptical of the potential delays, because we recorded significantly higher levels of CASP8 and ST1A1 in healthy control samples compared to inactive sJIA [85] (see **paper I**) and oligoJIA samples (see **paper II**).

Another limitation in **papers I and II** was the relatively small patient cohorts: in **paper I**, 21 sJIA patients and 30 plasma samples were included in the analysis; in **paper II**, 14 oligoJIA patients and 28 plasma samples were included in the analysis. In addition, in **paper I**, the included sJIA patients had various treatments making it difficult to stratify results with treatment effects. In **paper II**, we did not have access to the SF samples from non-arthritic healthy controls, limiting our analysis. This limitation is due to the ethical considerations with the collection of SF from healthy children. However, this does not undermine the value of the research, given that JIA is a rare disease with limited available information.

3.1.1.1 The importance of age- and sex-matched healthy control in pediatrics research

Evidence suggests that circulating plasma levels of cytokines are influenced by age in healthy children [160, 161]. Therefore, we initially investigated if the pattern of measured proteins (Inflammation panel, see 3.1.2.) showed age- and sex-associated differences using sixty samples from healthy children (Figure 7A). The results showed no separation between samples from females and males (Figure 7B), while samples from 4, 8 and 12-year-old children were partly separated (Figure 7C) in principle component analysis (PCA). Statistical analysis of the difference between sexes and ages revealed that 35 out of 92 proteins were significantly higher in normalized protein expression (NPX) values in 4 years old healthy children than in 12 years old healthy children (Table 2). Interestingly, no protein had significantly different expression levels when comparing 4- and 8-year-old or comparing 8- and 12-year-old children (Table 2). Based on these results, age- and sex-matched healthy control cohorts were used for the cross-sectional analysis between patients with JIA and healthy controls in **papers I** and **II**.

A

	4 years old	8 years old	12 years old
Number of samples	20	20	20
Sex (Female%)	60	50	75

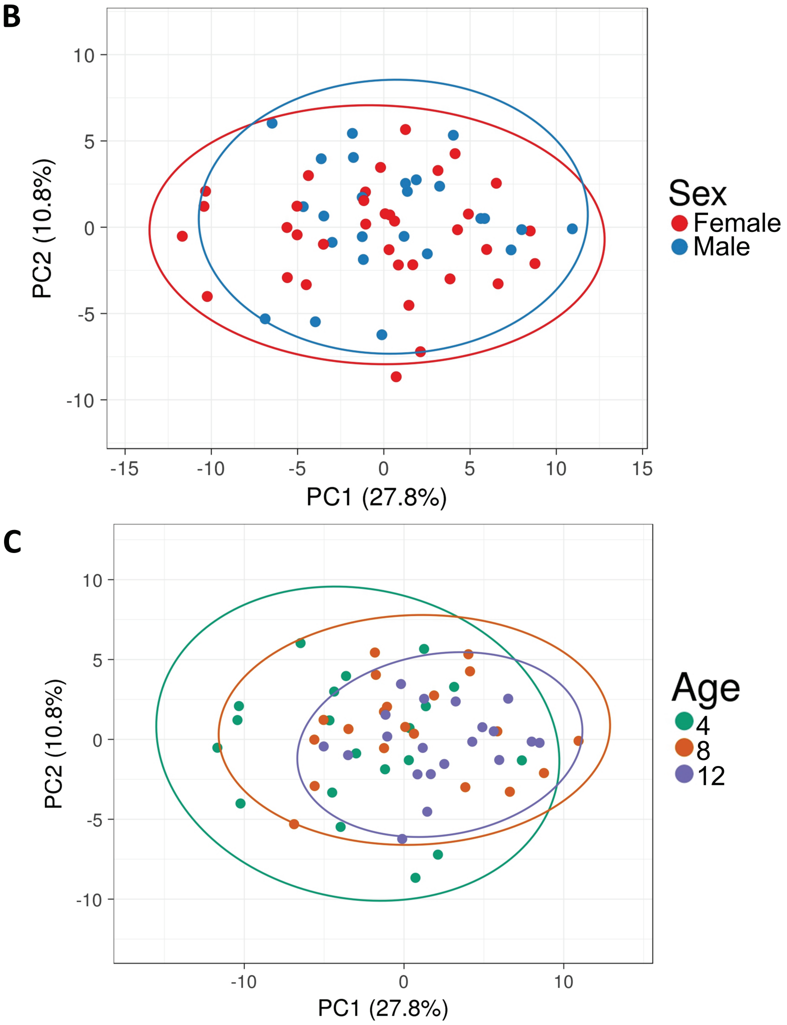


Figure 7. Age is a major confounding factor in plasma protein profiling. PCA analysis of the levels of 92 proteins in the Inflammation panel in plasma from sixty healthy control subjects based on their (B) sex and (C) age. The confidence level of the ellipses in (B) and (C) is 0.95.

Table 2. The average NPX values of each analyzed protein in healthy control groups with different ages. Statistics: Two-way ANOVA with correction of multiple comparisons by controlling the False Discovery Rate (FDR) of 5% via the two-stage step-up method of Benjamini, Krieger and Yekutieli. Significant differences are highlighted in pink colour.

	Mean of 4-year-old (NPX value)	Mean of 8-year-old (NPX value)	Mean of 12-year-old (NPX value)	4- v.s. 8-year-old Adjusted p-values	4- v.s. 12-year-old Adjusted p-values	8- v.s. 12-year-old Adjusted p-values
IL12B	7.16	7.00	6.64	0.3950	0.0040	0.2020
MMP10	7.85	7.62	7.03	0.5630	0.0040	0.2010
CD5	6.21	5.66	5.48	0.0900	0.0040	0.6120
CCL28	2.60	2.19	1.93	0.1870	0.0040	0.4250
IL17A	2.35	1.86	1.68	0.1010	0.0050	0.5860
IL8	5.88	5.46	5.01	0.3380	0.0060	0.4250
CCL4	6.61	6.24	5.91	0.3700	0.0060	0.5690
CD6	7.00	6.57	6.18	0.2500	0.0060	0.4640
CCL3	5.44	5.25	4.80	0.6170	0.0060	0.4190
TNFSF12	10.41	10.12	10.04	0.1870	0.0060	0.7890
TNFB	6.04	5.59	5.61	0.0610	0.0060	0.9640
DNER	9.62	9.41	9.36	0.0610	0.0060	0.7890
CXCL1	10.39	9.32	9.38	0.1110	0.0080	0.9640
TNFSF14	4.79	4.80	3.85	>0.999	0.0100	0.1660
IL7	4.42	3.64	3.41	0.2500	0.0110	0.8050
CASP8	4.08	3.04	2.31	0.3150	0.0120	0.4530
IL10	4.57	4.08	4.01	0.1120	0.0130	0.9010
CCL20	7.57	7.24	7.04	0.3150	0.0130	0.6030
CXCL6	10.30	9.50	9.03	0.3380	0.0140	0.6150
CXCL5	12.12	10.73	10.77	0.1560	0.0180	>0.999
STAMBP	7.35	6.14	5.77	0.2500	0.0200	0.7980
VEGFA	10.75	10.51	10.26	0.5250	0.0260	0.5860
SIRT2	7.33	5.57	5.45	0.1870	0.0260	0.9640
TGFB1	7.89	7.60	7.23	0.5540	0.0290	0.5390
CXCL9	6.99	6.88	6.48	0.7850	0.0290	0.4250
CST5	5.75	5.53	5.42	0.3950	0.0290	0.7890
IL18	9.38	9.11	8.85	0.3950	0.0290	0.5860
AXIN1	5.52	4.52	4.11	0.3590	0.0310	0.7890
CCL11	6.51	6.37	6.22	0.5840	0.0310	0.7300
SULT1A1	4.79	3.95	3.51	0.3660	0.0390	0.7300
IL15RA	1.45	1.21	1.31	0.0640	0.0420	0.5860
FLT3LG	9.14	8.88	8.91	0.2130	0.0420	0.9640
CCL19	9.95	9.69	9.66	0.3150	0.0430	0.9640
EIF4EBP1	9.35	8.26	8.25	0.2500	0.0480	>0.999
CCL13	13.80	13.21	13.06	0.3700	0.0500	0.9150
S100A12	3.70	3.67	3.03	>0.999	0.0540	0.4140
CD244	7.64	7.28	7.23	0.2500	0.0560	0.9640
OPG	10.01	9.84	9.82	0.2500	0.0560	0.9640
CD40	11.89	11.53	11.43	0.3560	0.0560	0.9010
CXCL11	8.84	8.68	8.12	0.8630	0.0820	0.5860
OSM	4.29	4.44	3.56	0.8570	0.0830	0.2020
HGF	8.76	8.58	8.50	0.4470	0.0830	0.8330
TNF	3.57	3.25	3.25	0.3660	0.0830	>0.999
ADA	6.27	6.11	5.85	0.6850	0.0830	0.4640
CSF1	10.02	10.11	10.14	0.4640	0.0830	0.9010
CCL8	9.43	8.98	8.92	0.3920	0.0890	0.9640
IL10RB	6.00	5.92	6.16	0.6720	0.0960	0.2020
FGF21	2.62	2.59	3.27	>0.999	0.1020	0.4640
IL6	2.98	3.23	3.29	0.5160	0.1230	0.9640
CX3CL1	6.22	6.32	6.38	0.6170	0.1860	0.9010
FGF23	2.85	2.91	2.95	0.6170	0.1980	0.8860
FGF19	8.41	7.81	8.02	0.2130	0.1990	0.7890
LIFR	3.66	3.71	3.76	0.6720	0.2090	0.8050
TNFRSF9	8.19	7.90	8.05	0.0990	0.2140	0.4930
PLAU	10.30	10.08	10.20	0.1000	0.2550	0.4640
SCF	9.53	9.54	9.67	>0.999	0.2850	0.4250
TNFSF11	6.31	5.88	6.17	0.0900	0.3010	0.4250
CD8A	10.62	10.37	10.45	0.4100	0.3070	0.9010
CCL2	10.96	10.78	10.87	0.3700	0.3330	0.7890
CD274	6.86	6.67	6.77	0.3150	0.3750	0.7890
NTF3	3.03	3.01	3.10	>0.999	0.4020	0.7890
CCL23	9.78	9.77	9.90	>0.999	0.4070	0.7890
CDCP1	3.04	2.98	2.97	0.7850	0.4360	>0.999
IL10RA	1.27	0.93	1.13	0.3660	0.4360	0.4820
MMP1	8.81	9.35	8.60	0.4100	0.4490	0.4250
CXCL10	9.52	9.50	9.46	>0.999	0.5550	0.9640
TGFA	3.75	3.75	3.72	>0.999	0.5640	0.9640
IL18R1	8.32	8.31	8.35	>0.999	0.6020	0.9480
IFNγ	6.59	6.74	6.67	0.8160	0.6020	0.9640
CCL25	5.72	5.64	5.70	0.8570	0.6560	0.9210
TNFSF10	8.30	8.18	8.31	0.3950	0.6620	0.4820

3.1.1.2 Impact of pre-treatment of synovial fluid on assay precision and reproducibility

SF is a difficult biological fluid to analyze because of its complex non-Newtonian nature [162]. SF can have up to 40 times higher viscosity than serum and plasma, contributing to poor assay reproducibility and potentially inefficient use of precious samples [163]. Two pre-treatment strategies, sample dilution and hyaluronidase (HAse) pre-treatment, and their effect on assay precision and reproduction are hence discussed.

Dilution

Dilution can reduce viscosity and improve the difficulty of handling synovial fluid samples. Dilution may also reduce the use of precious samples, especially important when SF from adolescents and children is valuable and limited. However, dilution can reduce the assay signal and render low-expressed proteins undetectable. Dilution may place the signal in the non-linear region at the low end of the standard curve, thereby increasing the coefficient of variation (CV). The data obtained from diluted SF samples may therefore not correlate with data obtained from undiluted SF samples.

To decide whether to dilute SF or not, the abundance of the studied protein in SF and the sensitivity of the assay need to be considered. For example, Struglics et al. [163] suggested diluting SF four times for use with the proximity extension assay (PEA) Inflammation panel from Olink. However, their justification for this recommendation needs to be clarified. Their data showed that only 44 out of 92 proteins measured were detected in their four-time diluted SF samples from healthy controls, and they did not show the detection rate from undiluted samples. In contrast, we detected 71 out of 92 proteins in the undiluted SF samples from patients with oligoJIA. Whether the difference was due to the JIA disease, sample dilution, or differences in the age or sex of the donors is unclear and warrants further investigation.

Hyaluronidase (HAse) pre-treatment

SF is a highly viscous biofluid containing many proteins and high amounts of hyaluronan (HA). HA is a glycosaminoglycan produced by synovial fibroblasts and forms a dense mesh matrix making SF viscous [164]. HAse can be used to degrade HA and reduce protein-matrix interactions. The benefits of HAse treatment on reproducibility and precision of measuring protein in synovial fluid have been demonstrated using the Luminex platforms [165]. However, the beneficial effects were not seen in all investigated platforms. For example, HAse pre-treatment did not impact protein detection in the Meso Scale Discovery platform [165] and Roche Cobas platform [166]. In addition to protein measurements, Brouwers et al. recently demonstrated that HAse treatment was essential for an unbiased analysis of the cell composition of SF [167]. They found that 0.8-70% (median 5%) of immune cells were missing in subsequent analyses when the cells were isolated from untreated SF [167].

SF samples used in **paper II** were not treated with HAse. Future tests are warranted to not only clarify the effects of HAse treatments of SF on PEA but also investigate the interfering mechanisms of HA on assay reproducibility and precision of different assays with distinct principles.

3.1.2 Proximity Extension Assay (PEA)

In **papers I** and **II**, we applied PEA (Olink, Uppsala, Sweden), which utilizes paired oligonucleotide antibody probes and qPCR for protein detection and quantification (Figure 8). Following the successful probe binding, hybridization and extension, the corresponding sequence was amplified and quantified by qPCR and relative log2 NPX measures. The multi-epitope binding design minimizes cross-reactivity, and the qPCR amplification allows for high sensitivity compared to other alternative techniques. Therefore, PEA makes the detection of low-expressed proteins possible.

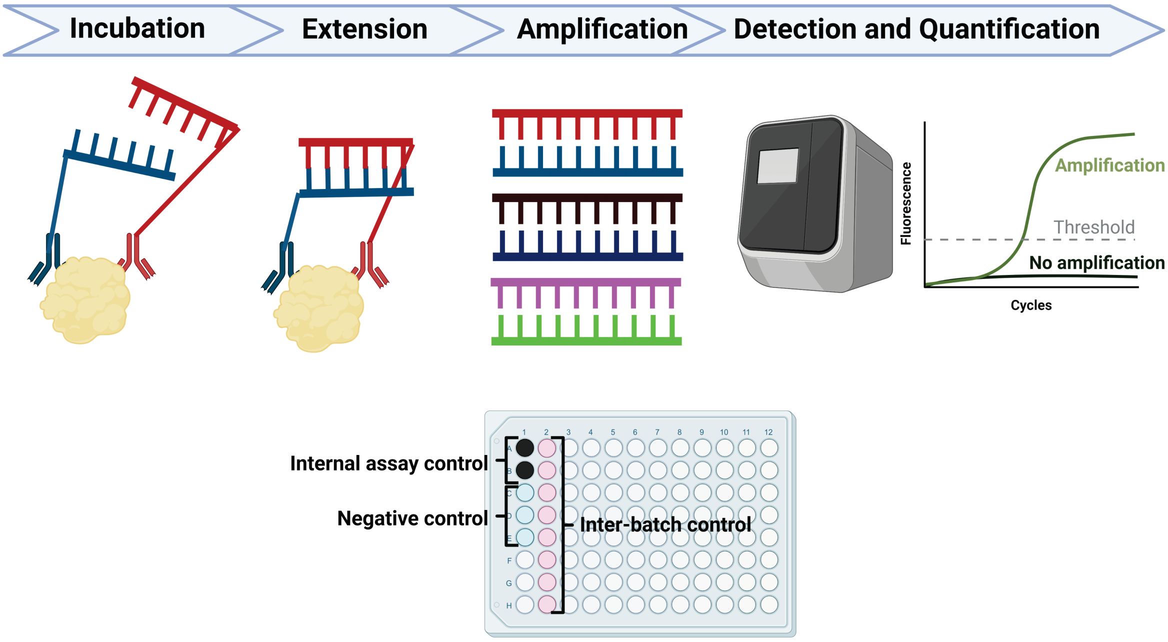

Incubation
Extension
Amplification
Detection and Quantification
Fluorescence
Amplification
Threshold
No amplification
Cycles
Internal assay control
Negative control
Inter-batch control
1
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Figure 8. Overview of the proximity extension assay technology. Detection and relative quantification are performed using qPCR. In each plate, there are two internal assay controls, three negative controls and eight inter-batch controls (also called bridging samples) to allow comparison across batches. This illustration was created with BioRender.com.

Two internal assay controls (one for extension steps and one for detection steps) were used to assess the quality of the reaction process (Figure 8). In addition, a triplicate of negative control samples and eight, for us, inter-batch controls were compared between runs/batches to assess batch effects and differences in sensitivity (Figure 8). Proteins with a low call rate (less than 20%), which mainly refers to measures below the limit of detection (LOD), were excluded prior to statistical analysis.

The pre-designed “Inflammation” assay panel from Olink was used and consists of 92 inflammation-related proteins, including cytokines, chemokines, enzymes, and growth factors, that regulate immune activation. In addition to common biomarkers for autoimmune and autoinflammatory diseases, the panel covers other mechanisms related to inflammation, such as neurodegeneration, oxidative stress, infection, transcription regulation and metabolism. Therefore, exploratory proteins with an unclear biological function or unknown disease involvement are included to identify potential novel biomarkers. A significant advantage of this technique over similar assays is that as little as one microliter of sample is needed for screening for all 92 proteins and hence enables measurement where sample amounts are limited.

Despite the high sensitivity and low cross-reactivity of the PEA technology, we still experienced some limitations in our project. Of the 92 proteins measured, 21 were not detectable in JIA plasma because of low call rate, and hence were excluded in the statistical analysis. Indeed, most of these 21 proteins, including MCP-3, GDNF, IL-20RA, IL-2RB, IL-1 alpha, IL2, IL-17C, TSLP, SLAMF1, FGF-5, IL-22 RA1, Beta-NGF, IL-24, IL13, ARTN, IL-20, IL33, IL4, LIF, NRTN and IL5, have been recorded at a low level in human plasma previously.

Additionally, only NPX values, rather than concentrations, are given as results. Other quantification methods, such as ELISA or Luminex, are needed to obtain the actual concentration. However, due to the sensitivity differences, not all results from PEA could be recalled by ELISA. For instance, the average level of GDNF in active sJIA was higher than matched healthy control according to PEA. We failed to record GDNF levels via commercial ELISA, as all the samples had GDNF concentrations lower than the lowest detection limitation (4.69 pg/mL) of the ELISA kit. On the other hand, the multi-epitope binding design of PEA is sometimes not comparable to ELISA. The capture antibodies of ELISA usually have a single epitope of the target protein.

Finally, batch differences of the panel should be stressed. The plasma samples included in **papers I and II** were run separately in two versions of the Inflammation panel (v.3012 and v.3021), with eight bridging samples for normalization between the two runs. Normalization was validated by checking for sample groupings according to the panel version using PCA before and after normalization (Figure 9). IFNγ and TNF were excluded from the bridging due to a change in antibody pairs between the two versions and a lack of agreement between control samples. In the future, ideally, as many samples as possible should be measured in a single run, avoiding bridging normalization, which is time-consuming and still not wholly controlled for batch issues.

Despite the limitations, we successfully recorded the elevation of classical sJIA activation biomarkers, such as IL6, IL18 and S100A12, indicating the validity of the PEA. More importantly, we did map several biomarkers that have not been well defined in sJIA.

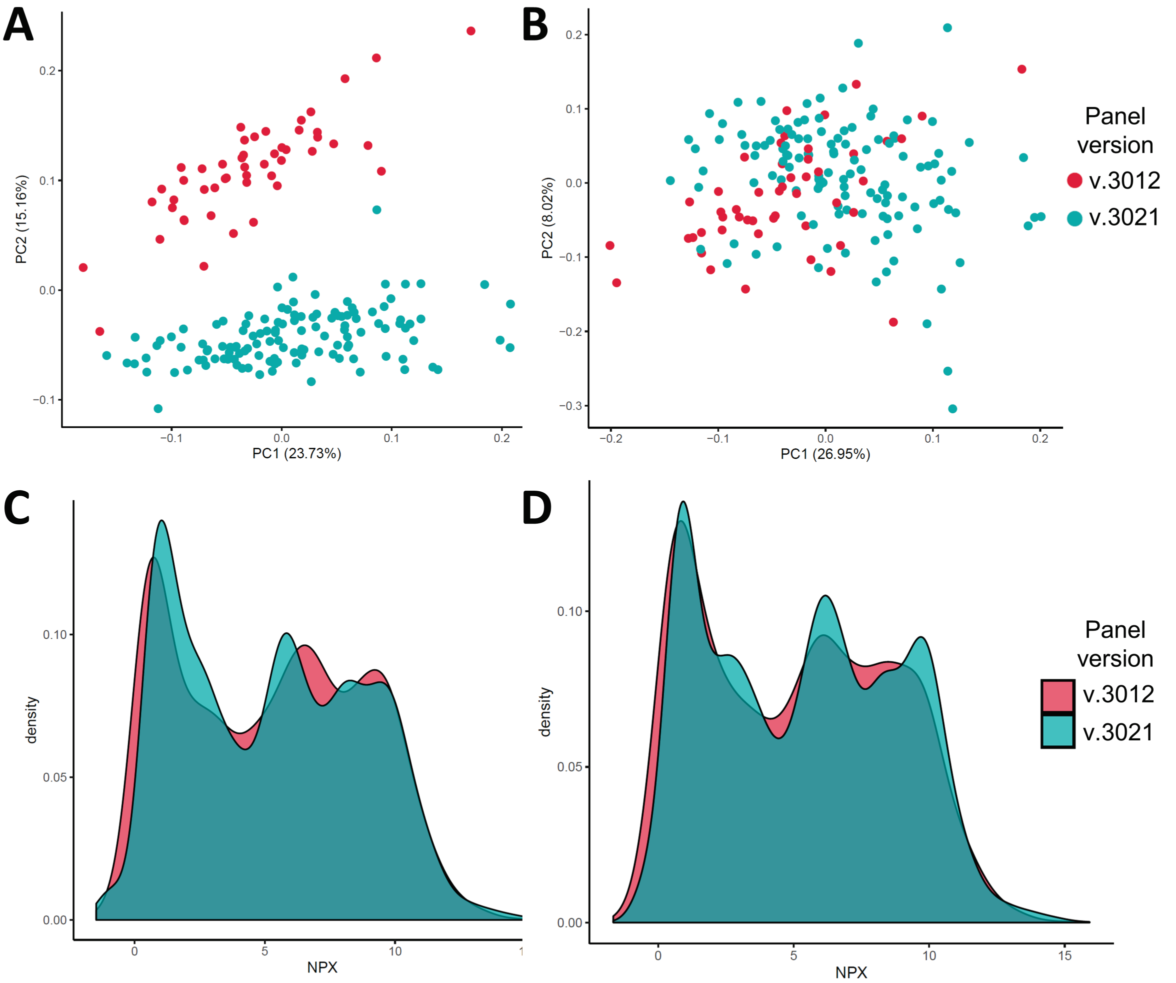


Figure 9. Normalization of two plasma datasets from different inflammation panel experimental batches. PCA plot of plasma samples before (A) and after (B) normalization colored by panel version. The percentage in parenthesis on the axis labels represents the percentage of variance explained by that principal component. Density plot of the NPX values from the two plasma datasets before (C) and after (D) normalization colored by panel version. The normalization removed most of the grouping in the PCA plots, while the changes in the distribution and density plots were not as obvious. Normalization was performed, and the figures were provided by Olink statistical service.

3.2 PAPERS III AND IV

3.2.1 In-house produced recombinant HMGB1

In **papers III** and **IV**, the recombinant HMGB1 we used was produced in-house (frHMGB1) or by Kevin Tracy (KT)'s laboratory at the Feinstein Institute for Medical Research in the USA (dsHMGB1). Briefly, HMGB1 DNA was sub-cloned into a pCAL/n vector with a calmodulin-binding protein (CBP) tag. A CBP tag was used as it has no known effect on the HMGB1 function [168]. *E.coli* BL21 (DE3) cells were transformed with the plasmid and cultured in 2-YT media. Protein expression was induced with the addition of 1 mM IPTG. HMGB1 was purified using calmodulin sepharose 4B resin (GE Healthcare). DNase I was added to remove any contaminating DNA, confirmed by GelRed staining of an agarose gel (Figure 10A). Protein purity was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis with Coomassie Blue staining (Figure 10B). To remove contaminating endotoxin, the protein was incubated with 5% Triton X114. Endotoxin levels were determined by the *Limulus* amoebocyte lysate assay at the clinical laboratory, Karolinska University Hospital. To produce frHMGB1, 5 mM DTT was added to all the buffers during protein purification, and the final storage buffer contained 0.5 mM DTT.

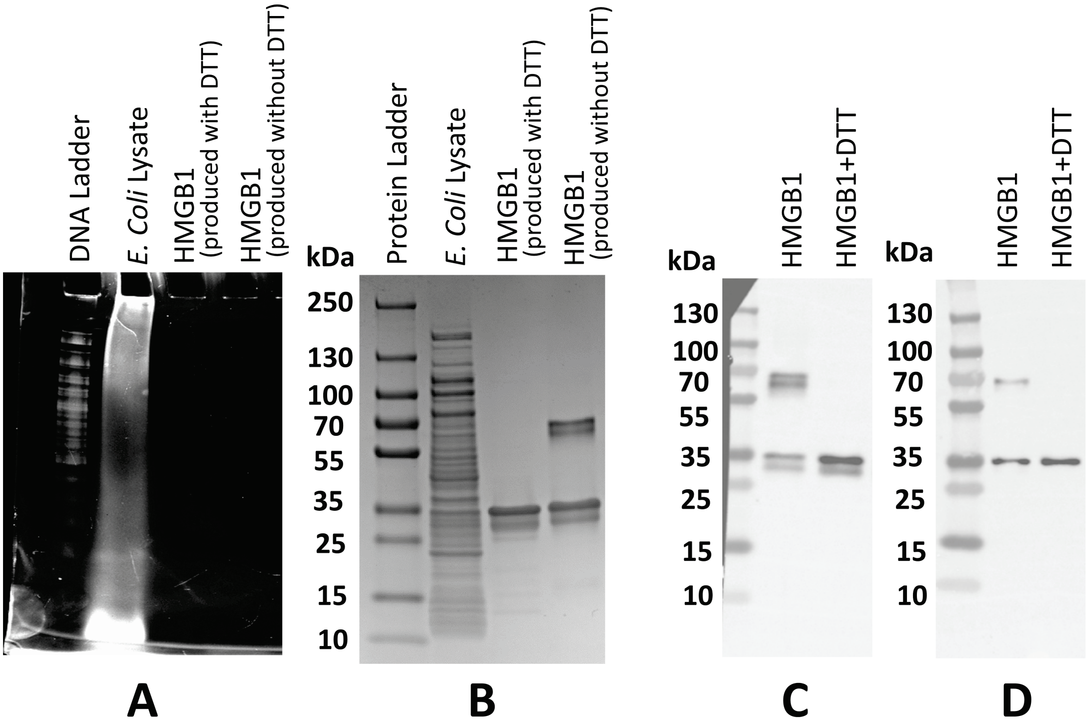

DNA Ladder
E. Coli Lysate
HMGB1 (produced with DTT)
HMGB1 (produced without DTT)
kDa
250
130
100
70
55
35
25
15
10
Protein Ladder
E. Coli Lysate
HMGB1 (produced with DTT)
HMGB1 (produced without DTT)
kDa
130
100
70
55
35
25
15
10
HMGB1
HMGB1+DTT
kDa
130
100
70
55
35
25
15
10
HMGB1
HMGB1+DTT
A
B
C
D

Figure 10. Example of validation of the in-house produced HMGB1 purity. (A) GelRed staining of an agarose gel was used to verify the DNA removal; *E.coli* lysate was used as the negative control. 5ug in-house produced HMGB1 was loaded in each lane. (B) Coomassie Blue staining of a SDS-PAGE gel was used to check the protein purity; *E.coli* lysate was used as the negative control. 5ug in-house produced HMGB1 was loaded in each lane. (C) Western blotting using a 2G7 primary antibody (anti-A-box of HMGB1) to blot HMGB1 produced without or with DTT. (D) Western blotting using anti-C-tail of HMGB1 primary antibody to blot HMGB1 produced without or with DTT.

However, despite protocol optimization, including removal of DTT during protein purification, adjusting the culturing temperature of *E. coli* and changing the pH of buffers, we were not able to produce functional dsHMGB1.

3.2.2 Distinguish the redox isoforms of HMGB1

Another obstacle for us is the need for a direct readout approach to distinguish the redox isoforms of HMGB1. Our lab exposes PBMCs to the produced HMGB1 in an overnight culture. The cytokine (usually IL6 and TNFα) production induced is quantified via commercial ELISA, and their levels are used to indicate dsHMGB1 indirectly. This is based on the fact that only dsHMGB1 can induce cytokine production from immune cells [31]. However, the method does not evaluate the purity of the redox isoform, as there are no standardized reference concentrations for pure dsHMGB1-induced cytokine from PBMCs. Even though cytokine release can be measured in cell supernatants, the HMGB1 can be a mixture of all isoforms.

Other labs have claimed that redox isoforms of HMGB1 can be identified by reading the shift of bands in western blot, as indicated when running SDS-PAGE without reducing reagent, where two bands with size approximately 25 kDa are present, the upper one being frHMGB1 and the lower one being dsHMGB1 [169, 170]. However, our in-house produced CBP-tagged HMGB1 always appears as two bands around 35kDa (Figure 10C) when blotting with an anti-A-box antibody (2G7), with the lower band disappeared when blotting with an anti-C-tail antibody (Figure 10D). The results indicated that the lower band was CBP-tagged HMGB1 without C-tail rather than dsHMGB1. Meanwhile, by exposing PBMCs to the in-house HMGB1, no TNFα or IL6 was produced by PBMCs. Therefore, we could conclude that our in-house produced HMGB1 was not dsHMGB1.

As mentioned in section 1.3.2, some chemical compounds are reactive to sulfhydryl (thiol/SH) groups, one being malPEG. Figure 11A illustrates the PEGylation of a single cysteine with malPEG. This protocol was tested during the last few months of my PhD study.

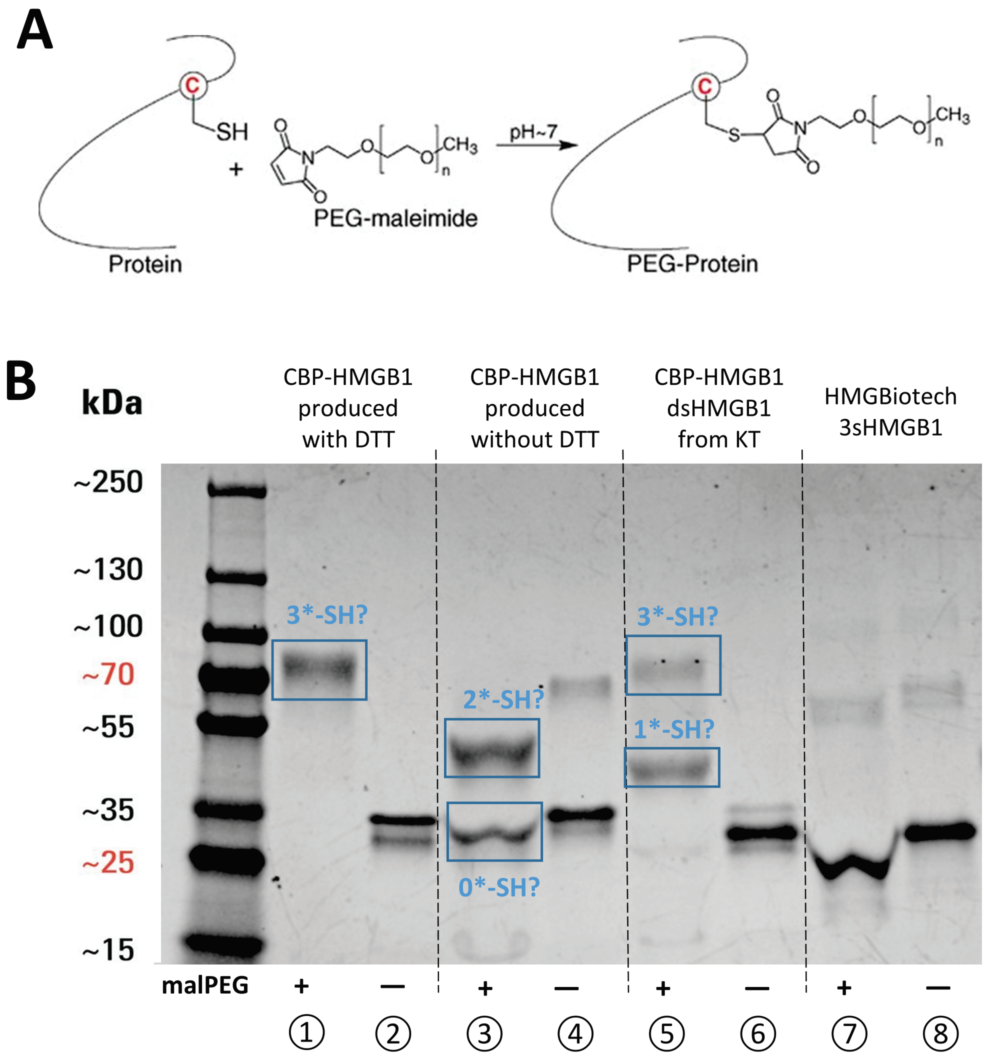

A
C
SH
+
PEG-maleimide
Protein
pH~7
CH3
n
S
N
O
PEG-Protein
B
kDa
CBP-HMGB1 produced with DTT
CBP-HMGB1 produced without DTT
CBP-HMGB1 dsHMGB1 from KT
HMGBiotech 3sHMGB1
~250
~130
~100
~70
~55
~35
~25
~15
3*-SH?
2*-SH?
0*-SH?
3*-SH?
1*-SH?
malPEG
+
—
+
—
+
—
+
—
①
②
③
④
⑤
⑥
⑦
⑧

Figure 11. MalPEG labels cysteine. (A) Illustration of the PEGylation process of a single cysteine in protein with malPEG. The figure was adapted from [171]. (B) HMGB1s were mixed with 5 mM malPEG in 1% SDS (pH = 7.5) at room temperature. Before mixing with the Laemmli buffer and boiling in 95 °C, 100 mM DTT was added to the HMGB1-malPEG mixture to quench the leftover free malPEG. The gel was stained in Coomassie Blue before imaging.

We first tested HMGB1-malPEG binding using four batches of HMGB1s from various resources (Table 3).

Table 3. Four different batches of HMGB1s from various resource

	Brief name	Descriptions
1	**CBP-HMGB1 produced with DTT**	- HMGB1 with CBP tag produced in-house, with 5mM DTT added in the purification process. The protein was finally maintained in PBS with 0.5 mM DTT. - We regard this batch as frHMGB1 as it is able to induce fibroblast migration with CXCL12. Meanwhile, this batch of HMGB1 was not cytokine-inducing.
2	**CBP-HMGB1 produced without DTT**	- HMGB1 with CBP tag produced in-house, without DTT added in the purification process. The protein was finally maintained in PBS without DTT. - This batch of HMGB1 was not cytokine-inducing.
3	**CBP-HMGB1 dsHMGB1 from KT**	- HMGB1 with CBP tag gifted by Kevin Tracy (KT)'s lab at Feinstein Institutes for Medical Research, US. - We regard this batch as dsHMGB1 as it is able to induce cytokines (including IL6, IL8 and TNFα) release from PBMCs.
4	**HMGBiotech 3sHMGB1**	- Tag-free HMGB1 purchased from HMGBiotech S.r.l. (Milano, Italy). - In the 3sHMGB1, also called non-oxidizable chemokine-HMGB1, all cysteines are replaced with serines. Therefore, malPEG should not be able to bind 3sHMGB1.

Interestingly, we were able to detect differences in band shifts between the batches (Figure 11B):

1) The CBP-HMGB1 produced and preserved with DTT showed the largest shift after malPEG incubation (lane ①), indicating three malPEG molecules attached.
2) The CBP-HMGB1 produced and preserved without DTT showed two bands after malPEG incubation (lane ③): one band is about the same size as the original CBP-HMGB1, indicating all three cysteines are in oxidized form; another band is about 45kDa, indicating two malPEG molecules attached.
3) The CBP-HMGB1 from KT's lab also showed two bands after malPEG incubation (lane ⑤): one was about the same size as the largest shift showed in lane 1, the other was between the size given two and zero malPEG molecules attached. The lower band in this lane was highly likely to be the malPEG-labelled dsHMGB1, which has a disulfide bond and a free thiol. On the other hand, with the two different-sized bands, we could also see that the CBP-tagged HMGB1 from KT's lab was not with a pure redox isoform.
4) The 3sHMGB1 from HMGBiotech showed no shift after malPEG incubation (lane ⑦). This confirmed the principle that without free thiols, malPEG does not bind HMGB1.

Although further tests of this assay in biological fluids, such as plasma and SF from patients with arthritis, were not conducted, this proof-of-concept assay did highlight an alternative way of distinguishing HMGB1 redox isoforms. However, one should consider the abundance of HMGB1 in the biofluid of interest. For example, in plasma from patients with active sJIA, the concentration of HMGB1 measured via commercial ELISA was only up to 10 pg/mL. The sensitivity of SDS-PAGE or western blot may not, therefore, be high enough to detect the HMGB1 and its redox isoform.

3.2.3 *In vitro* migration assays

In **paper III**, we demonstrated the migration-inducing role of dsHMGB1 on BMDMs in a wound-healing scratch assay. We used the standardized assay provided by IncuCyte® (Figure 12A). Compared to the conventional protocol using pipette tips to make wounds manually, the wound maker from IncuCyte® creates homogeneous, 700-800 µm wide scratch wounds in cell monolayers in one go on an IncuCyte® ImageLock 96-well microplates. Cell migration is monitored in real-time with live-cell imaging. The integrated analysis algorithm automatically masks each image and delineates cell-dense (unwounded) and cell-free zones (wounded). This system overcomes the limitations caused by user-induced bias in image acquisition and quantification. A more detailed protocol has been summarized by Sun et al. [172].

To exclude the confounding effects from cell proliferation, we pre-treated BMDMs with Actinomycin D (ACD), a proliferation inhibitor. However, ACD showed cytotoxicity to BMDMs after 24 hours, even at a reduced dose of 0.1 µM, which was at least five times lower than the suggested concentration [173, 174]. As 0.1 µM of ACD could successfully inhibit the proliferation of 3T3 fibroblast cells without cytotoxicity for up to 48 hours (data not shown), it is likely that primary cells are more sensitive to ACD. Instead, we measured the BMDM proliferation rate and the results showed that BMDMs did not proliferate differently between treated and control groups (data not shown). Therefore, we concluded that proliferation was not a confounding factor in the BMDM wound-healing scratch assay.

Another commonly used cell migration quantification method is the 3D trans-well assay (Figure 12B). Compared to the wound healing assay that assessed the horizontal cell motility under a particular stimulus, a trans-well assay assesses cell migration or invasion in response to a chemotactic gradient (Figure 12B). Most studies investigating HMGB1-induced cell migration were based on the 3D transwell assay [32, 35, 70].

More advanced systems, such as the Ibidi µ-Slide chemotaxis system (Figure 12C), is suitable for chemotaxis measurements of adherent and non-adherent cells [175]. By live cell imaging and individual cell tracking, this system enables detailed and defined analysis of the migration behavior of cells [175]. A recent study demonstrated HMGB1-CXCL12-CXCR4-induced cell migration using the Ibidi µ-Slide chemotaxis system [69].

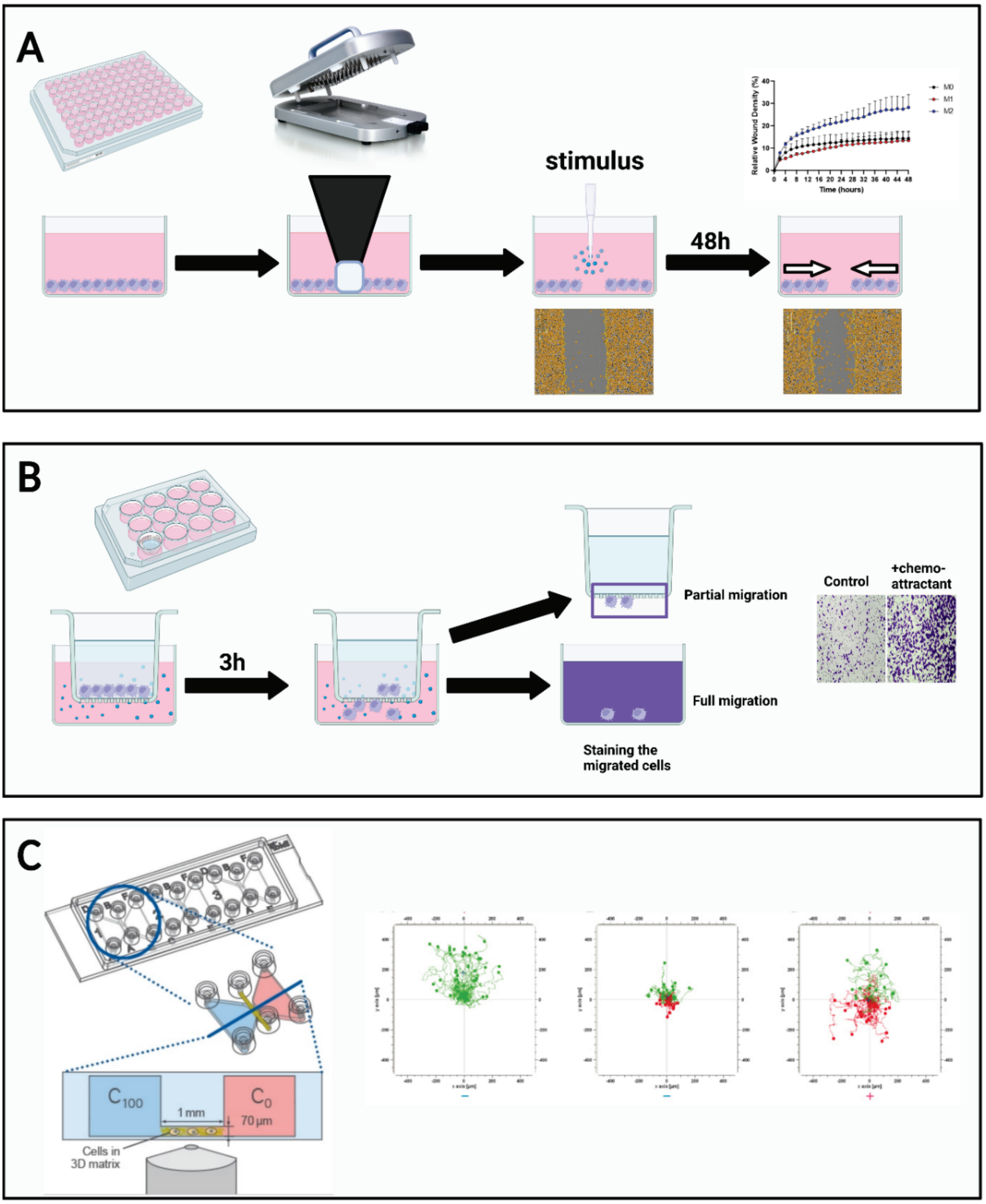

A
stimulus
48h
Relative Wound Density (%)
0
10
20
30
40
0 4 8 12 16 20 24 28 32 36 40 44 48
Time (hours)
M0
M1
M2
B
3h
Partial migration
Full migration
Staining the migrated cells
Control
+chemo-attractant
C
C_{100}
1 mm
C_0
70 µm
Cells in 3D matrix
x axis [µm]
y axis [µm]
−
−
+

Figure 12. Illustration of different migration assays. (A) Wound healing scratch assay, (B) Transwell assay, (C) Ibidi µ-Slide chemotaxis system (figures adapted from ibidi.com). This illustration was created with BioRender.com.

We did not confirm the results from the wound-healing scratch assay in other migration assays. Therefore, future validation is warranted. The following aspects should be taken into consideration when designing new migration experiments:

a) **Checking the pore size of the membrane in transwell assay**: too large or too small pores would interfere with the readout;

b) **Checking if cells are capable of invading the membrane and the Matrigel coating**: some cell types migrate horizontally very fast but cannot invade a pore membrane [176];

c) **Tracking individual cell migration**: by applying the Manual Tracking plugin of ImageJ/Fiji on living cell imaging, the average accumulated distance and velocity of cells can be quantified individually. This tracking method could be used to investigate the cell trajectory in an open area, better-defining cell motility without the cell density difference between unwounded and wounded areas in wound healing scratch assay.

3.3 PAPER V

3.3.1 Experimental arthritis models

In **paper V**, we investigated peripheral inflammation-induced neuroinflammation in two arthritis mouse models, CAIA and KRN T-cell-induced arthritis (KRN). Although the two models were induced via different immune mechanisms, they resulted in similar clinical symptoms of swollen, inflamed joints, the typical symptoms of human arthritis. Comparisons of the two used models and the commonly used CIA model with human arthritis are shown in Table 4.

Table 4. Comparison of the mouse arthritis models and human arthritis

	CIA	**CAIA**	**KRN**	**human arthritis**
Susceptible mouse strains	DBA1 B10.Q B10.RIII QB QD C3H.Q	Multiple strains	TCR KO mouse (C57BL/6 background)	
Developing time	4-5 weeks	10-15 days	10-15 days	Chronically, years
Main immune reaction	Active immunization, i.e. include a series of immune reaction including T- and B-cell response	Passive Collagen II antibodies-driven and formation of immune complexes (T and B cells are dispensable)	G6PI antibodies produced by B cells in recipient mice and formation of immune complexes	Active immunization
Specificity	Collagen II (with **CFA**)	Collagen II (with **LPS**)	G6PI	ACPAs RF G6PI HSP Collagen II
Important cells	Neutrophils Macrophages B and T cells	Neutrophils	Neutrophils Macrophages Mast cells	Neutrophils Macrophages CD4+ T cells B cells Dendritic cells
Important cytokines	TNF, IL6, GM-CSF, IL1B, G-CSF, MIF, IL17	TNF, IL1B, IL4	TNF, IL6, GM-CSF, IL1B, G-CSF, MIF	TNF, IL6, GM-CSF

Abbreviations: KRN, KRN T-cell-transfer arthritis; G6PI, glucose-6-phosphate isomerase; CFA, complete Freund's Adjuvant; HSP, heat shock proteins.

In CIA and CAIA models, anti-collagen II antibodies are pathogenic. In CIA, arthritis is induced by immunization with bovine or chicken collagen II in complete Freund's adjuvant (CFA), which is incomplete Freund's adjuvant (IFA) with Grind heat-killed *Mycobacterium tuberculosis*. The mice are boosted with collagen II emulsified in IFA four weeks later, with the onset of arthritis usually within two weeks after the boost. In CAIA, arthritis develops after the administration of a defined cocktail of anti-collagen type II monoclonal antibodies (mAbs) and inoculation with LPS three days after. LPS enhances the incidence and severity of the disease and reduces the amount of mAb required to induce arthritis. Compared to CAIA, CIA results from active immunization rather than the passive transfer of autoantibodies.

The KRN mouse line was proposed as a new model for human arthritis in 1996 [177]. The KRN mouse is a transgenic mouse line carrying rearranged T cell receptor (TCR) genes from the T cell hybridoma R28 [177]. The KRN T-cell arthritis is induced by transferring autoreactive CD4$^+$ T cells from a KRN mouse (C57BL/6 background) to a T cell-deficient I-A$^{g7+/-}$ I-A$^{b+/-}$ recipient mouse. The clinical score of the recipient mouse usually reaches a maximum score of 12 within two weeks after CD4$^+$ T cell transfer. The anti- Glucose-6-phosphate isomerase (G6PI) antibody level is upregulated within one week and maintained until the following week. The inflammation is driven by autoantibodies against the ubiquitously expressed self-antigen G6PI, leading to the formation of immune complexes that drive the activation of different innate immune cells such as neutrophils, macrophages and possibly mast cells [178].

Experimental arthritis models provide means to study aspects of arthritogenic mechanisms less feasible in humans, for example, time points before the clinical onset of disease, tissue-specific events and the impact of specific cell types or molecules. However, there are also limitations to all experimental models.

Foremost, compared to human arthritis, the time span between inciting event and the development of clinical disease signs are shorter in experimental models. CAIA and KRN models take less than two weeks after immunization to achieve the peak score; CIA also develops strong inflammation within two weeks after a booster injection of adjuvant. Although the life span of mice and humans are not comparable, it can still be problematic, especially when considering chronic peripheral inflammation-induced neuroinflammation. Meanwhile, arthritis development in both CIA and CAIA needs immune-stimulatory components, such as CFA in CIA and LPS in CAIA. It has been evident that the systemic administration of LPS can induce neuroinflammation and cognitive impairment in mice [179-181]. Therefore, whether the immune-stimulatory components would mask the neuroinflammation caused by joint inflammation should be carefully considered.

Additionally, the incidence rate of different models varies between different mouse strains. Both CAIA and CIA show lower incidence rates and less severity in the C57BL/6 strain than DBA/1 and BALB strains [182]. CIA is restricted to mice bearing the MHC class II H-2^q or H-2^r, but not H-2^b, haplotypes [183, 184], indicating the crucial role of T cells. Campbell et al. [185] and Kai et al. [186] demonstrated that by increasing *Mycobacterium tuberculosis* dose in immunization, MHC class II molecule I-A^b could also adequately present the collagen II peptide antigen to prime antigen-specific $CD4^+$ helper T cells and induce CIA. We tried to establish the CIA model on C57BL/6 mice four times, always observing less than a 30% incidence rate and mild symptoms (i.e. with arthritis scores only 1-2 out of 12). To optimize the protocol, we replaced bovine collagen with chicken collagen, and increased *Mycobacterium tuberculosis* dose up to 4 mg/mL, but we still observed little difference in the incidence rate and arthritis severity. Consequently, several mice had to be sacrificed due to persistent wounds at the injection site, indicating that a high *Mycobacterium tuberculosis* dose might lead to side effects besides arthritis development.

Lastly, no animal arthritis model completely reflects all features of human arthritis. CIA and CAIA are based on anti-collagen II antibodies; KRN T-cell-arthritis is based on anti-G6PI antibodies. Although collagen and G6PI autoantibodies are known to be present in human arthritis, there are more autoantigens and autoantibodies that define human arthritis. A typical example is ACPAs, which are present in up to 80% of patients with RA [187]. However, experiments in mice have shown that polyclonal and monoclonal ACPAs (defined as anti-CCP-2 IgG antibodies) induced only pain hypersensitivity and bone erosion, but no joint inflammation [188, 189]. This could be explained as human-derived antibodies were developed against human PTMs, and the same epitopes may not occur in mice [189]. Additionally, joint inflammation and pain hypersensitivity in human arthritis is complex, resulting from multiple autoantibodies and other mechanisms. In summary, we must be critical when interpreting arthritis-induced neuroinflammation in animal arthritis models.

3.4 ETHICAL CONSIDERATIONS

In this section, I address ethical considerations for my research work, which consists of wet lab work with primary cells and animals, as well as computer-based analysis of patient plasma proteomics datasets.

To address these considerations, we first assessed the importance of research on the pathogenesis of arthritis. Arthritis, a chronic inflammatory joint disease, affects both children and adults with a high prevalence. Although inflammatory and immune reactions are considered to be the cause and driver of arthritis, these three hallmarks of the disease (inflammation, pain and destruction) can appear independently of each other. The impact of therapy on these features can also differ. The underlying mechanisms are not fully understood, and validated biomarkers for prognostic and diagnostic purposes for each of these three hallmarks are lacking. HMGB1 is indicated as a mediator of multiple inflammatory diseases, including arthritis. Treatment targeting HMGB1 is beneficial in multiple disease models. Thus, there is a need to reveal the different inflammatory mechanisms involved in the initiation and perpetuation of each of these hallmarks as well as the need to develop a counteracting therapy, with a special focus on HMGB1. We aim to additionally delineate the mechanisms of inflammation, which is a core characteristic in a number of diseases, hoping that our findings will prove useful in many scientific areas.

Since most of our research involves laboratory animals, the biggest ethical consideration is whether the use of animals is justified. We evaluated the necessity of using animals over commercial cell lines or computer bioinformatics. After preliminary experiments with error, we concluded that animal-derived cells and arthritis animal models were necessary for the experiments. We follow the Swedish regulations for laboratory animals and try to implement the three Rs (Reduce, Replace, Refine) in the project as a whole and in each experiment. For example, we designed the experiment carefully with statistical power calculations to minimize the animal number; we precisely set the humane and experimental end-point to reduce animal suffering; we used well-established animal models, following the published protocol to avoid waste of animals for developing a novel protocol.

Another main ethical concern of my PhD research is the clinical research involving blood and SF from arthritis patients. We have established the JABBA biobank for over ten years, with a completed and updated ethical permit from Etikprövningsmyndigheten (EPN). All sample collections have consent from the children and their parents. SF is only taken from patients with swollen joints and trouble with movement, which is required for clinical purposes and done by clinical doctors. All samples are managed by professional staff. All usage of patient samples is recorded in detail. Patient data (for example, patient date of birth, disease onset date and treatments) is coded and never shared via email or external communication. The files are protected by PIN code if necessary. We host events to demonstrate the latest research projects and outcomes to the patients and their families, clearly and openly sharing the research aim, hypothesis, sample processing and achievements. Meanwhile, all the staff working on the projects are responsible for answering questions from the participants.

4 RESULTS AND DISCUSSION

4.1 SCF AS A NOVEL DIAGNOSIS BIOMARKER FOR SJIA

In **paper I**, we conducted a proteomics analysis of plasma from patients with active or inactive sJIA, as well as age- and sex-matched healthy controls. Using cross-sectional and paired analysis, we identified a series of 16 proteins that were significantly differentially expressed in sJIA. We not only confirmed the previously reported sJIA biomarkers IL6, IL18, S100A12 and OSM, but also identified new potential biomarkers CASP8, CCL23, CD6, CXCL1, CXCL11, CXCL5, EIF4EBP1, SCF, MMP1, SIRT2, SULT1A1 and TNFSF11 that characterized sJIA patients in active and inactive phases.

Among the newly identified biomarkers, SCF, also called c-kit ligand, was significantly lower in plasma from patients with active sJIA than patients with inactive sJIA and oligoJIA (Figure 13A). A study comparing multisystem inflammatory syndrome in children with COVID-19 and Kawasaki disease reported that compared to healthy control, the level of serum SCF was lower in children with Kawasaki disease but not in children with COVID-19 [190]. It appears that lower plasma levels of SCF relate to systemic inflammation, i.e. distinguishes sJIA from oligoJIA and could be a potential biomarker separating autoinflammatory diseases from infectious diseases.

4.1.1 Correlation of plasma SCF levels between PEA and ELISA

To further explore the level of SCF in sJIA and non-systemic JIA, we first quantified SCF levels in the plasma from patients with sJIA and oligoJIA with commercial ELISA. As NPX is the relative log2 normalized number, ELISA values were log2 transformed to be comparable to NPX values [191]. There was a weak correlation between the NPX value from PEA and the actual concentration from ELISA, with r=0.3310 (p-value = 0.0216) (Figure 13B). Meanwhile, using the same cohort, the significant differences from PEA (Figure 13A) were not confirmed by ELISA (Figure 13C). The Bland-Altman plot highlights that differences in measurement between the two assays were larger in samples with lower NPX values (Figure 13D), suggesting decreased accuracy at lower levels.

Disagreement between the two assays could result from differences in antibodies between each assay. In the Human SCF ELISA kit (R&D), the capture antibody and detection antibody are mouse monoclonal, and goat polyclonal, respectively; meanwhile; in PEA, both antibodies are goat polyclonal. Moreover, the manufacturer of the ELISA kit claimed that no epitope mapping had been done on the antibodies. In contrast, the PEA manufacturer claimed that the immunogen of their antibodies spanned most of the extracellular part of the SCF. The different domains of SCF might be present in varying concentrations due to cleavage, and hence disagreements between the two assays may occur due to differences in the targeted epitopes of primary antibodies. This is discussed in further detail in section 4.2.3.

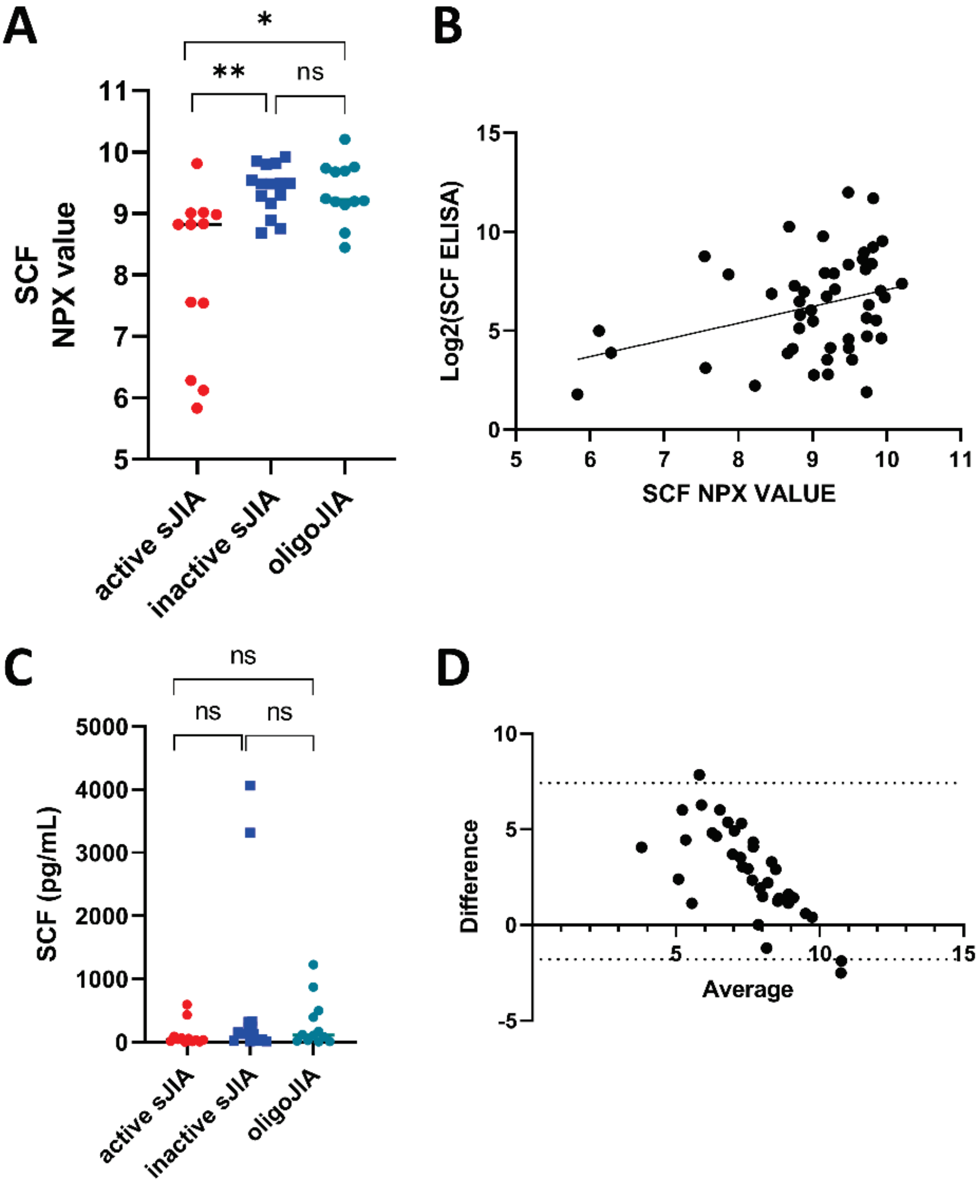


Figure 13. Quantification of plasma SCF in JIA patients via PEA and ELISA. (A) Data from PEA reveals that the level of SCF in plasma from patients with active sJIA is significantly higher than in plasma from patients with inactive sJIA and oligoJIA. (B) A weak but significant correlation was observed between PEA and ELISA (r=0.3310, p-value=0.0216). ELISA values were log2 transformed before the analysis to be comparable to NPX values. (C) ELISA data reveals no significant difference in the level of plasma SCF among patients with active sJIA, inactive sJIA and oligoJIA. (D) The Bland-Altman plot highlights inconsistent variability across the measurement range. Statistics: (A) and (C), Kruskal-Wallis test with correction of multiple comparisons by controlling the False Discovery Rate using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. *, $p<0.05$, ** $p<0.01$; (B), Pearson correlation.

Additionally, PEA reports higher sensitivity than ELISA, therefore we can expect more accurate results than ELISA when approaching its detection limit. The lower detection limit of PEA is an NPX value of 2.03, while all the sJIA samples had NPX values higher than 5.00. In contrast, the lower detection limit of ELISA is 31.3 pg/mL, and more than 30% of the measured samples have SCF concentrations lower than 31.3 pg/mL. Notably, most of the samples with low NPX values were below the detection limit of ELISA, impacting the correlation and cross-sectional analysis, and explaining the larger variation at lower NPX values in the Bland-Altman plot (Figure 13D).

As mentioned in section 3.1.2., we were not able to quantify GDNF via ELISA due to the detection limits. By referring to the publications that applied PEA technology, validating PEA results by other assays was not common. Thus, we decided to conduct further exploration solely based on results from PEA. In the future, one should consider the limitations of different assays when carrying out comparative analysis.

4.1.2 Plasma SCF, the disease duration and the severity of sJIA

Although active sJIA showed significantly lower levels of SCF in plasma in the cross-sectional analysis, we noticed that not all patients with active sJIA showed equally low SCF levels (Figure 13A). We further explored the medical history of the patients with lower SCF, using an NPX value of 8.500 as a cutoff. There were five and two samples from patients with active sJIA and oligoJIA with NPX value of SCF lower than 8.500, respectively. All samples from healthy controls and patients with inactive sJIA had an NPX value for SCF higher than 8.500.

A summary of the demographics and disease characteristics of the study subjects is outlined in Table 5.

Table 5. Clinical characteristics of the JIA patients at the sampling time points

Sample ID	SCF (NPX value)	Sampling Age (years)	Disease duration at sampling (months)	Diagnosis, disease activity	Symptoms at sampling	CRP (mg/dL)	Treatments at sampling	Treatment duration at sampling (months)
sJIA-1	5.832	10	1	sJIA, active	fever, rash	21	No treatment	-
sJIA-2	6.122	3	1	sJIA, active	arthritis	87	Ibuprofen	1
sJIA-3	6.285	4	4	sJIA, active	fever, arthritis	193	Prednisolone	4
sJIA-4	7.544	6	27	sJIA, active	arthritis, fever, hepatomegaly, splenomegaly	123	MTX, Prednisolone, Canakinumab	1, 1, 7
sJIA-5	7.560	9	1	sJIA, active	fever, splenomegaly, enthesopathy	52	Prednisolone	1
Oligo-1	8.126	3	10	Oligo, active	Arthritis, ANA+	NA	Ibuprofen	1
Oligo-2	8.449	2	1	Oligo, active	Arthritis, ANA+	NA	No treatment	-

Abbreviations: ANA+, Antinuclear Antibodies presented in the sample;CRP, C-reactive protein; MTX, Methotrexate; NA, data of CRP level was unavailable.

The five patients with sJIA had several clinical characteristics in common. All except sJIA-4 displayed relatively severe inflammation with CRP measurements up to 193 mg/dL at the sampling time and had disease durations of less than four months. The clinical cut-off of CRP used for children is over 10 mg/dL. Additionally, most sJIA patients studied here had fever at the sampling time. Both patients with oligoJIA had SF taken from the joints, indicating ongoing inflammation in the local tissues; however, no CRP data was available for these oligoJIA patients at the time of sampling, we were therefore unable to evaluate their systemic inflammation. As oligoJIA showed no significant difference in the average SCF level, we hypothesized that low SCF might be related to severe systemic inflammation with a short disease duration.

As mentioned previously, there has been a study about the multisystem inflammatory syndrome in children (MIS-C) with COVID-19 demonstrating that children with Kawasaki disease have significantly lower levels of plasma SCF compared with matched healthy controls; while no significant difference between children who tested positive for SARS-COV-2 or even who developed MIS-C [190]. Interestingly, the clinical parameters showed that the children with MIS-C and Kawasaki disease had average CRPs of 22.8 (18.2-26.5) and 11.3 (8.1-18.8) mg/dL, respectively [190], indicating systemic inflammation. These findings indicate that SCF could be used as a biomarker to distinguish acute autoimmune or autoinflammation diseases from infection or infection-induced systemic inflammation. However, further validation is needed in larger cohorts involving patients with both sterile and non-sterile inflammation.

4.1.3 SCF: What is known and what is new

SCF, also named ligand for the receptor-type protein-tyrosine kinase KIT (KITLG), can be produced by a variety of cells, including fibroblasts [192], smooth muscle cells [193] and endothelial cells [194, 195]. SCF plays an essential role in hematopoiesis and is critical for mast cell activation, expansion, differentiation and survival [196].

4.1.3.1 The two forms of SCF

Endogenous SCF occurs primarily in two forms, a 248 amino acid cleavable form (SCF^{248}) and a 220 amino acid “non-cleavable” form (SCF^{220}). The two forms differ by the presence or absence of exon 6 that encodes for protease cleavage site(s) (Figure 14A) [197]. SCF^{248} can be cleaved to generate a 165-amino acid soluble SCF (SCF^{165}) (Figure 14B), by proteases such as MMP9 [198], chymase 1 [199] and several members of the A desintegrin and metalloproteases (ADAMs) family [200, 201]. SCF^{165} can also form a dimer (Figure 14B).

SCF^{248} is more efficiently cleaved from the cell surface during inflammation [202]. SCF dimerization is correlated with its biological activity. Both membrane-bound SCF dimers (SCF^{220} dimers, mSCF) and soluble SCF dimers (SCF^{165} dimers, sSCF) bind to and activate the intrinsic tyrosine kinase activity of c-Kit. In contrast, monomer SCF^{165} cannot cross-link and activate c-kit [203].

There are both qualitative and quantitative differences in how SCF^{220} and SCF^{165} dimers signal. SCF^{220} dimers are associated with homeostasis, while SCF^{165} dimers are associated with peripheral immune responses [204]. Hsu et al. demonstrated that SCF dimers were dissociable under non-denaturing conditions [205]; they further verified *in vitro* that more than half of SCF existed as a monomer under physiological conditions [203]. Characterizing the structure of SCF in the plasma of JIA patients or healthy controls could be an exciting continuation of this project.

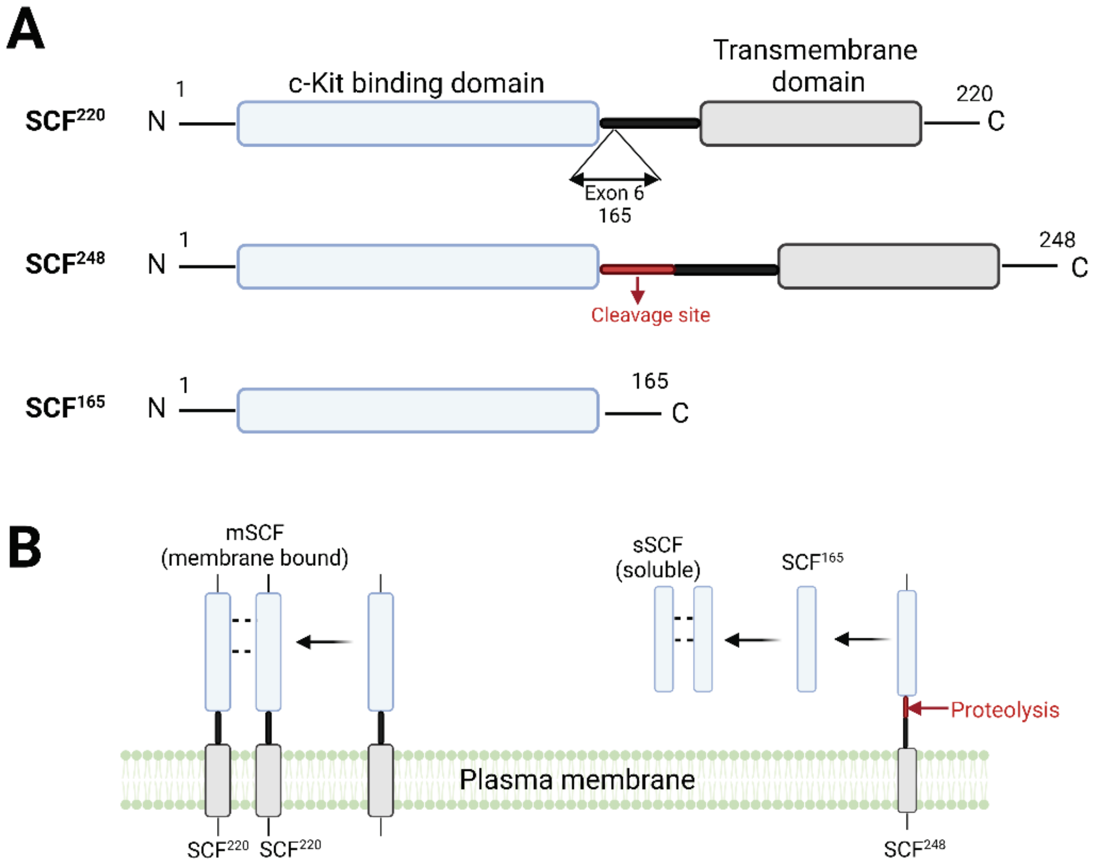


Figure 14. Schematic representation of SCF splice forms and protein processing. (A) SCF protein is produced as two transmembrane forms due to alternative splicing of exon 6, SCF^{220}, and SCF^{248}. In SCF^{248}, exon 6 is kept and encodes a proteolytic cleavage site, generating the soluble SCF^{165}. (B) SCF^{220} lacks the cleavage site and forms membrane-bound SCF dimers (mSCF), and SCF^{248} is processed to SCF^{165} that forms soluble SCF (sSCF). Dashed lines indicate that the SCF monomers are held together by noncovalent interactions. This illustration was created with BioRender.com.

4.1.3.2 Regulation of SCF production

SCF is upregulated *in vitro* by proinflammatory stimuli, such as TNFα, IL1β and phorbol 12-myristate 13-acetate (PMA) [196]. Mildly increased SCF gene expression can also be observed after stimulating 3T3 fibroblast cells with LPS *in vitro* (data unpublished). In contrast, glucocorticoids can downregulate SCF production [206], suggesting that SCF might be a good target for anti-inflammatory treatments. However, our observation of reduced SCF levels in patients with active sJIA and ongoing systemic inflammation indicates that the proinflammatory proteins in plasma did not upregulate SCF as *in vitro* experiments would suggest. On the other hand, 3 out of the 5 sJIA patients were treated with prednisolone (Table 5), and Otsuka et al. [207] reported that prednisolone inhibited SCF production from nasal epithelial cells from allergic patients. Therefore, it is likely that medication treatment can influence SCF expression. Future investigation in a larger controlled cohort is highly warranted.

SCF acts synergistically with other cytokines [196]. We correlated SCF with other proteins to explore potential mechanisms affecting plasma SCF levels. In active sJIA, 16 out of 68 proteins correlated with SCF (Figure 15). Only one protein, Fms-related tyrosine kinase 3 ligand

(FLT3LG), was positively correlated with SCF; the other 15 proteins were negatively correlated with SCF, among which VEGFA and TNF superfamily member 14 (TNFSF14) had the strongest relationship with R-values of less than -0.8000 (Figure 15).

	r	p-value	
SCF vs. VEGFA	**-0.8189**	**0.0006**	***
SCF vs. TNFSF14	**-0.8061**	**0.0009**	***
SCF vs. CXCL1	-0.7945	0.0012	**
SCF vs. CXCL5	-0.7855	0.0015	**
SCF vs. IL7	-0.7767	0.0018	**
SCF vs. IL18R1	-0.7345	0.0042	**
SCF vs. HGF	-0.7281	0.0048	**
SCF vs. CD40	-0.7256	0.0050	**
SCF vs. SULT1A1	-0.7172	0.0058	**
SCF vs. AXIN1	-0.6734	0.0116	*
SCF vs. CCL23	-0.6687	0.0124	*
SCF vs. S100A12	-0.6158	0.0250	*
SCF vs. FLT3LG	**0.6064**	**0.0280**	*
SCF vs. CD274	-0.6026	0.0293	*
SCF vs. CCL28	-0.5863	0.0352	*
SCF vs. CXCL11	-0.5583	0.0474	*

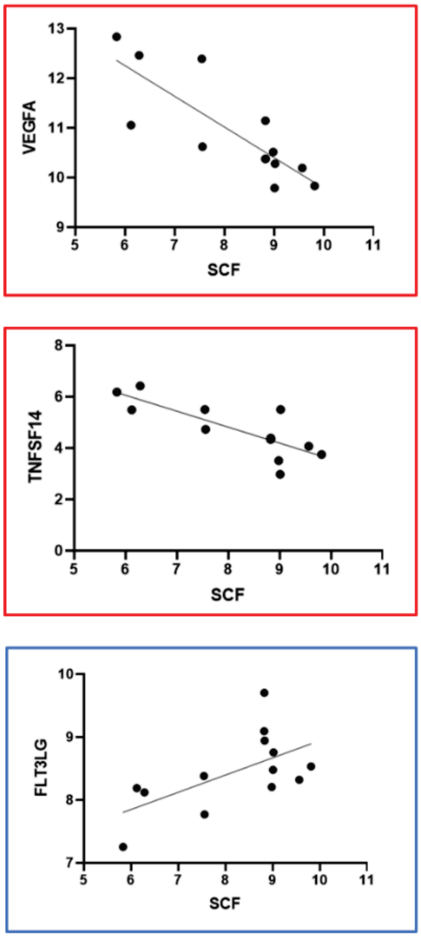


Figure 15. SCF is significantly correlated with 16 measured proteins in plasma from patients with active sJIA. Apart from FLT3LG, the other 15 proteins were negatively correlated with SCF. Statistics: Pearson correlation. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

The expression of both VEGFA and TNFSF14 have been related to the pathogenesis of RA [208, 209]. In contrast, there has been no study that connects the two proteins with SCF, except Feng et al. [210] reported that SCF-stimulated c-Kit signaling regulates VEGFA production via the Akt/mTOR pathway in rat insulinoma cell line. Interestingly, a structural biological study revealed the similarities among SCF, VEGF and FIT3LG, and suggested that the three proteins might bind to and activate PDGFR-like receptors in an equivalent manner [211]. This finding raised the possibility that the structural features allowed these proteins to be co-opted for their functions.

Although we measured 92 proteins, SCF may be correlated and affect other proteins not measured. SCF used as a potential diagnostic marker needs validation in larger cohorts. Further molecular and cellular studies are vital for better understanding the mechanism regulating SCF production and release.

4.2 HMGB1 INDUCED MACROPHAGE POLARIZATION

In **papers III** and **IV**, we investigated the role of HMGB1 in macrophage polarization. The main results are summarized in Figure 16. After maturing bone marrow cells to macrophages by stimulating with macrophage colony-stimulating factor (M-CSF) for 8-9 days, BMDMs were further polarized using frHMGB1 or dsHMGB1, or into pro-inflammatory M1 using IFNγ and LPS, or anti-inflammatory M2 using IL4, IL10 and transforming growth factor (TGF) β, which served as positive controls. Results from qPCR, RNA-Seq and ELISA revealed that dsHMGB1 induced a pro-inflammatory macrophage phenotype, sharing some similarities with M1 but without nitric oxide (NO) production. In contrast, results from scratch migration assay revealed that dsHMGB1 triggered an M2-like macrophage morphology and increased cell migration. Furthermore, inhibiting TLR4 alleviated dsHMGB1-induced cell migration, suggesting that the dsHMGB1-TLR4 signaling pathway not only induced pro-inflammatory cytokine secretion, but also contributed to cell migration.

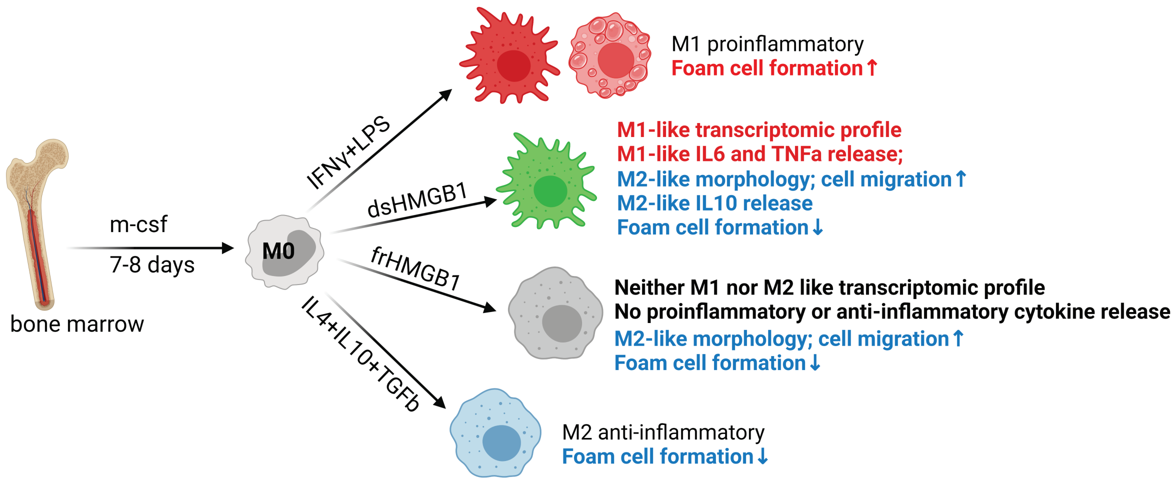
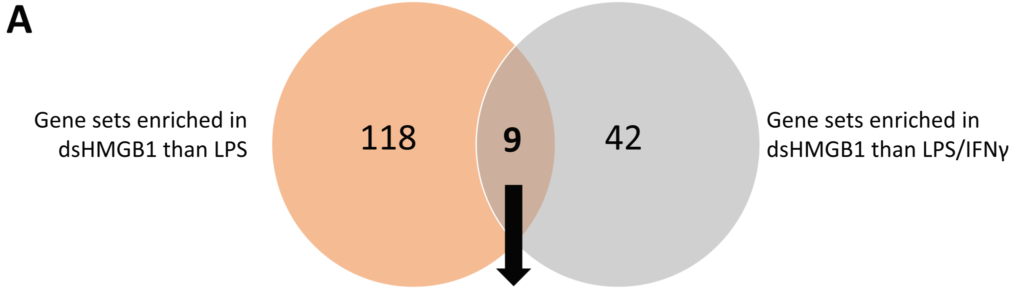

M1 proinflammatory
Foam cell formation ↑
IFNγ+LPS
M1-like transcriptomic profile
M1-like IL6 and TNFa release;
M2-like morphology; cell migration ↑
M2-like IL10 release
Foam cell formation ↓
dsHMGB1
m-csf
7-8 days
M0
bone marrow
frHMGB1
Neither M1 nor M2 like transcriptomic profile
No proinflammatory or anti-inflammatory cytokine release
M2-like morphology; cell migration ↑
Foam cell formation ↓
IL4+IL10+TGFb
M2 anti-inflammatory
Foam cell formation ↓

Figure 16. FrHMGB1 and dsHMGB1 play distinct roles in macrophage polarization. DsHMGB1 induced a proinflammatory macrophage phenotype, sharing similarities with M1 but without nitric oxide (NO) production and some M2 characteristics, with M2-like morphology and increased cell migration. FrHMGB1 induced neither an M1- nor an M2-like transcriptomic profile in BMDMs; but similar to dsHMGB1, frHMGB1 triggered an M2-like macrophage morphology and also increased cell migration. Neither dsHMGB1 nor frHMGB1 induced foam cell formation. This illustration was created with BioRender.com.

To further dissect the effects of HMGB1 isoforms on BMDM differentiation, RNA-Seq analysis was performed comparing the different polarization states after HMGB1 stimulations. RNA-Seq results in **Paper IV** were in accordance with findings in **Paper III** that dsHMGB1 induced an M1-like phenotype while frHMGB1 had limited effects on macrophage polarization. By performing gene set enrichment analysis (GSEA), we further identified the biological process enriched by dsHMGB1-stimulated BMDMs compared to LPS-stimulated BMDMs and M1. Nine gene sets were commonly enriched in both comparisons (Figure 17A). Interestingly, two of the nine commonly enriched gene sets were related to foam cell differentiation. The enrichment plot showed that dsHMGB1 resulted in foam cell differentiation gene set enrichment compared to the M1 (Figure 17B) and LPS (Figure 17C) groups.


A
Gene sets enriched in dsHMGB1 than LPS
118
9
42
Gene sets enriched in dsHMGB1 than LPS/IFNγ

Gene ontology biological process enriched in dsHMGB1 than LPS and LPS/IFNγ	NES (dsHMGB1 / LPS)	NES (dsHMGB1 / LPS/IFNγ)
Positive regulation of TLR4 signaling pathway	1.609	1.867
Peptidyl arginine N-methylation	1.785	1.720
Peptidyl arginine methylation	1.926	1.688
Antifungal innate immune response	1.607	1.667
Histone arginine methylation	1.919	1.653
Regulation of TLR7 signaling pathway	2.089	1.647
Regulation of macrophage derived foam cell differentiation	1.843	1.632
Regulation of TLR9 signaling pathway	1.940	1.631
Foam cell differentiation	**1.866**	**1.615**

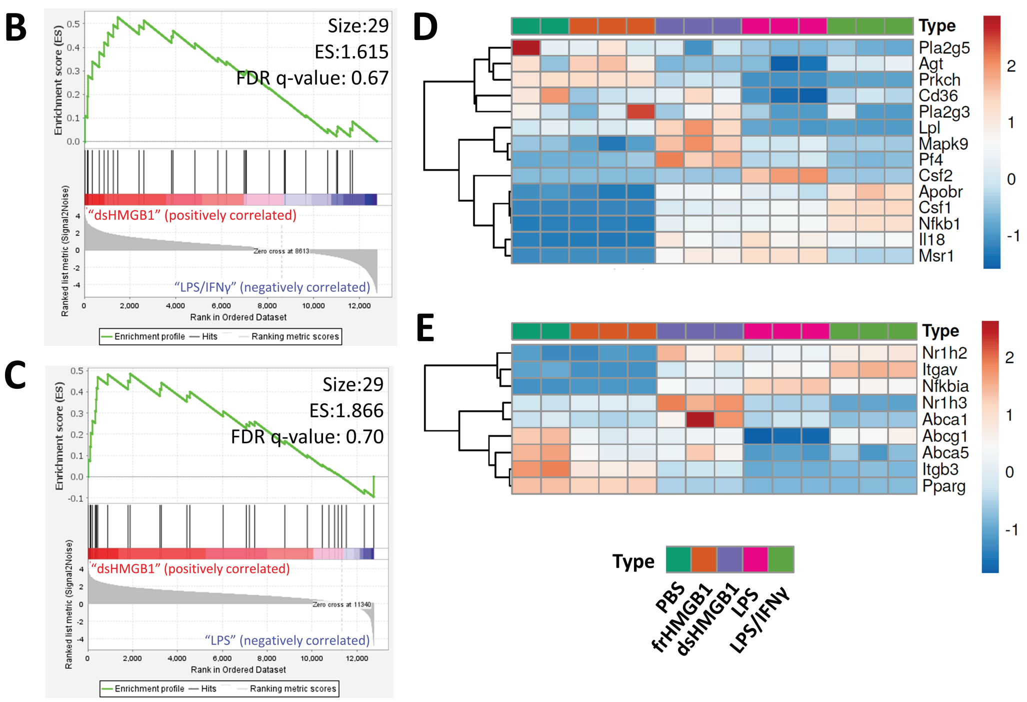


Figure 17. Foam cell differentiation-related pathways were enriched by dsHMGB1 than LPS and LPS/IFNγ. (A) GSEA analysis revealed that 127 gene sets were enriched by dsHMGB1 than LPS, 51 gene sets were enriched by dsHMGB1 than LPS/IFNγ (NES cut-off = 1.6). Nine sets were commonly enriched, among which two were related to foam cell differentiation. (B) Foam cell differentiation was enriched by dsHMGB1 (left, red) comparing with LPS/IFNγ (right, blue). (C) Foam cell differentiation was enriched by dsHMGB1 (left, red) comparing with LPS (right, blue). (D) The genes included in positive regulation of macrophage derived foam cell differentiation (GO:0010744) were listed and heatmap was created based on the normalized gene counts in each sample. (E) The genes included in negative regulation of macrophage derived foam cell differentiation (GO:0010745) were listed and heatmap was created based on the normalized gene counts in each sample. Abbreviation: ES, enrichment score; NES, normalized enrichment score; FDR, False Discovery Rate.

Foam cells in atherosclerotic lesions originate mainly from either circulating monocyte-derived macrophages or smooth muscle cells [212-214]. Foam cells are formed through dysregulated lipid metabolism resulting in the intracellular storage of lipid droplets [215]. HMGB1 is elevated in atherosclerotic lesions, indicating its possible involvement in the progression of atherosclerosis [216-218]. Discerning the role of HMGB1 in atherosclerosis pathogenesis, including interactions with lipoproteins, lipid uptake, and foam cell formation may benefit future therapies.

To further explore the involvement of dsHMGB1 in foam cell differentiation, we stimulated BMDMs with oxidized low-density lipoprotein (oxLDL), a commonly used molecule to induce *in vitro* foam cell formation. We initially followed the 'add-later' protocol, i.e., priming BMDMs with HMGB1/LPS/IFNγ for two hours, then adding oxLDL into the cell culture and incubating for 24 hours (Figure 18, 'Add-later' method). The results showed almost no oil red positive staining in frHMGB1- or dsHMGB1-stimulated BMDMs, regardless of oxLDL stimulation; in contrast, lipid droplet accumulation was observed in control, LPS, and M1 demonstrated an equal oxLDL uptake in M1- and LPS-stimulated BMDMs and a lower uptake in control BMDMs (Figure 6B in **Paper IV**).

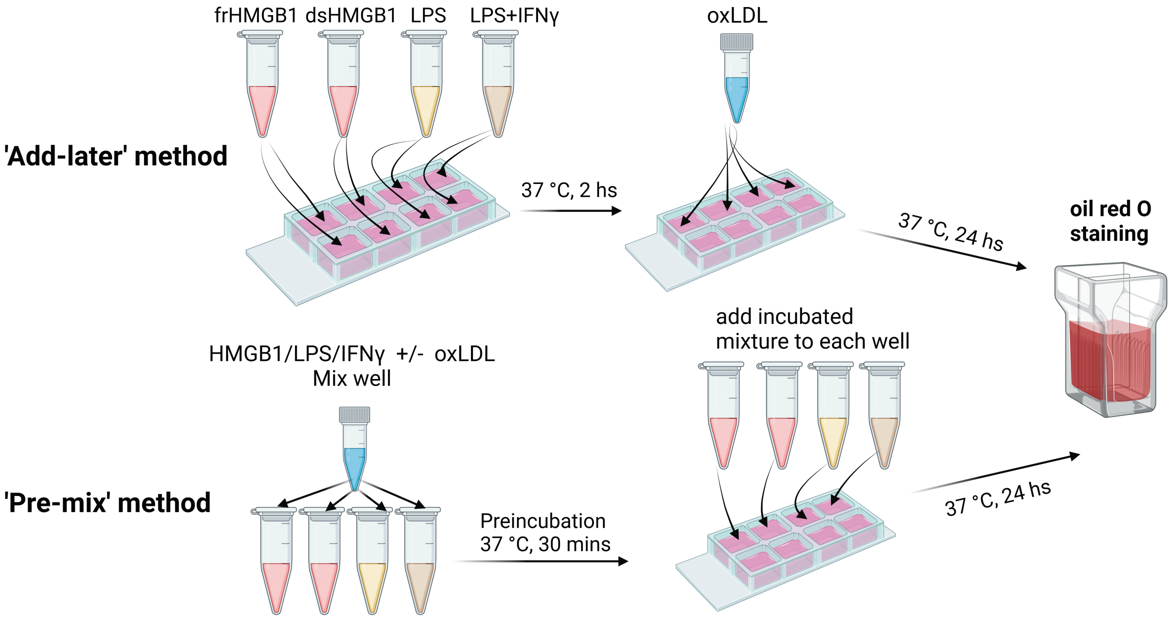

frHMGB1
dsHMGB1
LPS
LPS+IFNγ
oxLDL
'Add-later' method
37 °C, 2 hs
37 °C, 24 hs
oil red O staining
HMGB1/LPS/IFNγ +/- oxLDL
Mix well
add incubated mixture to each well
'Pre-mix' method
Preincubation
37 °C, 30 mins
37 °C, 24 hs

Figure 18. Illustration of the two protocols in oxLDL-induced foam cell formation. This illustration was created with BioRender.com.

Therefore, we hypothesized that both frHMGB1 and dsHMGB1 acted as an antagonist of the oxLDL receptors and blocked the intake of oxLDL by BMDMs. To further characterize this mechanism, we tried another protocol named "pre-mix", i.e., incubating HMGB1/LPS/IFNγ with oxLDL for 30 minutes at 37 °C, then adding the mixture into cell culture and incubating for 24 hours (Figure 18, ‘Pre-mix’ method). Interestingly, compared to the ‘add-later’ protocol, pre-mixing LPS/IFNγ with oxLDL significantly attenuated oxLDL intake (Figure 19). In contrast, pre-mixing frHMGB1 and dsHMGB1 with oxLDL resulted in no difference in oxLDL intake (Figure 19).

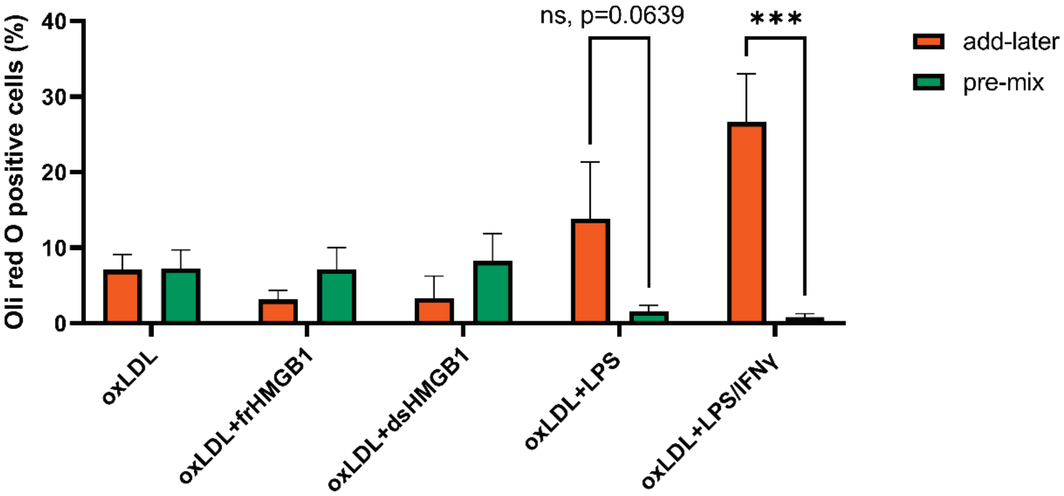


Figure 19. Different oxLDL treatment protocols resulted in distinct foam cell formation percentages. Average was calculated from the three pictures taken from each cell culture well, n = 4 mice, mean of 4 + SEM. Statistics: Statistical comparisons were performed using two-way ANOVA with correction of multiple comparisons by controlling the False Discovery Rate using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, ***, $p < 0.001$.

Our results contradict those from previous publications reporting that HMGB1 induces foam cell formation, promotes cholesterol accumulation in vascular smooth muscle cells [219] and accelerates oxLDL-induced foam cell formation and apoptosis in RAW264.7 cells [220]. Further, Lee et al. [221] demonstrated that HMGB1 enhanced oxLDL uptake by induction of low-density lipoprotein receptor-1 (LOX-1) in human coronary artery endothelial cells. LOX-1 is upregulated after exposure to proinflammatory and pro-atherogenic stimuli and can be detected in atherosclerotic lesions [222]. However, our RNA-Seq results showed that LPS/IFNγ- but not dsHMGB1-stimulated BMDMs had higher expression of *Olr*.

One explanation for these contradictory findings could be the difference in cell types (mouse BMDM compared to vascular smooth muscle cells, RAW264.7 cells and coronary artery endothelial cells). We also performed the *in vitro* verification based on the GSEA of the RNA-Seq results. The transcriptomic changes may not correspond to the proteomic and metabolic changes, so the differences observed from RNA-Seq may only partially represent the effects of DAMP and PAMP stimulation.

Overall, neither frHMGB1 nor dsHMGB1 induced oxLDL intake or foam cell formation in BMDMs, regardless of the treatment protocol. Despite the contrary results from the *in vitro* assay to GSEA, there were differences between LPS/IFNγ and dsHMGB1 in oxLDL intake, indicating that dsHMGB1, as a DAMP, induces macrophage polarization different from the classical M1 phenotype.

Transcriptomic profiling by RNA-Seq could not provide answers regarding differences in the NO production and migration induction observed in **paper III**. It is possible that RNA expression changes occurring at earlier or later time points that were not investigated may be consequences of the recorded differences. For instance, we previously identified the *Il6* expression peak after dsHMGB1 stimulation at 7 hours. Alternatively, not all protein expression and PTMs are reflected in their mRNA levels. Other readouts, for example, proteomic quantification, may be better suited to dissect the effects of HMGB1 stimulation.

4.3 MECHANISMS BEHIND ARTHRITIS-INDUCED NEUROINFLAMMATION

As discussed in section 3.3.1, there is no single mouse arthritis model that fully resembles human arthritis. Therefore, we tested our hypothesis that arthritis could induce inflammation in CNS in two available arthritis mouse models, CAIA and KRN. This data is presented in **paper V**. Our data suggest that arthritis can lead to neuroinflammation, significantly upregulating proinflammatory gene expression and interfering with hippocampal neurogenesis and proliferation.

In the following section, I discuss additional findings from the CAIA model, which were observed both during the experimental development of the study set-up and actual experiments presented in **paper V**.

4.3.1 Kinetic inflammatory marker changes in the hippocampus of CAIA mice

One feature of the CAIA model is the transient joint inflammation, with pain-like behaviors observed prior to, and outlasting, the clinical signs of arthritis [223]. Correspondingly, in patients with arthritis, though the disease-modifying anti-rheumatic drugs (DMARDs) effectively control joint inflammation, one out of four arthritis patients continue suffering from persistent pain, indicating arthritis-associated pain is not simply an outcome of inflammation [224-226]. Whether persistent pain could also affect central neuroinflammation in the brain is yet unknown. Therefore, we wanted to explore the kinetics of inflammatory markers in the hippocampus of CAIA mice.

The experimental protocol is described in Figure 20A. Mice brain tissues were isolated on day 0 prior to collagen II antibody injection (naïve control), day 3 prior to LPS injection, day 4, day 7, day 14 and day 21. The arthritis scores peaked on day 10-12 (Figure 20B), and mouse body weights were significantly decreased the day after the LPS injection (Figure 20C). It should be noted that in this batch of CAIA study, a slightly lower dose of LPS was injected compared to the batch of CAIA study presented in **Paper V**, explaining the generally lower peak scores.

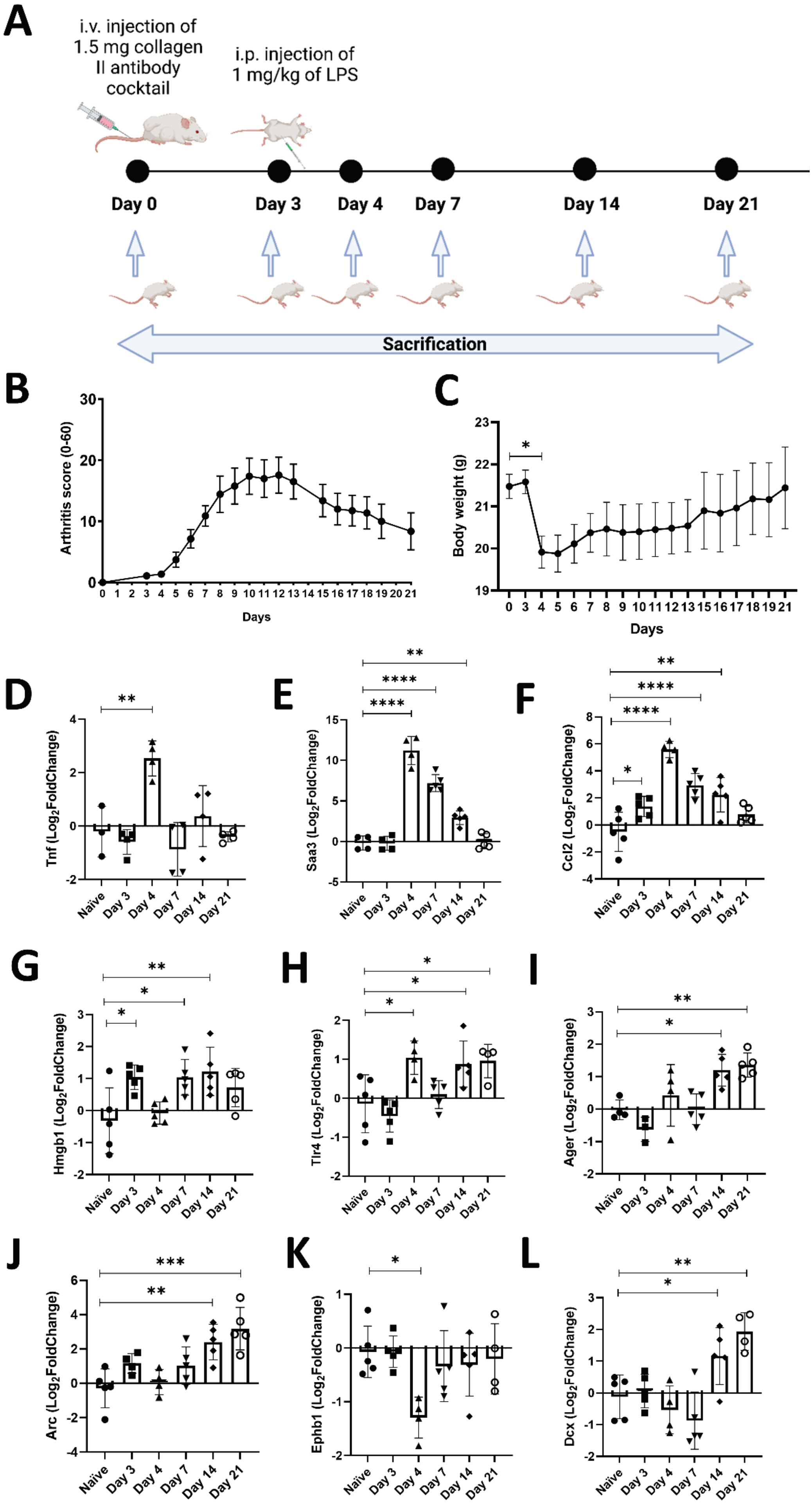

A
i.v. injection of 1.5 mg collagen II antibody cocktail
i.p. injection of 1 mg/kg of LPS
Day 0
Day 3
Day 4
Day 7
Day 14
Day 21
Sacrification
B
Arthritis score (0-60)
Days
C
Body weight (g)
Days
*
D
Tnf (Log2FoldChange)
**
E
Saa3 (Log2FoldChange)
**
****
****
F
Ccl2 (Log2FoldChange)
**
****
****
*
G
Hmgb1 (Log2FoldChange)
**
*
*
H
Tlr4 (Log2FoldChange)
*
*
*
I
Ager (Log2FoldChange)
**
*
J
Arc (Log2FoldChange)
***
**
K
Ephb1 (Log2FoldChange)
*
L
Dcx (Log2FoldChange)
**
*
Naïve
Day 3
Day 4
Day 7
Day 14
Day 21

Figure 20. Inflammatory markers kinetics in the hippocampus of CAIA mice. (A) Illustration of experimental design. This illustration was created with BioRender.com. (B) Average arthritis scores of CAIA mice, data were presented as mean ± SEM. (C) Average body weight of CAIA mice, data were presented as mean ± SEM. Statistics: ordinary one-way ANOVA comparing each group with the control group. (D-M) Expression of Tnf (D), Saa3 (E), Ccl2 (F), Hmgb1 (G), Tlr4 (H), Ager (I), Arc (J), Ephb1 (K) and Dcx (L) were determined using qPCR on the total RNA isolated from mouse hippocampus, n = 4-5 mice per group, 2 replicates/sample/condition. Gene expression is represented as Log2FoldChange (Log2FC) relative to the mean of the naïve group, and the scale bars represent the standard deviations (SD). Statistical comparisons were performed using RM-one way ANOVA with Turkey's multiple comparisons test on data that are normally distributed or using Friedman test with Dunn's multiple comparisons on data that are not normally distributed. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

qPCR was performed on total RNA extracted from the hippocampus. A series of inflammatory markers were selected based on literature searches and our research interests. *Tnf*, *Saa3* and *Ccl2* are inflammation markers that can be regarded as positive controls for systemic inflammation. Expression of these three genes peaked on day 4 (Figure 20D-20F). Interestingly, the expression of *Ccl2* had already been increased prior to LPS injection, indicating that the collagen II antibodies could induce inflammation.

HMGB1 can initiate CNS inflammation and cause brain damage by disrupting BBB and inducing the expression of proinflammatory factor IL1β and apoptosis [34]. Mildly increased *Hmgb1* expressions were measured on day 3, day 7 and day 14 (Figure 20G). Additionally, *Tlr4* and *Ager*, well-known receptors of HMGB1, were expressed at higher levels on day 14 and 21 (Figure 20H-20I). These results indicate ongoing neuroinflammation even during the remission of joint inflammation.

We then analyzed the markers with described relevance in cognitive disorders and neurogenesis. *Arc* encodes the protein activity-regulated cytoskeleton-associated protein (ARC). ARC regulates glutamatergic synapse plasticity and mood-related behavior [227] and is required for numerous learning and memory tasks [228]. Further, ARC has been linked to neuropsychiatric illness; stress increased *Arc* gene expression, and *Arc*-deficient mice show reduced anxiety-like behaviors, depressive-like behaviors and novelty discrimination [229]. We observed significant upregulation of *Arc* on day 14 and 21 (Figure 20J). These results potentially indicate an increased stress level of CAIA mice during the later stage of inflammation.

Ephb1 encodes the protein named ephrin type-B receptor (EphB) 1. EphB1 and EphB3 cooperatively regulate the proliferation and migration of neural progenitors in the hippocampus [230]. LPS injection resulted in systemic inflammation, transiently leading to *Ephb1* down-regulation in the hippocampus (Figure 20K). No difference was shown in *Ephb1* expression at later time points.

Dcx encodes the protein DCX, an important regulator of hippocampal neurogenesis, implicated in memory function, mood and cognitive disorders [145-147]. Sutthiwarotamakun et al. observed a significant reduction of DCX-positive cells in CIA DBA/1 mice brains on day 42 after immunization, regardless of disease severity [98]. However, Wolf et al. [148] reported an increasing number of DCX-positive cells and hippocampus neurogenesis in AIA C57BL/6 mice on day 7. These contradictory results could be due to different arthritis-inducing procedures for CIA and AIA. The immune response of CIA involves both T- and B-cells, while the AIA model is Th1-cell and neutrophil dependent. Different inflammatory profiles may cause different effects on hippocampus neurogenesis. In CAIA, we measured an upregulation of *Dcx* on day 14 and 21 (Figure 20L). It is likely that during the inflammation remission period, neuro cells were also undergoing regeneration from the previous damage.

In summary, by recording the kinetics of inflammatory marker changes in CAIA, we confirmed that the CNS was also affected by the induced peripheral inflammation. LPS injection directly resulted in the up-regulation of proinflammatory gene expression, but long-term inflammation may also play a role in neuro-regeneration and cognitive disorders.

5 CONCLUSION

Despite recent progress in JIA research, the understanding of the pathogenesis is still incomplete, hampering the development of targeted therapies. Therefore, a deeper understanding of the molecular mechanisms and key drivers of disease is needed. Immunoprofiling of JIA and subgrouping based on immune profiles provide means to both better understand the mechanisms behind disease flares, but also to develop better therapeutic options and monitoring tools suited for specific individuals. In **paper I**, we identified a novel set of biomarkers distinguishing active sJIA from inactive sJIA or healthy controls. Our findings enable a better understanding of the immune mechanisms active in sJIA and aid the development of future diagnostic and therapeutic strategies. In **paper II**, we found that in contrast to sJIA, the plasma immunoprofiles of patients with oligoJIA did not differ from their age and sex-matched healthy controls. To investigate if the inflammatory profile changed over time, we conducted a longitudinal analysis of a series of plasma and SF samples from an individual oligoJIA case. The data displayed a persistent inflammation over time, with a transient decrease in inflammation due to MTX treatment, which increased after two weeks when the medication changed. Our method reflected the clinical findings. However, when comparing the early and persistent disease phases in a cohort of patients with oligoJIA, we found that levels of chemokines were reduced as the disease progressed. Therefore, we proposed chemokines as potential JIA therapeutic targets, especially during the early disease phase.

HMGB1 acts as an alarmin when released from stressed or dying cells, with functions highly dependent on its complex PTMs and redox isoforms. In line with previous findings on the role of HMGB1 in arthritis pathogenesis, in **paper I**, plasma HMGB1 levels were found to be higher in sJIA patients during active disease than during the inactive disease phase. In **papers III** and **IV**, we investigated the effects of frHMGB1 and dsHMGB1 on macrophage polarization *in vitro* to explore the role of different redox isoforms of HMGB1 in detail. Our results revealed that dsHMGB1 could not only induce an M1-like proinflammatory phenotype and genotype but also increase cell migration. FrHMGB1 showed limited effects on macrophage polarization, with only cell migration induced by frHMGB1. It is interesting to speculate that dsHMGB1, as a DAMP, induces a macrophage phenotype on the M1 end of the spectrum but is more fine-tuned to sterile inflammatory conditions. This is strengthened by its suggested role in TLR7 and TLR9 regulation and retained migratory capacity, more prone to deal with sterile inflammatory features, including cell death, tissue injury and subsequent regeneration, rather than defense against pathogens. Finally, our study also suggested that both frHMGB1 and dsHMGB1 mitigated the formation of foam cells from BMDMs.

Peripheral inflammation-induced neuro-inflammation is a complex and important subject. In **paper V**, we investigated the occurrence of arthritis-induced neuroinflammation in two arthritic mouse models, CAIA and KRN. Our data suggest that arthritis can lead to neuroinflammation, significantly upregulating proinflammatory gene expression and interfering with hippocampal neurogenesis and proliferation. Different disease phases should be investigated to elucidate the potential regulatory mechanisms.

In conclusion, there is robust clinical evidence for HMGB1 as a potential biomarker for early prediction or progression of various diseases, including JIA. Furthermore, HMGB1 neutralization has shown effects in several pre-clinical models, including sepsis, arthritis and neuroinflammation, but no clinical studies of HMGB1 neutralization therapy have yet been performed. This could be due to the lack of convincing results from pre-clinical laboratory studies, or that neutralizing HMGB1 can cause unexpected side effects. Regardless, efforts should be made to understand HMGB1 release patterns, PTMs, and quaternary structures, in different microenvironments. Further, understanding the interaction between HMGB1 and its receptors is needed to achieve more precise HMGB1 targeting.

Understanding the pathogenesis of cognitive decline and neuropsychiatric comorbidities in arthritis is important for developing novel potential biomarkers for early diagnosis and prognosis, as well as new therapeutic strategies. Regarding our research interests in JIA, longitudinal follow-up of the children’s psychological and emotional well-being and performance in education could also facilitate our understanding of how chronic inflammation affects cognitive function.

6 POINTS OF PERSPECTIVE

My PhD work is divided into three directions: novel biomarkers in JIA, the role of different redox isoforms of HMGB1 in macrophage polarization, and peripheral inflammation-induced neuroinflammation. All are important for understanding the ongoing inflammatory mechanisms active during JIA and their potential clinical use. In this section, I address my perspective on each direction separately.

6.1 NOVEL DIAGNOSTIC AND PATHOGENETIC BIOMARKERS FOR JIA

Functional investigation of the hits we found in **papers I and II** would be of interest both for understanding the pathogenesis and aiding in diagnostic and prognostic biomarker development. There are still unmet needs in JIA diagnosis, prediction of disease progression and treatments.

At present, no single test can confirm an sJIA diagnosis. Increased CRP and ESR levels are found in up to 95% of sJIA cases [231]. However, before confirming the diagnosis, it is necessary to exclude other severe systemic illnesses, such as viral and bacterial infection and acute lymphoblastic leukaemia, which can mimic sJIA and its concomitant MAS [232]. Extra assays, such as microbiological cultures, viral panels, peripheral blood smears, bone marrow aspiration, genetic studies, echocardiography and chest X-ray, could be done to increase the accuracy of diagnosis [233]. In addition, S100 proteins, including S100A8/9 and S100A12, could distinguish sJIA from non-systemic JIA, other defined autoinflammatory syndromes (AID) and systemic undifferentiated recurring fever syndromes (SURFS) [234]. SCF could also be of use to distinguish sJIA from other non-systemic JIA and viral infections. Reproducing our SCF findings with clinical samples from larger and different cohorts would increase the validity of the study. Meanwhile, quantifying SCF via different techniques could also optimize and ensure an accurate readout.

The main treatment options for sJIA include glucocorticoids (GCs), DMARDs, non-steroidal anti-inflammatory drugs (NSAIDs) and methotrexate (MTX) [235, 236]. There is no cure for sJIA as of today. The overarching goal of sJIA treatment is to achieve clinically inactive disease and clinical remission. GCs are effective in treating the initial phase of sJIA, but a long-term treatment of GCs can result in several side effects, such as post-steroid obesity and osteoporosis [237]. IL1 blockers (anakinra and canakinumab) and IL6 blockers (tocilizumab) provide reliable evidence of both efficacy and safety [238-240], but in practice, there are still cases where treatments are not effective.

Compared with sJIA, oligoJIA often presents with milder systemic inflammation. One of the challenges in the clinical treatment of oligoJIA is disease persistence and its unresponsiveness to drug treatment. For example, the oligoJIA case we discussed in **paper II** showed MTX resistance, which can occur in up to 65% of patients with JIA [241]. Reasons behind the MTX intolerance and resistance could be the long disease duration before MTX administration [242], low baseline cytokine (for example, S100A12 [243]) concentration, and single nucleotide polymorphisms (SNPs) in genes involved in the mechanism of action of MTX [244, 245]. Even though that numerous studies investigating MTX intolerance and resistance mechanisms, a specific marker predicting MTX efficacy or adverse events has yet to be identified. Therefore, a quick and accessible prediction model is needed to assist clinicians in making individualized treatment decisions. The lesson from this case study was that biomarkers that correlate between SF and plasma would be useful for clinicians to adjust medicine prescriptions in time to improve the clinical outcome of the disease more efficiently.

To sum up, treatment for patients by JIA could be improved by identifying predictors of a good response to the currently available treatments; alternatively, drugs could be repurposed and novel drugs could be developed by targeting identified biomarkers involved in pathogenesis.

6.2 HMGB1 BIOLOGY

Redox isoforms of HMGB1 and their functional importance were proposed in the last decade. Despite extensive research on the proinflammatory role of dsHMGB1 and the migration-inducing role of frHMGB1, there is no valid or widely accepted strategy for detecting redox isoforms of HMGB1. Consequently, this limited our translational studies of the redox isoforms of HMGB1 *in vivo*. Future research would benefit from establishing a reproducible protocol for redox isoform detection, for example, by mass spectrometry, ELISA or typical antibodies or biosensors that can distinguish disulfide bonds and/or reduced cysteine.

In **paper III**, the induction of migration by dsHMGB1 was an interesting observation, as HMGB1-induced migration has been previously demonstrated as a feature of frHMGB1 [31]. We demonstrated that TLR4 blockade attenuated the migration of BMDMs induced by dsHMGB1, indicating that dsHMGB1-TLR4 signaling mediated the BMDM migration. We did not find a significant effect of knocking out RAGE on BMDM migration, suggesting that RAGE might not play a significant role as TLR4 in dsHMGB1-induced BMDM migration. To better understand the contribution of receptors TLR4 and RAGE to dsHMGB1-induced cell migration, one should test in different cell types than BMDMs, as we recorded relatively low *Rage* expression in BMDMs compared with *Tlr4*. Meanwhile, the scratch assay used in **paper III** differs from the chemotaxis assay used in previous studies [32, 35, 70]. It would be interesting to test BMDM chemotaxis in the 3D trans-well assay under the same treatment condition, making our findings comparable to the previous findings by others.

The acidic C-tail in HMGB1 easily becomes truncated due to the long stretch of repeating glutamic and aspartic acid residues in C-tail domain. Indeed, truncated HMGB1 is usually produced during our *E. coli* HMGB1 production process. The C-tail can interact with both A- and B-boxes, as well as the linker regions [246, 247]. The binding of the C-tail to the specific residues within A- and B-boxes results in HMGB1 stabilization [246]. The binding can also modulate the interaction of HMGB1 with other molecules, such as DNA, and regulate the PTMs [248, 249]. With regard to the extracellular situations, Gong et al. [250] showed that amino acid residues 201-205 in C-tail played a crucial role in antibacterial activity; Banerjee et al. [251] demonstrated that the C-tail was essential for HMGB1-RAGE binding and the downstream efferocytotic and apoptotic activities; our group previously reported that the C-tail negatively regulated HMGB1-TLR2 binding without interfering IL6, IL8 or CXCL1 secretion [252]. Recently, Borde et al. [253] reported that deleting the C-tail of HMGB1 reduced the viability of cancer cells but not of normal immortalized fibroblasts.

Because of the consequence of truncation on biological function, it is of great biological relevance to map distribution and localization of truncated HMGB1 *in vivo*, not limited to C-tail truncation but also other HMGB1 subparts. Such research into the distribution and localization would greatly expand our knowledge of the role of HMGB1.

6.3 DIFFERENCES BETWEEN HMGB1 AND LPS IN IMMUNE ACTIVATION

In **paper IV**, we conducted an investigation to discern differences in macrophage polarization between HMGB1, a prototypic DAMP, and LPS, a PAMP. Both dsHMGB1 and LPS are ligands for TLR4, though via different epitopes [43], and exerted similar proinflammatory downstream responses, which aligns with our findings. The difference we mapped is that LPS and dsHMGB1 play different roles in oxLDL intake by BMDMs. I would hypothesize that the difference we saw was independent of the TLR4 signaling pathway but more related to the oxLDL receptors, i.e. scavenger receptors. HMGB1 may be able to bind to oxLDL and hide its scavenger receptor binding epitope, or HMGB1 acts as an antagonist by direct binding to the scavenger receptor resulting in less opportunity for oxLDL-scavenger receptor interaction.

It should be noted that the cells used in **papers III** and **IV** were BMDMs from female mice with a C57BL/6 background. Sex differences in immunity are widely recognized, and confirmed experimentally [62, 254, 255]. Future studies should therefore take sex differences into account and validate concepts in both sexes if needed. In addition, macrophages are only a small part of the immune cell family. The inflammation and immune activation also involve monocytes, natural killer cells, dendritic cells and other lymphoid cells. Therefore, cell-specific differences in differentiation and proliferation induction by DAMPs and PAMPs are of interest.

6.4 ARTHRITIS-INDUCED NEUROINFLAMMATION

Arthritis-induced and peripheral inflammation-induced cognitive disorders have attracted greater attention. Although the association of JIA with psychiatric comorbidity remains controversial, children with other chronic diseases, including childhood-onset inflammatory bowel disease [256] and celiac disease [257], are reported to have an increased risk of psychiatric comorbidity, highlighting a connection between chronic inflammation and psychiatric comorbidity.

Recently, Berthold et al. [121] reported that the risk of depression and anxiety was not increased in individuals with JIA (n=400) compared to a reference group of individuals free from JIA (n=3200), matched for sex, year of birth and residential region. However, their results may have been impacted by several confounding factors in data analysis, including follow-up time, the coverage of the diagnosis codes and the validation through medical record review or personal interview. Further, JIA has distinct subtypes that involve systemic inflammation to varying degrees. One may expect to see the difference between patients with sJIA and oligoJIA in proinflammatory protein levels in plasma. Thus, researchers can consider separating JIA patients based on their diagnosed subtypes and do data analysis separately for each subtype to draw a more accurate conclusion.

In **paper V**, we investigated the involvement of HMGB1 in arthritis-induced neuroinflammation. Increasing levels of extracellular HMGB1 were observed in clinical and preclinical studies on nervous system diseases, including Parkinson's disease [258], autism [259] and depression [260]. Further, by targeting extracellular HMGB1 with antagonists, improvements have been achieved in animal models of nervous system diseases [261, 262], though relevant clinical studies are still lacking. In **paper V**, no significant differences in gene expression or nuclear-cytoplasm translocation of HMGB1 were found. Interestingly, though not significant, levels of HMGB1 in the hippocampus, as indicated by immunofluorescence, were reduced in arthritic mice (KRN and CAIA) compared with control mice in both models, indicating some potential HMGB1 secretion or release from the cells. The occurrence of oxidative stress in CNS disease-causing cell death and its facilitation of HMGB1 releases may be a good avenue to explore. In addition, immunohistochemical staining might not be sensitive enough to detect the translocation of HMGB1. Alternative approaches include extracting and isolating nucleic and cytoplasmic protein separately and quantifying HMGB1 in different fractions. Meanwhile, one could consider the indirect roles of HMGB1 in neuroinflammation: instead of being overexpressed by local neuro cells in the brain, circulating HMGB1 could induce BBB disruption and neuronal cell polarization. For example, our group has previously shown that the intracerebral injection of dsHMGB1 upregulated MHC class II expression in rat brains [263].

Several reasons may explain the inability of researchers to find a mechanism behind arthritis-induced neuroinflammation. One main reason is the lack of reliable methods and tools to assess posed hypothesis. As discussed in section 3.3.1, there is no single arthritic mouse model that accurately resembles human arthritis. The complexity of human social interaction makes cognitive disorder even harder to interpret. Despite thesis limitations, future studies should pay attention to the following aspects:

a) **Larger and more representative cohorts:** Statistical power of a study should be calculated; for non-hypothesis-driven studies or exploratory studies, balancing group size between healthy control and patients is vital to draw reliable conclusions.
b) **Reuse of publically available omics datasets:** Where applicable, time and money can be saved through the reuse of data. Regarding my thesis, I was unsuccessful in finding relevant open-access sequencing data of brain tissue from arthritis models, indicating few research groups are actively investigating arthritis-induced neuroinflammation.
c) **Objective interpretation:** One reason behind contradicting results from different studies was the need for unified criteria for patient inclusion, data collection and classification. Additionally, scientists often only report statistically significant findings. Full disclosure of all results is beneficial to allow readers access to all information required for accurate and objective interpretation. Meanwhile, one should make efforts

to study design before carrying out the analysis, rather than simply discussing the limitations in the publications.

d) **The translational study from animals to humans:** Currently, most studies investigating systemic inflammation-induced neuroinflammation and cognitive decline use animal models. This is due to the accessibility of animal tissues and the simplicity of interpreting animal behaviour. However, researchers must always consider and emphasize the clinical relevance of research based on animals. It is always optimal if the concepts adapted from animals are applicable to humans, though this is often difficult.

Overall, inflammation appears to be the main actor in this scenario. Indeed, there is some evidence that DMARDs can protect patients with arthritis from developing cognitive disorders, including dementia [264, 265], though some others reported controversial results or no relationship between neurological outcomes and classical DMARDs [266-268]. In the future, a multidisciplinary approach to assess all variables involved would be informative. The possibility of evaluating novel pharmacological classes to select the most effective ones and eventually identify new possible synergistic co-treatments could be promising.

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