School of Medicine
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INVESTIGATION OF THE ROLE OF ENDOGENOUS LIGANDS FOR TOLL-LIKE RECEPTORS IN THE INCREASED RISK OF CARDIOVASCULAR DISEASE IN PATIENTS WITH CHRONIC KIDNEY DISEASE

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Mazzarino M., Cetin E., Bartosova M., Marinovic I., Ipseiz N., Hughes T., Schmitt C., Ramji D., Labéta M., Raby A-C. "Identification of Damage-associated Molecular Patterns as Therapeutic Targets to Reduce Key Atherogenic Responses in Chronic Kidney Disease".

ABSTRACT

Chronic Kidney Disease (CKD) is associated with markedly increased cardiovascular (CV) morbidity and mortality, but the mechanisms are not fully understood. Notably, atherosclerosis, an inflammation-driven thickening of the vascular wall which underlies most CV events, is aggravated in CKD. This work identified 4 known endogenous TLR agonists, Damage-Associated Molecular Patterns (DAMPs), namely Hsp70, Calprotectin, Hyaluronic acid and HMGB-1, elevated in CKD plasma and demonstrated that these DAMPs can differentially drive key cellular functions and responses in endothelial cells, monocytes and macrophages that are associated with worsening of atherosclerosis. Specifically, CKD-associated DAMPs induced loss of trans-endothelial resistance, enhanced chemokine-driven monocyte migration, increased cytokine production and atherosclerosis-associated gene expression by macrophages, and promoted foam cell formation by reducing cholesterol efflux. These activities were mostly mediated by TLR2 and TLR4. In mice, CKD induction also led to increased DAMP plasma levels and induced a range of short and long-term systemic inflammatory, immune and atherosclerosis-promoting responses that are known to drive CVD. *In vivo,* both a multi-TLR inhibition approach and a Calprotectin-specific targeting approach reduced or prevented CKD-induced systemic inflammatory and pro-atherogenic responses, such as cytokine production, gene expression and increase inflammatory leukocyte proportions. Furthermore, Calprotectin inhibition robustly reduced the CKD-induced increased aortic expression of atherosclerotic-promoting genes. These findings identify specific DAMPs elevated in CKD that can critically promote pro-inflammatory and atherogenic responses and demonstrate that specific targeting within the DAMP/TLR pathway is a promising therapeutic strategy to reduce chronic inflammation and lower the atherosclerotic burden in CKD, ultimately reducing CV risk in this patient population.

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List of Abbreviations

α-SMA: α -smooth muscle actin

AA: Aristolochic Acid

AAN: Aristolochic Acid-induced Nephropathy

ABCA1: ATP-binding cassette subfamily A member 1 transporter

ACE: Angiotensin converting enzyme

AcLDL: Acetylated LDL

ACR: Albumin-to-creatinine ratio

AKI: Acute Kidney Injury

AP-1: Activating protein-1

ApoE$^{-/-}$: Apolipoprotein E deficient

Bcl: B-cell lymphoma

BODIPY: Sterol dipyrromethene boron difluoride

C. pneumonia: Chlamydia pneumoniae

CCL: Chemokine C-C motif ligand

CCR: C-C Motif Chemokine Receptor

CD: Cluster of Differentiation

CKD: Chronic kidney disease

CMV: Cytomegalovirus

CRS: CardioRenal Syndrome

CV: Cardiovascular

CVD: Cardiovascular disease

CXCL: C-X-C Motif Chemokine Ligand

CXCR: C-X-C Motif Chemokine Receptor

DAMPs: Damage-Associated Molecular Patterns

DCs: Dendritic cells

DD: Death Domain

EDN: Eosinophil-derived neurotoxin

ERK: Extracellular signal-regulated kinase

ESRD: End-Stage Renal Disease

FcγRs: Fcγ Receptors

FEEL-1: Link Domain-Containing Scavenger Receptor-1

G-CSF: Granulocyte colony stimulating factor

GFR: Glomerular filtration rate

GM-CSF: Granulocyte-Macrophage colony-stimulating factor

GPI: Glycosyl-phosphatydil-inositol

H. pylori: Helicobacter pylori

HA: Hyaluronan

HAEC: Human Aortic Endothelial Cells

HAS: HA synthase

HD: Hemodialysis

HDL: High-Density Lipoprotein

HIV: Human Immunodeficiency virus

HMGB-1: High-mobility Group Box 1 Protein

HMGN1: High Mobility Group Nucleosome Binding Domain 1

HMW: High molecular weight

Hsp: Heat-shock protein

HSV: Herpes Simplex Virus

HUAEC: Human umbilical arterial endothelial cells

I-κB: Inhibitor of NF-κB

I/R: Ischemia/reperfusion

ICAM-1: Intercellular adhesion molecule 1

IFN: Interferon

IKK: I-κB kinases complex

IL: Interleukin

IL-1R: Interleukin-1 Receptor

IRAK-4: Interleukin-receptor-associated-kinase-4

IRFs: Interferon-response factors

JNK: C-jun-N-terminal kinase

KLF4: Krüppel-like factor-4

L+MMW: Low and Medium Molecular weight

LAM: Lipoarabinomannan

Lama1: Laminin Subunit Alpha 1

LBP: LPS-Binding Protein

LDL: Low-Density Lipoprotein

LOX-1: Lectin-like OxLDL receptor-1

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

LRR: Leucine-Rich Repeat

M-CSF: Macrophage colony-stimulating factor

MAL: MyD88 adaptor-like

MAPKs: Mitogen-activated protein kinases

MCP-1: Monocyte Chemoattractant Protein-1

MD-2: Myeloid differentiation factor 2

MKKs: MAPK kinases

MM6: MonoMac 6

MMPs: Matrix metalloproteinases

MyD88: Myeloid Differentiation Primary Response protein 88

NF-κB: Nuclear Factor-κB

NFR2: Nuclear Factor Erythroid 2-related factor 2

NK: Natural Killer

OxLDL: Oxidized LDL

P_3C: Pam_3CSK_4

P. gingivalis: Porphyromonas gingivalis

PAMPs: Pathogen Associated Molecular Patterns

PBMC: Peripheral blood mononuclear cells

PD: Peritoneal dialysis

Pdgfb: Platelet-Derived Growth Factor Subunit B

PGN: Peptidoglycan

PPAR- γ: Peroxisome proliferator activated receptor- γ

PRR: Pattern Recognition Receptors

PSGL-1: P-selectin glycoprotein ligand 1

qPCR: Quantitative polymerase chain reaction

RAGE: Receptor for Advanced Glycation End-products

RANTES: Regulated on Activation, Normal T Expressed and Secreted

RHAMM: Receptor of HA motility

RIN: RNA integrity number

ROS: Reactive oxygen species

RP105: Radioprotective 105

Rs-LPS*: R. sphaeroides LPS*

RT: Reverse transcription

RTT: Renal replacement therapy

SARM: Sterile- α and HEAT-Armadillo motifs-containing protein

SIGIRR: Single Immunoglobulin IL-1R-related molecule

SLE: Systemic Lupus Erythematosus

SMCs: Smooth muscle cells

SNS: Sympathetic Nervous System

Sp-1: Signal protein-1

SR-A: Scavenger receptor A

SREC-1: Scavenger Receptor Expressed by Endothelial Cells-I

sTLR2: Soluble TLR2

TAB: TAK-1 binding proteins

TAK-1: TGF-β-activated kinase-1

TER: Trans-endothelial electrical resistance

TFIII: Tissue Factor III

TGF-β: Transforming growth factor-β

Th1: Type 1 T helper

Th2: Type 2 T helper

TIR: Toll/IL-1R

TLR: Toll-like receptors

TNF: Tumor Necrosis Factor

Treg: Regulatory T-cells

TRAM: TRIF-related adaptor molecule

TRAF-6: TNFα-receptor-associated factor-6

TRAILR: TNF-related apoptosis-inducing ligand receptor

TRIF: TIR domain-containing adaptor inducing IFN-β

TRIL: TLR4 Interactor with LRR

VCAM-1: Vascular cell adhesion molecule 1

VSMCs: Vascular smooth muscle cells

ZO-1: Zonula occludens-1

I- INTRODUCTION

I-1. Chronic kidney disease (CKD) and cardiovascular (CV) complications

I-1.1. Definition of CKD

CKD is a major public health problem worldwide, affecting up to 15% of the global population, with a particularly high incidence in high-income countries (1). In the UK, the NHS spent an estimated £1.45 billion on CKD in 2009/2010 for a prevalence of renal replacement therapy (RTT) of 832 per million population (2). At the end of 2017, the prevalence of CKD in the UK reached 983 per million population as reported by the UK Renal Registry (3) with a prevalence of stage 3-5 of 4.1% in UK population aged more than 18 years old in 2016/2017 (4). CKD is characterised by renal dysfunction and is associated with high morbidity and mortality.

CKD is classified into 5 stages according to its severity (**Table 1**), which is determined by a combination of the estimated glomerular filtration rate (GFR) and the presence of markers of renal injury, such as raised albuminuria, for a duration of more than 3 months (5-7).

- Stages 1-2 are typically asymptomatic with a high to normal GFR (≥60 ml/min/1.73 m^2). At these stages, there is no CKD in the absence of markers of kidney damage.
- Stage 3 is considered early CKD. The GFR rate displays a moderate to severe reduction (30-59 ml/min/1.73 m^2). If CKD is due to external factors, the disease may still be reversed or stopped at these stages. Treatment will certainly include a change of lifestyle, with notably a change of diet.
- Stages 4-5 are characterised by a severe reduction in GFR (≤29 ml/min/1.73 m^2) and are non-reversible. Proper management of lifestyle is not sufficient to ameliorate the patient conditions. Stage 5 (GFR <15 ml/min/1.73 $m^{2)}$, also called End-Stage Renal

Disease (ESRD), is the stage of kidney failure. Stage 5 CKD patients need RTT, either dialysis (hemo- or peritoneal dialysis), or a kidney transplant.

Table 1. Classification of CKD stages according to GFR and protein albumin-to-creatinine ratio (ACR)

GFR and ACR categories and risk of adverse outcomes		ACR categories (mg/mmol), description and range		
		<3 Normal to mildly increased	3-30 Moderately increased	>30 Severely increased
		A1	A2	A3
≥90% Normal and high kidney function	G1	**Low risk of disease progression**	**Moderately increased risk**	**High risk**
60%-89% Mild reduction of kidney function related to normal range for a young adult	G2	**Low risk of disease progression**	**Moderately increased risk**	**High risk**
45%-59% Mild-moderate reduction of kidney function	G3a	**Moderately increased risk**	**High risk**	**Very high risk**
30%-44% Moderate-severe loss of kidney function	G3b	**High risk**	**Very high risk**	**Very high risk**
15%-29% Severe loss of kidney function	G4	**Very high risk**	**Very high risk**	**Very high risk**
<15% of function remaining Kidney failure	G5	**Very high risk**	**Very high risk**	**Very high risk**

Adapted from Levey AS &al, 2011 (8)

Not considered as CKD unless there is other evidence of kidney damage
Mild CKD due to mildly or moderately changing kidney function and urine protein levels
CKD
End-stage kidney disease

I-1.2. Causes and management of CKD

The exact causes of CKD are not always clear and are often complex and this multivariate aetiology leads to different progression rates and prognoses. There are several well-accepted risk factors for the development of CKD, including:

- Monogenic disorders e.g. autosomal dominant polycystic kidney disease, Fabry disease, Alport syndrome, atypical haemolytic-uraemic syndrome (9). These conditions are mostly inherited and kidney disease progress is not reversible/ stoppable.
- Congenital abnormalities such as, congenital anomalies of the kidney and the urinary tract (10);
- Co-morbidities: Age, diabetes, hypertension, obesity;
- Prolonged exposure to medication, notably nephrotoxic nonsteroidal anti-inflammatory drugs or chemotherapy;
- Infections: e.g. Human immunodeficiency virus (HIV), hepatitis virus, malaria, bacterial infections (11);
- Kidney malignancy (12);
- Episode of Acute Kidney Injury (AKI), which, because of a maladaptive repair process, may lead to chronic damage (13-17).

No curative treatment for CKD exists at present, but guidelines and medication exist to control the co-morbidities and symptoms associated with CKD (18, 19) :

- Reversing or stopping CKD progression: As previously mentioned, moderate stages of CKD may still be reversed with lifestyle changes such as adopting a healthy and

balanced diet, quitting smoking, lowering alcohol intake, exercising regularly and avoiding nonsteroidal anti-inflammatory drugs (20, 21).

- RRT: In patients reaching stage 5 CKD, the last option of treatment is RRT to replace kidney function, or a renal transplant (see next Section I-1.3 for details).
- CV pathologies: CV mortality is the leading cause of death in CKD patients (see Section I-1.4 for details). Angiotensin converting enzyme (ACE) inhibitors and diuretics to reduce water retention may be used to help control hypertension as a result of hypervolemia. Lipid lowering drugs, such as statins, will reduce high cholesterol (22).
- Anaemia: In more advanced stages of CKD, a large number of patients develop anaemia and injection of erythropoietin (to stimulate the production of red blood cells) and iron supplements can be prescribed (23).
- Hyperphosphatemia: Loss of kidney function leads to a build-up of metabolic waste products, such as phosphate, which is detrimental if unbalanced. In that case, calcium acetate or calcium carbonate, that are classified as phosphate binders, will be given (24).
- Vitamin D deficiency will be treated with supplements deficiency (25).
- Glomerulonephritis, an inflammation of glomerulus, will typically be treated with steroids or cyclophosphamide (26).

Given the lack of cure for CKD, in addition to CKD increasing in occurrence, it is also on the rise as a cause of mortality and now contributes in 1.35% of the global burden of disability life years lost, growing at a rate of 1% per year (6). Premature mortality increases with decreasing GFR and increasing albuminuria and is highest in patients on RRT. Life

expectancy for those on dialysis is roughly one-third of that of the general population (age- and sex-matched).

I-1.3. RRT and kidney transplant in ESRD

When ESRD is reached, RRT is required for patients to remain alive, either while waiting for a kidney transplant or as the only treatment option in patients who cannot have or do not want a transplant. Currently, there is an estimated 3.8 million people worldwide who rely on one or another form of dialysis (27). As stated previously, two modes of dialysis exist: peritoneal dialysis (PD) and hemodialysis (HD). Both have advantages and drawbacks.

a. Peritoneal Dialysis

PD uses the peritoneal membrane as a dialysis membrane: a hypertonic dialysis fluid is instilled into the peritoneal cavity with the aim to create a pressure gradient between the blood from peritoneal capillaries and the dialysate. Consequently, water and waste products are drawn out from the blood into the dialysate. The dirty dialysate is removed and replaced with clean PD solution. The number of times that this process is repeated daily varies with remaining kidney function: exchange may only be performed overnight if there is still a substantial residual kidney function, or several times daily when kidney function is low (28). PD is often implemented before HD, if there is no medical contraindication, especially in developed countries, as it is much cheaper than HD. It also does not require a healthcare setting and is typically performed at home by the patient. For this reason, PD is often associated with better quality of life and gives the patient more freedom than HD (29). A major complication of PD is the occurrence of infectious peritonitis (30), which is most commonly driven by gram-positive bacteria (e.g. *S.epidermidis*) and less often gram-negative

bacteria (e.g. *E.coli*). Fungal peritonitis may also happen, and although less common it is the most threatening, as it cannot be treated with antibiotics and catheter removal is needed (31, 32).

b. Hemodialysis

HD is based on a hydrostatic pressure being used to create an external blood circuit and divert the whole blood volume of the patient into a machine acting as an artificial kidney. Blood will then be filtered and returned to the body. This type of dialysis is very efficient and, unlike PD, does not need to be performed several times daily. HD is typically performed 3 times a week, and a session will last about 3h. On HD-free days, patients will need to thoroughly control their diet and fluid intake. HD is typically performed in a hospital ward, although technical improvements have made it more and more possible to carry out at home in recent years, thus significantly improving patients' quality of life. A downside of HD is the high infection rate and its severe consequences (33). In the HD patient population, infection is the second leading cause of mortality, after CV death, with 169 per 1000 patient-years at risk (34). The pathogens can be exogenous or endogenous and cause different types of infections:

- Bloodstream infections: They are the most common type among HD patients and represent a leading cause of hospitalisations, morbidity and mortality. Bloodstream bacterial infections are closely linked to the vascular access site, notably the majority of vascular access-associated bloodstream infections occur in patients dialysing with central vein catheters compared to grafts or fistulas (35). These infections can cause disseminated bacteremia or loss of the vascular access which are major problems. As observed in PD, gram-positive bacteria (e.g. *S. aureus and* coagulase negative

staphylococci) are the most commonly reported pathogen to trigger bloodstream infections and access-related bloodstream infections (32% to 53% of cases and 20% to 32% of cases, respectively) in HD (36). Interestingly, a study reported that 28% of infections in HD patients involved the vascular access and 25% the lung showing the urge to reduce access-related bloodstream and respiratory infections in this population (37).

- Respiratory-track infections: In HD patients, hospital admissions for pneumonia are 102% higher compared with the general population (38). Patients with pneumonia present a poor prognosis (0.17 survival probability after 5 years), and it is often the antecedent of CV death (39). Other respiratory infections include seasonal influenza with complications, tuberculosis infection, or more recently Middle East respiratory syndrome caused by corona virus. However, with an exception for pneumonia, other respiratory infections can be effectively prevented by vaccination of patients and health workers (33).
- Viral infections: Outbreaks of transmission of Hepatitis B, C and HIV have been observed in the dialysis setting, mostly because of failure to adhere to infection control practices (33).

c. Kidney transplant

The first successful kidney transplantation was performed by Joseph Murray in 1954, has been greatly improved and has become the treatment of choice for ESRD since (40). ESRD patients have better long-term survival (about 10 years), as well as improved quality of life if they undergo a kidney transplantation, when possible, than those who stay on dialysis (41). Kidney recipients do not have to have dialysis anymore. They also present a reduced long-

term risk of myocardial infarction compared with dialysis patients (42). The ESRD patients who benefit the most from a kidney transplant in terms of years of life gain are young patients with diabetes. In this sub-population, aged from 20 to 39, patients on dialysis live about 8 years, while after a transplant expectancy increases to 25 years (43). However, the number of patients on the kidney waiting list rapidly increased in the past years while the number of transplants performed each year has remained the same (43). Although kidney transplant is considered the best treatment option for ESRD patients, it also presents complications. The procedure itself is associated with immediate or short-term risks, among them haemorrhage, thrombosis, infections as patients are heavily immune-suppressed during the first 3-6 months post-operation, disruption of lymphatics (lymphocele) and disruption of the urinary collecting system (urinoma) (40). A major long-term complication is chronic kidney transplant rejection triggered by both immunologic and non-immunologic factors leading to late allograft loss (43, 44). Understanding of the molecular and cellular mechanisms underlying rejection is improving, but remains to be translated in better early detection and effective therapies.

I-1.4. Cardiovascular disease (CVD) in CKD

CVD is the leading cause of death worldwide, irrespective of race and ethnicity (22). As for CKD, CVD is the consequence of multifactorial risk factors, both traditional (Framingham) risk factors including ageing, hypertension, diabetes, smoking, dyslipidemia and obesity, homocysteine blood levels and left ventricular hypertrophy, as well as non-traditional risk factors. An increase in CV mortality was first observed in patients undertaking HD by Lindner and colleagues in 1974. Since then, studies around this issue have been increasing and it is now well accepted that CVD is more frequent and severe in patients with CKD than in the general population (45). CVD risk is increased by CKD by 2 to 20 times

depending on CKD stage (46). In patients with stage 3 CKD, CV mortality is about twice as high as in individuals with normal kidney function. However, in ESRD patients (Stage 5 CKD), the risk of CV mortality increases 10 to 20-fold compared to the normal population of the same age, race and sex, accounting for around 50% of overall mortality among these patients (45, 47). This strong interrelation between CVD and CKD is often referred to as CardioRenal Syndrome (CRS) (48), and is clinically characterised by the presence of lung rales, increased jugular venous pressure and tachypnea, while systolic blood pressure is > 160mmHg without fatigue. The relationship between the two pathologies is complex, and the increased CV risk in CKD patients is in part due to the high prevalence of multifactorial risks described previously, which are classical risk factors for both CKD and CVD (22, 49). However, traditional risk factors are insufficient to explain the high incidence of CVD in CKD patients. This is highlighted by the fact that prevention of CVD by controlling traditional risk factors, such as smoking, obesity, cholesterol levels and hypertension, although effective in the general population, does not significantly lower CVD mortality in CKD patients (50). This suggests that other links exist between CKD and CVD that need to be investigated and that more innovative therapeutical strategies need to be evaluated (22, 45). In 1990, Guyton proposed that CKD-specific factors were emerging as causes for the co-existence of renal failure and heart disease (51). His theory was that regulation of extracellular volume, blood pressure and renal sodium handling, controlled through mediators such as the renin-angiotensin system and endothelin, are the major parameters responsible for the crosstalk between both diseases. Since this description, the model had been refined and extrapolated, and the non-traditional, CKD-specific, risk factors of CVD now accepted include:

- Mineral abnormalities, e.g. reduced calcitriol activation for control of calcium levels and impaired phosphorus removal. This in turn promotes arterial vascular

calcification, the predominant form of vascular disease found in CKD patients and is associated with poor cardiovascular outcomes (22, 52).

- Oxidative stress, due to accumulation of uremic toxins because of deficient renal clearance. This is notably believed to lead to abnormal lipid modifications, such as excessive oxidation of Low-Density Lipoprotein (LDL) cholesterol in CKD patients (45), a key step in the initiation of atherosclerotic disease.
- Volume overload, due to reduced urine output. This will lead to hypertension.
- Malnutrition, e.g. increased protein catabolism and Vitamin D deficiency due to kidney dysfunction.
- Endothelial dysfunction, characterised by phenotypic changes, decreased viability and senescence of endothelial cells, notably as a consequence of increased uremic toxins which activate pro-inflammatory and fibrogenic factors.
- Sympathetic nervous system (SNS) excessive activation, which may induce cardiac myocytes apoptosis, myocardium hypertrophy and focal myocardial necrosis (53), as well as dysregulated lipid metabolism (54, 55) and enhanced renin release in patients with renal failure (56). Notably, SNS activation can also induce inflammation via increased cytokine production from both the liver and the heart, and potentially promotes the formation of atheromatous plaques in the carotid artery indirectly (48).
- Chronic inflammation. Persistent, low-grade inflammation is now considered a hallmark feature of CKD, as demonstrated by the presence of modest to moderate levels of circulating inflammatory markers in CKD patients (57-59). The causes of the inflammatory burden in CKD are not fully understood but higher systemic inflammation levels have been linked to worst patient outcomes in general (58-61) and CVD mortality in particular (60, 62). Epidemiological and clinical studies have

shown strong and consistent relationships between markers of inflammation and risk of CV events (63). Inflammation underlies many aspects of CVD, and notably plays a crucial role in all stages of atherothrombosis (63-65) (see Section I-3.1 for details).

I-2. Toll-like receptors (TLRs), major mediators of inflammation

I-2.1. TLRs as microbial sensors

TLRs are a class of Pattern Recognition Receptors (PRRs) that are part of the innate immune system and were first described for their critical role in initiating and mediating inflammation in response to infections (66, 67). TLRs are thus named because they are homologous to the Toll-receptors first identified in the fruit fly, *Drosophila Melanogaster* and found to play a critical role in the insect's immunity to infections (68). TLRs are type I transmembrane receptors with an extracellular domain rich in Leucine-Rich Repeat (LRR) domains responsible for ligand recognition and co-receptor interactions, and an intracytoplasmic domain homologous to that of Interleukin-1 Receptor (IL-1R), termed Toll/IL-1R (TIR) domain. TLRs extracellular domain is responsible for recognition of microbial components, termed Pathogen Associated Molecular Patterns (PAMPs) expressed by microbes such as bacteria, viruses or fungi (69-73). Ten TLRs have been identified in human, each capable of recognising different PAMPs from different pathogens, including bacteria, viruses and fungi (**Table 2**):

- TLR2 and TLR4 are probably the best characterised TLRs and are major players in bacterial recognition. TLR2 senses mainly cell wall components from gram-positive bacteria, such as peptidoglycan (PGN) and acylated lipopeptides, while TLR4 is the main receptor for

lipopolysaccharide (LPS, also known as endotoxin) from gram-negative bacteria (74). Both TLR2 and TLR4 can also sense some viral proteins.

- TLR5 mostly senses flagellin, a major protein constituent of microbial flagella.

- TLR3/7/8 sense viral nucleic acids.

- TLR9 senses unmethylated CpG DNA, mostly from bacteria and viruses.

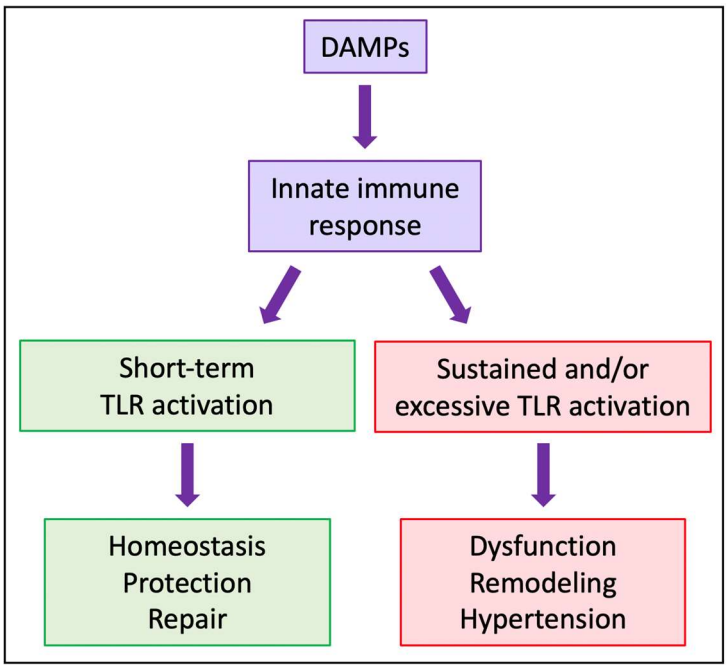
- TLR10 remains an orphan receptor, with no well described ligand and function. It is believed to either have pro-infectious/inflammatory role in some cases notably when it forms heterodimers with TLR1 or TLR6, or on the contrary be an anti-inflammatory receptor, notably via dampening other TLRs' responses. This is particularly true via its binding to some TLR2 ligands (75-78).

Table 2. Human TLRs and their PAMP ligands

TLRs	Ligands	Sources
TLR2 (dimerisation with TLR1 or TLR6)	Lipoprotein/lipopeptides	Bacteria, mycobacteria
	Triacylated lipopeptides (P_3CSK_4)	Bacteria, mycobacteria
	Diacylated lipopeptides	Mycoplasma
	Peptidoglycan (PGN)	Gram-positive bacteria
	Lipoteichoic acid (LTA)	Gram-positive bacteria
	Lipoarabinomannan (LAM)	Mycobacteria
	Zymosan	Fungi
	Hemagglutinin	Measles virus
TLR3	dsRNA	Viruses
TLR4	Lipopolysaccharide (LPS)	Gram-negative bacteria
	Fusion protein	Respiratory syncytial virus
	Envelope proteins	Mouse mammary tumor virus
TLR5	Flagellin	Bacteria
TLR7	ssRNA	Viruses
TLR8	ssRNA	Viruses
TLR9	Unmethylated CpG DNA	Bacteria, virus, yeast, insects
TLR10	Structural proteins	HIV-1 virus
	Triacylated lipopeptides (P_3CSK_4)	Bacteria, mycobacteria
	Synthetic diacylated lipopeptides (FSL-1)	Mycoplasma
	LPS	Gram-negative bacteria
	Borrelia burgdorferi,	Anaerobic bacteria
	Listeria monocytogenes	Spirochete bacteria
	Helicobacter pylori	Gram-negative bacteria
	Herpes Simplex Keratitis	Virus
	H1N1	Virus
	RNA-protein complexes	H5N1 influenza virus
	dsRNA	Viruses
	Flagellin	Bacteria

I-2.2. TLRs as sensors of sterile damage

In addition to microbial PAMPs, an increasing number of endogenous agonists, or so called Damage-Associated Molecular Patterns (DAMPs), are being reported as ligands of TLRs (79-82) (**Table 3**). DAMPs are endogenous host-derived components that are typically not accessible to TLRs due to their localisation, but become so as they are released by necrotic cells or damaged tissues. In normal physiological conditions, most DAMPs are expressed ubiquitously in a variety of cells and tissues and carry out essential cellular maintenance functions without being detected by the immune system, notably because they are not localised in the same cellular compartment as their receptors. Early during an injury, DAMPs are released from intracellular compartments or from the extracellular matrix and can engage TLRs (mostly TLR2, TLR4 and TLR9), which leads to sterile inflammatory response. Of note, some reports have shown that DAMPs may use the same binding sites as PAMPs (83), however, they may also use different accessory proteins to bind to TLRs, therefore triggering different, sometimes opposite, downstream signaling pathways. Therefore, different DAMPs may induce very different responses while binding to the same TLR (84). In health, DAMPs contribute to the initiation of normal repair processes and tissue healing. However, in pathological conditions, release of endogenous molecules become misallocated, and can lead to a systemic distribution and ultimately, to a state of chronic inflammation because of a maladaptive inflammatory response (67, 85-87) (**Figure 1**).



Adapted from McCarthy CG. et al., 2013 (88)

Figure 1. Paradoxical effects of TLRs activation
Short-term and controlled TLR activation by their endogenous ligands on innate immune system cells initiates an inflammatory response which play a major role in homeostasis maintenance and wound healing. However, excessive and dysregulated TLR activation due to the chronic presence of DAMPs abolish beneficial effects and leads to a pro-inflammatory state, blood pressure elevation and maladaptive tissue repair.

Examples of the best described DAMPs ligands for TLRs include:

High-mobility Group Box 1 Protein (HMGB-1): HMGB-1 is one of the best characterised DAMP and is one rare endogenous molecule to have the ability to activate TLR2, TLR4 and TLR9 (89-93). Structurally, HMGB-1 is a 25-30 kDa nuclear nonhistone DNA-binding protein that acts as a regulator for gene transcription and DNA repair. It is expressed ubiquitously and in its fully reduced form in all nucleated cells (94). During injury, cellular stress or necrosis, HMGB-1 is released extracellularly, either passively by necrotic, apoptotic or injured cells (95) or actively by activated immune cells such as monocytes and macrophages (96). Depending on its release route, HMGB-1 can initiate or promote inflammation, via cytokine production, chemotaxis

and dendritic cells (DCs) maturation (97-99), thereby initiating or contributing to inflammatory and immune responses. These responses can be mediated via HMGB-1 interaction with different receptors, namely TLR2, TLR4, TLR9 and the Receptor for Advanced Glycation End-products (RAGE) (100-102). RAGE is a multifunctional transmembrane protein of the immunoglobulin superfamily, expressed at low baseline levels in the majority of tissue and increased under inflammatory conditions (103). It was the first receptor described for HMGB-1 (100). HMGB-1 binding to RAGE leads to the activation of Ras, PI3K, and Rho, which trigger nuclear factor-κB (NF-κB) pathway activation (104). Moreover, the activation of RAGE by HMGB-1 also triggers the activation of the Mitogen-activated protein kinases (MAPKs)/Extracellular signal-regulated kinase (ERK) pathway, which is important in cell migration, tumor proliferation and invasion, and expression of matrix metalloproteinases (MMPs). The implication of HMGB-1/RAGE in cell recruitment and migration is directly mediated by the increase in adhesion molecule expression such as Vascular cell adhesion molecule 1 (VCAM-1) and Intercellular adhesion molecule 1 (ICAM-1) (93), or indirectly by inducing secretion of chemokines, in particular chemokine C-X-C Motif Chemokine Ligand (CXCL) 12 (105). In addition, HMGB-1 binding with RAGE can induce autophagy (106). In addition to RAGE, HMGB-1 has been reported to directly interact with TLR2, TLR4 and TLR9, as well as to act as a co-receptor for these TLRs (107). For example, HMGB-1 can enhance TLR9 sensitivity towards CpG-DNA by forming complexes with it, which will lead to increase cytokine production in plasmacytoid DCs (108), and HMGB-1 binding to nucleosomes from apoptotic cells activates macrophages and DCs through TLR2 (109). However, the responses activated by the HMGB-1/TLR4 pathway have been the main research focus, notably because of its wide and well-described contribution in inflammation and immune regulation (110-112). As mentioned above for HMGB-1/RAGE signaling, NF-κB is activated when HMGB-1

interacts with TLRs, with Cluster of Differentiation (CD) 14 being mandatory for activation in the case of TLR4 (108, 113-115). More recently, numerous reports demonstrated a critical importance of the redox state of cysteines in the structure of HMGB-1 in the binding to its inflammatory receptors, and therefore in its function.

HMGB-1 contains 3 cysteines: C23 and C45 can form a disulfide bond, and C106 is unpaired. These cysteines are modified by post-translational modifications leading to 3 different isoforms: i) “fully reduced HMGB-1” for the all-thiol form, ii) “disulfide HMGB-1” for the partially oxidized form, and iii) “sulfonyl HMGB-1” for the terminally oxidized form. When fully reduced, HMGB-1 interacts with CXCL12 to form a heterocomplex which binds to CXCL12 receptor, C-X-C Motif Chemokine Receptor (CXCR) 4, with increased affinity to promote cell migration. Immune cells secrete CXCL12 after NF-κB activation, and HMGB-1 itself is able to induce CXCL12 production via RAGE engagement (116). When released and/or secreted extracellularly, oxidation of C23 and C45 of the fully reduced HMGB-1 results in disulfide HMGB-1. The extracellular TLR4 adaptor myeloid differentiation factor 2 (MD-2) only binds specifically to this isoform, and TLR4/MD-2 complex induces cytokines/chemokines production (117). Finally, all 3 cysteines being terminally oxidized to sulfonates, sulfonyl HMGB-1 does not present any pro-inflammatory or chemoattractant activities. This indicates that the disulfide bond between C23 and C45 is essential to the inflammatory activity of HMGB-1 (118). Interestingly, because of the nature of redox modifications caused by reactive oxygen species (ROS), the immune activities of HMGB-1 at any location can vary over time and space and according to intra- and extracellular events e.g. cell stress amounts and intercurrent events as in ischemia/reperfusion (I/R) can affect HMGB-1 to mediate from chemotaxis activity to cytokine production to inactivity during the course of the same inflammatory response (119).

HMGB-1 has widely been reported as an important player in promoting tissue regeneration after acute inflammation in different models of tissue injury including spinal cord, skin, muscle and heart (118, 120, 121). Of note, a selective suppressive mechanism for immune responses induced by HMGB-1 has been identified by its interaction with another receptor complex, namely CD24 associated with Siglec-10, a sialic acid-binding Ig-like lectin (122, 123). However, HMGB-1-driven responses can become dysregulated and HMGB-1 has been shown to act as a late-phase pro-inflammatory mediator in a variety of pathological conditions such as sepsis and sterile inflammatory conditions (124, 125), rheumatoid arthritis, Systemic Lupus Erythematosus (SLE), Cutaneous SLE, scleroderma, Alzheimer's disease (126, 127). HMGB-1 blocking was found to have beneficial effects in different animal models of spinal cord, liver, brain and myocardial damage after I/R injury (128). However, other studies have reported beneficial effects of HMGB-1 administration, notably in acute coronary syndromes, including better outcomes and recovery after myocardial infarction in the presence of HMGB-1, although it is considered to be one of the mediators in ischemic heart disease. HMGB-1 injection to the myocardium, from as soon as 1 minute to 3 weeks after injury (e.g. coronary ligation or acute global I/R) resulted in the development of new myocytes and reduced DC numbers, cardiomyocyte hypertrophy and extra-cellular collagen deposition at the injured site, for a better recovery (100, 121). Transgenic mice with cardiac overexpression of HMGB-1 were able to release more systemic HMGB-1 after ligation of the left anterior descending coronary artery, and had smaller infarcted areas, improved cardiac function and higher survival rates compared to control mice (129). Discrepancies between reports may in part be from the redox state of HMGB-1, which was not discussed in the studies, or the fact that the fully reduced and the disulfide forms can interconvert once added *in vitro* or *in vivo*, even when the fully reduced recombinant form is used. Of note, the

presence of disulfide HMGB-1 has been correlated by several studies with the onset of pathologies such as brain injury, liver damage, myositis, and juvenile idiopathic arthritis (126, 130-132). Interestingly, patients with early macrophage activation syndrome (affecting about 10% of children with juvenile idiopathic arthritis) have dramatically increased systemic levels of disulfide HMGB-1 isoform (126, 131-133). Moreover, disulfide HMGB-1 (and no other isoforms) is able to activate TLR4 in order to contribute to the transmission of nociceptive signals (134). It has been recently reported that blockade of HMGB-1 release with Ethyl Pyruvate, or inhibition of its activity with a neutralising antibody, lead to decreased liver inflammation as well as ameliorated liver injury in a model of heatstroke-induced liver injury requiring Nlrp3 inflammasome, thus demonstrating a role for HMGB-1 in mediating activation of inflammasome (135). Of particular relevance to our study, HMGB-1 expression is quickly up-regulated in renal and hepatic I/R injury and is believed to mediate a strong necrosis-induced inflammatory response via TLR4-signaling predominantly (89, 91, 136, 137). There is also evidence of HMGB-1 presence in serum of patients with glomerulonephritis (85, 138), and increased HMGB-1 levels correlate in CKD with pro-inflammatory markers and declining kidney function (139-141). HMGB-1 serum levels are also significantly higher in both diabetic and non-diabetic patients with acute coronary syndromes compared to those without acute coronary syndromes (142). Atherosclerosis-focused studies also reported significantly increased expression of HMGB-1 in the nuclei and the cytoplasm of macrophages and smooth muscle cells (SMCs) localised near the intima in human atherosclerotic lesions from the aorta, carotid and coronary arteries compared to normal human arteries, as well as in areas adjacent to the necrotic core of atherosclerotic lesions (143). Another study using atherosclerosis-prone animals, apolipoprotein E deficient ($ApoE^{-/-}$) mice fed with high-fat diet, has demonstrated the pro-atherogenic effect of HMGB-1 by administration of neutralising

antibodies for HMGB-1 which resulted in a decrease in atherosclerosis by 55%, notably via a decrease in macrophages, DCs, and $CD4^+$ T-cell accumulation in atherosclerosis lesions and a reduced expression of VCAM-1 and Monocyte Chemoattractant Protein-1 (MCP-1) (144). Consistent with these results, other reports using recombinant HMGB-1 demonstrated its ability to activate vascular endothelial cells leading to the expression and secretion of ICAM-1, VCAM-1, E-selectin, granulocyte colony stimulating factor (G-SCF), RAGE, Tumor Necrosis Factor (TNF) α, MCP-1, interleukin (IL)-8, plasminogen activator inhibitor 1, and tissue plasminogen activator (145).

Heat-shock Proteins (Hsps): Hsps are a group of proteins that are evolutionarily conserved and show high sequence homology between species, from bacteria to humans. They are classified in 6 families based on their approximate molecular weights: small Hsps, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp110. In physiological conditions, Hsps are intracellular chaperones that guide newly synthetised polypeptide chains to prevent aggregation and misfolding and mediate the refolding and stabilisation of damaged polypeptides. They are present in the cytoplasm and nucleus of eukaryotic cells, except Hsp60 and small Hsps which are mostly located in mitochondria and less so in the cytoplasm. Hsps can also be released extracellularly via an active route using non-classical pathways in both physiological and stress conditions, or passively by necrotic cells. Endothelial and other cells in the vessel wall notably are able to upregulate their expression of Hsps under cellular stress. Once in the extracellular compartment, Hsps act as DAMPs which are able to trigger a wide range of immunological effects via their interaction with several receptors, including PRRs which causes pro-inflammatory cytokine and chemokine release (146). Hsps can also serve as chemoattractants, and can promote maturation of antigen-presenting cells. Hsps, especially

Hsp70, have also been demonstrated to help antigen presentation by binding and presenting antigens to cell surface MHC class I molecules (85, 147, 148). Of note, there is evidence for a role of Hsps as major antigens and very strong inducers of humoral and cellular immune responses in the context of infectious diseases from studies revealing increased levels anti-Hsps antibodies in patients or experimental disease models. However, more recently it has been suggested that the phylogenetically conserved nature of Hsps across species could facilitate immunological cross-reactions between pathogen and self-Hsps potentially resulting in autoimmune diseases. This hypothesis has been widely studied in experimental models of arthritis and patients with rheumatoid arthritis, and vascular diseases. In addition, increased levels of Hsp70 have notably been found to circulate in sepsis and septic shock and are believed to contribute to the pathology (149, 150). Hsps are also thought to play a role in kidney disease, as Hsp70 is up-regulated after I/R injury in rat kidneys (151), and levels of Hsp-90α are higher in plasma from children and young adults with CKD (152). Furthermore, differential or opposite roles have been reported in disease for different Hsps. Indeed, Hsp22, Hsp27 and Hsp32 have been shown to have potential protective effects in heart infarction, atherosclerosis and oxidative stress respectively, while major pathogenic roles have been related to Hsp60, Hsp70 and Hsp90 in CVD. Notably, soluble Hsp60 is increased in patients with early carotid atherosclerosis and has been shown to promote atherosclerosis via its binding to TLR4 leading to the ERK/MAPKs pathway activation inducing SMCs migration (153, 154). In another study, human Hsp60 increased SMCs proliferation *in vitro* in a dose-dependent manner via its interaction with TLR2 and TLR4 (155). It was even suggested that atherosclerosis is initiated as a result of the inflammatory processes caused by autoimmunity to Hsps (156, 157). On the other hand, despite their pro-inflammatory properties, Hsps, notably Hsp70, Hsp40 and Hsp90, have neuroprotective role in neurodegenerative disorders.

Indeed, recombinant Hsp70/Hsp40 and Hsp90 were able to block the aggregation of amyloid-β proteins, a major trigger in Alzheimer's disease, and virally-induced Hsp70 overexpression rescued neurons culture from toxicity induced by amyloid-β aggregation *in vitro* for example (158, 159). These apparent discrepancies may be the results of several factors, such as the formation of immune complexes (Hsp-Hsp antibody) or differential receptor engagement by different Hsps or in different locations or pathological scenarios. Of particular relevance to this study, Hsp70 has been shown to induce inflammatory responses via interaction with TLR2 and TLR4, and the co-receptor CD14 (149, 160). Extracellular Hsp70 has also been observed to engage scavenger receptors, such as Lectin-like Oxidized LDL (OxLDL) receptor-1 (LOX-1), Scavenger Receptor expressed by Endothelial Cells-I (SREC-1), and Link Domain-Containing Scavenger Receptor-1 (FEEL-1), although the functional consequences on immune responses is still unclear. However, the association of CD91, another well-established receptor for Hsp70, with members of the scavenger receptors family, has been shown not only to enhance the proliferation of antigen-specific human $CD4^+$ T-cells but also to promote the uptake of antigenic peptides (here tetanus toxin and influenza hemagglutinin) by monocytes upon exposure to Hsp70 *in vitro*. These results were also confirmed in the same study using a CD91 knockdown *in vitro* model with addition of scavenger receptor antagonists in order to block their binding site, resulting in the reduction of Hsp70-induced proliferation of $CD4^+$ T-cells confirming the role of Hsp70-CD91-scavenger receptors complex in immunological memory (161). Consistently, HEK293T cells transfected with CD40, a cell surface protein crucial for B-cells function and autoimmunity, and exposed to Hsp70 displayed the ability to uptake Hsp70-bound peptide (here using peptide C) suggesting a role of CD40-Hsp70 interaction in the cross-presentation of Hsp70-peptides complexes towards MHC class I molecules (162). In addition, Hsp70 has been described as a ligand for both Siglec-5 and Siglec-14, which share

identical ligand-binding domains, and are expressed on monocytes and neutrophils. The signal delivered through binding of Hsp70 to Siglec-5 was found to be anti-inflammatory, while Siglec-14 engagement was mostly pro-inflammatory, revealing a dual effect of Hsp70 depending on its receptor, its localisation, and the intracellular signaling pathway associated. Moreover, some studies also reported a crosstalk between Siglecs and TLRs reinforcing the hypothesis of Siglecs as immunomodulatory receptors (163-166).

S100A8/S100A9 (Calprotectin): The S100 proteins family is a large family of calcium-binding proteins implicated in a variety of normal cellular functions, such as protein phosphorylation, Ca^{2+} homeostasis, cytoskeleton dynamics and cell growth (167), exclusively expressed in vertebrates. Among S100 proteins, S100A8 and S100A9 are small cytoplasmic proteins expressed abundantly by neutrophils and monocytes. These proteins exist as homodimers, but preferably form more stable heterodimers, notably S100A8/A9, also called Calprotectin, formed in presence of calcium and zinc (168). When released extracellularly following cellular stress or necrosis, some members of the S100 family can function as DAMP and trigger immune responses, normal and pathological, via TLR4 and RAGE (169-172). A relatively new hypothesis regarding the effect of S100 family in promoting inflammation is the interaction with CD33 as a receptor, notably recognising S100A9 (173). CD33 is part of the Siglec receptors family and was implicated in certain cancers and Alzheimer's disease (174). S100 proteins have notably been associated with cancer, neurodegenerative disorders, and diseases of the kidney, heart, joints and lungs (168, 175-179). On the contrary, anti-inflammatory and tissue protective functions (e.g. oxidant scavenging and inhibition of MMPs and ROS production in phagocytes) have also been reported for S100 proteins, notably S100A8 and S100A9, in complex or not. It is therefore speculated that the functions of these

proteins may be concentration-dependent and influenced by the cellular and biochemical composition of the local milieu (180, 181). As mentioned above, Calprotectin is the active S100A8/S100A9 complex, a 36 kDa molecule expressed at low levels constitutively. However, it becomes significantly upregulated and is released by phagocytes (mostly), especially neutrophils, in response to cellular stress. It contributes to pathological inflammatory processes (182, 183). For example, mice deficient in S100A9 were less severely affected and survived significantly longer to a lethal endotoxin-induced shock, while intravenous injection of Calprotectin markedly shortened the mice survival after shock, notably in part via release of TNFα which demonstrate a role for Calprotectin in endotoxin-induced septic shock via its binding with TLR4 (184). Calprotectin is also believed to contribute significantly to acute coronary syndrome pathogenesis (185) as well as the pathology of sepsis in humans (184, 186) via its interaction with TLR4 alone and TLR4-RAGE, respectively. RAGE signaling and subsequent activation of NF-κB have been shown, in blocking experiments *in vitro*, to promotes tumor cell growth in the presence of low concentrations of Calprotectin (187). Calprotectin secretion can also be triggered by cellular interaction during neutrophil rolling and its release induces VCAM-1 and ICAM-1 expression in endothelial cells and increase the capacity of leukocyte Mac-1 (an adhesion molecule heterodimer formed by CD11b and CD18) to bind endothelial ICAM-1 in a TLR4-mediated pathway. This process results in reducing rolling velocity and faster adhesion for trans-endothelial migration (188). Furthermore, Calprotectin levels in various fluids and tissues have been investigated as potential disease biomarkers. In particular, fecal Calprotectin has typically been associated with intestinal inflammation and is a predictor of the course of inflammatory bowel disease (189, 190), one of the main risk factors for colorectal cancer. In colorectal cancer, clinical and experimental studies have suggested that malignant cells and tissues overexpress both S100A8 and S100A9

separately, and Calprotectin. Therefore, the Calprotectin in stool has been classified as a reliable and sensitive marker for the diagnosis of bowel cancer and postoperative evaluation of patients, but not for disease progression (191). Elevated serum levels of Calprotectin have also been identified in patients suffering from a number of infections and chronic inflammatory pathologies such as cystic fibrosis, tuberculosis, bronchitis and rheumatoid arthritis which suggest an active role in disproportioned inflammatory reactions. High levels of Calprotectin have also been found in the synovial fluid and plasma from patients with rheumatoid arthritis and gout.

Of particular relevance to this study, clinical studies have positively correlated Calprotectin levels in the atherosclerotic plaque with plaque vulnerability (192, 193). Calprotectin has also been used as a biomarker of diabetes mellitus, and it has been suggested that high plasma levels may be associated with atherosclerosis in diabetic patients (193, 194). Furthermore, S100A8 and S100A9 are present in atherosclerotic plaques of both mice and humans, and can activate neutrophils and monocytes in arterial lesions, thus participating to atherogenesis (195). Consistently, elevated plasma Calprotectin levels have been described and associated with the development of larger atherosclerotic lesions in an experimental model of diabetic ApoE$^{-/-}$ mice (196). Indeed, plaque size reduction was demonstrated in experimental models of diabetes deficient in TLR4 or RAGE suggesting important roles for their endogenous ligands, notably Calprotectin, in the accelerated atherogenesis associated with diabetes (196, 197). In addition, inhibition of Calprotectin's activity with Paquinimod (ABR-215757) resulted in vascular protection in diabetes-related CVD, which led the way towards the approvals of drugs associated with Calprotectin for clinical testing (168).

Hyaluronan (HA): HA, a proteoglycan that is ubiquitously and highly expressed, is a structural component of the extracellular matrix, especially prominent in soft connective tissue and the vasculature. It is also the largest polysaccharide found in vertebrate, and the only one produced at the cell surface and released into the extracellular matrix as a High Molecular Weight (HMW, 1,000 - 6,000 kDa) glycosaminoglycan. In healthy cells, HA is a critical regulator of cell phenotype in the context of wound healing and recovery (98, 198). Indeed, in case of epidermal barrier injury, HMW HA interacts with its main cell surface receptor, CD44, to promote keratinocyte differentiation, lamellar body formation, and permeability barrier homeostasis. Injured or necrotic cells can cleave HA, via the promotion of ROS, leading to the production and release of degradation products known as Low and Medium Molecular Weight (L+MMW, 10 - 500 kDa). While full length HA is involved in normal cellular function and has anti-inflammatory properties, such as inhibiting cell growth and division, L+MMW forms have been reported to serve as DAMP and trigger robust immune responses (199, 200), notably by promoting cell migration and differentiation. A role of HA in modulating phagocytosis has also been demonstrated *in vitro*: mouse peritoneal macrophages cultured in the presence of LMW HA (< 100 kDa), phagocytosed larger numbers of latex beads, while HMW HA (1,000 - 2,000 kDa) markedly inhibited phagocytosis (201).

Mechanistically, CD44 is considered the main receptor for both HA full length and fragmented HAs, and its engagement results in different signaling pathways depending on HA molecular weight (202, 203). In addition, there is evidence for L+MMW HA interaction with TLR2 or TLR4, alone or in a complex with CD44 (202, 204-207). This interaction led to the expression of inflammatory cytokines by DCs and macrophages and promoted cell maturation. HA was found at increased levels in serum or tissues in a variety of diseases, notably lung or liver fibrosis, diabetes, cancer, vascular pathologies and kidney diseases (208-

214). In the context of lung injury, a HA/TLR2/TLR4 pathway has been clearly identified. Peritoneal macrophages from wild-type mice were able to release TNFα after stimulation with HA MMW fragments (about 135 kDa), while the effect was lost in macrophages from either $TLR2^{-/-}$ or $TLR4^{-/-}$ mice (215). In the cardiovascular context, transgenic-induced HA overproduction targeted to the SMCs in atherosclerosis-prone animals ($ApoE^{-/-}$ mice) led to the thinning of the elastic membranes in the aorta, potentially promoting vessel stiffness, and to increased aortic HA fragments deposition, suggesting that accumulation of HA fragments accelerates the progression of aortic atherosclerosis (216). However, the mechanisms behind HA involvement in CV pathologies does not appear to have received as much focus as some other DAMPs, and its implication is still mostly indirectly suggested rather than strictly demonstrated. For example, the use of models deficient for both ApoE and HA synthase (HAS) 3, one of the 3 enzymes responsible for HA synthesis, show an inhibition of atherosclerotic plaques development and plaque inflammation (217). Indirect evidence of the implication of HA in atherosclerosis was also gained via the investigation of its receptor CD44 and Receptor of HA motility (RHAMM). In particular, the absence of CD44 in $ApoE^{-/-}$ mice inhibited the inflammation caused by macrophages. This study also demonstrated the crucial role of HA via its binding to RHAMM in the migration and proliferation of SMCs, typically found in advanced atherosclerotic plaques (218). Regarding a potential role for HA in renal diseases, increased accumulation of HA and HAS expression has been reported in autoimmune renal injury, and serum HA levels were correlated with the degree of impaired renal function in advanced stages of CKD (219). Furthermore, increased serum HA levels in dialysis patients were found to correlate with increased disease severity and mortality (220, 221).

Table 3. Human DAMP ligands, their TLR receptors and other receptors

TLRs	Major DAMPs	Other immune receptors	Origin
TLR2 (dimerisation with TLR1 or TLR6)	Biglycan	NLRP3	Extracellular matrix
	Decorin	Not reported	
	Versican	Not reported	
	LMW Hyaluronan	NLRP3, CD44	
	S100 proteins	RAGE, CD33	Cytosol
	Heat shock proteins	CD91, LOX-1, SREC-1, FEEL-1, Siglec-5, Siglec-14	
	Aβ	NLRP1, NLRP3, CD36, RAGE	
	Oxidized LDL (OxLDL)	LOX-1, CD36, SR-A	
	Histones	NLRP3	Nuclear
	HMGB-1	RAGE, CD24, Siglec-10	
	Eosinophil-derived neurotoxin (EDN)	Not reported	Granule
TLR3	RNA	RIG-I, MDA5	Nuclear
TLR4	Biglycan	NLRP3	Extracellular matrix
	Decorin	Not reported	
	LMW Hyaluronan	NLRP3, CD44	
	Heparan sulfate	Not reported	
	Fibronectin (EDA domain)	Not reported	
	Fibrinogen	Not reported	
	Tenascin C	Not reported	
	S100 proteins	RAGE, CD33	Cytosol
	Heat shock proteins	CD91, LOX-1, SREC-1, FEEL-1, Siglec-5, Siglec-14	
	Modified LDL	SR-A	
	Histones	NLRP3	Nuclear
	HMGB-1	RAGE, CD24, Siglec-10	
	High Mobility Group Nucleosome Binding Domain 1	Not reported	
	Defensins	Not reported	Granule
	Granulysin	Not reported	
	Syndecans	β4 integrins, receptor tyrosine kinase	Plasma membrane
	Glypicans	Not reported	
TLR7	RNA	RIG-I, MDA5	Nuclear
TLR8	RNA	RIG-I, MDA5	Nuclear
TLR9	HMGB-1	RAGE, CD24, Siglec-10	Nuclear
	DNA	AIM2	
	mtDNA	cGAS, NLRP3, NLRC4, AIM2	Mitochondria

Adapted from Roh J. et al., 2018 (195), Schaefer L., 2014 (83), Babelova A. et al., 2009 (222) and Kim S. et al., 2009 (223).

AIM2, Absent in melanoma 2; FEEL-1, Fasciclin EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1; LOX-1, Leptin-like OxLDL receptor-1; MDA5, Melanoma differentiation-associated protein 5; NLRC4, Nucleotide oligomerisation domain (NOD)-like receptors family Caspase activation and recruitment domains (CARDs) domain-containing protein 4; NLRP1, NOD-like receptor family pyrin domain containing 1; NLRP3, NOD-like receptor family pyrin domain containing 3; RAGE, Receptor for advanced glycation end product; RIG-I, Retinoid acid-inducible gene I; Siglec-5, Sialic-acid binding immunoglobulin-like lectins 5; Siglec-10, Sialic-acid binding immunoglobulin-like lectins 10; Siglec-14, Sialic-acid binding immunoglobulin-like lectins 14; SR-A, Scavenger receptor A; SREC-1, Scavenger receptor expressed by endothelial cells 1.

I-2.3. TLR tissue and cellular distribution

Consistent with their function in pathogen recognition and as a first line of defense, TLRs are widely expressed by immune cells and cells in tissues potentially exposed to infecting microorganisms, such as epithelial or endothelial cells. At a subcellular level, TLRs are found in 2 different locations: TLR1, 2, 4, 5 and 6, which recognise mostly components from extracellular pathogens such as bacteria or fungi, and extracellular DAMPs, are mainly expressed at the cell surface, while TLR3, 7, 8 and 9, involved mostly in viral, bacterial and host nucleic acid recognition, are mostly localised in intracellular compartments (224). Among blood leukocytes, TLR expression is greatest on innate immune cells and mRNA for all TLR members, except TLR3, are expressed in phagocytes, such as neutrophils, monocytes and macrophages (225, 226).

In immune cells, TLR3 is expressed only in myeloid DCs, notably in the professional antigen-presenting $CD141^+$ DCs subset, and macrophages (227, 228). Myeloid cells have also been shown to express TLR1-6, 8 and 10 (229). On the contrary, plasmacytoid DCs lack TLR3 expression, but do express high levels of TLR7 and TLR9 leading to the secretion of large amounts of interferon (IFN)-α upon activation, as well as lower levels of TLR1 and TLR10 (230). Discrepancies exist on TLR3 expression related to adaptive immunity, especially in some T-cells subsets and Natural Killer (NK) cells, although NK cells are major players in the antiviral response so TLR3 expression by these cells would be logical (225, 231-233). However, many other cell types express TLR3 as fibroblasts and a variety of epithelial cells including airway,

corneal, cervical, biliary, and intestinal cells, which are target sites of virus infection (234). TLR3 is also expressed in the kidney and on glomerular mesangial cells in physiological conditions and it has been demonstrated that its expression is increased in mesangial cells and macrophages infiltrating the nephritic kidney in a lupus-prone mice model compared to mice with healthy kidney (235, 236).

Eosinophils express TLR1, 3, 4, 6, 7, 9 and 10 mRNAs constitutively, whereas basophils express TLR2 and TLR4, and mast cells TLR1, 2 and 6 mRNAs.

TLRs are also expressed by lymphocytes, which are part of the adaptive immune system, and they participate in the regulation of responses to antigens. TLR2, 5, and 9 are expressed by NK cells. In addition, B-lymphocytes display high expression levels of TLR1, 6, 9 and 10, together with lower levels of TLR2, 4 and 7. T-cells show differential TLR expression depending on the subset. Indeed, TLR3, 5, 6, 7 and 9 mRNAs have been detected in $CD4^+$ T-cells, and TLR2 and TLR4 proteins surface expression can be detected at the surface of activated and memory T-cells while naïve $CD4^+$ cells do not show significant levels of protein expression. On the other hand, mRNA and protein expression for TLR2, TLR3, TLR4 and TLR5 were reported in human $CD8^+$ T-cells. TLR2, TLR3 and TLR5 protein expression levels have also been found higher in activated $CD8^+$ T-cells than whose found on $CD4^+$ T-cells. Consequently, TLR2 co-stimulation drives more proliferation in CD8 T-cells than in CD4 T-cells. In addition, TLR2 was found constitutively expressed on Listeria-specific CD8 memory T-cells. Furthermore, several studies reported a role for TLRs in the direct modulation – both up and downregulation – of regulatory T-cells (Treg) activity *in vitro* and *in vivo* (237-241).

Besides immune cells, TLRs are expressed in tissues where they play a major role in stopping the entry of pathogens in the host organism. In this regard, TLR2 and TLR4 display a particularly wide/spread localisation, in line with their ability to recognise a large array of

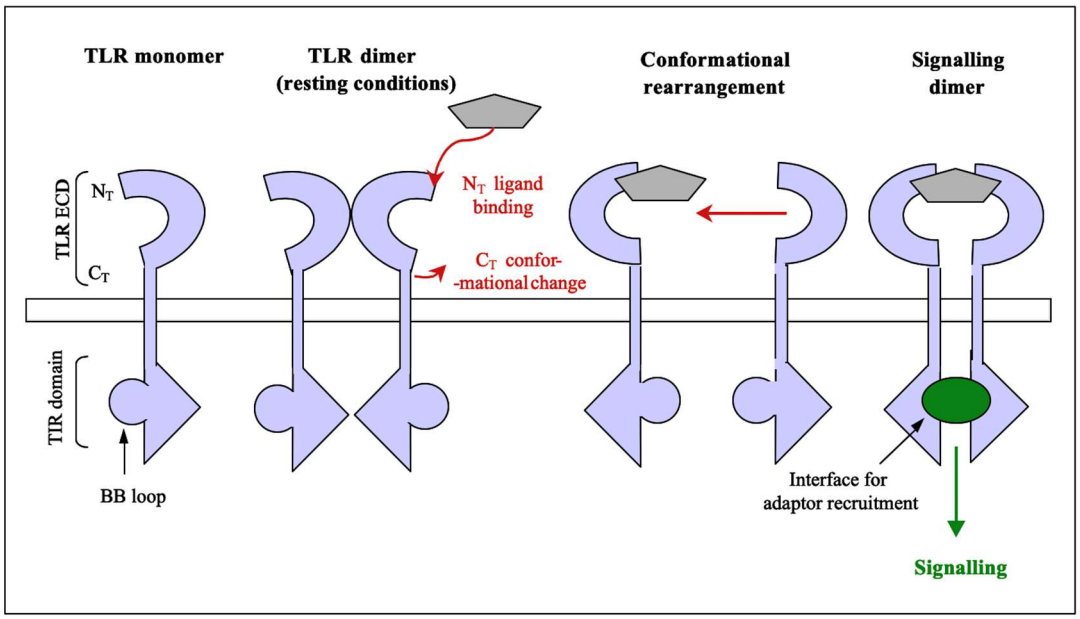
ligands, either PAMPs or DAMPs. Indeed, mRNAs for TLR2 and TLR4 were detected in nasal mucosa, adenoids and salivary glands and studies using immunohistochemical staining of human airways reported surface expression of TLR2 throughout the epithelium, and TLR4 expression in pulmonary epithelial cells as well as corneal epithelium (226, 242). Interestingly, mRNA for every TLR was detected in several layers of the eye, namely cornea, conjunctiva and retina while only mRNA for TLR4 is also detected in both the uvea, also expressing TLR4 protein, and sclera (243). Keratinocytes express TLR1-5 constitutively (242), and TLR1, 2, 3, 5 and 6 mRNAs were found in the lower female genital tract.

Of particular relevance to this study, endothelial cells for both large vessels and micro-vasculature, have been reported to express all TLRs, except TLR8 (244-248). Compared with leukocytes, endothelial cells express relatively low levels of most of the TLRs at baseline. However, TLR3 is highly expressed in human coronary artery endothelial cells compared to monocytes surface expression level, and both TLR3 and TLR4 are highly expressed in human micro-vascular endothelial cells from different locations (150). Notably, despite a lower expression at baseline, TLR2 was found strongly upregulated following stimulation of endothelial cells with bacterial lipopeptide, a TLR2 agonist (249, 250). This phenomenon has not been observed in human leukocytes suggesting that endothelial TLR2 serves as a host's response calibrator to different levels of infectious threats or injuries (150, 251, 252). TLR2 expression has also been shown to be increased in endothelial cells located in regions of disturbed flow, such as vessel bifurcations, thus promoting atherosclerosis by exacerbating the local inflammatory process in response to its wide range of agonists, both endogenous or exogenous (253-256). An *in vitro* investigation demonstrated that this differential TLR2 expression in the arterial tree, both at mRNA and protein levels, was due to the phosphorylation of the transcription factor signal protein-1 (Sp-1) in laminar flow regions,

thereby blocking its binding to the TLR2 promoter required for TLR2 expression (257). Another example of differential TLR expression by the same cell type depending on localisation includes intestinal epithelial cells which mostly express TLR2 and TLR4, and TLR5 on the basolateral surface but not the apical side (258). Consequently, only pathogens invading the basolateral compartment of the epithelium elicit an inflammatory response, protecting the host from unwanted inflammatory responses to the natural gut flora.

I-2.4. TLR signaling

Most TLRs function mostly as homodimers, with the exception of TLR2 which forms heterodimers with TLR1 or TLR6, depending on the ligand being recognised (86, 259, 260). However, dimerisation is not sufficient to ensure signal transduction, receptors need to respect a correct orientation. Gay and co-workers, following previous experiments on Toll receptors, proposed a model of sequential activation of TLRs in which ligands binding at the N-terminus induces a conformational change at the C-terminal region of the TLR extracellular domain, and this change allows for a stable receptor-receptor interaction. This, in turn, promotes the rearrangement of the transmembrane helices of the receptor dimer allowing the initiation of the downstream signaling (261) (**Figure 2**).



<u>Figure 2.</u> Ligand-induced TLR conformational changes initiating signaling
Binding of the ligand to the N-terminus domain of a TLR is thought to induce a conformational change in the C-terminus part of the ectodomain that allows for a successful contact between the 2 TLRs TIR domains. The BB loop of the signaling TIR domain is likely to be involved in receptor-receptor contacts. Formation of the signaling complex is proposed to generate a new interface for the recruitment of signal adaptors. ECD, Ectodomain; C_T/N_T, C/N-terminus.

To achieve their optimal function, most TLR dimers also need the presence of co-receptors. A major co-receptor utilised by most TLRs is CD14, a 55 kDa protein which can be anchored to the cellular membrane or found as a soluble form, sCD14 (262). mCD14 is mostly expressed at the surface of myeloid cells, and to a lesser extent, on B-lymphocytes and monocytes while sCD14 is present in different body fluids, notably in plasma, to enhance TLRs signaling by cells lacking mCD14 expression (263-265). Instead, CD14 binds to a wide variety of TLR ligands, exogenous and endogenous, via its LRR domains and then forms a complex with the TLR to allow for the ligand to be transferred from CD14 to the TLR (266). The requirement for CD14 varies with the ligand and the TLR, but CD14 enhancing effect has been reported for most TLRs (113, 267-272). In line with the dual implication of TLRs in immunity,

CD14, as essential accessory molecule with the ability to modulate TLR signaling, has been reported to exert significant impact in pathogenesis of several disease, notably in CVD and ischemic stroke (273-275).

Another essential co-receptor for TLR4 signaling is MD-2. Even in the presence of CD14, TLR4 is unable to trigger a response to ligand binding if not complexed with MD-2 and, therefore, no physiological role of TLR4 has been demonstrated in the absence of MD-2. MD-2 is a 18-25 kDa membrane-bound protein that binds to the ectodomain of TLR4 and can also be secreted as a soluble molecule. Interestingly, in atherosclerosis MD-2 has been demonstrated as critical for Ox-LDL-induced TLR4 dimerisation in monocytes and macrophages, downstream activation of NF-κB and subsequent production of pro-inflammatory cytokines. In addition, MD-2 deficiency leads to reduced atherosclerotic plaques through reduced lesional macrophage content and expression of inflammatory cytokines (276).

CD36, a member of the class B family of scavenger receptors, is also an accessory molecule for TLRs. It is a 88 kDa membrane glycoprotein considered to be the main receptor to mediate phagocytosis of OxLDL. In line with this primary role, CD36 is expressed in various cell types including monocytes, macrophages, DCs, platelets, microglia, cardiovascular cells and adipocytes (277), and is involved in different pathogenesis as inflammation, lipid metabolism and atherosclerosis progression (278). Furthermore, CD36 participates to the assembly of the TLR2/6 heterodimers following stimulation with bacterial lipoteichoic acid. More recently, Stewart *et al.* showed the inability of macrophages from TLR4 or TLR6-deficient mice to express IL-1β mRNA following OxLDL stimulation. Moreover, using transfections experiments in HEK293 cells, they demonstrated that only cells transfected with both TLR4 and TLR6, as well as CD36, were able to trigger OxLDL or β-amyloid-induced NF-κB

expression. These findings suggested the formation of a CD36/TLR4/6 heterotrimer, initiated following OxLDL and β-amyloid binding to CD36, therefore inducing pro-inflammatory mediators production, such as IL-1β and NF-κB, and contributing to atherosclerosis and Alzheimer's disease progression (279).

CD14, MD-2 and CD36 are the 3 main co-receptors for TLRs but there is more evidence for TLRs collaboration with other PRRs or proteins, to enhance ligand recognition. A non-exhaustive list includes notably the LPS-Binding Protein (LBP), TLR4 Interactor with LRR (TRIL), radioprotective 105 (RP105), MD-1, Dectin-1, Vitronectin, CD44, RAGE, B-cells receptor (280). The large number of co-receptors and accessory molecules employed by TLRs underlies the ability of these receptors to bind wide variability of ligands as well as the complexity of the regulation of the subsequent signaling cascade. This concept is core to explaining the wide range of functions, and sometimes differential effects, mediated by TLR activation.

Ligand binding initiates changes in TLR conformation from monomers to dimers resulting in the recruitment of specific signaling adaptors to the TLRs' cytoplasmic TIR domain (281). Of note, the BB loops of TIR domains have a particular importance for TIR interaction between two different TLRs and their homo- or heterodimerisation (282, 283). In addition, it is now well established that the TIR-TIR platform formed by the dimerisation of two TLRs promotes homotypic protein-protein interactions with additional cytoplasmic adapter molecules, resulting in an active signaling complex. These adaptors initiate a chain of phosphorylation and ubiquitination events that lead to the activation of the transcription factor NF-κB, MAPKs, and interferon-response factors (IRFs) (284).There are 5 TLRs adaptor proteins which have been identified:

- Myeloid Differentiation Primary Response protein 88 (MyD88),

- MyD88 adaptor-like (MAL),
- TIR domain-containing adaptor inducing IFN-β (TRIF),
- TRIF-related adaptor molecule (TRAM),
- Sterile-α and HEAT-Armadillo motifs-containing protein (SARM).

Depending on the adaptor molecules involved, two signaling pathways have been described, the MyD88 dependent pathway and the MyD88 independent pathway also known as TRIF dependent pathway (285, 286) (**Figure 3**).

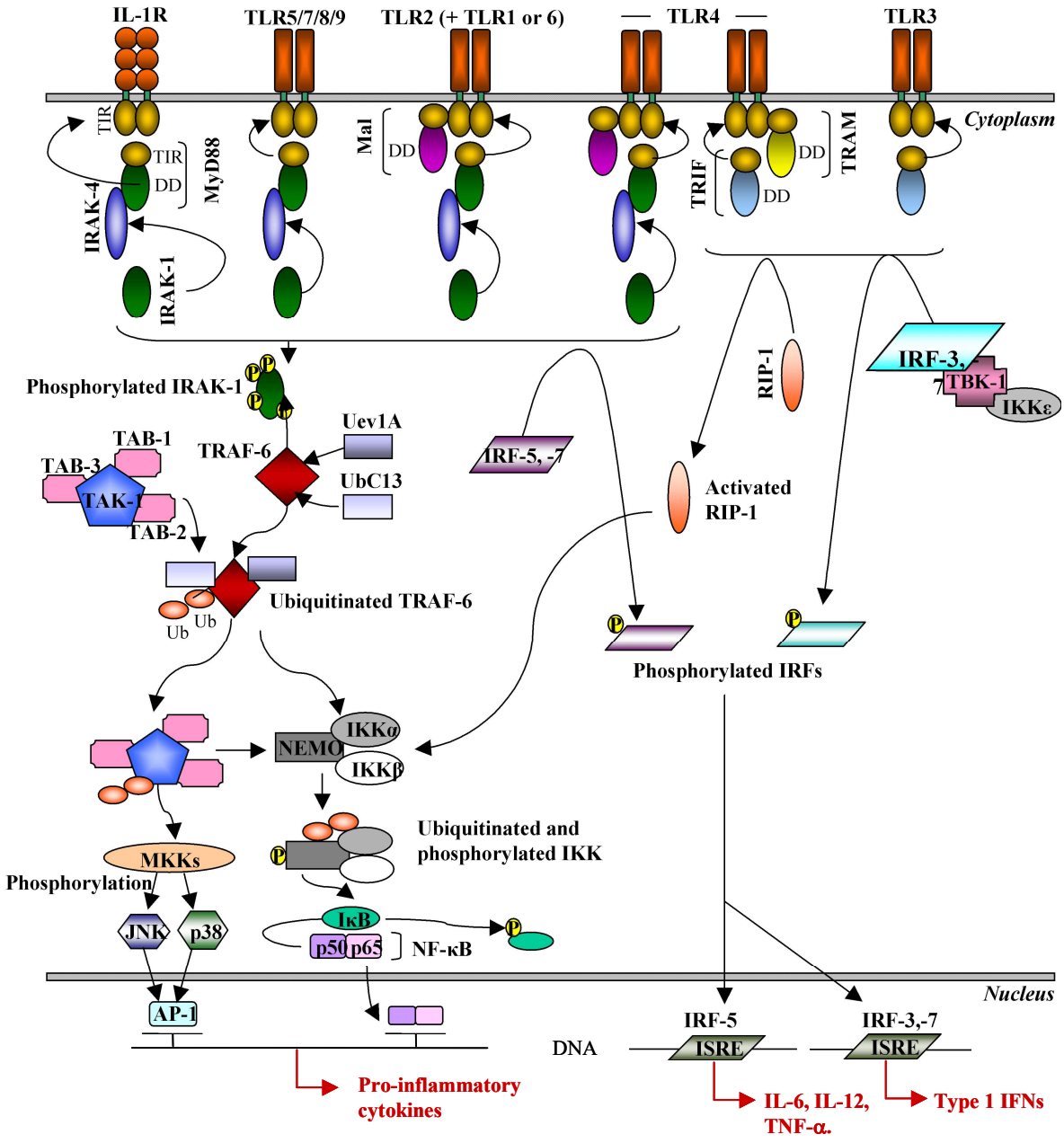


Figure 3. TLR signaling pathways

Schematic representation of the MyD88- and TRIF-dependent pathways for TLR signaling (see description in the text). AP-1, activating protein-1; DD, death domain; IκB, inhibitor of NF-κB; IKK, IkB kinases; IL, interleukin; IL-1R, interleukin-1 receptor; INF, interferon; IRAK, interleukin-receptor-associated kinase; IRF, interferon-response factor; ISRE, IFN-stimulated response element motifs JNK, c-jun-N-terminal kinase; Mal, MyD88 adaptor-like; MKKs, mitogen-activated protein-kinase kinases; MyD88, myeloid differentiation primary response protein 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-kappa B; RIP-1, receptor interacting protein-1; TAB, TAK-1 binding protein; TAK-1, TGF-b-activated kinase-1; TBK-1, TRAF-family member-associated-NF-κB activator (TANK)-binding kinase-1; TIR, Toll/interleukin-1 receptor domain; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α; TRAF, TNF-α-receptor-associated factor-6 TRAM, TRIF-related adaptor molecule; TRIF, TIR domain containing adaptor inducing INF-β; Ub C13, ubiquitin-conjugating enzyme 13; Uev 1A, ubiquitin-conjugating enzyme E2 variant 1.

MyD88 is a universal adaptor molecule used by almost all TLRs, except TLR3, and plays a crucial role in TLRs transduction signal. It was the first adaptor described as a member of "shared" signaling pathway induced by TLRs as well as by the IL-1R family. At its carboxy-terminal, MyD88 possesses a TIR domain, and a Death Domain (DD) at its N-terminal. TLR ligand binding causes association of the TIR domains of MyD88 and the TLR which, followed by the recruitment of the first kinase of the signaling cascade, interleukin-receptor-associated-kinase-4 (IRAK-4). The complex constituted of TLR/MyD88/IRAK-1/IRAK-4 is called Myddosome complex. IRAK-4 activates IRAK-1 which is then auto-phosphorylated at several sites. Phosphorylated IRAK-1 dissociates from the Myddosome complex, and associates with TNFα-receptor-associated factor-6 (TRAF-6). TRAF-6 links the IL-1R/IRAKs or TLR/IRAKs complexes with the activation of the NF-κB and MAPKs cascade. Once activated via poly-ubiquitination and oligomerisation, TRAF-6 associates with four downstream proteins: Transforming growth factor-β (TGF-β)-activated kinase-1 (TAK-1), and the TAK-1 binding proteins-1, 2 and 3 (TAB-1, TAB-2, TAB-3). Activation of the TAK-1/TAB complex enhances the activity of the inhibitor of NF-κB (I-κB) kinases (IKK) complex. The IKK complex subsequently phosphorylates I-κB proteins that are associated with NF-κB in the cytoplasm of resting cells and degrades them which has as consequence to liberate NF-κB. The release of NF-κB leads ultimately to its nuclear translocation and the promotion of genes responsible for inflammatory responses such as TNFα, IL-1β, IL-6, IL-8 and MCP-1.

TAK-1 also activates the MAPK pathway. TAK-1 phosphorylates MAPK kinases (MKKs), which in turn phosphorylate and activate the MAPKs p38 and c-jun-N-terminal kinase (JNK). Activated p38 and JNK then activate the transcription factor, activating protein-1 (AP-1). Similarly to NF-κB, AP-1 initiates the transcription of genes coding for pro-inflammatory cytokines e.g IL-2, IL-8 and TNFα (287).

MyD88-dependent pathway can also activate members of the IRF family, notably IRF-5, which in turn induces the production of TNFα, as well as IL-6 and IL-12 (288).

TLR3 is the only member of the TLR family known to signal exclusively via the TRIF-dependent pathway (289). As with the MyD88-pathway, TRIF-dependent signaling leads to NF-κB activation but also to activation of the transcription factors IRF-3 and IRF-7, which activate the interferon IFN-β promotors, leading to several IFN-inducible genes responsible for the expression of type I IFNs as IFN-α and IFN-β. Consistent with the function of TLR3 as a sensor of viral nucleic acids, type I IFNs stimulate both macrophages and natural killer cells to elicit strong anti-viral responses. In addition to TLR3, it is now well accepted that TLR4 can also utilise the TRIF pathway, making it the only TLR able to activate both pathways. While TLR3 interacts with TRIF directly, TLR4 needs the TRAM molecule to complex with TRIF (289, 290).

SARM is unlike the other TLR adaptors because it acts as a negative regulator of signaling via inhibition of NF-κB and IRF activation in response to TLR triggering (291), notably by interacting with TRIF.

I-2.5. Regulation of TLR activation

The regulation of the activation of the immune system is crucial as illustrated in this introduction with all the pathologies derived from excessive immune responses. Therefore, TLR activity has to be tightly regulated and a number of modulatory mechanisms exist to control it. These include:

- Apoptosis of the activated cells, as TLRs can also function as death receptors (292);

- Reduction of TLR expression, which can be achieved by protein degradation or inhibition of their expression, notably by anti-inflammatory cytokines (293, 294);
- Prevention of ligand binding to its TLR, mostly via the expression of soluble forms of TLRs or accessory molecules, which acts as ligand decoy receptors. Soluble forms of TLR2 (295), TLR3 (296) and TLR4 (297), consisting in the extracellular domains of these receptors, have been reported to inhibit TLR activation (298), although the presence of sTLR3 *in vivo* has not been demonstrated;
- Inhibitors of TLR signaling cascade, such as SARM, the Single Immunoglobulin IL-1R-related molecule (SIGIRR), ST-2, TNF-related apoptosis-inducing ligand receptor (TRAILR), and a number of others (299-301).

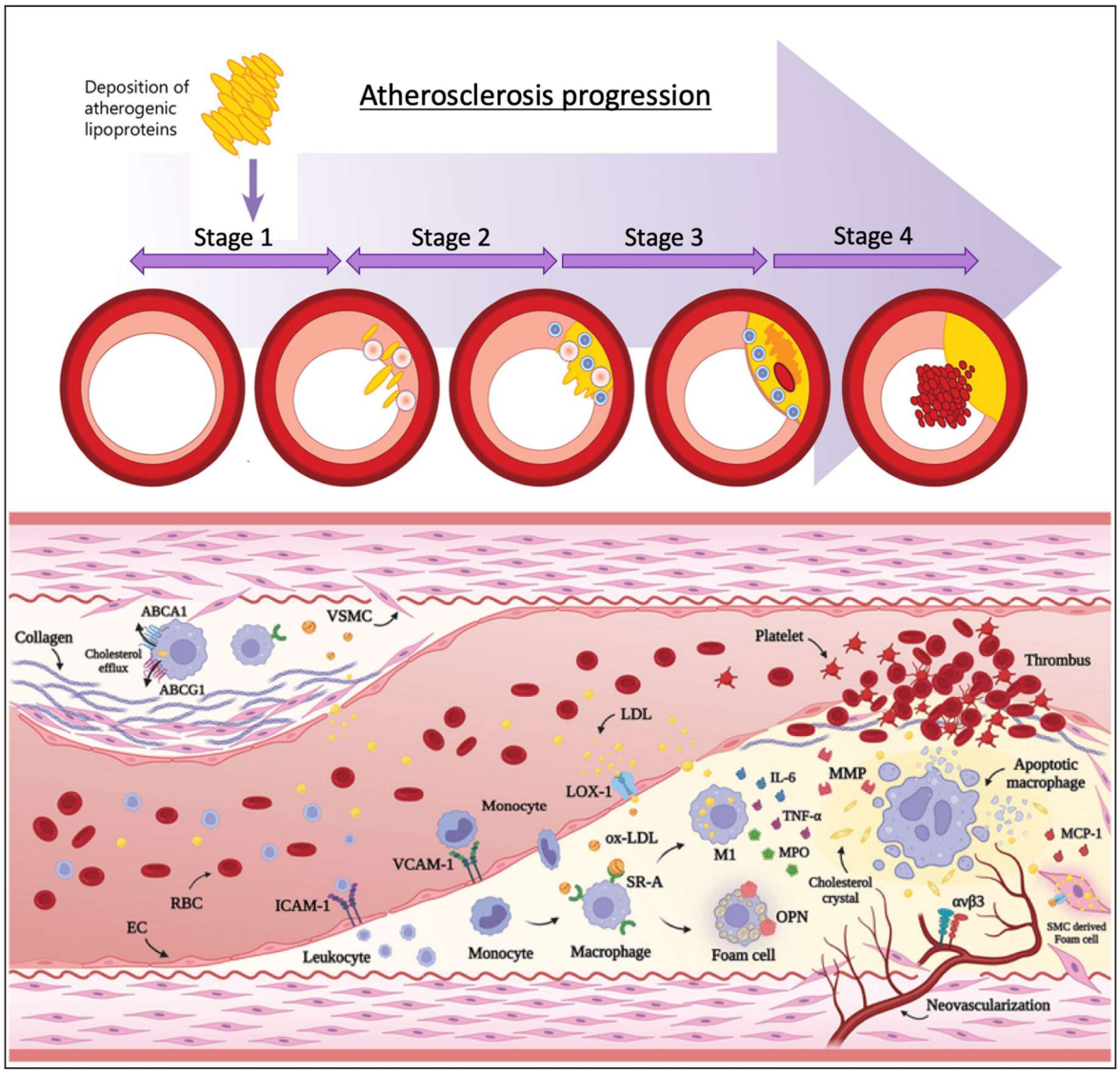
I-3. Inflammation and CVD

Chronic inflammation is a major contributor to CVD, notably by contributing to the development and progression of atherosclerosis, a lipid-driven chronic inflammatory disease of the arterial wall (302-304) which is the most common underlying CV pathology. Plaque rupture followed by thrombosis lead to myocardial infarction and stroke, the most common causes of death worldwide (305, 306).

I-3.1. Aetiology of atherosclerosis

Atherothrombosis, characterised by atherosclerotic lesion disruption with superimposed thrombus formation is the primary cause of heart disease and stroke. Over the past few decades, the viewpoint of atherosclerosis as an exclusively lipid-driven disease, has been gradually replaced by the concept of a chronic low-grade inflammatory process of the

arterial wall (307). While inflammation in itself is not sufficient to cause atherosclerosis in the absence of atherosclerotic lipids, it induces a series of responses that link lipid metabolism, with atherosclerosis plaque development (308, 309). Indeed, inflammation arises from an imbalance in lipid metabolism which leads to a subsequent accumulation of lipoproteins in the subendothelial space, followed by a maladaptive immune response. The arterial wall is composed of 3 layers which are, starting from the arterial lumen: i) the intima, lined and separated from bloodstream by endothelial cells composing the endothelium, ii) the tunica media composed mostly of SMCs, and iii) the adventitia. In normal conditions, resting endothelial cells do not attract blood leukocytes (308). An event (or association of several), such as injury, damage or stress to the endothelium, notably as a result of high circulating levels of cholesterol or persistent hypertension serves as the initial trigger for atherogenesis. Atherosclerosis evolves through several stages, each involving changes in the arterial wall (**Figure 4**).



Adapted from Nayor M. et al., 2021 (310) and Zhang S. et al., 2022 (311)

ABCA1, ATP-binding cassette subfamily A member 1 transporter; ABCG1, ATP-binding cassette transporter G1; EC, Endothelial cell; ICAM-1, Intercellular adhesion molecule 1; IL-6, Interleukin 6; LDL, Low density lipoprotein; LOX-1, Lectin-like OxLDL receptor-1; MCP-1, Monocyte chemoattractant protein-1; MMP, Matrix metalloproteinases; MPO, Myeloperoxidase; OPN, Osteopontin; OxLDL, Oxidized LDL; RBC, Red blood cell; SMC, Smooth muscle cell; SR-A, Scavenger receptor A; TNF-α, Tumor necrosis factor-α ; VCAM-1, Vascular cell adhesion protein 1; VSMC, Vascular smooth muscle cell.

Figure 4. Schematic illustration of atherosclerosis progression and pathological mechanisms associated

Top: The 4 stages of pathological progression of atherosclerosis. Initiation of atherosclerosis is triggered by a proatherogenic, lipid rich, inflammatory milieu leading to focal inflammation, maladaptive intimal and medial thickening, and endothelial damage. As atherosclerosis progresses, fatty streaks develop into atherosclerotic plaques. Bottom: This process is heavily influenced by different leukocytes species 'functions, including macrophage polarisation and transition to foam cells. At the bottom right, micro-calcifications and secondary necrosis, together with cholesterol, inflammatory cells and cytokines accumulation, promote a lipid-rich necrotic core, which, coupled with a thin fibrous cap, are characteristic of vulnerable plaques more prone to rupture compared with stable plaque (at the middle left). Complications of atherosclerosis primarily occur as a result of critical blockages and plaque rupture events leading to thrombosis and acute organ ischemia.

- First stage: Initiation of atherosclerosis associated with recruitment of immune cells

The endothelial lining of arteries responds to mechanical and molecular stimuli, notably inflammation, throughout the circulation. As previously mentioned (Introduction, Section I-2.3), endothelial cell activation is more likely to occur in regions of arterial curves because of disturbed blood flow and low shear stress. These are also regions of facilitated accumulation and retention of blood circulating factors, such as cholesterol-rich LDL, a major atherogenic lipoprotein, rendering these regions atherosclerosis-prone areas (312). Once the endothelium has been activated by stress or injury, it will upregulate extracellular matrix components, notably proteoglycan, which has a high affinity for LDL. Consequently, LDL will aggregate at the lesion site. Accumulated LDL particles suffer modifications from the milieu environment such as oxidation leading to the formation of OxLDL (313, 314). LDL is notably also able to infiltrate into the endothelium via endocytosis leading to increased levels in the intima (315, 316). The accumulation of OxLDL in turn stimulates endothelial cells to produce pro-inflammatory cytokines and chemokines to attract circulating leukocytes to the lesion site (317, 318). Of particular importance in this process is MCP-1, which attracts monocytes to the lesion, but also direct the migration into the intima following a concentration gradient, after monocyte adhesion to the endothelium (305). MCP-1 is expressed in all steps of atherosclerosis and OxLDL has been shown to upregulate its mRNA levels in endothelial cells and SMCs (319, 320). Activated endothelial cells also upregulate the expression of adhesion molecules on their surface, notably VCAM-1 and ICAM-1 (321, 322). These adhesion molecules will interact with their ligands expressed on the surface of blood leukocytes (such as Mac-1 (CD11b/CD18) and integrin $\alpha 4\beta 1$ on monocytes) to promote recruitment from the blood into the artery wall. The recruitment of immune cells follows 3 main steps, namely

rolling and firm adhesion to the endothelium via adhesion molecules interaction, then extravasation through the endothelial layer which displays an increased paracellular permeability (318, 323). The first cell type to be recruited to the site of OxLDL accumulation are monocytes, followed by lymphocytes (324). However, more recently some studies also showed evidence for neutrophils implication in atherosclerosis suggesting that they are also recruited (325, 326).

- Second stage: Formation of fatty streaks

Once the endothelial barrier is crossed, monocytes differentiate into macrophages in the intima (327). Interestingly, a specific pro-inflammatory subset of monocytes has been identified to trigger lesional macrophages, Ly6C^{high} monocytes, in part because they express higher levels of adhesion molecules than Ly6C^{low} monocytes and are therefore preferentially recruited (328). Macrophage colony-stimulating factor (M-CSF), highly expressed at plaque site, is a major driver of monocyte to macrophage differentiation in atherosclerosis (329). Macrophages have the ability to uptake LDL either by phagocytosis, which is performed via scavenger receptors (317, 330), or by macropinocytosis which is a non-selective form of endocytosis (331). Initially, phagocytes may have anti-atherogenic effect as they uptake and process excess lipids from the extracellular space, and may then leave the arterial wall (327). However, because of the accumulation of OxLDL and ongoing inflammation, notably chemoattractant secretion (332), macrophages keep accumulating and remain trapped in the arterial wall. In addition, it also has been demonstrated that they can proliferate into the intimal layer (333). Importantly, scavenger receptor A (SR-A) (334) and CD36 (335), expressed by macrophages, are two major receptors for modified LDL, which, in contrast to other LDL receptors, are not down-regulated in response to rising intracellular cholesterol content

(336). The excessive uptake of OxLDL by macrophages results in cholesterol-engorged macrophages, called foam cells (337, 338). Foam-cells in turn secrete inflammatory mediators and ROS, leading to the maintenance, rather than resolution, of inflammation (339). In addition, cholesterol-engorged phagocytes become unable to perform efficient efferocytosis, the clearance of apoptotic or necrotic cells. As a consequence, most apoptotic foam cells in the plaque will not be efficiently cleared and will undergo secondary necrosis. Necrotic foam cells then release their lipid-filled contents further contributing to the inflammatory burden as well as formation of lipid streaks which constitute the beginning of the plaque formation (340-342). It also has been recently observed *in vivo* that foam cells found at the lesion site could also be of SMC, rather than macrophage, origin, but both types of foam cells present markers in common (343, 344).

Monocytes and lymphocytes are still recruited from the bloodstream at all stages of atherosclerosis. Monocytes and macrophages keep producing inflammatory mediators such as TNF, IL-6, IL-8 or MCP-1, growth factors and free radicals which leads to more recruitment, metabolic changes associated with more LDL oxidation, and more damage to the endothelium (345). T-lymphocytes, fewer in number than myeloid cells, drive the adaptive immune response but also interact with innate immune cells via production of mediators that notably control many of their functions (346).

Fatty streaks do not result in clinical complications and can even undergo regression with lifestyle changes or treatments. However, once SMCs infiltrate, and the lesions become more advanced, regression is less likely to occur (312).

- Third stage: Plaque growth leading to atheroma formation

Over time, fatty streaks will increase in size and may merge to become a larger plaque (336). Of note, the release of lipids by necrotic foam cells plays a major role in the growth of the pro-thrombotic necrotic core of the lesion which is where the plaque is thicker and more vulnerable (347). The cooperation between innate and adaptive immunity stimulate the production of pro-inflammatory cytokines that sustain and amplify the local inflammatory response (348, 349). Of note, DCs are key players in this cooperation, as they are found in the intima of normal arteries and accumulate in atherosclerotic lesions, inducing T-cell activation via antigen presentation, notably that of antigens specific to the atherosclerosis process, such as modified LDL (340). In response to pro-inflammatory mediator production, especially IL-1β, TNF and TGF-β, SMCs from the tunica media migrate into the intima where they synthetise and promote the accumulation of extracellular matrix (308, 317). These mechanisms lead to i) the expansion of the intima towards the vascular lumen which reduce the diameter available for blood flow and ii) the formation of the fibrous cap on the developing plaque. The fibrous cap is composed of collagen-rich fiber tissues, SMCs, macrophages and T lymphocytes which stabilise the plaque and prevent it from rupturing into the bloodstream (350, 351). The association of necrotic core and fibrous cap forms the mature atherosclerosis plaque (327). Small intimal calcifications are also found in more advanced plaques (352), as a result of SMCs osteogenic differentiation and mineralisation of the extracellular matrix in response to exposure to inflammatory cytokines (353, 354). Developed atherosclerotic plaques are harder than immature ones, thus leading to stiffer arteries.

- Fourth stage: Plaque rupture

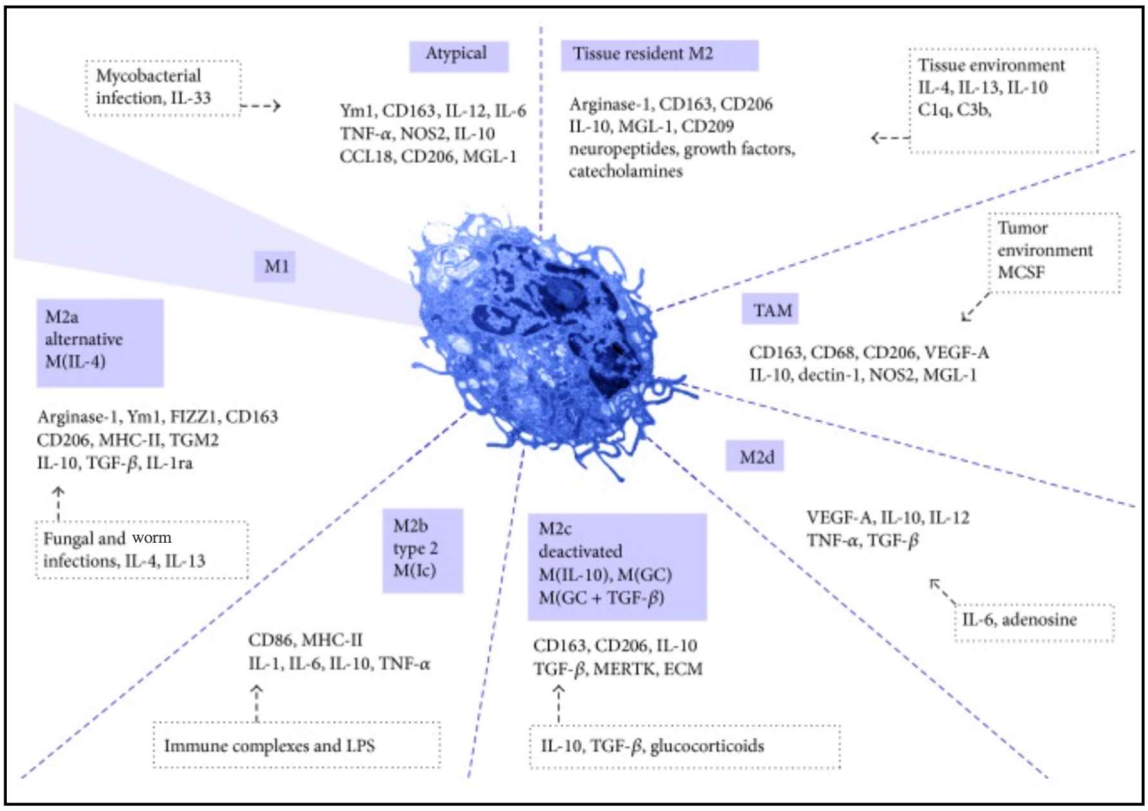
The cycle of chronic inflammation and cell death transform the stable plaque into a vulnerable plaque triggering an acute clinical event by plaque rupture and thrombosis (305, 355-357). A first step towards plaque rupture is the thining of the fibrous cap, which is driven by the same cell types that have promoted its formation. Notably, foam cells, macrophages, SMCs and endothelial cells secrete MMPs which contribute to the lysis of extracellular matrix (targeting collagen especially), and activated T-cells prevents the collagen synthesis by SMCs via TNFα production (358-360). As a result of fibrous cap degradation, lipids and collagen are released in the systemic circulation which contributes to the accumulation and adhesion of platelets at plaque site, promoting blood clot formation (361-363). This clot, also known as thrombus, can block the blood flow and trigger acute cardiac events, such as a heart attack or a stroke (350, 364-366).

I-3.2. Macrophage heterogeneity in atherosclerosis

Following activation of the endothelium by deposition of modified LDL, monocytes are reported as the first cell type to be recruited to the intima, where they differentiate into macrophages and contribute to the maintenance of chronic inflammation, leading to a worsening of the atherosclerotic disease. However, plaque macrophages also exert anti-inflammatory functions that can contribute to disease resolution or plaque stabilisation and the mechanisms controlling macrophage phenotypes and functions are not fully understood (367). Macrophages are a very heterogeneous cell population and several phenotypes typically co-exist, whether in the plaque or in other organs, in health or in disease. In an attempt to explain the diversity of macrophage phenotypes identified *in vivo*, a conceptual

framework evolved that classified macrophages as belonging to either an “M1” or “M2” phenotype analogous to the type 1 T helper (Th1)/type 2 T helper (Th2) concept that was dominating T-cell biology (**Figure 5** (368)), and which display antagonist functions. Several ways to classify macrophages have been used, based on:

- Their role in key macrophage homeostatic activities, namely host defense, wound healing, and immune regulation. This method distinguishes activated M1 macrophages, whose major role is in pathogen killing and promote, and are promoted, by Th1 cellular immune responses, from wound healing macrophages - M2a-, and regulatory macrophages (both M2b and M2c subclasses) which also preferentially induce Th2-type humoral-immune responses to antigen. (369)
- Their expression of cell surface markers and the production of specific factors, mainly levels and types of cytokines produced (370). This classification recognises, at least, M1, M2a, M2b and M2c distinct subclasses. M1 macrophages secreting pro-inflammatory cytokines, and M2 macrophages overall presenting anti-inflammatory profiles, but with slight differences between each subtype.
- The polarisation and activation pathways specific of each subtype. This is often useful in conjunction with the classification methods described above. For instance, M2a macrophages can be discriminate from M2c ones because they are induced by Th2 cytokines, IL-4 and IL-13, whereas M2c macrophages are induced by glucocorticoids. (356, 370-372).



Adapted from Röszer T. (2015) (368)

<u>Figure 5.</u> Overview of macrophage phenotypes
Macrophage activation is widely considered as a polarisation towards M1 or M2 states. However, the M2 activation state involves heterogenous and functionally distincts macrophages. The diagram represents the most prevalent examples of the M2 activation and lists the markers associated with the distinct activation phenotypes. The upstream signals are labeled in dotted frames.

Broadly, M1 macrophages, also known as “classically activated macrophages”, are described as pro-inflammatory, while M2 macrophages, “alternatively activated macrophages”, show anti-inflammatory properties (368, 373-378). The main role of M1 macrophages is believed to be the clearance of pathogens during infection through efficient phagocytosis, the generation of ROS and the production of pro-inflammatory cytokines such as TNF, IL-6 and IL-1β (379, 380). On the other hand, an essential function of M2 macrophages is to promote

tissue repair and wound healing following an infection or injury (381-383). To perform this role, M2 macrophages are notably able to produce anti-inflammatory cytokines such as IL-10 and TGF-β, thus defining them as potent inhibitors of acute inflammatory responses to bacterial ligands. In contrast to M1 macrophages, M2 macrophages are described as ineffective at intracellular pathogen killing (384, 385), but have a crucial role in the uptake of apoptotic cells, termed efferocytosis, and debris (340, 386, 387). Moreover, an ability of M2 macrophages to produce several extracellular matrix components, such as fibronectin, or polyamine and proline promoting collagen formation, has been described, consistent with their role in wound healing (382, 388, 389). Notably, according to the M1/M2 paradigm, tissue resident macrophages in health are classified as M2 macrophages given their role in maintaining tissue homeostasis, notably by performing efferocytosis during routine cell turnover.

The polarisation pathway undergone by macrophages depends in a very significant part on their microenvironment (371, 372, 390). Both tissue resident macrophages or monocytes-derived macrophages can become M1 or M2 macrophages (391, 392). In physiological conditions, the sensing of a threat such as an external pathogen, bacterial LPS, or sterile inflammatory signals, such as DAMPs, by macrophages triggers their polarisation towards an M1 phenotype. Given the potent pro-inflammatory activities of M1 macrophages, their numbers and activation must be spatially and temporally regulated to prevent uncontrolled tissue damage to the host. Regulation can come from the M1 macrophage itself, mostly via apoptosis, or from surrounding cells, notably by concomitant M2 polarisation. In a positive feedback loop, M2 macrophages stimulate T-cells to produce IL-4, which in turns lead to more M2 polarisation. M2 macrophages in turn secrete immunoregulatory cytokines,

especially TGF-β and IL-10, which dampen macrophage activation and therefore help to dampen the pro-inflammatory damage induced by M1 macrophages. Hence, the coexistence of both macrophages phenotypes creates a balance that aims at efficient pathogen clearance followed by then tissue healing (371, 393, 394).

However, chronic inflammation may be associated with an imbalance between M1 and M2 macrophages which promotes the chronic activation of the pro-inflammatory, M1, macrophages leading to tissue damage and impairment of wound healing (395, 396).

Consistently, atherosclerosis was initially thought to be a mostly M1-driven chronic inflammatory condition of the arterial wall. Although both M1 and M2 macrophages have been detected in the plaque, the initial simplistic viewpoint was that pro-inflammatory, M1, macrophages are pro-atherosclerotic, while anti-inflammatory, M2, macrophages are anti-atherogenic. This hypothesis was supported by several observations:

- The recruitment and retention of macrophages is dramatically increased at the lesion site, besides an enrichment in M1 phenotype in advanced plaques (392, 397). Hence, the M1:M2 balance appears as a major component of atherosclerosis progression (333, 398).
- M1 macrophages have been associated with symptomatic, unstable and progressing plaques, whereas M2 macrophages are found in more stable, asymptomatic or even regressing plaques (399-401).
- The position of M1 and M2 macrophages within the plaque. Macrophages expressing mainly M1 polarisation markers have been located in one of the most unstable areas within the plaque called shoulder, whereas the fibrous cap which is a more stable location within the plaques contains both M1 and M2 phenotypes in similar numbers

(398, 402-404). This observation raised several questions about spatiotemporal monocytes recruitment and differentiation into macrophages. Notably, the fact that M1:M2 balance may depend on factors other than the microenvironment. It has been hypothesised that polarisation can also be influenced by macrophage origins, with circulating monocytes and tissue-resident macrophages not differentiating into the same phenotypes, although it is still a subject of debate (391, 404-408). Moreover, it has also been evoked that polarisation may depend on intrinsic cell properties, such as the levels of marker Ly6C expressed by recruited monocytes, with $Ly6C^{high}$ monocytes differentiating preferentially into M1, and $Ly6C^{low}$ into M2 macrophages (328, 333, 392, 409-411). Interestingly, if the microenvironment is able to influence macrophages polarisation, the contrary is also true. Therefore, M1:M2 macrophages ratio has the ability to influence the plaque structure i.e the position of macrophages within the plaque which is a major component of the lesion phenotype and its evolution.

- Inducing an aggressive lowering of lipids or raising High-Density Lipoprotein (HDL) levels triggered regression of atherosclerosis in mice. This effect was associated with a plaque enrichment in M2 macrophages, consistent with an atheroprotective function. This finding also suggested that the HDL:LDL ratio could also be a macrophage polarisation factor (412, 413).

However, the simplistic M1 are pro-inflammatory and M2 are anti-inflammatory paradigm cannot fully explain atherosclerosis progression and does not take into consideration emerging evidence for more diverse roles within a same macrophage phenotype. Notably, anti-inflammatory – M2 – macrophages, originally described as anti-

atherogenic, are now thought to have possible implications in atherosclerosis progression under specific conditions and depending on the stimuli they receive. For example, particular M2 macrophages subtypes have been found able to secrete large amounts of pro-inflammatory cytokines (414-416). It has also been described that M2-like macrophages may be more prone to foam cells formation, or that they can secrete large amounts of MCP-1 when loaded with OxLDL. Moreover, a M2 subtype has been associated in hemorrhagic areas within the plaque which correlate with plaque progression (338, 415-418). Therefore, more recent studies argue for a greater complexity than the restricted paradigm of 2 main types of macrophages: M1 vs M2. As mentioned before, the M2 classification already regroups three subtypes (M2a, M2b and M2c), identified in both humans and mice based on differences in functions and expression markers. Besides, in the context of atherosclerosis in particular, extensive *in vivo* studies lead to the discovery of a wide range of intermediate macrophage phenotypes shown to be to some extent involved in plaques progression (**Figure 6** (356)), notably:

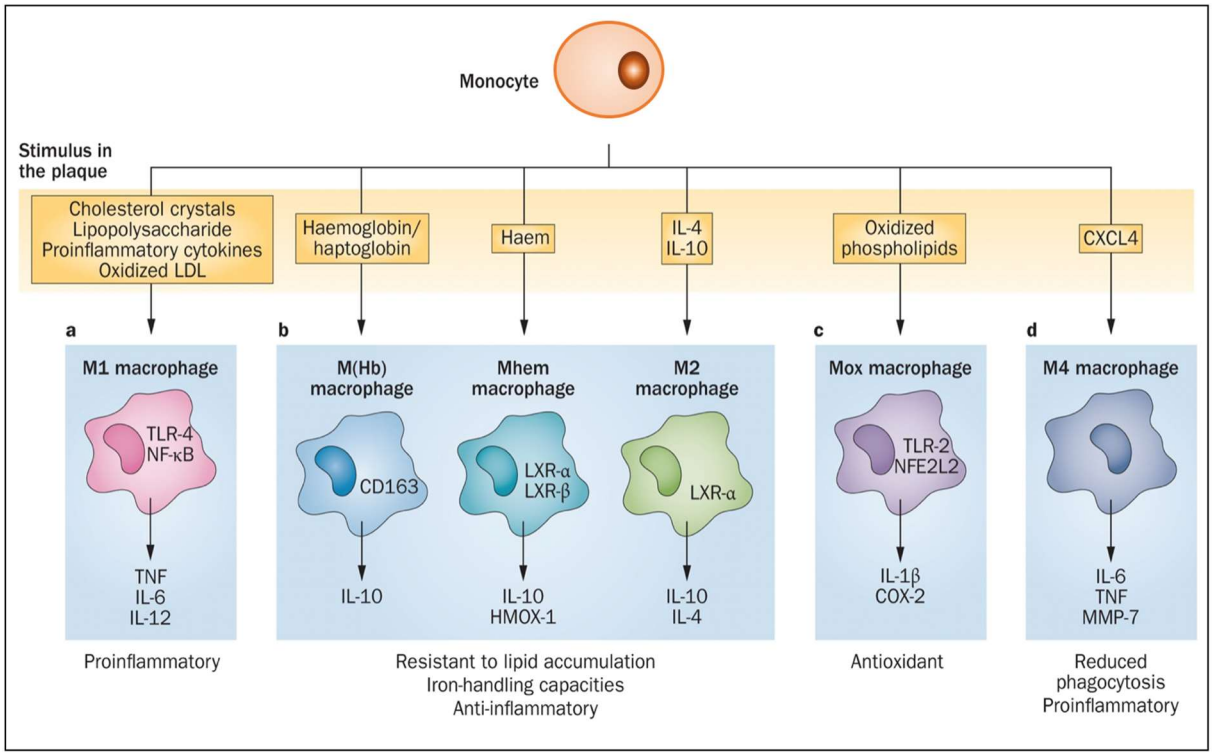
- Mox macrophages are characterised by increased expression of Nuclear Factor Erythroid 2-related factor 2 (NFR2) as well as reduced phagocytic and chemotactic capacities (419). They are thought to be induced by exposure to oxidized phospholipids present in OxLDL.
- Mhem macrophages are polarised by exposure to heme, a precursor of hemoglobin, and display reduced levels of oxidative stress and lipid accumulation along with a reduced capacity for foam cells formation (420-422).
- M(Hb) macrophages have characteristics very close to those of Mhem macrophages, but are induced by exposure to haemoglobin-haptoglobin complexes rather than to heme (423, 424).

- M4 macrophages are thus named because they are polarised by CXCL4. They are found in human atherosclerotic plaques and completely lack the capacity for phagocytosis but might prevent the development of Mhem phenotype in atherosclerotic plaques (425, 426).

Interestingly, most of the described alternative macrophage phenotypes seem to display more M2 than M1 markers and functions. This is consistent with the large number of pathways leading to M2 polarisation but it also suggests that alternative macrophages might represent different stages of differentiation of the same cell population (356). For example, the very close phenotypes displayed by Mhem and M(Hb) macrophages and the fact that they are polarised by closely related factors, heme and hemoglobin, support this concept. In contrast, M1 macrophages polarisation pathway seems to be simpler and well established, and intermediate M1 phenotypes have not been described to date. (392, 413, 427, 428)

This complex diversity is thought to be partly due to the complexity of the plaque microenvironment (355, 356, 429). For instance, a single factor can lead to both M1 and M2 polarisation: in the plaque, OxLDL, a straightforward cue for pro-inflammatory polarisation, can lead to M1 phenotypes via TLR-signaling, but also induces expression of M2 macrophages phenotypic markers, notably arginase 1 via the activation of peroxisome proliferator activated receptor-γ (PPARγ) (430). Another example is that Granulocyte-Macrophage colony-stimulating factor (GM-CSF) and M-CSF, known to promote monocyte differentiation into opposing macrophage phenotypes, are both present in the atherosclerotic plaque (431, 432). In addition to the increasing number of macrophage phenotypes being characterised *in vivo,* different studies have also reported macrophage plasticity, or the ability to switch

between phenotypes, during the course of atherosclerosis (425, 426). In mice, macrophages plasticity was observed during plaque regression, with a switch from M1 to M2 phenotype, suggesting that the stage of the disease may control both macrophages polarisation and plasticity (412, 413). In another study, pro-inflammatory cytokines and oxidized phospholipids found in the plaque could reduce macrophage expression of Krüppel-like factor-4 (KLF4), a transcription factor promoting M2 polarisation suggesting that the M2 phenotype may be repressed during atherogenesis contributing to disease progression when pro-inflammatory signals predominate (400). Thus, it is now admitted that macrophages are extremely plastic cells and that their polarisation is not definitive. Phenotype switching may occur depending on complex environmental cues, contributing to the wide spectrum of plaque macrophages which are able to further evolve with disease progression or regression.



Adapted from Chinetti-Gbaguidi, G. et al. (2014) (356)

Figure 6. Main macrophage subtypes found in atherosclerotic lesions
Stimuli present in atherosclerotic lesions drive the differentiation of monocytes towards different macrophage phenotypes. **a.** M1 macrophages release pro-inflammatory cytokines. **b.** M(Hb), Mhem, and M2 macrophages are resistant to lipid accumulation, possess iron-handling capacities, and have anti-inflammatory effects. **c.** Mox macrophages display an antioxidant gene expression profile. **d.** M4 macrophages, like M1 macrophages, are pro-inflammatory but lack the capacity for phagocytosis.

The *in vivo* complexity of the plaque microenvironment is currently impossible to reproduce *in vitro*, thereby limiting the relevance of *in vitro* only polarisation studies. However, various protocols have been described to promote the M1-like or M2-like phenotypes *in vitro*. Briefly, an M1-like phenotype can be obtained by:

- Macrophage exposure to a combination of two signals: IFN-γ and TNFα. The IFN-γ cytokine does not activate cells but is needed to prime them (433) along with exogenous TNFα, or an inducer of TNFα. Classically, microbes or microbial products such as LPS are very potent TNFα inducers and lead to M1 macrophage polarisation

(371, 373). Moreover, DAMPs that act as TLRs ligands have also been shown to promote a M1 phenotype (86, 204, 279). For example, in the context of atherosclerosis, increased OxLDL/Ox phospholipids concentration leading to TLRs signaling is a M1-activation signal (339, 434, 435).

- In addition to the manipulation of the extracellular milieu, the more recent identification of transcriptional programs that regulate macrophage polarisation, has led to the development of other techniques to differentiate macrophages *in vitro*. For example, targeted depletion of the transcriptional factor KLF4, which promotes M2 and inhibits M1 polarisation, leads to a M1-like macrophage phenotype (400, 436).

On the other hand, *in vitro* conditions to obtain M2-like macrophages include:

- Exposure of murine peritoneal macrophages to IL-4. This protocol results from the first description of "alternatively activated" – M2 – macrophages by *Gordon and al.*, who observed an "alternative activation phenotype" when cells were exposed to IL-4 (437).
- A two-signal polarisation pathway. It requires the ligation of Fcγ Receptors (FcγRs) coupled with a macrophage stimulatory signal to influence cytokine production induced by TLRs (LPS stimulation), CD40 (Lipoteichoic acid stimulation) or CD44 (HA stimulation) engagement. Without FcγRs ligation, all these stimuli induced pro-inflammatory profiles, but with the ligation, authors noticed the induction of high levels of IL-10 and abrogation of IL-12 production. However, in contrast to M1 polarisation, macrophages do not need to be IFN-γ primed (371, 393, 438).
- Combined exposure to IL-4 and IL-13 (M2a differentiation) (369, 429).
- Exposure of cells to classical activating signals – TNF, LPS, IL-1β – in the presence of immunoglobulin G immune complexes (M2b differentiation) (356, 414).

- Exposure to IL-10, TGF-β, IL-13 or glucocorticoids (M2c macrophages) (370, 439).

A widely-used technique to differentiate primary blood monocytes into macrophages *in vitro* is by addition of growth factors, either GM-CSF, or M-CSF (415, 431, 440, 441). Macrophages express receptors for both GM-CSF and M-CSF, which trigger signaling pathway for cell survival, proliferation, differentiation and activation (442, 443). In physiological conditions, basal levels of GM-CSF are low but become dramatically elevated during inflammation, while M-CSF is found ubiquitously in many tissues and controls the number and function of resident macrophages (444). In atherosclerosis, the GM-CSF:M-CSF ratio changes with disease progression. M-CSF is expressed in both healthy arteries and atherosclerotic lesions, while GM-CSF expression is very low in healthy vessels but increases in SMCs and endothelial cells during disease progression and macrophage accumulation (431, 432). In various studies, GM-CSF has been shown to trigger formation of cells with antigen-presenting properties, and M-CSF-differentiated cells were found to be involved in inflammation resolution (445-447). Taken together, these observations led to the description of GM-CSF or M-CSF *in vitro* differentiated-macrophages as models of M1 or M2 macrophages respectively (441). However, an increasing number of studies describes that differentiation with GM-CSF/M-CSF only does not induce true M1/M2 phenotypes and that they should be used in combination with other factors to provide more physiologically relevant biological models. For example, both GM-CSF and M-CSF-derived macrophages can behave in an M1-like manner if stimulated with TLR ligands while M-CSF differentiation requires addition of key cytokines, such as IL-4 or IL-13 to obtain a more physiological M2 phenotype (448).

Therefore, although *in vitro* experiments are essential and integral parts of studies aimed at deciphering macrophage-related immune mechanisms, it is important to bear in mind that no *in vitro* model of macrophage, no matter how complex, can fully represent the diversity, complexity and plasticity of primary macrophages in the i*n vivo* setting, especially in an evolving disease scenario such as atherosclerosis.

I-3.3. TLRs in atherosclerosis

The role of TLRs in mediating and orchestrating the initiation and progression of atherosclerosis is now well accepted (307, 449, 450). An increase in TLR2 and TLR4 expressions have been observed in monocytes from patients and mice with coronary artery disease (451, 452). Furthermore, TLR1, 2 and 4 expression levels are upregulated in human atheroma compared with healthy vessels, and NF-κB colocalises with both TLR2 and TLR4 in the plaque (254). Functionally, TLRs are implicated in a wide range of pro-atherogenic mechanisms, directly or indirectly, such as pro-inflammatory mediators' production and secretion, macrophages recruitment, foam cells formation and subsequent differentiation of T-cells towards Th1 against Th2 phenotype (453). Therefore, the role of TLRs in mediating atherosclerosis has been the subject of intense study and it was shown that:

- Knocking-out of MyD88, the main TLR signaling adaptor, reduced early atherosclerotic lesion development in hyperlipidemic mice via impairment of macrophage recruitment into the artery wall (454);
- Deletion of TLR2 in $LDLR^{-/-}$ mice on a high fat diet reduced atherogenesis compared to $LDLR^{-/-}$ mice after 10 and 14 weeks on a high fat diet (455). Interestingly, in bone marrow transplantation experiments, loss of TLR2 expression in bone marrow-derived cells only did not reduce the atherosclerotic burden. This suggested that an unknown

endogenous TLR2 agonist influenced lesion progression by activating TLR2 in cells that were not of bone marrow origin. However, atherosclerosis was dramatically worsened by administration of the TLR2 agonist, Pam_3CSK4 (P_3C), a synthetic bacterial lipopeptide, and this effect could be reversed in full in $LDLR^{-/-}$ mice completely deficient for TLR2, or in bone-marrow only TLR2-deficient mice. These observations suggested a differential involvement of TLR2 on atherosclerosis initiation and progression or worsening in response to infections, depending on its tissue distribution (455).

- TLR4 deficiency was associated with reduced aortic atherosclerosis burden in $ApoE^{-/-}$ mice which accompanied decreased lipid content in the plaque and lower levels of circulatory pro-inflammatory cytokines (197).

Mechanistically, it was found that the stimulation of human macrophages with OxLDL induced foam cell formation, cytokine secretion, HLA-DR and CD86 expression and T-cell proliferation *in vitro*. Interestingly, antibody blocking of TLR2, TLR4 and CD36 reduced the secretion of IL-1β, IL-6 and IL-8, the expression of HLA-DR and CD86, T-cell proliferation and foam cell formation. However, the blockade of TLR2 did not affect the formation of foam cells (456). Stewart *et al.* also demonstrated that a complex composed of TLR4, TLR6 and CD36 could recognise OxLDL and promote sterile inflammation (279). Interestingly, other forms of atherogenic lipids, such as minimally modified LDL, which are not recognised by scavenger receptors, can also activate TLRs, notably TLR4, with the classical involvement of the co-receptors CD14 and MD-2 (457).

Aside from oxidized/modified LDL recognition, TLRs also promote atherosclerosis

development in response to infections, in line with their long-described ability to sense microbial components (458). Infectious agents that have been linked to atherosclerotic disease include, but not limited to, Chlamydia pneumoniae (*C. pneumonia*), Porphyromonas gingivalis (*P. gingivalis*), Helicobacter pylori (*H. pylori*), influenza A virus, cytomegalovirus (CMV), Epstein-Barr virus, HIV, herpes simplex virus (HSV)-1 and 2, and hepatitis C virus (458, 459). TLR2 and TLR4 were found involved in endothelium activation, leukocyte recruitment, foam cell formation and lesion rupture in atherosclerosis promoted by the bacteria *C. pneumonia*, *P. gingivalis*, *H. pylori*; while the intracellular TLR3, 7, 8 and 9 were involved in mediating the promoting effect of CMV, HIV and HSV (458).

In terms of therapeutic exploitation of the role of TLRs in atherosclerosis, Arslan *et al.* demonstrated that a monoclonal antibody against TLR2 (OPN-301) resulted in reduced neutrophil, macrophage, and T-lymphocyte infiltration, and reduced the production of pro-inflammatory TNF-α, IL-1α and GM-CSF in mice (460). The same group later described the first humanised anti-TLR2 antibody, OPN-305, which reduced infarct size, preserved systolic function and eventually prevented myocardial damage in a model of I/R Injury (461). Additionally, an established TLR4 antagonist, *R. sphaeroides* LPS (Rs-LPS), prevented the expression of the pro-atherogenic factors IL-6 and MMP-9, as well as macrophage accumulation in atherosclerotic plaques in $ApoE^{-/-}$ mice (462). However, the therapeutic options existing so far to target TLRs are quite limited and further preclinical development is needed. Although the evidence for TLR2 inhibition is somewhat stronger than for TLR4, this could simply be due to the existence of a powerful blocking antibody approved for use in clinical trials.

Of note, TLR activation is not always detrimental to atherosclerosis progression. Endosomal TLRs, TLR3 in particular, have been shown to play protective functions. *In vivo,* neointima formation following perivascular collar-induced injury model was reduced after administration of the TLR3 synthetic analog PolyI:C in $TLR3^{+/+}ApoE^{-/-}$ mice compared with $TLR3^{-/-}ApoE^{-/-}$ mice. Moreover, $TLR3^{-/-}ApoE^{-/-}$ mice had earlier atherosclerosis than their $TLR3^{+/+}ApoE^{-/-}$ counterparts, suggesting that TLR3 is protective. The TLR3 downstream signaling cascade activates TRIF. $LDLR^{-/-}$ mice with lack-of-function mutation in TRIF (Lps2) were significantly protected from atherosclerosis, where the mice displayed fewer observed lesional macrophage (307). TLR7 was found to play a protective role by constraining monocyte/macrophage pro-inflammatory activity. $TLR7^{-/-}ApoE^{-/-}$ mice displayed elevated levels of necrotic core formation, lipid deposition, macrophage infiltration, and pro-inflammatory cytokine production, and reduced presence of SMC and collagen. It was suggested that TLR7 hinders the expression of inflammatory $Ly6C^{high}$ monocytes and inflammatory M1 macrophages, a MCP-1-mediated process, possibly triggered by the pathogenic TLR2 and TLR4. The mechanisms behind the atheroprotective effects of TLR7 are not well understood (307). Of note, extracellular TLRs may also mediate atheroprotective responses, notably via their ability to modulate the adaptive immune response. For example, MyD88-mediated DC activation provides atheroprotection by promoting Treg generation. Tregs, in turn, abolish T effector cells, inflammatory macrophages and attenuate monocyte recruitment by suppressing MCP-1 production in a TGF-β dependent manner (307). For example, $ApoE^{-/-}$ mice deficient in TLR4 infected with *P. gingivalis* were paradoxically more susceptible for developing atherosclerosis, presenting increased levels of inflammatory Th17 cells.

Therefore, although TLRs are widely considered to be atherogenic, the resulting effect of TLR activation on the pathology is difficult to predict and will likely depend on the stage of atherosclerosis and the nature of the TLR ligands encountered (endogenous or exogenous, bacterial or viral).

I-3.4. Atherosclerosis in CKD

The first CV changes observed in patients with CKD are: i) arteriosclerosis, which is characterised by arterial stiffening and loss of cushioning function, often caused by atherosclerotic plaques as a result of dyslipidemia, ii) altered ventricular diastolic function, and iii) left ventricular hypertrophy. Overall, in patients suffering with CKD, atherosclerosis is worsened compared with non-CKD patient. This was confirmed in postmortem studies, which have reported extensive coronary artery disease in CKD patients, characterised by an increased number of plaques, as well as by their morphology which appears to be thicker and more often calcified (463-465). As mentioned before, a number of CKD-specific risk factors have been described as risk factors for increased atherosclerosis and CV risk (Introduction, Section I-1.2). Vitamin D deficiency has been associated with increased adhesion molecules expression in endothelial cells and the stimulation of a senescent phenotype in SMCs, which has notably been found in the neointima of atheroma plaques (466, 467). Reports have also linked vitamin D deficiency with an increased CV mortality and morbidity including vascular calcification and stiffness (468, 469). Of note, calcification is typically exacerbated in CKD patients, as calcification of atherosclerotic plaques is both more frequent and accelerated in CKD patients compared to non-CKD population (470-474). In addition to intimal calcification associated with the atherosclerosis plaque, which is also observed in non-CKD patients, medial calcification has been described specifically in CKD and can be found even outside of

plaque sites (475, 476). This was also observed in post-mortem studies, which reported a thickening of the arterial media and the presence of medial calcification in CKD compared with patients without impaired kidney function (464, 465, 477). This suggests a tendency to more rapid and more severe calcification of the vasculature in general in CKD patients. Medial calcification, in contrast to intimal calcification does not lead to obstruction of the lumen but still results in vascular stiffness, arteriosclerosis, and decreases the compliance of blood vessels which leads to hypertension (478) and left ventricular hypertrophy (479). Medial calcification is also considered a strong marker of future CV risk in patients suffering diabetes mellitus (480), as well as a powerful diagnostic marker for all-cause and CV mortality in patients receiving HD (481). Interestingly, medial calcification identified in renal failure patients present similarities with bone formation. Consistent with this finding, some reports have described the vessel wall calcification as an actively regulated process sharing similarities with osteogenesis, not directly specific to the uremic state, but rather due to a perturbed phosphate-calcium imbalance common in CKD patients (482, 483).

Recent reports also described higher prevalence and occurrence of plaques in carotid and femoral arteries, characterised by intima-media thickness, in CKD patients on dialysis compared with predialysis patients or general population respectively, indicating a worsening atherosclerosis burden with CKD progression (484-486). Interestingly, there is some evidence for slower progression of atherosclerosis in advanced stage of CKD, however the CV risk is still increased compared to non-CKD patients (486). This may be the result of additional changes to the uremic milieu that increase the risk of an acute CV event once atherosclerosis is developed (for example by promoting fibrous cap thining), or because of non-atherosclerotic CV injury (50). Indeed, as mentioned, atherosclerotic plaques of CKD patients display more complex features that especially suggest greater instability such as lipid content, widespread

necrosis, disruption of the extracellular matrix, and enhanced inflammation notably increased by oxidative stress that develops early in CKD, persists through the disease and is not improved by dialysis treatment (487-489). Consistently, intravascular ultrasound assessments have showed that coronary plaques in CKD are characterised by a higher lipid core and a lower fibrous volume, creating less stable plaques with greater chance of rupture and acute clinical event (489-491). Interestingly, the composition of coronary plaques in CKD evolves with renal impairment progression, from necrotic core-rich to extremely calcium-rich (492). As described above, macrophages are involved, directly or indirectly, throughout atherosclerotic progression. A number of their atherosclerosis-associated functions have been shown to be affected by CKD, notably i) higher migratory activity in response to chemoattractants, (493, 494), ii) impaired cellular mobility and sub-endothelial trapping and subsequent accumulation (495), iii) increased foam cell formation ability, due to upregulation of surface scavenger receptors levels (496) and downregulation of ATP-binding cassette subfamily A member 1 transporter (ABCA1) function (497), which is a major macrophages transporter for cholesterol efflux, thus leading to a higher cellular accumulation of cholesterol (494).

II- AIMS OF THE STUDY

Beside shared traditional risk factors, the hallmark of both CKD and CVD is chronic inflammation. Tissue injury during CKD has been shown to lead to the increased release of TLR DAMPs in the kidney, contributing to the maintenance of the local chronic inflammatory state and thus to the progression of kidney damage. Dialysis treatments for CKD have also been shown to lead to the release of DAMPs in the peritoneal cavity (PD) (498) and the blood (HD) (499, 500). The concept that endogenous ligands for TLRs or other PRRs may mediate increased cardiovascular risk in CKD has been suggested increasingly in recent years. However, the specific mechanisms behind this concept are very poorly described. This study aims to:

1- *Identify the specific TLR DAMPs that are elevated systemically in patients with CKD*

2- *Determine the ability of the DAMPs elevated systemically in CKD (identified in 1) to affect key pro-atherogenic responses and cellular functions*

3- *Verify the contribution of TLRs to the atherogenic responses triggered by the DAMPs (described in 2)*

4- *Determine the potential of the DAMP/TLR pathway as a therapeutic target to reduce the atherosclerotic burden in CKD*

The overarching goal of this project is to identify promising therapeutic targets to pave the way towards the development of treatments/management/prevention options to lower CV risk in patients with CKD that are more effective than the currently available ones.

III- RESULTS

III-1. Plasma levels of endogenous ligands for TLRs in CKD patients

III-1.1. Elevated levels of TLR DAMPs in CKD patients compared to healthy donors

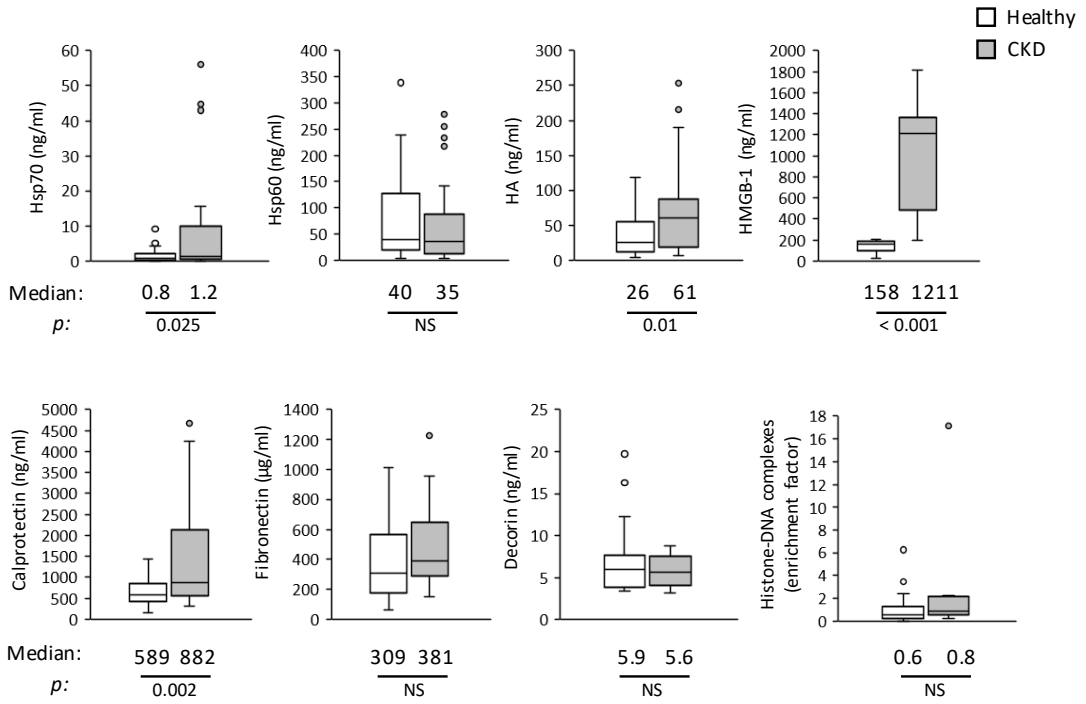
In order to identify DAMPs potentially involved in driving CVD in CKD, we obtained plasma samples from CKD patients (n=35, stage 5 on PD), and quantified the levels of known TLR DAMPs by ELISA. Plasma samples from age-matched (CKD: 66 (52-71); Healthy: 64 (49–68)) healthy donors (n=30) were used as reference to identify which DAMPs may be increased systemically due to CKD. CKD patients with diabetes, cancer, prior CV event or diagnosed CVD were excluded from the analysis. The DAMPs to test were selected to encompass ligands for various TLRs and to represent a range of well-described DAMP families:

- Chaperone proteins: Hsp60 and Hsp70 (TLR2 and TLR4 agonists);
- DNA-binding proteins: HMGB-1 (TLR2 and TLR4 agonist), Histone H3 (TLR2 agonist);
- Extracellular matrix components: HA, Fibronectin and Decorin (TLR2 and TLR4 agonists);
- Ca^{2+} binding proteins: Calprotectin (S100A8/S100A9, a TLR4 agonist);
- Self-nucleic acids: Histone-DNA complexes (TLR9 agonists).

Out of the 9 known TLR DAMPs tested, 8 could be detected and quantified in most samples (Hsp60, Hsp70, HA, HMGB-1, Calprotectin, Fibronectin, Decorin and Histone-DNA) in healthy donors and CKD patients (**Figure 7**). Histone H3 was below the detection threshold for both groups. Four DAMPs were found significantly elevated in the plasma of patients compared to controls, namely Hsp70, HA, HMGB-1 and Calprotectin.

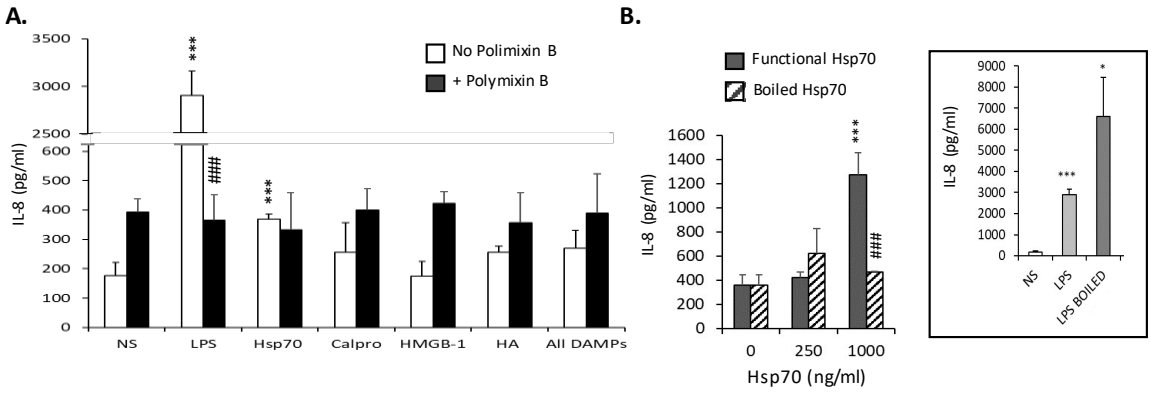
These findings are in line with our working hypothesis that TLR activation by their endogenous ligands may aggravate CV risk in patients with CKD. However, different TLR

DAMPs have been shown to have different protective or detrimental effects on different cardiovascular pathologies (280). This is in part because different TLR ligands may induce qualitatively and quantitatively different responses (501-503), and different DAMPs may involve different TLR co-receptors, and have different additional immune receptors other than TLRs (504-506). Therefore, to assess a potential role for these particular CKD-associated TLR DAMPs in driving CV risk, their effect *in vitro* on key cell functions critical to atherosclerosis formation was evaluated next.



<u>Figure 7.</u> Plasma levels of TLR DAMPs in CKD patients and healthy individuals
Concentrations of TLR DAMPs were determined by ELISA performed on triplicate aliquots of plasma from healthy donors (white) or Stage 5 CKD patients (grey). Horizontal lines in boxes denote the median value, Open circles denote outliers which were defined as data points localised outside of the lower or upper lower range limits (1.5 times the inner quarter range). Hsp70, Hsp60, HA, Calprotectin, Fibronectin: Healthy n=30, CKD n=35; Decorin: Healthy n=20, CKD n=25; HMGB-1, Histone-DNA complexes: Healthy n=12, CKD n=15.
*, $p<0.05$; ***, $p<0.005$, CKD patients *vs* healthy donors.

Prior to the start of these *in vitro* experiments, it was first confirmed that the purified DAMP preparations were not contaminated with functionally significant levels of endotoxin. Specifically, only Hsp70 exposure led to a measurable increase in IL-8 production by human monocytes, although these cells were sensitive to stimulation with low amounts of purified LPS (10 ng/ml, **Figure 8.A**). The Hsp70-induced IL-8 production could not be inhibited by the LPS antagonist Polymixin B but was abrogated following denaturation by boiling (**Figure 8.B**), which preserves endotoxin. Our LAL measurements confirmed the very low endotoxin content of the DAMP preparations (**Figure 8.C**).



C.

	Endotoxin concentration (EU)	Endotoxin concentration (pg/ml)
Hsp70	*undetected*	*undetected*
Calprotectin	*undetected*	*undetected*
HMGB-1	0.001	0.15
HA	*undetected*	*undetected*

<u>Figure 8.</u> The preparations of CKD-associated DAMPs are not contaminated with significant amounts of endotoxin

A-B. Triplicate cultures of human MonoMac6 (MM6) monocytes were stimulated (18 hours, 37°C) with LPS (10 ng/ml) or the indicated DAMPs (1 µg/ml) in the presence or absence of Polymixin B (5 µg/ml, **A**), or before and after boiling (95°C, 10 minutes). Results shown are mean +/- SD of 2 experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$, TLR ligand vs no stimulation (NS) or #, +Polymixin B/Boiling vs No Polymixin B/no boiling. **C.** Endotoxin measurements in DAMP preparations by the LAL method.

III-1.2. Discussion

This first part of the work identified 4 TLR DAMPs elevated in plasma from Stage 5 CKD patients compared to healthy donors. Donors in both groups were age-matched (Healthy: 64 (49–68), CKD: 66 (52-71)) and CKD patients with diagnosed CVD, diabetes and malignancy were excluded from the analysis to avoid the confounding factor of additional chronic inflammation likely induced by these co-morbidities, which may also lead to increased DAMP plasma levels. However, the following factors could not be controlled for:

- Sex: Healthy donors plasma samples were from a previous study, therefore it was not possible to fully (age and sex) match the healthy to the CKD samples. Age-matching was prioritised over sex-matching, as it was not expected that the male:female proportion would be significantly different between the healthy and the CKD cohort, while the CKD cohort may be expected to be older than the healthy cohort.
- Co-morbidities: While CKD patients with diagnosed co-morbidities were excluded from the analysis, it is possible that some patients with underlying undiagnosed conditions were included in the analysis. Because CKD patients on Stage 5 have regular health checks, this proportion is probably minimal and the same issue may also apply to the healthy cohort.
- Treatments: It is likely that the CKD population was receiving more treatment interventions than the healthy population, likely including lipid-lowering (e.g. statins) or blood-pressure reducing (e.g. ACE inhibitors) drugs. However, most of these treatments are anti-inflammatory by nature (507, 508) and are therefore not expected to confound the analysis by increasing DAMPs plasma levels.
- Dialysis: Blood samples are easier to obtain from CKD patients who routinely attend hospital-based clinics. For this reason, our cohort of patients are Stage 5 CKD patients,

who were on PD at the time of sampling. Therefore, we cannot exclude a contribution of PD, in addition to CKD itself, to the overall elevation of DAMPs plasma levels. However, this does not invalidate the relevance of the findings, as dialysis (PD or HD) is an integral part of higher stage CKD and is believed to further drive CV risk (509). Of note, it is possible that other or different DAMPs would be found elevated in patients receiving HD rather than PD.

Following this comparison, Hsp70, Calprotectin, HMGB-1 and HA were selected for *in vitro* evaluation of their ability to drive typical pro-atherogenic responses. As described in the Introduction (Section I-2.2, p.37), HA comes in a wide range of molecular weights, with lower molecular weights driving pro-inflammatory responses while high-molecular weight HA is typically anti-inflammatory. It is relevant to note that it cannot be identified by ELISA which molecular weights are elevated in CKD plasma. However, it is expected that HA forms released from the extracellular matrix will have lower molecular weights, while large forms will remain embedded in the matrix. For this reason, a mix of low (15-40 kDa) and medium (75-350 kDa), but not high, molecular weight HAs were used in the *in vitro* experiments described here. Similarly, HMGB-1's function varies with its redox state (Introduction, Section I-2.2, p.26), which was not controlled for here. Therefore, it is possible that the redox state of CKD plasma HMGB-1 is not fully similar to that of the purified recombinant HMGB-1 used in the *in vitro* studies.

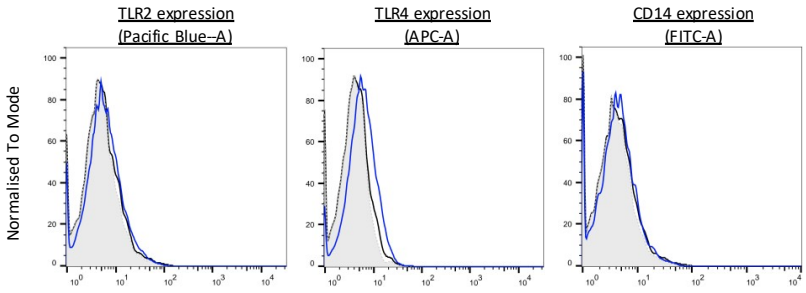
III-2. Effect of CKD-associated DAMPs on typical endothelial dysfunction-associated response by endothelial cells *in vitro*

Endothelial cells are reportedly the first cell type to be stimulated by an increased concentration of modified LDL, which leads to the loosening of permeable tight intercellular junctions, and to the release of pro-inflammatory cytokines and chemokines, promoting the recruitment of circulating leukocytes to the intima. Therefore, the ability of the 4 identified CKD-associated DAMPs to trigger pro-atherosclerotic responses was first evaluated on human endothelial cells.

III-2.1. Model selection

The Human Aortic Endothelial Cell (HAEC) line was selected over other available models as they were generated from primary cells that: i) are isolated from healthy adults, and therefore display a fully mature phenotype, notably in terms of immune responses. This is of particularly relevance for research into endothelial dysfunction and atherosclerosis, which are mostly conditions of adulthood, and ii) are of arterial, rather than venous, origin, making them more relevant to the study of atherosclerosis-associated responses.

Prior to testing the effect of DAMPs on these cells, we verified that TLR2, TLR4 and CD14 expression by HAEC was as reported for endothelial cells. As expected, expression levels were low altogether, with non-detectable levels of CD14, very low levels of TLR2 and moderate levels of TLR4 (**Figure 9**). This result is in line with reports of low baseline expression of TLRs (150).



Samples / MFI	TLR2	TLR4	CD14
Unstained	6.93	4.62	5.13
Isotype control	7.58	5.10	5.64
Specific antibody	8.26	16.5	5.72

Figure 9. TLR2, TLR4 and C14 expression by HAEC
Resting HAEC were analysed by flow cytometry for cell-surface expression of TLR2, TLR4 and CD14. Table shows the MFI values of at least 10,000 cells/condition for each receptor.

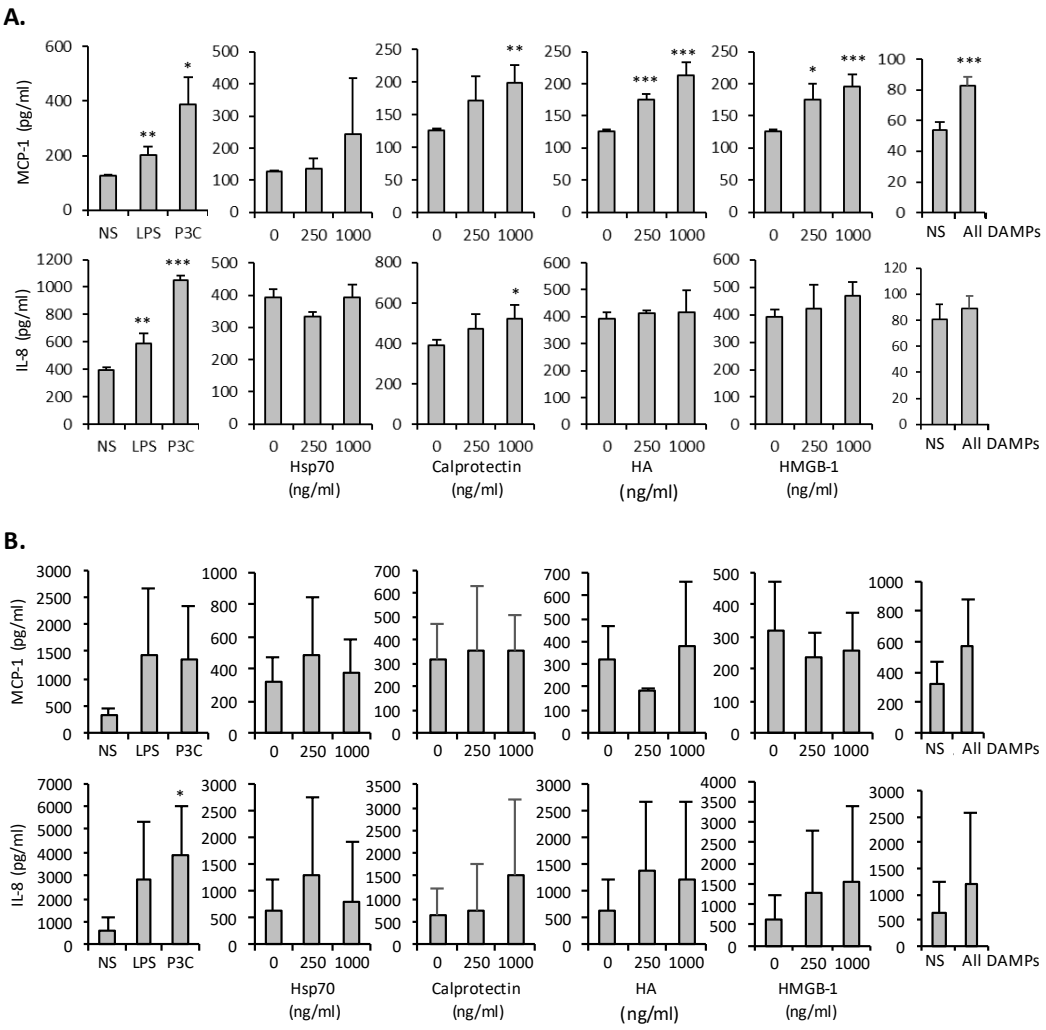
III-2.2. Release of pro-atherosclerotic mediators by HAEC

As mentioned previously (Introduction, Section I-3.1, p.54), the production of pro-inflammatory mediators by endothelial cells is an important initial step in the recruitment of monocytes to the lesion site. Hence, the effect of CKD-associated DAMPs on pro-inflammatory and pro-atherogenic mediators production by HAEC was investigated. Specifically, the following cytokines were selected for testing:

- MCP-1, well documented for its role in atherosclerosis by promoting recruitment of monocytes to the lesion site (410, 510).
- IL-8, a potent neutrophil chemoattractant whose receptor (CXCR2) is also expressed at high levels in plaque macrophages and is believed to be critical to retention of lesion macrophages in atherosclerotic plaques as well as being an important modulator of monocyte-endothelial interaction under flow conditions (511-513).
- IL-6, a potent inflammatory cytokine, is highly expressed in the plaque and elevated plasma levels have been associated with increased cardiovascular risk (514, 515).

Exposure of HAEC to Calprotectin, HA (Low and Medium Molecular weights combined) and HMGB-1 but not Hsp70 or to all CKD-associated DAMPs combined (**Figure 10.A**), led to a moderate increase in MCP-1 production, and Calprotectin also induced the modest but significant release of IL-8, which was otherwise mostly unaffected by exposure to the DAMPs (**Figure 10.A**). IL-6 was undetectable in these culture supernatants. This trend remained when pooling data from independent experiments (**Figure 10.B**), although statistical significance was lost due to heterogeneity in responses across experiments and the moderate extent of the responses.

Confirming that HAEC are sensitive to TLR2 and TLR4 stimulation, HAEC released larger amounts of both MCP-1 and IL-8 in response to a purified TLR2 bacterial agonist, Pam_3CSK_4 (P_3C), and a TLR4 bacterial agonist, LPS (**Figure 10.A and B**)**.** The strong response to P_3C despite undetectable levels of TLR2 at rest is consistent with literature showing that TLR2 is strongly upregulated following stimulation of endothelial cells with bacterial TLR ligands. This difference in response to bacterial ligands and DAMPS is not surprising as bacterial agonists are typically more potent than endogenous ones at inducing TLR mediated responses.

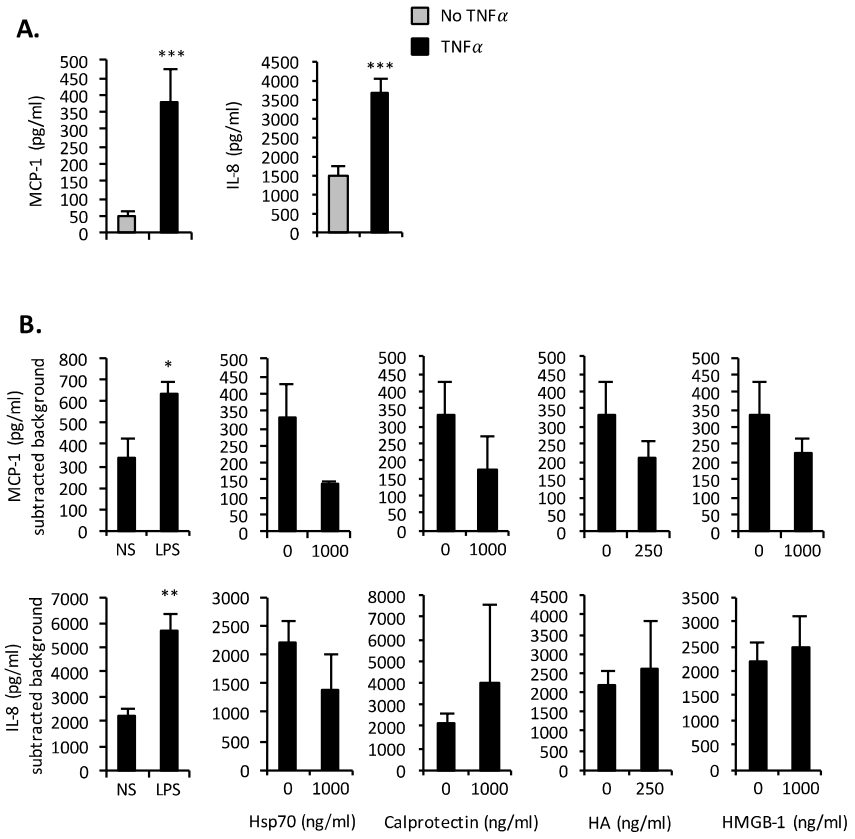


<u>Figure 10.</u> CKD-associated DAMPs induce very modest pro-inflammatory and atherosclerotic cytokine release by HAEC

Triplicate cultures of HAEC were stimulated (18 hours) with LPS (10 ng/ml), Pam_3CSK_4 (P3C, 500 ng/ml) or the indicated concentrations (or 1 µg/ml,) of Hsp70, Calprotectin, HA, or HMGB-1, alone or combined. Cytokine levels in culture supernatants are shown as mean +/- SD from 1 typical experiment out of 6 run (**A**) or from pooled data from 6 independent experiments (**B**).

*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$, Stimulation vs no stimulation.

In the context of chronic inflammatory conditions, such as CKD, high plasma levels of pro-inflammatory mediators can affect the extent and quality of subsequent immune responses. For example, higher plasma levels of TNFα have been found in CKD patients (516) and TNFα has the capacity to upregulate TLR expression, in particular TLR2 in immune cells (517, 518) and TLR4 in HAEC (519). Moreover, TNFα is overproduced at atherosclerotic plaque sites and plays a crucial role in the development of early lesions (520). Therefore, it was evaluated whether a TNFα priming step would affect the response of HAEC to CKD-associated DAMPs. However, although TNFα was able to induce the release of both MCP-1 and IL-8 by HAEC (**Figure 11.A**), it did not potentiate the response to TLR ligands, either LPS or DAMPs (**Figure 11.B**). On the contrary, the modest response seen before to some of the DAMPs was lost when cells were pre-exposed to TNFα, although primed HAEC were still able to respond to LPS stimulation. This suggest that TNFα exposure initiated some negative feedback loop that reduced, but did not abrogate, TLR responsiveness to their ligands. Consequently, the modest DAMP-induced responses were lost while the more robust LPS-induced ones were still detectable.

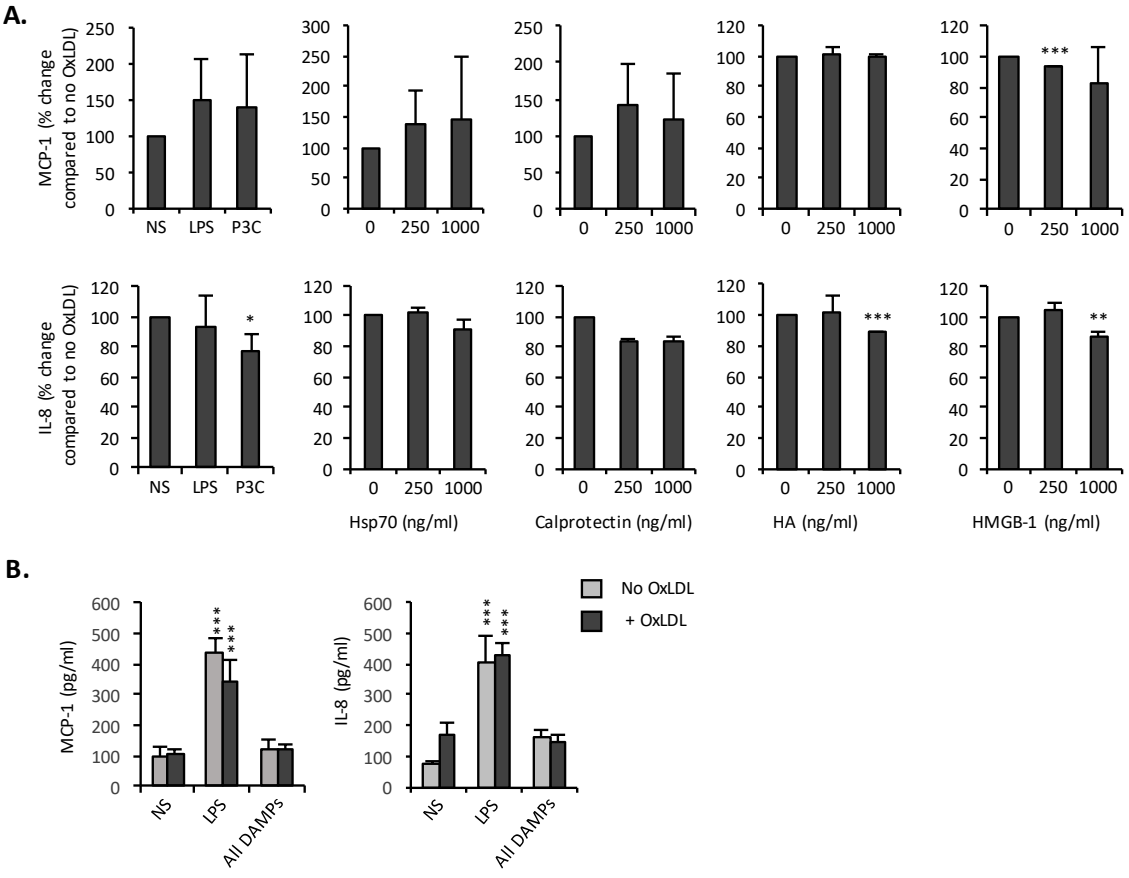


<u>Figure 11.</u> TNFα pre-exposure does not potentiate DAMPs-induced pro-atherogenic cytokines release by HAEC

Triplicate cultures of HAEC were pre-exposed (4 hours, **A and B**) or not (**A**) to TNFα (10 ng/ml), prior to stimulation (18 hours, **B**) or not (NS, **A and B**) with LPS (10 ng/ml) or the indicated concentrations of Hsp70, Calprotectin, HA, or HMGB-1 (**B**). Cytokine levels in culture supernatants are shown as mean +/- SD from 1 experiment.

*, p<0.05; **, p<0.01; ***, p<0.005, TNFα (**A**) or DAMPs (**B**) Stimulation vs no stimulation.

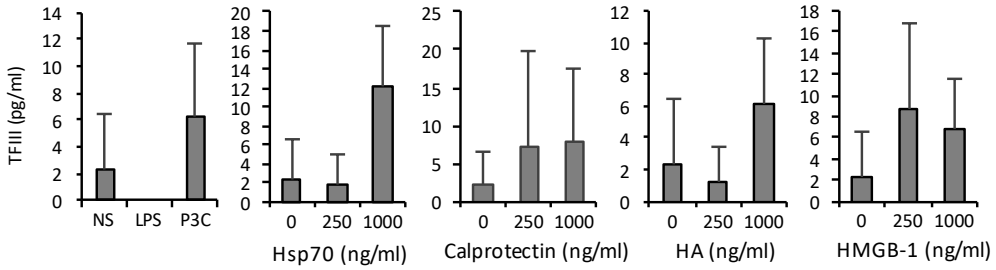
In the context of atherosclerosis, exposure to modified LDL also activates endothelial cells and may modify their inflammatory responses. Therefore, a possible potentiating effect of OxLDL on HAEC was also investigated. However, pre-exposure to OxLDL did not alter the DAMPs response profile (**Figure 12.A and B**).



<u>Figure 12.</u> Co-exposure to OxLDL does not increase DAMP-induced pro-inflammatory mediator production by HAEC

Triplicate cultures of HAEC were stimulated (18 hours) with LPS (10 ng/ml), Pam_3CSK_4 (P3C, 500 ng/ml) or the indicated concentrations (or 1 µg/ml, **B**) of Hsp70, Calprotectin, HA or HMGB-1, separately (**A**) or together (**B**) in the presence (black bars) or absence (light grey bars) of OxLDL (10 µg/ml). Cytokine levels in culture supernatants are shown as mean of % change in the presence vs the absence of OxLDL +/- SD from 3 independent experiments (**A**), or cytokine concentrations from 1 experiment (**B**).
*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; *, DAMP/LPS/P3C Stimulation vs no stimulation.

The release of Tissue Factor III (TFIII) by endothelial cells is a key initiator of the coagulation cascade (521), which can also contribute to vascular pathologies, notably the progression of atherosclerosis and thrombi. Therefore, the ability of CKD-associated DAMPs to induce TFIII release was investigated next. Basal expression levels of TFIII were either undetectable or very low and exposure of HAEC to the CKD-associated DAMPs did not lead to a significant increase in TFIII production (**Figure 13**).

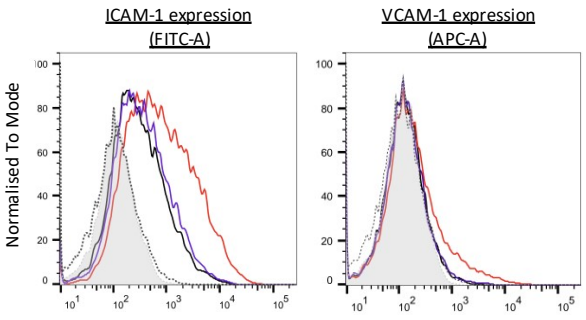


<u>Figure 13.</u> CKD-associated DAMPs do not induce Tissue Factor III (TFIII) release by HAEC
Triplicate cultures of HAEC were stimulated (18 hours) with LPS (10 ng/ml), Pam_3CSK_4 (P3C, 500 ng/ml) or the indicated concentrations of Hsp70, Calprotectin, HA, or HMGB-1. Cytokine levels in culture supernatants are shown as mean +/- SD from 1 experiment. TFIII was undetectable in a further 2 experiments.

III-2.3. Adhesion molecule expression by HAEC

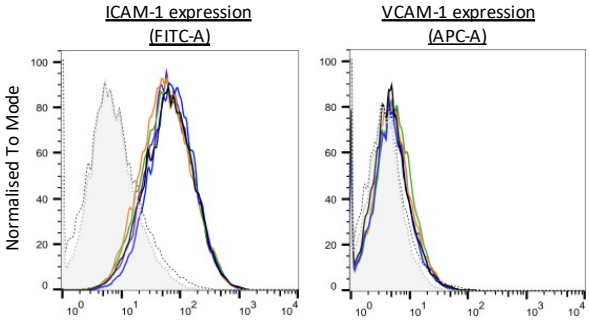
As an early step in the atherosclerotic process, modified LDL-activated endothelial cells upregulate surface expression of adhesion molecules to facilitate monocyte extravasation into the sub-endothelial space. VCAM-1 and ICAM-1 are major endothelial adhesion molecules of the Ig gene superfamily that participate in atherogenesis by promoting monocyte recruitment to the arterial intima (522), via interaction with α4β1 (e.g. VLA-4) and β2 (e.g. LFA-1: CD11a/CD18; Mac-1: CD11b/CD18) integrins on monocytes, respectively. ICAM-1 was expressed at moderate levels on the surface of resting HAEC, while VCAM-1 was undetectable (**Figure 14**). This is consistent with reports that ICAM-1 is expressed very broadly throughout the vasculature, in atherosclerotic plaques as well as in healthy vessels, while VCAM-1 expression is restricted to regions predisposed to atherosclerosis and at the periphery of established lesions (321). LPS, was able to increase both ICAM-1 and VCAM-1 expression but exposure to CKD-associated DAMPs, alone (**Figure 14.B**) or combined (**Figure 14.A**), did not.

A.



Samples / MFI	ICAM-1	VCAM-1
Unstained	151	154
Isotype control	184	179
Non-stimulated	641	195
LPS	2066	567
All DAMPs	757	219

B.



Samples / MFI	ICAM-1	VCAM-1
Unstained	11.4	4.65
Isotype control	13.3	6.07
Non-stimulated	100	7.02
Hsp70	106	6.82
Calprotectin	92	7.03
HA	96.6	7.21
HMGB-1	98.1	7.06

Figure 14. CKD-associated DAMPs do not affect adhesion molecules expression by HAEC
HAEC were stimulated or not (18 hours), with LPS (10 ng/ml, **A**); or 1 µg/ml of Hsp70, Calprotectin, HA, or HMGB-1, alone (**B**) or combined (**A**), and ICAM-1 and VCAM-1 surface expression levels were analysed by flow cytometry. Tables show the MFI values of at least 10,000 cells/condition for each receptor.

III-2.4. Atherosclerosis-related gene expression by HAEC

In order to gain a broader understanding of the potential effects of CKD-associated DAMPs on endothelial responses, an atherosclerosis-focused gene array was performed. Consistent with our previous results, exposure of HAEC to the CKD-associated DAMPs (combined) only had a very minor effect on the expression profile of genes typically associated with atherosclerosis (**Figure 15.A**). Out of 84 genes tested, only 2 were found significantly downregulated ($p<0.05$, fold change ≤ 0.5) following exposure to the DAMPs: Laminin Subunit Alpha 1 (*Lama1*) and Platelet-Derived Growth Factor Subunit B (*Pdgfb*). By contrast, stimulation with LPS led to a number of genes being significantly up (fold change ≥ 2) or down-regulated, confirming sensitivity of the cells to TLR stimulation (**Figure 15.B, Table 4**). This reduced effect of the DAMPs on gene expression by HAEC is in line with the very modest effect on cytokine production and lack of effect on TFIII production and adhesion molecule expression.

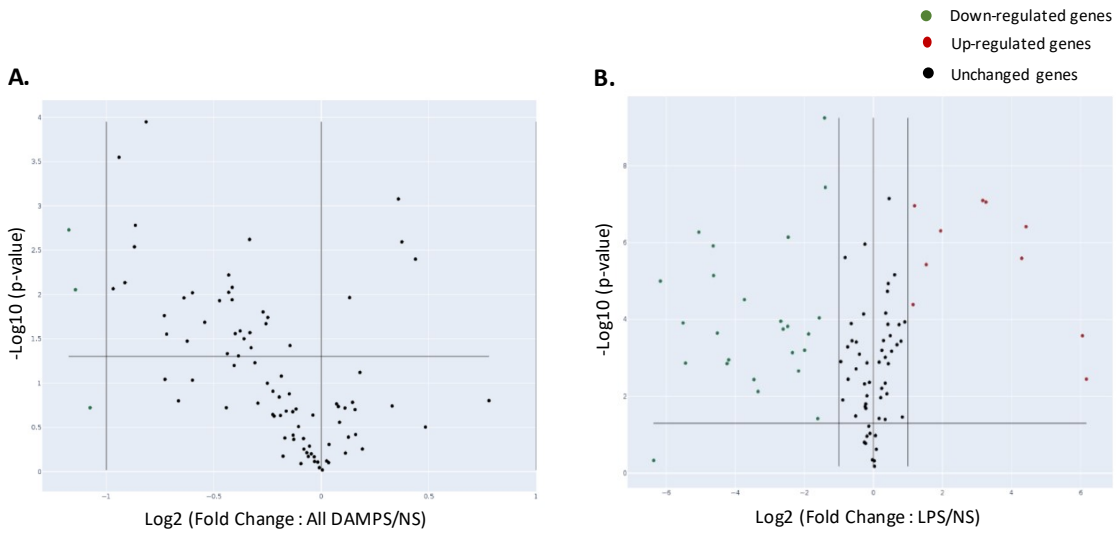


Figure 15. CKD-associated DAMPs do not robustly modulate atherosclerosis-related gene expression in HAEC

HAEC were stimulated (6 hours) with a combination of all DAMPs (1 µg/ml, **A**) or LPS (10 ng/ml, **B**) prior to atherosclerosis-focused gene array. Red (upregulated, fold change ≥ 2) and green (downregulated, fold change ≤ 0.5) circles represent single genes significantly affected ($p<0.05$, represented by the horizontal line) by the DAMPs (**A**), or LPS (**B**) treatment.

Table 4. Changes in atherosclerosis-related gene expression in HAEC stimulated with LPS for 6 hours*
*Only statistically significant (p<0.05) fold changes ≤ 0.5 (in green) or ≥ 2 (in red) are shown

Gene symbol	Description	Fold change	*p* value
Birc3	Baculoviral IAP repeat containing 3	9.56	*0.0001*
Ccl2	Chemokine (C-C motif) ligand 2	71.85	*0.0036*
Ccl5	Chemokine (C-C motif) ligand 5	2.28	*0.0001*
Csf1	Colony stimulating factor 1 (macrophage)	2.22	*0.0001*
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	21.38	*0.0001*
Icam1	Intercellular adhesion molecule 1	8.96	*0.0001*
Il1a	Interleukin 1, alpha	2.88	*0.0001*
Sele	Selectin E	66.35	*0.0003*
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	3.85	*0.0001*
Vcam1	Vascular cell adhesion molecule 1	19.60	*0.0001*
Ace	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	-5.60	*0.0002*
Ccr1	Chemokine (C-C motif) receptor 1	-22.97	*0.0002*
Ccr2	Chemokine (C-C motif) receptor 2	-18.91	*0.0014*
Col3a1	Collagen, type III, alpha 1	-1.20	*0.0048*
Eln	Elastin	-10.14	*0.0075*
Fga	Fibrinogen alpha chain	-13.33	*0.0001*
Ifng	Interferon, gamma	-33.27	*0.0001*
Il1r2	Interleukin 1 receptor, type II	-3.68	*0.0002*
Il2	Interleukin 2	-45.67	*0.0001*
Il3	Interleukin 3 (colony-stimulating factor, multiple)	-24.95	*0.0001*
Il4	Interleukin 4	-18.30	*0.0011*
Il5	Interleukin 5 (colony-stimulating factor, eosinophil)	-43.48	*0.0014*
Itgax	Integrin, alpha X (complement component 3 receptor 4 subunit)	-24.76	*0.0001*
Itgb2	Integrin, beta 2 (complement component 3 receptor 3 and 4	-5.54	*0.0001*
Lpa	Lipoprotein, Lp(a)	-72.01	*0.0001*
Lpl	Lipoprotein lipase	-3.98	*0.0006*
Mmp3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	-6.13	*0.0002*
Msr1	Macrophage scavenger receptor 1	-2.97	*0.0001*
Nos3	Nitric oxide synthase 3 (endothelial cell)	-2.67	*0.0001*
Npy	Neuropeptide Y	-3.06	*0.0381*
Pdgfrb	Platelet-derived growth factor receptor, beta polypeptide	-5.09	*0.0007*
Ptgs1	Prostaglandin-endoperoxide synthase 1	-2.63	*0.0001*
Spp1	Secreted phosphoprotein 1	-11.01	*0.0037*
Thbs4	Thrombospondin 4	-4.50	*0.0022*

III-2.5. Trans-endothelial resistance

A key function of endothelial cells is the maintenance of the endothelial barrier and the regulation of endothelium permeability. Increased vascular permeability promotes local inflammation and atherosclerosis development and occurs at a very early stage of the disease (523, 524). Using an *in vitro* model of trans-endothelial electrical resistance (TER) measurements previously described (525) we found that exposure of endothelial cells to our positive control LPS, Hsp70 or Calprotectin (**Figure 16.A**) or to a combination of CKD-associated DAMPs (**Figure 16.B**) resulted in a significant drop in TER, starting between 1 hour and 5 hours following exposure to DAMPs (**Figure 16.B**). This decrease was associated with a reduction in cell-to-cell contacts levels of Zonula occludens-1 (ZO-1), a key scaffold protein involved in endothelial tight junctions (525) (**Figure 16.C**). This suggests an overall loss in ZO-1 expression and/or ZO-1 relocation from the cell surface to the cytoplasm following DAMPs treatment, as previously reported (525) and consistent with the observed drop in TER.

Thus, while the CKD-associated DAMPs did not induce robust inflammatory responses by endothelial cells, they were capable of disturbing the endothelial barrier, a first step towards endothelial dysfunction, which in turn facilitates atherosclerosis development.

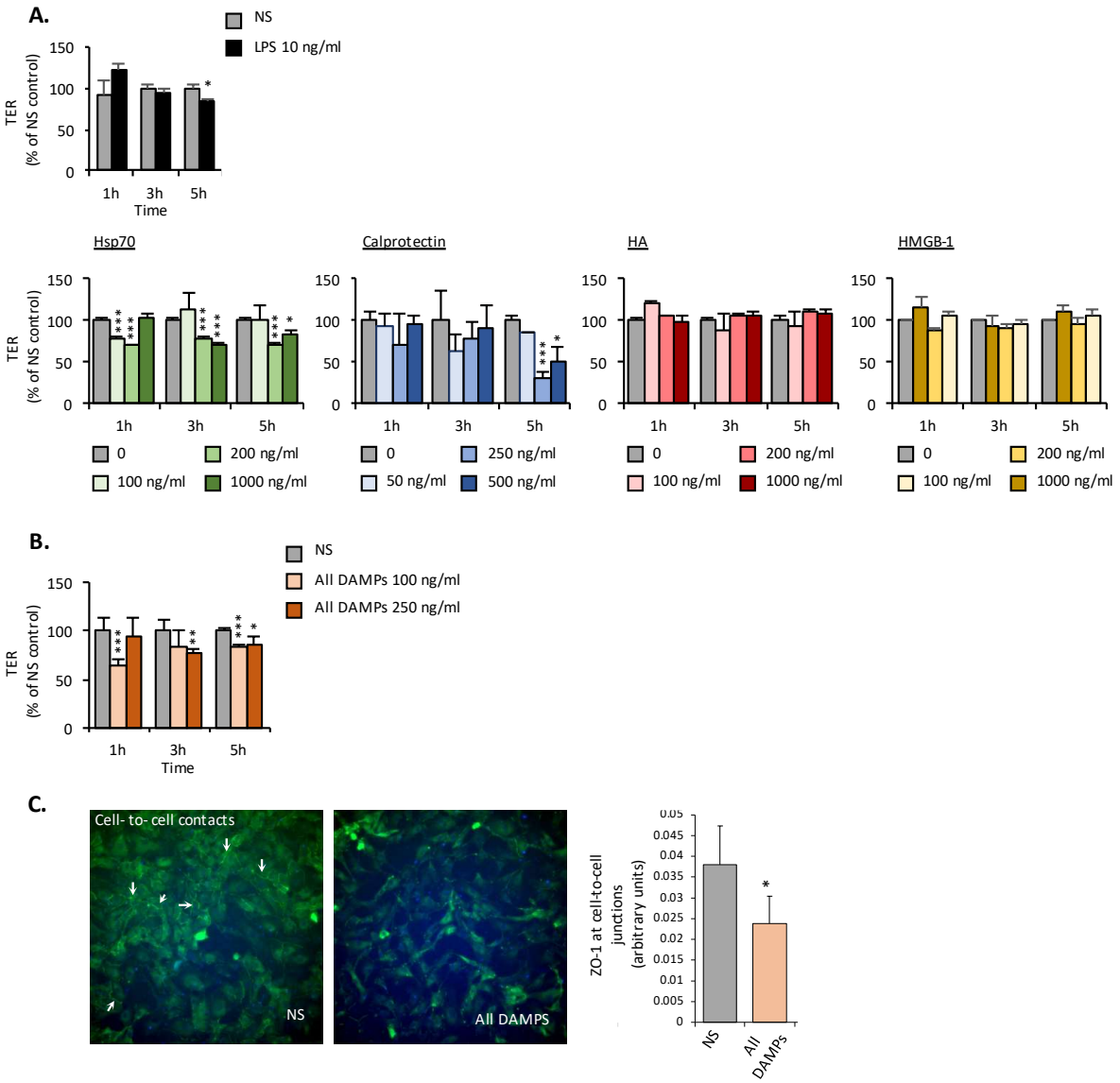


Figure 16. CKD-associated DAMPs disturb the endothelial barrier

Triplicate cultures of Human Umbilical Arterial Endothelial Cells (HUAEC) were grown to confluence prior to stimulation or not (NS) with LPS (10 ng/ml) or with single (**A**) or combined (**B and C**) CKD-associated DAMPs at the indicated concentrations (**A and B**) or 100 ng/ml (**C**). Trans-endothelial electrical resistance (TER) was measured at the indicated time points (**A and B**) and ZO-1 expression levels at cell-to-cell junctions (**C, arrows indicate examples**) were visualised and quantified 5 hours following DAMPs stimulation; Histograms show mean +/- SD from 3 independent experiments (**A and B**) or 5 independent fields of view (**C**).

*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$, Stimulation vs no stimulation.

III-2.6. Discussion

Endothelial cells have a complex and dual role in the function of the vascular system. They are able to actively control vascular permeability, coagulation, blood pressure and angiogenesis but also trigger and regulate inflammation (526, 527). In this Chapter, we demonstrate that *in vitro* exposure of aortic endothelial cells to Hsp70, Calprotectin or a combination of all 4 CKD-associated DAMPs led to a significant and immediate loss of trans-endothelial resistance. This is expected to lead to an increase in vascular permeability, which can be assessed *in vitro* by measuring dextran transport across the endothelial cell monolayer in a dual chamber system. Unfortunately, it was not possible to run these experiments due to time and cost limitations, especially since the model used here was developed in Heidelberg, Germany, which is where the experiments were performed. However, it is expected that the decrease in resistance will coincide with an increase in permeability, as widely accepted (528). Of note, the loss of endothelial resistance was of only transient, typically peaking at around 5 hours and coming back up to more normal levels at 24 hours. This is line with what has been described previously for this model (525) and is consistent with the fact that loss of vascular integrity is transient in the case of an acute inflammatory response (528). However, the endothelium of patients with CKD is chronically exposed to DAMPs and other inflammatory mediators, and chronic inflammation has been associated with sustained loss of resistance and increased permeability, which do not resolve spontaneously (528). Therefore, although this was not reproduced in our *in vitro* model, we speculate that continuous exposure to increased levels of DAMPs may lead to maintained loss of endothelial cell barrier and increased permeability in patients with CKD.

The responses of endothelial cells to CKD-associated DAMPs in terms of cytokine production and gene expression changes were found to be modest at best, or minimal. Explanations behind this observation likely include:

- Expected lower sensitivity of endothelial cells to TLR stimulation:

  As shown above (**Figure 9**), HAEC expression levels of TLRs were low overall. This result is in line with reports of low baseline expression of TLRs (150). In addition, the expression of TLR2 and TLR4 was found to be lower in endothelial cells originating from large vessels compared to the microvasculature (529, 530). Therefore, it is expected that TLR-mediated responses by aortic endothelial cells may be more modest than that of immune cells or endothelial cells of other origins, that express higher levels of TLRs. Of note, the TLR co-receptor CD14 was not detected in our HAEC model, also in line with previous reports (531, 532). *In vivo*, the lack of membrane CD14 on endothelial cells is compensated for by the presence of soluble CD14 in human plasma, released by cell types that express the membrane bound form. This was reproduced in our *in vitro* settings by the addition of 10% FCS, which may not match the physiological concentrations of soluble CD14 and therefore may not provide the optimal amount of CD14. However, HAEC were able to mount stronger responses, in terms of cytokine production and gene expression changes, to the purified bacterial TLR ligands LPS and P_3C. This suggests that TLR engagement by DAMPs may be counterbalanced by other DAMP receptors on HAEC (Introduction, Sections I-2.2 and I-2.3), although this issue was not investigated.

- Model limitations:

It is likely that the low number of HAEC used in these experiments may in part explain the low levels of cytokines produced following DAMP stimulation. For example, typical stimulation experiments were run with 5,000 HAEC/well of a 96-well plate, while 15,000 macrophages would be used for the same culture volume and area (Section III-3. Effect of CKD-associated DAMPs on atherosclerotic functions by monocytes/macrophages *in vitro*). This was dictated by the size of the cells. It is also relevant to mention that TLR2 levels have been shown to be increased in endothelial cells located in regions of disturbed flow (533), a condition that is difficult to reproduce *in vitro.* As mentioned above, endothelial cells of other origins may have responded better to DAMP stimulation. However, because atherosclerosis is most common the large arteries of adults, HAEC were selected over other models of endothelial cells, notably cells of venous or embryonic origins.

III-3. Effect of CKD-associated DAMPs on atherosclerotic functions by monocytes/macrophages *in vitro*

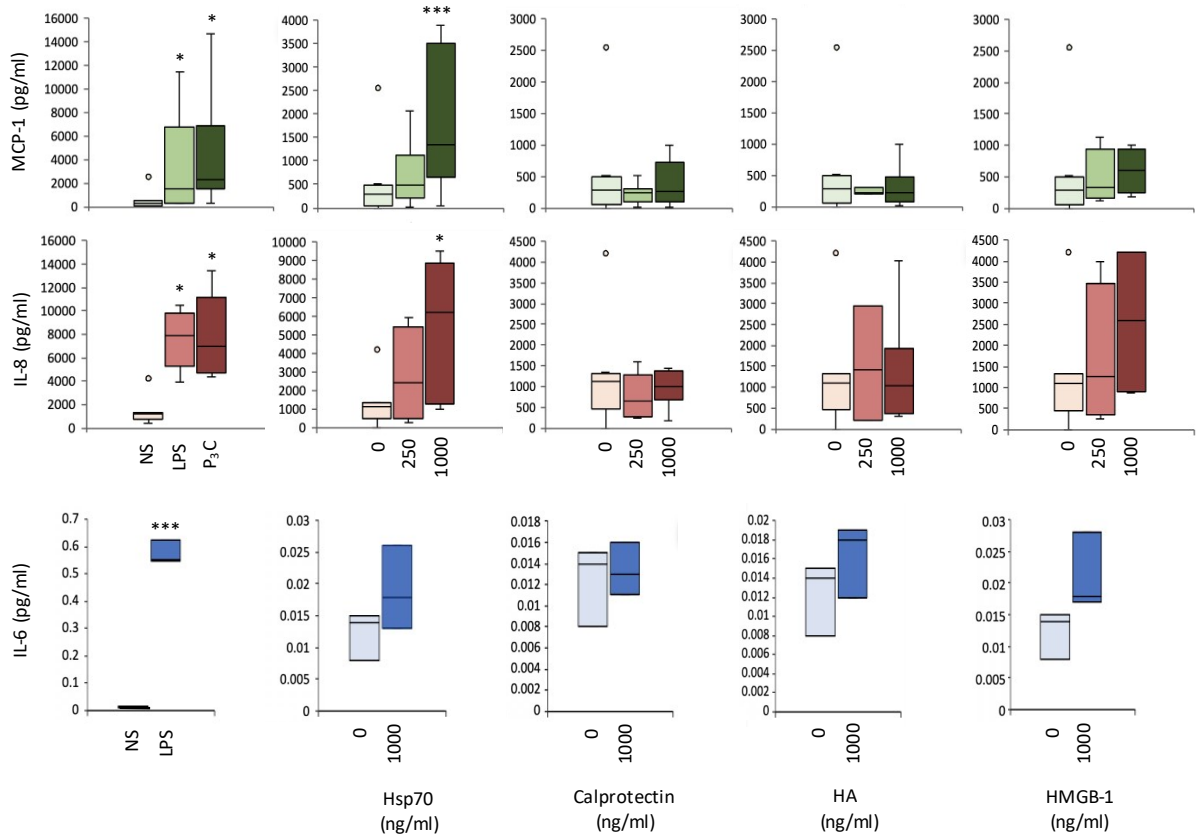
As described above (Introduction, Section I-3.1), monocytes are major players in atherosclerosis initiation and progression, as they are among the first immune cells to be recruited to the intima, where they differentiate into macrophages.

III-3.A. Monocytes

III-3.A.1. Atherogenic and inflammatory mediator production by monocytes

Activated monocytes secrete large amounts of pro-inflammatory mediators. Continued or repeated activation of blood monocytes is expected to induce or worsen the development of a chronic inflammatory state, which will ultimately promote CV disease (Introduction, Section I-3.1, p.51). In the case of monocytes newly recruited to the plaque site, production of chemoattractants will increase the recruitment of more monocytes or other immune cells which will contribute to atherosclerosis progression. Therefore, the ability of CKD-associated DAMPs to induce the release of pro-inflammatory and pro-atherosclerotic mediators by monocytes was investigated. Exposure to Hsp70 led to the significant release of MCP-1 and IL-8 by human peripheral mononuclear cells in a dose-dependent manner (**Figure 17**). This was true when pooling data from all donors (shown) and for all donors tested (individual donors not shown). When pooling data from all donors, Calprotectin, HA and HMGB-1 were not able to induce significant cytokine production although the response varied greatly with the donor tested, with these DAMPs inducing modest but significant mediator production in some donors but not others (data not shown). DAMPs exposure did not lead to the release of detectable levels of IL-6 although LPS stimulation did. This could in part be because the time point of culture surpernatants collection was not optimal for IL-6, which tends to be released somewhat later than IL-8 in our experience. Of note, Peripheral blood mononuclear cells (PBMCs), as opposed to purified monocytes were used in these experiments. However, we speculate that monocytes were the main source of cytokines as: i) the expression of TLRs by lymphocytes is minimal overall and ii) the mediators tested are not typically associated with lymphocyte responses. In addition, the conclusions drawn from these experiments regarding the overall effect of CKD-associated DAMPs on systemic pro-

inflammatory/atherogenic mediators' production would remain valid in the case of a contribution of lymphocytes towards the response.



<u>Figure 17.</u> Hsp70 induces pro-inflammatory mediator production by monocytes
Triplicate cultures of primary derived monocytes were stimulated (18 hours) with LPS (10 ng/ml), Pam_3CSK_4 (P3C, 500 ng/ml) or the indicated concentrations (or 1 µg/ml) of Hsp70, Calprotectin, HA, or HMGB-1 separately. Cytokine levels in culture supernatants are from 7 experiments (MCP-1, IL-8) or 3 (IL-6) performed with cells from different donors. Horizontal lines in boxes denote the median value. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$, Stimulation vs no stimulation.

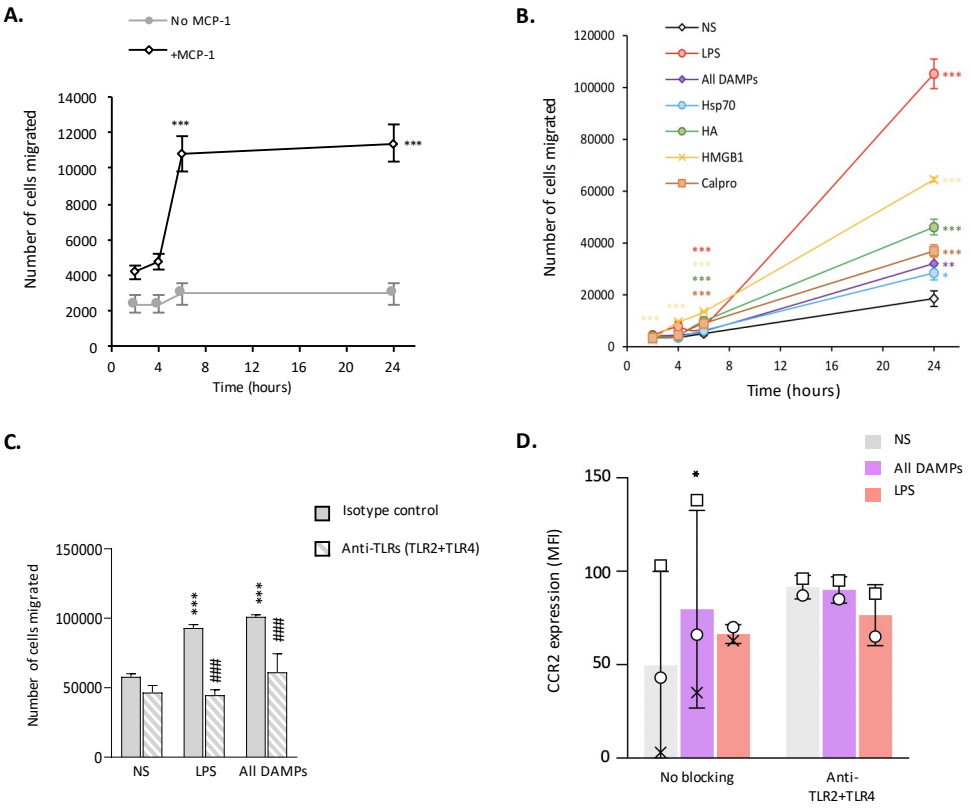
III-3.A.2. Monocyte migration

MCP-1 is the main monocyte chemoattractant expressed at the atherosclerotic plaque site. Therefore, the capacity of the 4 CKD-associated DAMPs to modulate the capacity of monocytes to migrate towards MCP-1 was evaluated next. Briefly, MonoMac 6 (MM6) monocytic cells were pre-stimulated with the DAMPs, alone or in combination, before seeding in the top chamber of a trans-well. MCP-1 was used as a chemoattractant in the bottom chamber. The number of monocytes in the bottom chamber was counted at the indicated time points (typically 2, 4, 6 and 24 hours). As expected, the number of cells migrated increased steadily until 24 hours, when MCP-1 was present in the bottom chamber, but not in its absence (**Figure 18.A**). As expected, MM6 pre-stimulation with LPS led to a dramatic increase in the number of cells migrated (**Figure 18.B**). In addition, pre-exposure to all CKD-associated DAMPs, either alone or in combination, also significantly increased the monocytic migratory capacity.

In order to confirm that DAMP-induced increase in monocyte migration was mediated by TLRs, blocking experiments with specific TLRs neutralising antibodies were performed. As the 4 CKD-associated DAMPs identified are TLR2 and/or TLR4 ligands, a combination of anti-TLR2 and anti-TLR4 antibodies was used. As expected, pre-exposure to the antibodies successfully inhibited the increase in migration induced by LPS. In addition, the combination of antibodies also inhibited almost completely the increase in monocyte migratory capacity induced by the CKD DAMPs (**Figure 18.C**), demonstrating that the effect of DAMPs on monocyte migration is mediated, in the most part, via TLR2/4 signaling.

Mechanistically, the DAMPs were found to upregulate the expression of the main MCP-1 receptor, C-C Motif Chemokine Receptor (CCR)2, and this effect was prevented by pre-exposure to a combination of blocking TLR2 and TLR4 antibodies (**Figure 18.D**). This is

consistent with our findings that monocyte migration is increased by CKD DAMPs in a TLR2/4 dependent manner.



<u>Figure 18.</u> CKD-associated DAMPs increase the migratory capacity of monocytes towards MCP-1 in a TLR dependent manner

Triplicate cultures of MonoMac6 monocytes were stimulated or not (18 hours, 37°C) with LPS (10 ng/ml) or the indicated DAMPs (1 µg/ml), alone or in combination, after pre-exposure (1 hour, 37°C) or not (**A and B**) to a combination of anti-TLR2 and anti-TLR4 blocking antibodies (5 µg/ml) or the relevant isotype control (10 µg/ml). **A-C.** Cells were then starved in serum-free medium for 1 hour prior to seeding (200,000 cells, in triplicates) in the top chamber of 8 μm pores transwells. The bottom compartment was filled with RPMI + 10% serum + MCP-1 (50 ng/ml) (A-**C**), or not (**A**, control experiment). Cell numbers were counted in the bottom compartment after 2 hours, 4 hours, 6 hours (**A and B**) and 24 hours (**A-C**). Results shown are mean +/- SD from 1 experiment representative of 7 experiments (**B**, LPS and all DAMPs combined) or 3 experiments (**B**, single DAMPs, **A, C and D**). *, p<0.5; **, p<0.01; ***, p<0.005; *, Stimulation vs no stimulation or #, anti-TLR antibodies vs isotype control, unpaired Student t-test. **D**. Cells were then analysed by flow cytometry for the levels of CCR2. Results are shown as MFI for at least 10,000 cells/condition from 2 or 3 independent experiments (open circles denote individual experiments). Identical symbols identify paired results. *, p<0.05, All DAMPs vs no stimulation (NS), paired Student t-test.

III-3.A.3. Adhesion molecules expression by monocytes

Monocyte migration to the inflamed intima involves notably: i) the rolling of monocytes along the endothelial cell surface, which occurs through interaction between P-selectin glycoprotein ligand 1 (PSGL-1) and selectins expressed by endothelial cells, ii) the initial firm binding of monocytes to endothelial cells promoted notably by the interaction between Mac-1 (CD11b/CD18) on monocytes and ICAM-1 on endothelial cells, iii) the stable arrest and subsequent transmigration of monocytes, partly mediated by integrin α4 and its binding to VCAM-1 (Introduction, Section I-3.1, p.54) (534, 535). Therefore, the effect of the CKD-associated DAMPs on the expression of integrins PSGL-1, CD11b and α4 by monocytes was investigated.

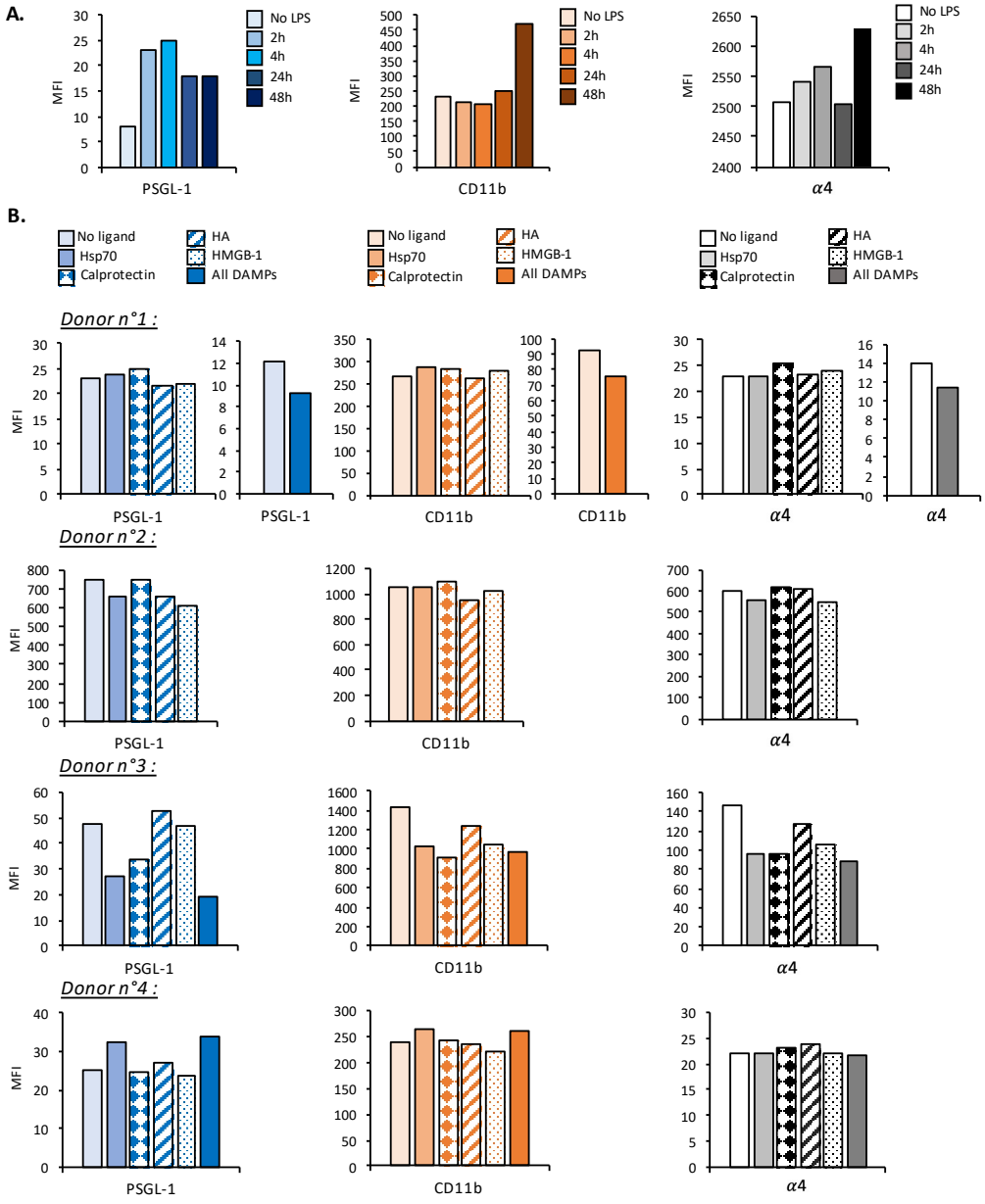


Figure 19. CKD-associated DAMPs do not robustly modulate adhesion molecules expression by primary monocytes

MM6 cells (**A**) or PBMC (**B**) were stimulated for the indicated duration (**A**) or 48 hours (**B**) with LPS (10 ng/ml, **A and B**) or 1 μg/ml of Hsp70, Calprotectin, HA or HMGB-1, alone or all DAMPs combined (**B**) prior to flow cytometry analysis for cell surface expression of CD11b, PSGL-1 and α4. Results are MFI of at least 10,000 cells/condition with subtracted background fluorescence.

To determine the best time-point at which to measure changes in expression in response to TLR triggering, a time course of LPS stimulation was carried out (**Figure 19.A**). Exposure of MM6 to LPS led to an increase in the expression of all adhesion molecules by monocytes (gated based on their FSC-SSC profile and expression of CD11b), but the timing of this effect varied. As the 3 adhesion molecules were found elevated after 48 hours of stimulation, this time point was selected to look at the effect of the DAMPs. Overall, there was no consistent and robust effect of DAMPs stimulation on adhesion molecule expression. Out of 4 monocyte donors tested, DAMPs stimulation did not lead to significant changes in expression of adhesion molecules in 2 donors (Donors 1 and 2, **Figure 19.B**), although there was a trend to slightly lower levels following stimulation with all DAMPs combined in Donor 1. Hsp70, Calprotectin and all CKD-DAMPs stimulation of Donor 3's cells led to a robust decrease in all 3 adhesion molecules, while HMGB-1 and HA had a more modest effect. In contrast, exposure to Hsp70 and all DAMPs combined led to the modest and very modest upregulation of PSGL-1 and CD11b, respectively in Donor 4. Therefore, the effect of CKD-associated DAMPs on adhesion molecule expression by monocytes appears to vary with the individual and the adhesion molecule tested. To avoid the confounding factor of donors' heterogeneity, these experiments were repeated in the monocytic cell line MM6. Exposure to DAMPs did not lead to significant changes in any of the 3 adhesion molecules tested in this model (**Figure 20**).

The lack of robust DAMPs effect is in apparent contradiction with the effect of TLR4 stimulation triggered by LPS (**Figure 19.A**). This highlights the fact that the resulting effect of TLRs ligation depends on the TLR ligand, as discussed previously (Results, I-1. p.75). It is also possible that the time point selected following LPS stimulation (48 hours) is not optimal to look at changes induced by DAMPs, because the signaling pathways involved will likely be, at

least in part, different. However, taking all results together we conclude that the effect of DAMPs on the expression of adhesion molecules, if any, will be very modest at most and is unlikely to affect the majority of the patients.

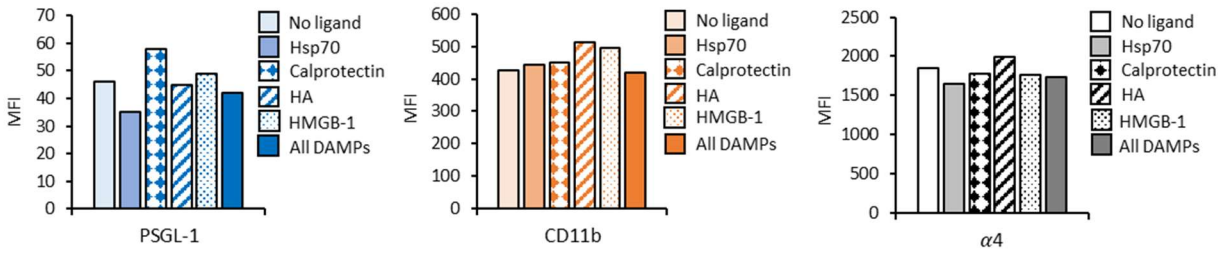


Figure 20. CKD-associated DAMPs do not robustly modulate adhesion molecules expression by a monocytic cell line
MM6 were stimulated for 48 hours with Hsp70, Calprotectin, HA or HMGB-1, alone or all DAMPs combined (1 μg/ml) prior to flow cytometry analysis for cell surface expression of PSGL-1, CD11b or α4. Results are MFI of at least 10,000 cells/condition with subtracted background fluorescence.

Thus, the CKD-associated DAMPs could induce pro-inflammatory mediators' production by monocytes and promote their chemokine-induced migration by increasing the expression of a chemoattractant receptor critical to atherosclerosis development, and without affecting their adhesion capacity.

III-3.B. Macrophages

III-3.B.1. Macrophage model selection

Tissue macrophages are typically heterogeneous and plastic populations, and this is also the case at the atherosclerotic plaque site (Introduction, Section I-3.2, p.58). Fully reproducing this heterogeneity *in vitro* is not possible, but to ensure the relevance of the findings described in this section, the appropriateness to evaluate our hypothesis of two *in vitro* models of macrophages was evaluated.

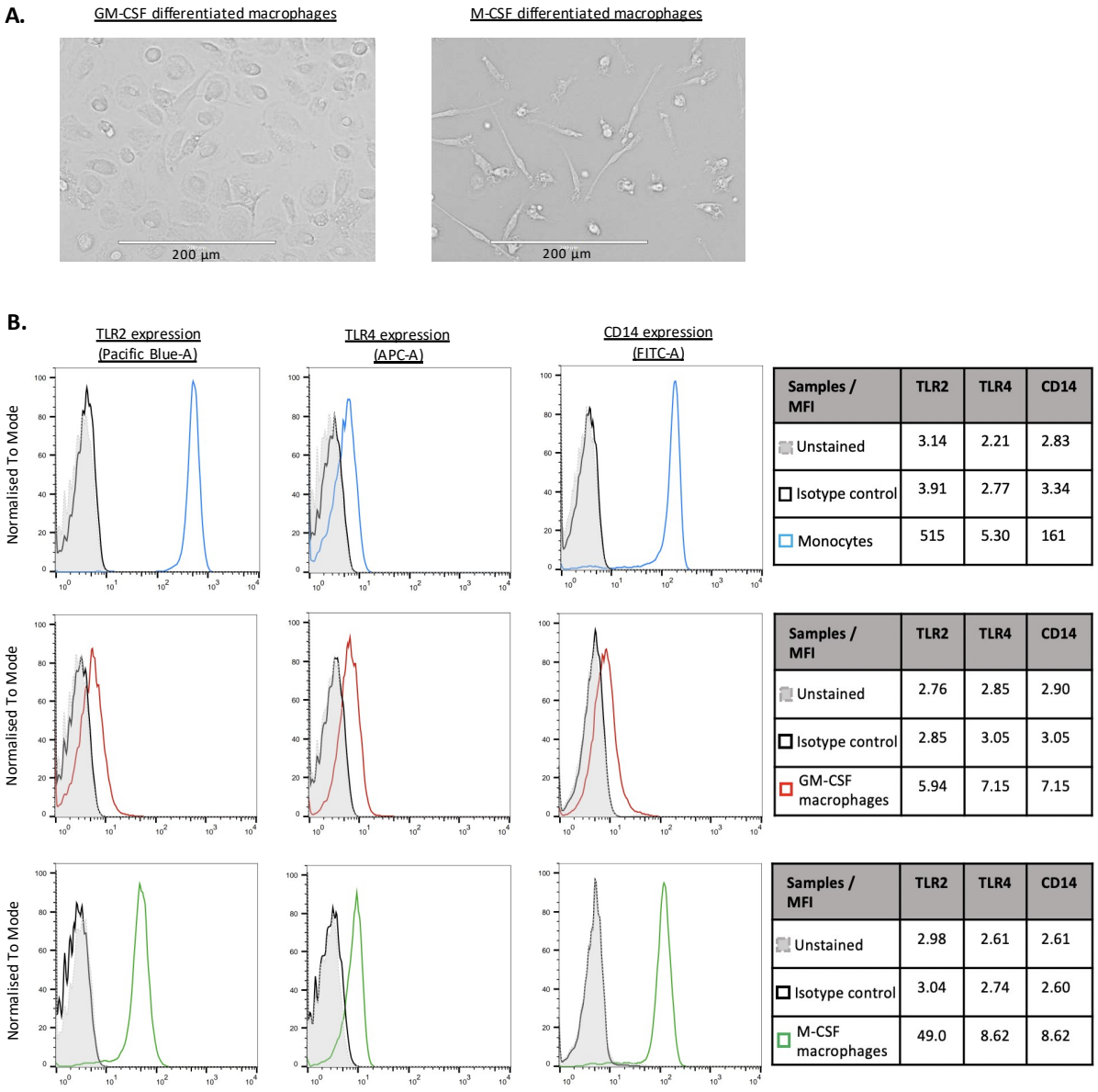
There is evidence that exposure of monocytes to GM-CSF leads to a M1-like phenotype while culture with M-CSF leads to M2-like characteristics (415, 441). Therefore, two models of primary monocyte-derived macrophages, differentiated by exposure to GM-CSF or M-CSF were characterised and compared.

a. Morphological features

Blood monocytes were obtained by adhesion of freshly isolated PBMC and differentiated into macrophages by addition of M-CSF (10 ng/ml) or GM-CSF (80 IU/ml). After 7 days of differentiation, cells were strongly adherent and exhibited a change from the monocytic morphology with either M-CSF or GM-CSF. Consistent with the literature (536, 537), microscopic examination revealed that GM-CSF-differentiated macrophages were round in shape with a big cytoplasm presenting inclusions (often referred to as “fried-egg shape”) while M-CSF-differentiated macrophages had a more elongated shape with less apparent cytoplasmic inclusions (**Figure 21.A**).

b. TLR expression

Given their critical relevance to this study, TLR2, TLR4 and CD14 expression by both types of macrophage was determined by flow cytometry. Both TLR2 and CD14 expression were found decreased in either M-CSF or GM-CSF-differentiated macrophages compared to monocytes, as expected (538). However, M-CSF macrophages were found to express higher levels of TLR2, TLR4 and CD14 than GM-CSF ones (**Figure 21.B**), which was unexpected and may appear inconsistent with the description of GM-CSF macrophages as an *in vitro* model for M1 – or pro-inflammatory – macrophages and M-CSF macrophages as M2 – or anti-inflammatory – macrophages. However, higher levels of TLR expression does not necessarily equate with higher pro-inflammatory responses, as TLRs activation also induces anti-inflammatory responses in a negative feedback mechanism. Therefore, differences in pro-inflammatory and pro-atherosclerotic responses between GM-CSF and M-CSF macrophages were evaluated next.



Samples / MFI	TLR2	TLR4	CD14
Unstained	3.14	2.21	2.83
Isotype control	3.91	2.77	3.34
Monocytes	515	5.30	161

Samples / MFI	TLR2	TLR4	CD14
Unstained	2.76	2.85	2.90
Isotype control	2.85	3.05	3.05
GM-CSF macrophages	5.94	7.15	7.15

Samples / MFI	TLR2	TLR4	CD14
Unstained	2.98	2.61	2.61
Isotype control	3.04	2.74	2.60
M-CSF macrophages	49.0	8.62	8.62

Figure 21. Characterisation of GM-CSF vs M-CSF-differentiated macrophages

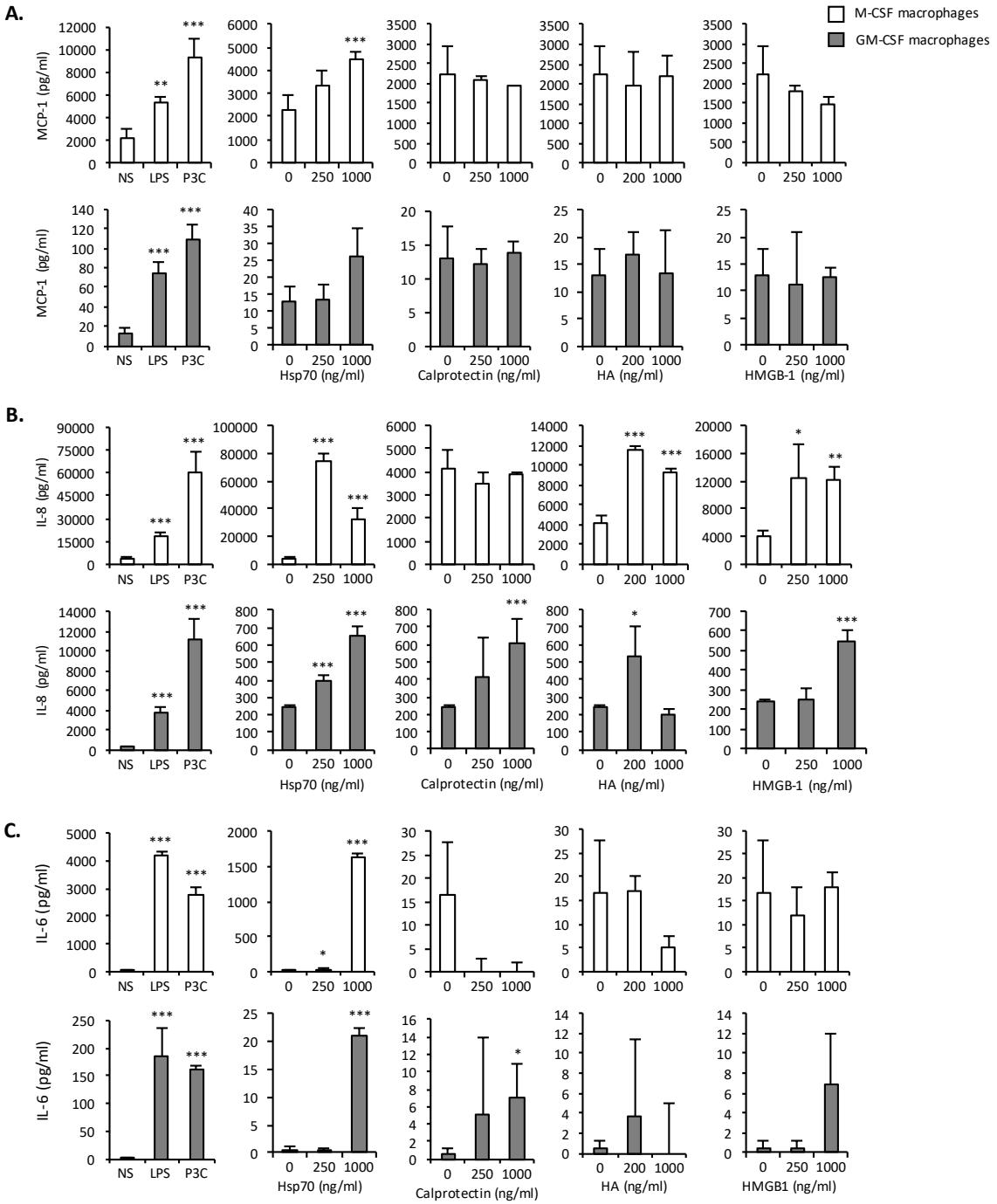
A. Light microscopy images of monocyte-derived macrophages differentiated with GM-CSF (80 IU/ml) or M-CSF (10 ng/ml) for 7 days. Pictures were taken with a 20x magnification. **B.** Monocytes (blue), GM-CSF (red) and M-CSF (green)-differentiated macrophages were analysed by flow cytometry for cell surface expression of TLR2, TLR4 and CD14 (unstained control in filled grey and isotype control in open black). Table shows the MFI values for each differentiation condition and each receptor. MFI were obtained from at least 10,000 cells/condition.

c. *Atherosclerosis-associated mediators production*

To evaluate potential differences in function between GM-CSF and M-CSF macrophages, both sets were differentiated from the same monocyte preparation before stimulation with TLR bacterial agonists (LPS and P_3C) or with CKD-associated DAMPs and the release of both pro- and anti-inflammatory cytokines known to play a role in atherosclerosis development was compared.

Of the 4 CKD-associated DAMPs, only Hsp70 was able to stimulate significantly the release of MCP-1 (**Figure 22.A**), IL-8 (**Figure 22.B**) and IL-6 (**Figure 22.C**) in both M-CSF (white histograms) and GM-CSF macrophages (grey histograms). This is consistent with the findings made in monocytes (Results, Section III-3.A.1, **Figure 17**). In addition to Hsp70, all 3, Calprotectin, HA and HMGB-1 also appeared to have the ability to induce the release of IL-8 (**Figure 22.B**), but not MCP-1 or IL-6. However, this effect was not reproduced in the 3 donors tested (not shown).

Of note, M-CSF-differentiated released much larger amounts (up to hundred times more) of every pro-inflammatory cytokine tested, not only after Hsp70 stimulation, but also in response to bacterial ligands LPS and P_3C. This suggests that M-CSF macrophages respond significantly more potently to TLR stimulation than GM-CSF macrophages, which is consistent with the higher TLR expression levels described in **Figure 21.B**.



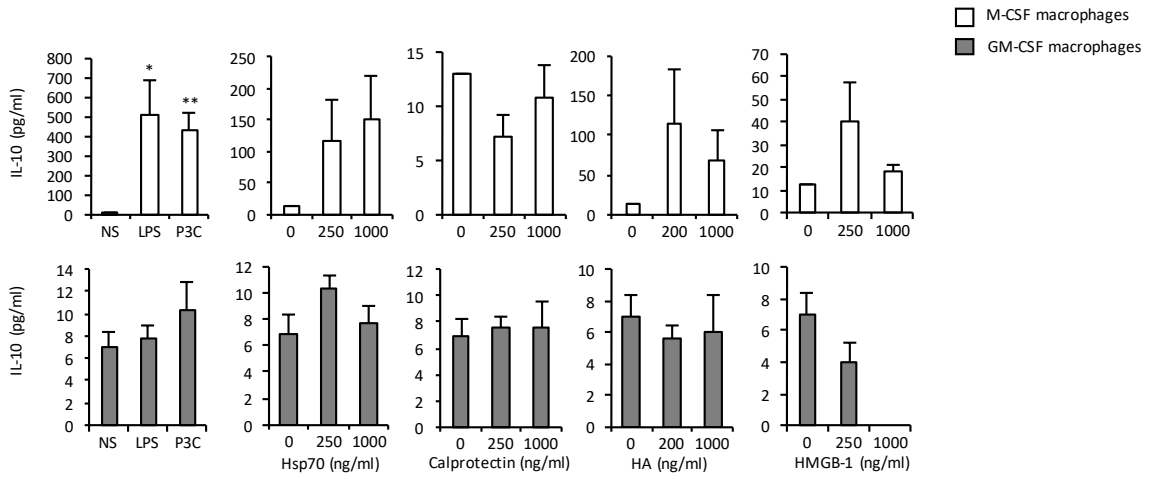
<u>Figure 22.</u> Comparison of DAMP-induced pro-atherogenic cytokine release by GM-CSF and M-CSF-derived macrophages

Triplicate cultures of GM-CSF or M-CSF macrophages were stimulated (18 hours), or not (NS), with LPS (10 ng/ml), Pam_3CSK_4 (P3C, 500 ng/ml) or the indicated concentrations of Hsp70, Calprotectin, HA, or HMGB-1.

Results shown are mean +/- SD from 1 experiment representative of at least 2 independent experiments (GM-CSF macrophages: IL-8 and MCP-1 n=5; IL-6 n=2; M-CSF macrophages: IL-8 and MCP-1 n=9; IL-6 n=2).

*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$, Stimulation vs no stimulation.

IL-10 is a potent anti-inflammatory cytokine described to be protective in atherosclerosis and believed to be produced mostly by M2-type macrophages (539, 540). M-CSF macrophages, but not GM-CSF macrophages, were able to release significant amounts of IL-10 in response to both TLR2 and TLR4 bacterial ligands as well as following stimulation with Hsp70 (**Figure 23**). This is in line with the reports that M-CSF differentiation induces a M2-like phenotype.



<u>Figure 23.</u> Comparison of DAMP-induced release of IL-10 by GM-CSF and M-CSF-derived macrophages

Triplicate cultures of GM-CSF or M-CSF macrophages were stimulated (18 hours) with LPS (10 ng/ml), Pam_3CSK_4 (P3C, 500 ng/ml) or the indicated concentrations of Hsp70, Calprotectin, HA, or HMGB-1. Results shown are mean +/- SEM from 3 independent experiments.

*, p<0.05; **, p<0.01; ***, p<0.005, Stimulation vs no stimulation.

d. Cholesterol uptake

Cholesterol handling by plaque macrophages has a key impact on the development of atherosclerosis. Notably, LDL uptake is a driving mechanism of the pathology. Therefore, it was important to compare GM-CSF and M-CSF-differentiated macrophages for their ability to uptake modified LDL. Scavenger receptors are one main route of modified LDL uptake, notably SR-A and CD36. Therefore, their expression in both types of macrophages was compared. While SR-A and CD36 were detected on both macrophage types, M-CSF macrophages expressed much higher levels of SR-A but significantly less CD36 than GM-CSF (**Figure 24.A**). The resulting effect of this differential expression on modified LDL uptake is therefore difficult to predict. Modified LDL uptake by both macrophage types was therefore quantitated next. Cells were starved in serum-free medium for 18 hours before incubation with increasing concentration of fluorescent (DiI) acetylated LDL (AcLDL) for 24 hours prior to quantify modified LDL uptake using flow cytometry. Both GM-CSF and M-CSF macrophages were able to uptake cholesterol in a dose-dependent manner to very similar extents (**Figure 24.B**). To confirm that the fluorescent signal observed is indeed from AcLDL uptake, rather than binding to the cell surface, the assay was repeated on ice (4°C), a temperature that allows binding but not internalisation. As significant cell death was likely to occur during a 24 hours' incubation on ice, the incubation time was reduced to 2 hours for the purpose of this experiment. Similar to the cells not incubated with AcLDL used as control, no increase in fluorescence was detected when incubation was performed at 4°C, as opposed to 37°C (**Figure 24.C**). This confirmed that the fluorescent signal observed is a result of LDL uptake, rather than surface binding.

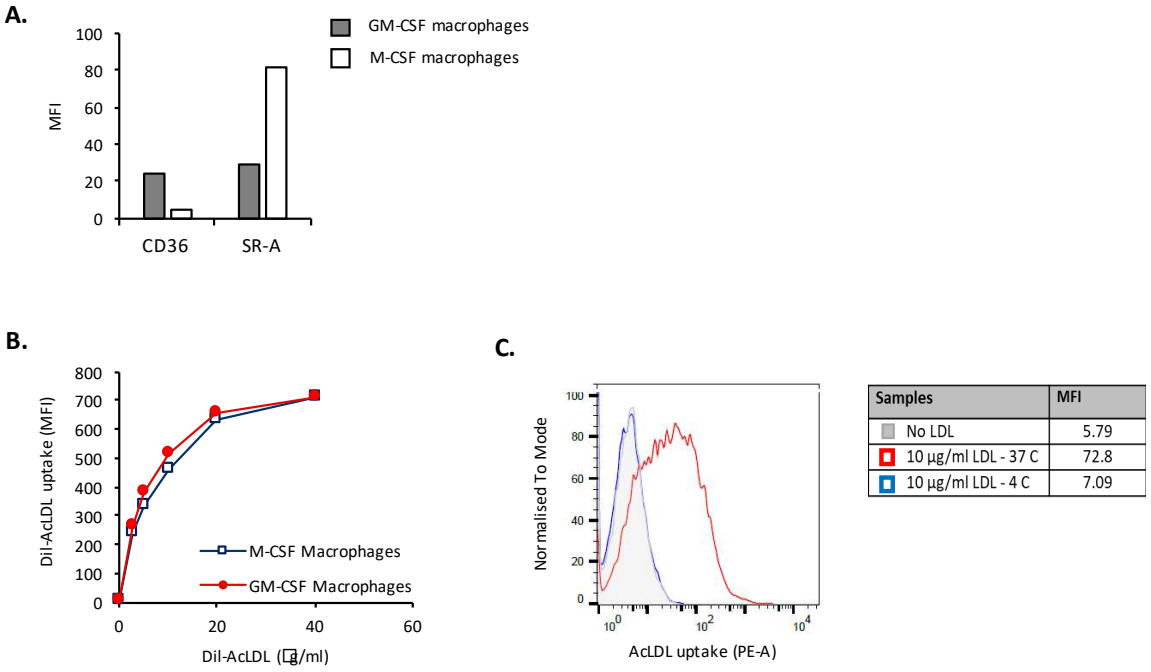


Figure 24. Comparison of modified LDL scavenger receptors expression and uptake by GM-CSF vs M-CSF-differentiated macrophages

A. Flow cytometry analysis of cell surface expression of scavenger receptors CD36 and SR-A in GM-CSF and M-CSF-differentiated macrophages (150,000 cells/condition). Results are MFI of at least 10,000 cells/condition. **B.** GM-CSF and M-CSF macrophages (40,000 cells/condition) were starved for 18 hours by culturing in serum free medium before addition of the indicated concentrations (**B**) or 10 µg/ml (**C**) of Dil-AcLDL. Phagocytosis was performed at 37°C for 24 hours (**B**) or for 2 hours at 4°C or 37 °C (**C**). Cells were then analysed by flow cytometry and results are shown as MFI of at least 10,000 cells/condition.

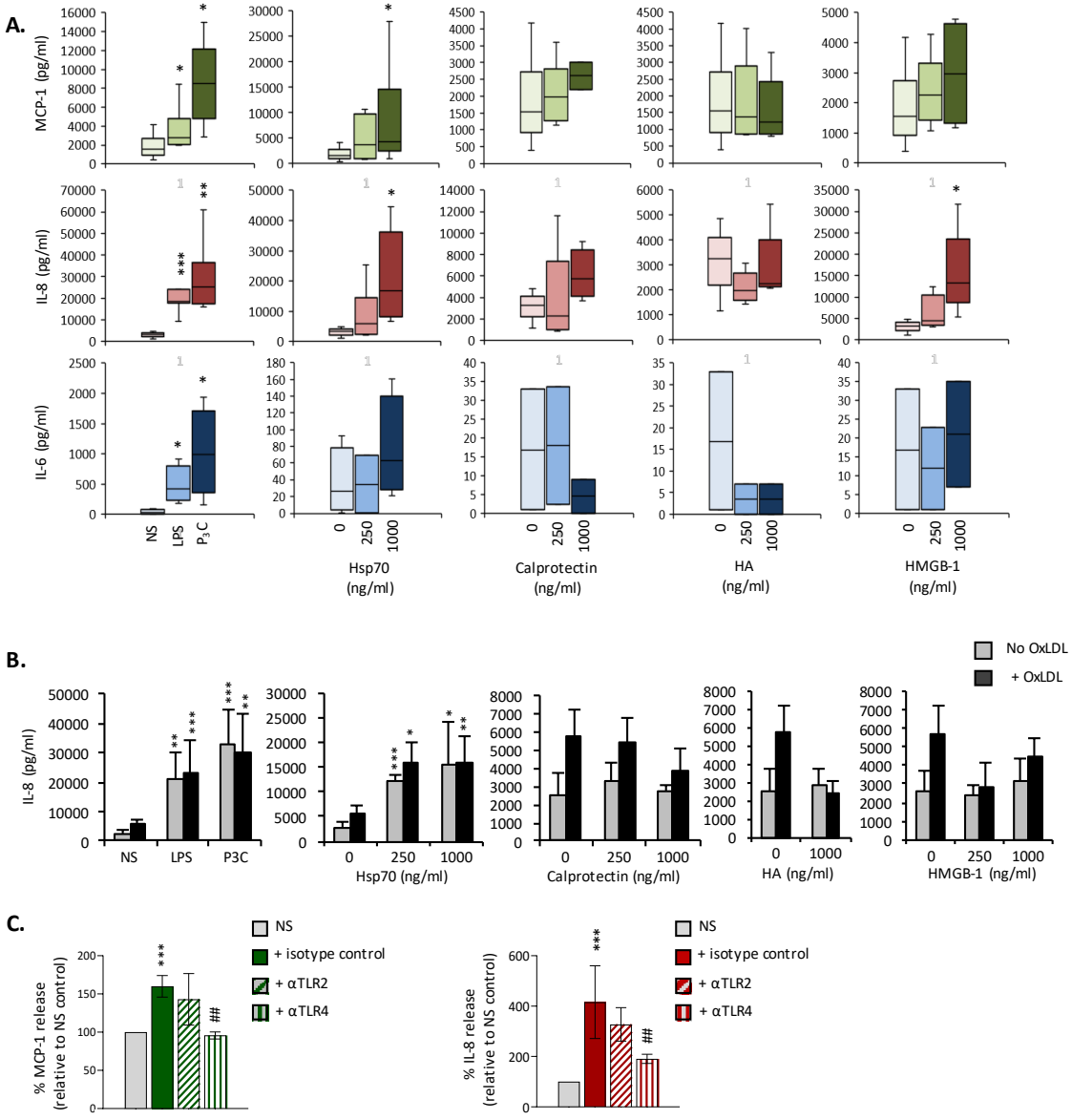
Altogether, the characterisation and comparison of the 2 *in vitro* macrophage models showed that: i) M-CSF-differentiated macrophages displayed higher expression of TLR2 and TLR4, the main TLR receptors for the 4 CKD-associated DAMPs identified, and of CD14, a critical TLR co-receptor; ii) M-CSF differentiated macrophages were more sensitive to stimulation with TLR bacterial ligands and Hsp70, and produced much higher levels of both pro- and anti-inflammatory/atherosclerotic mediators, and iii) M-CSF and GM-CSF macrophages were similarly able to uptake modified LDL, a key function in atherosclerosis

development. In addition, the role of M-CSF in plaque progression is very well described (431, 541, 542), confirming its relevance as a macrophage differentiation factor in atherosclerotic disease. Therefore, M-CSF-differentiated macrophages were selected as macrophage model for the remainder of the *in vitro* studies.

III-3.B.2. Atherogenic mediator production by macrophages

The ability of the CKD-associated DAMPs to induce pro-atherogenic cytokine production in M-CSF macrophages was further evaluated by testing more donors and evaluating the contribution of TLRs to these responses. As observed before, when looking at pooled data obtained from 6 different donors, only Hsp70 was able to stimulate the release of all 3 cytokines tested, although HMGB-1 could also induce the modest release of IL-8 (**Figure 25.A**). Of note, Hsp70-induced IL-6 production was not statistically significant when pooling all donors, because of large donor variability in background levels of IL-6 and responses to Hsp70. However, it was significant for 3 out of 6 donors tested (not shown). As observed before for endothelial cells, co-stimulation with OxLDL did not alter the cytokine response to the TLR agonists, either bacterial or the DAMPs (**Figure 25.B**).

In addition to TLR2 and TLR4, other non-TLR receptors for extracellular Hsp70 have been reported (Introduction, Section I-2.2, p.31). Therefore, to assess the contribution of TLRs to the Hsp70-induced release of pro-atherosclerotic mediators by macrophages observed here, specific blocking experiments were carried out using neutralising antibodies for TLR2 and TLR4. Hsp70-induced MCP-1 and IL-8 release was almost completely abrogated by co-stimulation with the anti-TLR4 antibody, while it remained unaffected by the blocking of TLR2, indicating that TLR4 mediates most of the Hsp-70-induced release of pro-atherosclerotic mediators while TLR2 does not appear to be significantly involved (**Figure 25.C**).



<u>Figure 25.</u> Hsp70 induces pro-inflammatory mediator production by macrophages in a TLR4-dependent manner

A-B. Triplicate cultures of M-CSF-differentiated macrophages were stimulated (18 hours, 37°C) in the absence (**A and B**), or presence (**B**) of OxLDL (10 μg/ml) with LPS (10 ng/ml), Pam_3CSK_4 (P3C, 500 ng/ml) or the indicated concentrations of Hsp70, Calprotectin, HA or HMGB-1. **C.** Triplicate cultures of macrophages were pre-exposed (1 hour, 37°C) to anti-TLR2 or anti-TLR4 blocking antibodies or the relevant isotype control (10 µg/ml) prior to stimulation (18 hours, 37°C) with Hsp70 (1000 ng/ml).
Results shown in **A** are from 6 experiments performed with cells from different donors. Horizontal lines in boxes denote the median value. Results shown in **B and C** are the results of 3 experiments (+/- SEM) performed with cells from different donors.
*, p<0.05; **, p<0.01; ***, p<0.005. (**A and B**) Stimulation vs no stimulation (**C**) *, stimulation vs no stimulation; #, anti-TLR2/TLR vs isotype control.

III-3.B.3. Atherosclerosis-related gene expression by macrophages

To further assess the range of Hsp70's atherosclerosis-related effects, an atherosclerosis-focused gene array was performed following macrophages stimulation (or not) with Hsp70. Out of the 84 genes tested, 56 were found significantly upregulated (fold change ≥ 2, $p \leq 0.05$) by treatment of macrophages with Hsp70 (**Figure 26 and Table 5,** genes indicated in red). They were involved not only in inflammatory cytokine/chemokine production (e.g. *Nfkb1*) but also in leukocyte recruitment (e.g., *Mcp-1*, *Chemokine C-C motif ligand (Ccl) 5*), monocyte to macrophage differentiation (e.g. *M-csf, Gm-csf*), macrophages retention in the plaque, lipid metabolism (e.g. *Apoe, Ldlr*), cell death (e.g. *B-cell lymphoma (Bcl) 2*, *Fas*) and matrix remodeling (e.g. *Mmp1, Mmp3*). In addition, 6 genes were found significantly downregulated (fold change ≤ 0.5, $p \leq 0.05$, genes indicated in green) by Hsp70, notably *Pparg,* a receptor whose activation leads to potent anti-atherosclerotic responses and *TgfB2*, a well-described anti-inflammatory mediator which can also promote an M2 macrophage phenotype (543), suggesting that stimulation with Hsp70 can lead to the downregulation of anti-atherosclerotic pathways. These results also indicate that Hsp70 does not only affect cytokines production but also potentially a wide variety of functions associated with atherosclerosis.

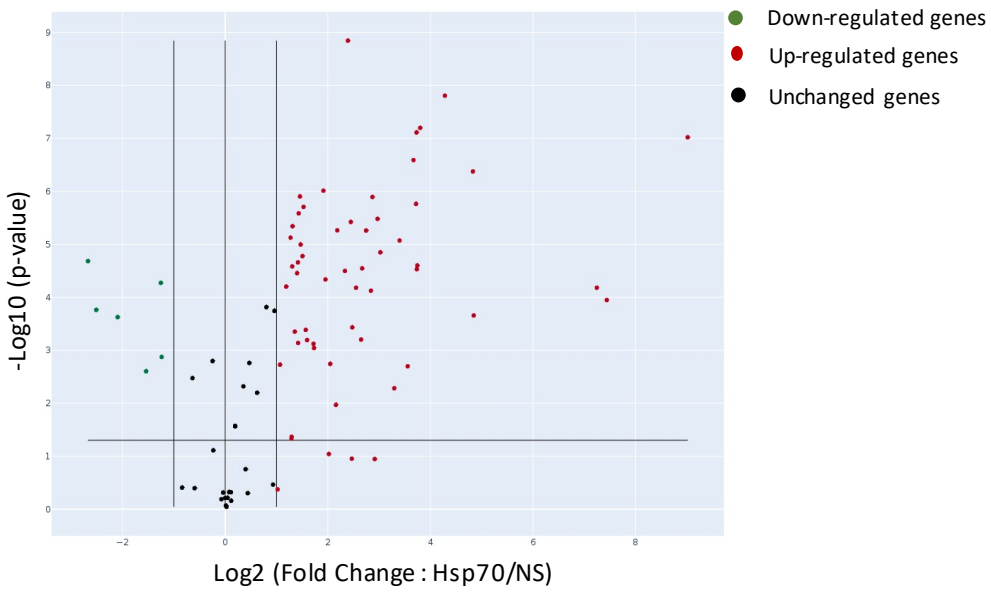


Figure 26. Hsp70 modulates the expression of a wide range of atherosclerosis-related genes by macrophages

M-CSF-differentiated macrophages were stimulated (18 hours) with Hsp70 (1,000 ng/ml) prior to RNA extraction and atherosclerosis-focused gene array (RT-qPCR analysis). Red (upregulated, fold change ≥ 2) and green (downregulated, fold change ≤ 0.5) circles represent single genes significantly affected (p<0.05, represented by the horizontal line) by the Hsp70 treatment.

Table 5. Changes in atherosclerosis-related gene expression in macrophages stimulated with Hsp70 for 18 hours. *
*Only statistically significant (p<0.05) fold changes ≤ 0.5 (in green) or ≥ 2 (in red) are shown.

Gene symbol	Description	Fold change	*p* value
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	**3.30**	*0.0008*
Ace	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	**2.56**	*0.0004*
Apoe	Apolipoprotein E	**2.74**	*0.0001*
Bax	BCL2-associated X protein	**2.77**	*0.0001*
Bcl2	B-cell CLL/lymphoma 2	**7.16**	*0.0001*
Bcl2a1	BCL2-related protein A1	**3.02**	*0.0006*
Bcl2l1	BCL2-like 1	**2.10**	*0.0019*
Bid	BH3 interacting domain death agonist	**7.30**	*0.0001*
Birc3	Baculoviral IAP repeat containing 3	**13.31**	*0.0001*
Ccl2	Chemokine (C-C motif) ligand 2	**28.38**	*0.0001*
Ccl5	Chemokine (C-C motif) ligand 5	**516.28**	*0.0001*
Ccr1	Chemokine (C-C motif) receptor 1	**5.45**	*0.0001*
Ccr2	Chemokine (C-C motif) receptor 2	**2.45**	*0.0464*
Cd44	CD44 molecule (Indian blood group)	**12.72**	*0.0001*
Cdh5	Cadherin 5, type 2 (vascular endothelium)	**8.13**	*0.0001*
Cflar	CASP8 and FADD-like apoptosis regulator	**10.55**	*0.0001*
Csf1	Colony stimulating factor 1 (macrophage)	**2.88**	*0.0001*
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	**2.45**	*0.0430*
Eng	Endoglin	**2.48**	*0.0001*
Fas	Fas (TNF receptor superfamily, member 6)	**13.15**	*0.0001*
Fgf2	Fibroblast growth factor 2 (basic)	**5.53**	*0.1115*
Fn1	Fibronectin 1	**2.47**	*0.0001*
Icam1	Intercellular adhesion molecule 1	**13.93**	*0.0001*
Ifnar2	Interferon (alpha, beta and omega) receptor 2	**3.32**	*0.0009*
Il1a	Interleukin 1, alpha	**151.38**	*0.0001*
Il1r2	Interleukin 1 receptor, type II	**5.04**	*0.0001*
Il4	Interleukin 4	**2.03**	*0.4233*
Il5	Interleukin 5 (colony-stimulating factor, eosinophil)	**7.54**	*0.1133*
Itga2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	**5.25**	*0.0001*
Itga5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	**6.27**	*0.0006*
Itgb2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	**2.97**	*0.0004*
Kdr	Kinase insert domain receptor (a type III receptor tyrosine kinase)	**4.06**	*0.0911*
Ldlr	Low density lipoprotein receptor	**5.56**	*0.0004*
Lif	Leukemia inhibitory factor (cholinergic differentiation factor)	**2.68**	*0.0007*
Lpl	Lipoprotein lipase	**2.42**	*0.0001*
Mmp1	Matrix metallopeptidase 1 (interstitial collagenase)	**173.22**	*0.0001*
Mmp3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	**4.46**	*0.0108*
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	**4.13**	*0.0018*
Nr1h3	Nuclear receptor subfamily 1, group H, member 3	**6.70**	*0.0001*
Pdgfa	Platelet-derived growth factor alpha polypeptide	**13.25**	*0.0001*
Pdgfb	Platelet-derived growth factor beta polypeptide	**5.85**	*0.0001*
Pdgfrb	Platelet-derived growth factor receptor, beta polypeptide	**9.81**	*0.0052*
Ppard	Peroxisome proliferator-activated receptor delta	**3.87**	*0.0001*

Gene symbol	Description	Fold change	*p* value
Sell	Selectin L	**11.76**	*0.0020*
Serpine1	Serpin peptidase inhibitor, clade E, member 1	**4.55**	*0.0001*
Spp1	Secreted phosphoprotein 1	**2.67**	*0.0001*
Tgfb1	Transforming growth factor, beta 1	**2.70**	*0.0001*
Tnc	Tenascin C	**2.64**	*0.0001*
Tnf	Tumor necrosis factor	**28.70**	*0.0002*
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	**19.45**	*0.0001*
Vcam1	Vascular cell adhesion molecule 1	**13.40**	*0.0001*
Vegfa	Vascular endothelial growth factor A	**6.36**	*0.0001*
Col3a1	Collagen, type III, alpha 1	**-2.91**	*0.0025*
Egr1	Early growth response 1	**-2.39**	*0.0001*
Il1r1	Interleukin 1 receptor, type I	**-6.39**	*0.0001*
Pparg	Peroxisome proliferator-activated receptor gamma	**-2.36**	*0.0013*
Tgfb2	Transforming growth factor, beta 2	**-5.71**	*0.0002*
Thbs4	Thrombospondin 4	**-4.28**	*0.0002*

III-3.B.4. Foam cells formation by macrophages

Foam cells are disease macrophages unique to the atherosclerotic plaque, which become engorged with cholesterol. Foam cells formation is the result of excessive intake and/or reduced efflux of cholesterol. As described in the Introduction (Section I-3.1, p.56), the transition from macrophage to foam cell is a hallmark of the atherosclerosis process and foam cells are major contributors towards disease progression. Therefore, the effect of CKD-associated DAMPs on foam cell formation, modified LDL uptake and cholesterol efflux was investigated.

a. Foam cell formation

To induce foam cell formation, macrophages were exposed to large amounts of LDL (25 μg/ml) for 24 hours, in the presence or absence of CKD-associated DAMPs, prior to staining of intracellular neutral lipids with Oil Red-O followed by microscopy analysis, as previously described (544). As a measure of foam cell formation, the percentage of foam cells

was determined for each condition. To avoid experimenter's bias, the first 20 cells from the top left corner of each picture were selected for analysis. Cells with detectable intracellular red staining were considered foam cells, while cells without staining were considered normal macrophages. The process was repeated with the 5 pictures taken for each condition (total of 100 cells counted/condition). Exposure to LDL alone resulted in a modest rise in the number of foam cells which was significantly increased by co-treatment with LPS (**Figure 27.A**). Co-exposure of macrophages to LDL and CKD-associated DAMPs, either alone or in combination, also led to a robust increase in foam cell formation compared to LDL exposure alone (**Figure 27.B**). Although this remains to be confirmed in a higher number of donors, stimulation with all DAMPs combined did not appear to increase foam cells formation significantly more than most individual DAMPs (Donor 1). If confirmed, this may suggest that i) TLR triggering can only increase foam cells formation to a certain extent, which cannot be exceeded with further triggering and/or ii) the amount of LDL used here cannot induce foam cells formation past the observed threshold.

TLR involvement in the CKD-DAMPs promotion of foam cell formation was assessed by specific TLR2 and TLR4 blocking experiments using neutralising antibodies, which revealed that the increase in foam cell formation induced by the CKD-DAMPs was mediated by TLR2 and TLR4, the latter having seemingly a bigger involvement (**Figure 27.C**).

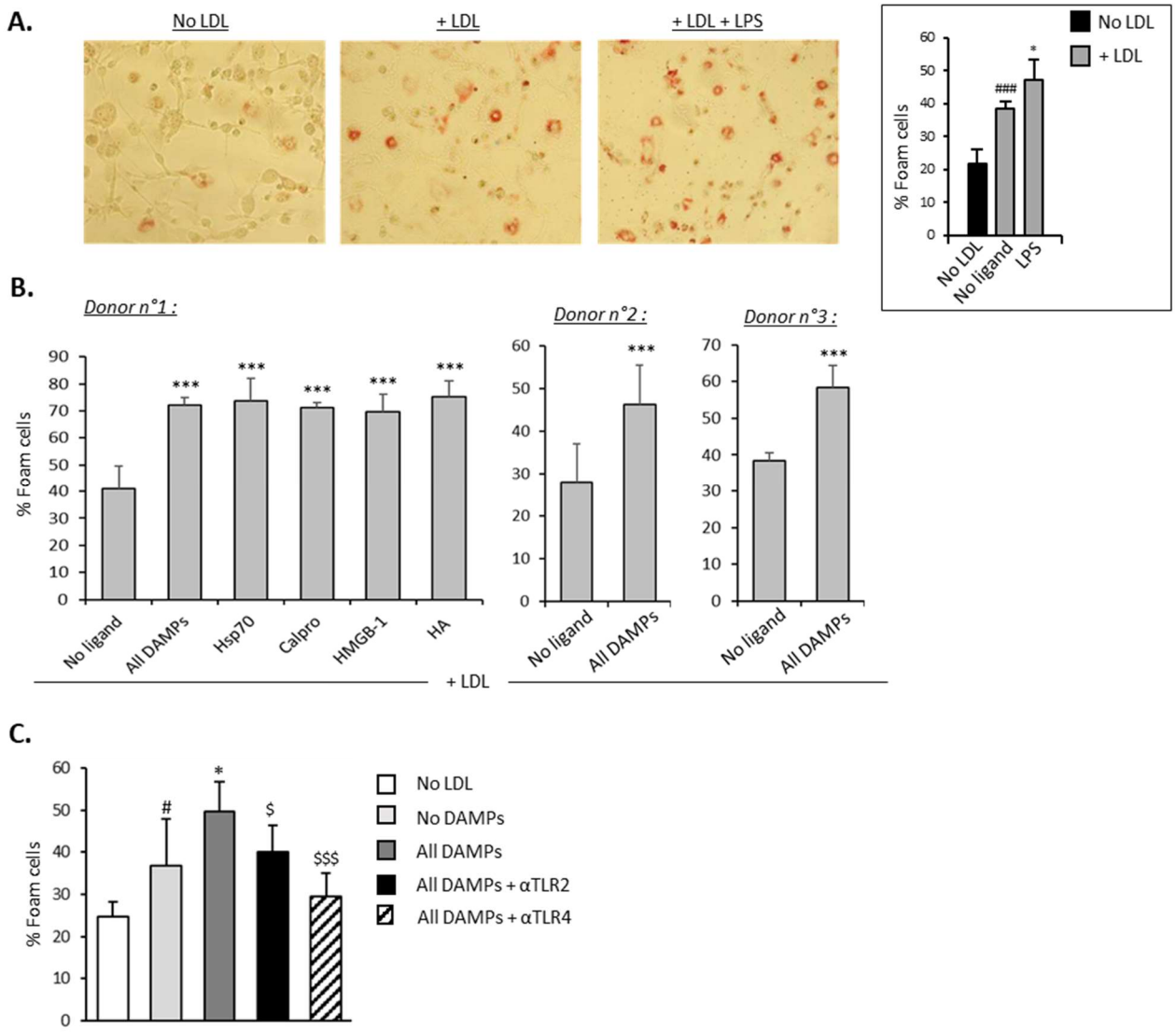


Figure 27. Effect of CKD-associated DAMPs on foam cell formation and cholesterol handling by macrophages

A-C. Primary monocyte M-CSF-derived macrophages were exposed (24 hours) to LDL (25 µg/ml) in the presence or absence of LPS (10 ng/ml, **A and B**) or the indicated DAMPs (**B**), alone or in combination (1 µg/ml), and of the indicated anti-TLR blocking antibodies (10 µg/ml, **C**) prior to staining with Oil Red-O for lipid visualisation by light microscopy (representative images shown). Histograms show the percentage of foam cells in each condition. Results are mean +/- SD run with macrophages prepared from 3 different donors.

#, $p<0.05$; ###, $p<0.005$, LDL vs no LDL.

*, $p<0.05$; ***, $p<0.005$, LPS or DAMP stimulation vs no ligand.

$, $p<0.05$; $$$, $p<0.005$, αTLR antibodies vs LDL+DAMPs stimulation.

Foam cells formation is the result of excessive intake and reduced efflux of cholesterol. Therefore, the effect of the CKD-associated DAMPs on these macrophage functions was evaluated to investigate the mechanisms behind their ability to increase foam cells formation.

b. Modified LDL uptake and scavenger receptors expression by macrophages

A potential effect of CKD-associated DAMPs effect on AcLDL uptake was analysed by flow cytometry as described above (**Figure 24.B**). Based on the titration shown in **Figure 24.B**, a suboptimal concentration of 10 µg/ml of Dil-AcLDL was selected for these experiments, to allow for the detection of potential up or down modulation of AcLDL uptake following DAMPs treatment. Macrophages were starved in medium supplemented with 0.2% fatty-acid free BSA (18 hours) and pre-exposed, or not, to the 4 CKD-associated DAMPS identified, prior to 24 hours' incubation with Dil-AcLDL (10 µg/ml). When DAMPs were added in macrophages culture during the starvation step, and although each batch of macrophages responded slightly differently, repeated experiments carried out with macrophages isolated from 4 different donors indicated that most DAMPs, apart from HA and as opposed to LPS, induced a slight reduction in AcLDL uptake. This effect was significant for HMGB-1 or a combination of all DAMPs (**Figure 28.A**). Given that the combination of all DAMPs did not lead to a stronger inhibition than HMGB-1 alone, and the fact that other single DAMP failed to show a significant effect on AcLDL uptake, we speculate that the effect observed with all DAMPs is mostly due to the activity of HMGB-1. Although a reduction of around 10% in AcLDL uptake may not seem biologically relevant, this value is in within the range of what is regarded as noteworthy in published findings (545). It is important to consider that, while our experiments run over a short period of time, CKD patients' macrophages are exposed to DAMPs for months or years,

and even a moderate reduction in AcLDL uptake may have a biological relevance if maintained over time.

Uptake of maximally modified LDL is mostly driven by interaction with SR-A and CD36. Thus, the effect of CKD-DAMPs exposure on expression of these scavenger receptors was analysed as a first step to investigate the mechanisms behind reduced cholesterol uptake.

CD36 expression by macrophages remained mostly unaffected by stimulation with LPS or the CKD-associated DAMPs, alone or combined, although Hsp70 induced its modest upregulation. On the contrary, SR-A expression was modestly decreased following stimulation with LPS as well as the 4 CKD-associated DAMPs, alone (but Hsp70) or in combination (**Figure 28.B**).

As uptake of modified cholesterol is a driver of foam cell formation, the reduction in uptake induced by the combination of DAMPs may be thought to be atheroprotective. However, uptake of modified LDL can be both atheroprotective and atherogenic depending notably on the type of macrophage involved, namely healthy macrophage or foam cell (303, 546). Briefly, uptake of modified LDL by healthy macrophages, which are able to process and efficiently clear it, limits LDL accumulation in the intima, therefore avoiding formation of foam cells which are detrimental and major players in maintaining chronic inflammation and worsening atherosclerosis. On the other hand, increased uptake of modified LDL by foam cells, which are already engorged with cholesterol and no longer able to process it, will favor the maintenance of chronic inflammation in the intima and promote plaque development by promoting foam cell secondary necrosis and the formation of more foam cells as neighboring phagocytes attempt to clear the cholesterol burden released by dying foam cells. For this reason, the effect of the DAMPs on modified LDL uptake by foam cells was investigated next.

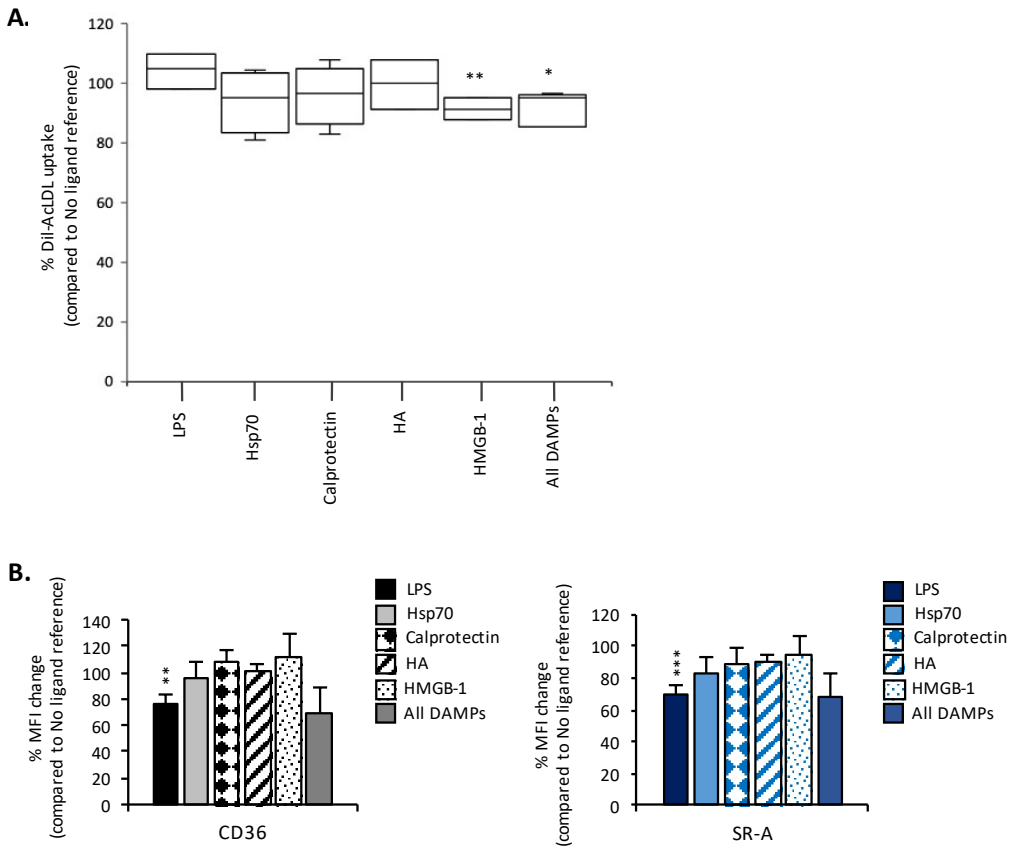


Figure 28. CKD-associated DAMPs moderately reduce modified cholesterol uptake by healthy macrophages

A. M-CSF-differentiated macrophages were starved in medium supplemented with 0.2% fatty-acid free BSA (18 hours) in the presence or absence or the indicated DAMPs, alone or in combination (1 μg/ml) before addition of DiI-AcLDL (10 µg/ml). After 24 hours, internalised DiI-AcLDL was quantified by flow cytometry (at least 10,000 cells/condition, MFI shown). Results are the average (+/- SEM) of 4 independent experiments. **B.** M-CSF-differentiated macrophages were stimulated (18 hours) with LPS (10 ng/ml), or Hsp70, Calprotectin, HA or HMGB-1, alone or combined (1 μg/ml) prior to flow cytometry analysis of cell surface expression of scavenger receptors CD36 and SR-A (at least 10,000 cells/condition, MFI shown). Results are shown as percentage change compared to the no ligand reference expression and are the average (+/- SEM) of 4 independent experiments.

*, p<0.05; **, p<0.01; ***, p<0.005 DAMPs/LPS Stimulation vs no stimulation.

c. Modified LDL uptake and scavenger receptors expression by foam cells

To set up a foam cell model *in vitro*, monocytes were differentiated into macrophages with addition of M-CSF as before. After 7 days, a large concentration of OxLDL (50 µg/ml) was added in the culture medium (48 hours) for the macrophages to uptake and become foam cells, as previously described by others (544, 547). OxLDL, rather than AcLDL or LDL, was used as it is harder to degrade and therefore is more efficient at inducing cholesterol engorgement and foam cell formation (548). The medium with OxLDL was then removed and Dil-AcLDL uptake and CD36 and SR-A expression were analysed as before.

These experiments showed that foam cells uptake significantly larger amounts of AcLDL than healthy macrophages (**Figure 29.A**). However, as opposed to what was observed for healthy macrophages, exposure of foam cells to all DAMPs, either alone or in combination, did not reduce foam cell formation to a significant extent (**Figure 29.B**). Although there was a statistically significant reduction with both HA, and HMGB-1, it only averaged to 4.18% and 3.98%, therefore is unlikely to be biologically relevant (**Figure 29.B**).

In apparent opposition with the lack of effect on modified LDL uptake, DAMPs stimulation upregulated the expression of both scavenger receptors. Specifically, CD36 (**Figure 29.C**) was strongly upregulated following activation with Hsp70, HMGB-1 and a combination of all DAMPs, moderately with LPS and Calprotectin, and remained not affected by exposure to HA. The expression of SR-A (**Figure 29.C**) in foam cells was found moderately increased following exposure to all CKD DAMPs, alone or combined, or to LPS. Importantly, these results need to be confirmed in foam cells generated from monocytes from other donors, as only 1 donor was tested here. If confirmed, this increase in scavenger receptor expression would be in opposition with what was observed in healthy macrophages (**Figure 28.B**)**,** suggesting a differential effect of DAMPs on both cell types. As a first step towards

investigating the mechanisms behind this difference, it may be interesting to compare TLR expression levels between healthy macrophages and foam cells.

Therefore, the results of the experiments described here suggest that DAMPs exposure may inhibit beneficial LDL clearance by healthy macrophages at the start of the atherosclerotic process but do not inhibit detrimental uptake by foam cells.

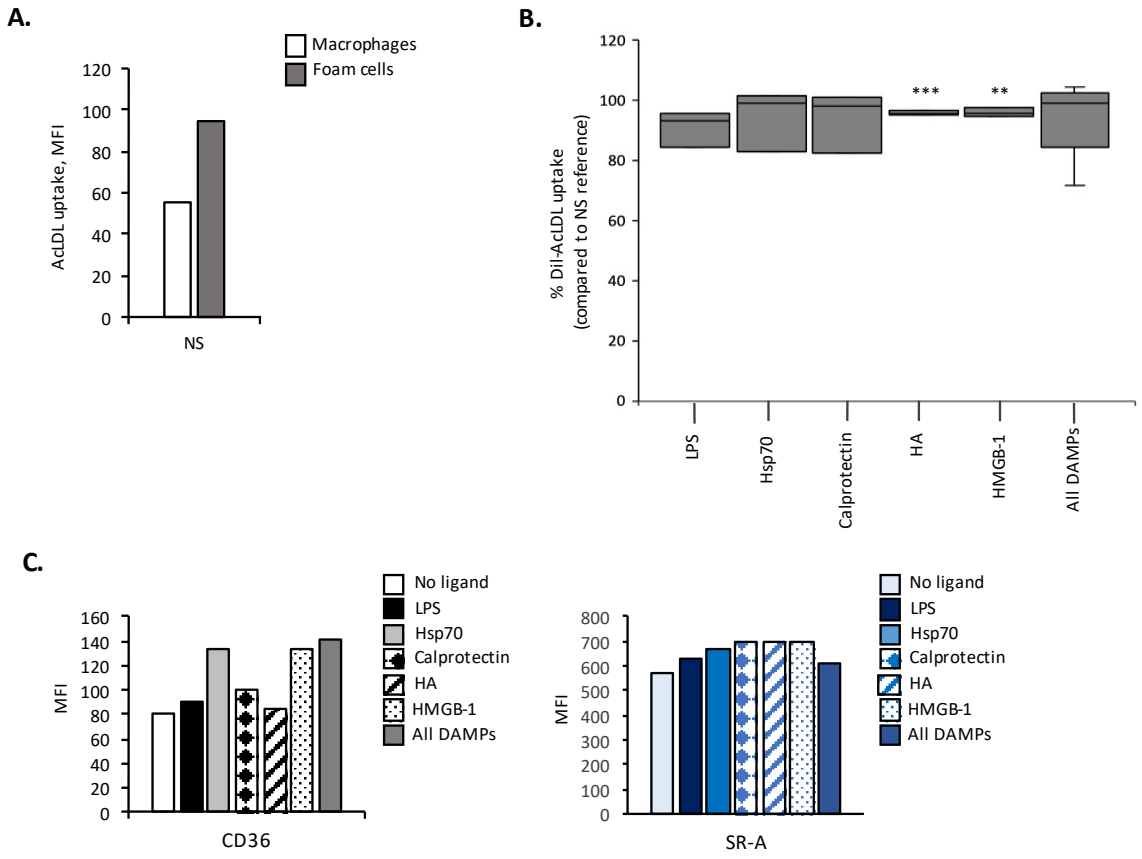


Figure 29. CKD-associated DAMPs do not affect modified cholesterol uptake by foam cells

A. M-CSF-differentiated macrophages (**A**) or foam cells (**A and B**) were starved in medium supplemented with 0.2% fatty-acid free BSA (18 hours) in the presence (**B**) or absence (**A and B**) or the indicated DAMPs, alone or in combination (1 μg/ml) before addition of DiI-AcLDL (10 µg/ml). After 24 hours, internalised DiI-AcLDL was quantified by flow cytometry (at least 10,000 cells/condition, MFI shown). Results in **A** are from 1 experiment representative of 2. Results in **B** are the average (+/- SEM) of 3 independent experiments. **C.** M-CSF-differentiated foam cells were stimulated (18 hours) with LPS (10 ng/ml), or Hsp70, Calprotectin, HA or HMGB-1, alone or combined (1 μg/ml) prior to flow cytometry analysis of cell surface expression of scavenger receptors CD36 and SR-A. Results are MFI of at least 10,000 cells/condition from 1 donor, with subtracted background fluorescence.
**, p<0.01; ***, p<0.005; DAMPs/LPS Stimulation vs no stimulation.

d. Cholesterol efflux by macrophages

Cholesterol efflux was measured using the fluorescent sterol dipyrromethene boron difluoride (BODIPY) cholesterol (549). Briefly, macrophages were exposed to BODIPY cholesterol in the absence of DAMPs (18 hours, RMPI 1640 supplemented with 0.2% fatty-acid free BSA) prior to medium removal and equilibration (1 hour) with or without DAMPs (no cholesterol, serum-free medium). Medium was removed once more and replaced with medium containing 10% FCS as a cholesterol acceptor, again with or without DAMPs (Material and Methods, Section V-9.). At the indicated time points between 1 hour and 24 hours, cholesterol efflux was measured by detection of BODIPY fluorescence in the culture supernatants. In macrophages from all donors tested, all CKD-associated DAMPs, either alone or in combination, significantly reduced the efflux of cholesterol (**Figure 30**). The extent and timing of the reduction varied with the donor tested.

Thus, the experiments shown in this section indicate that CKD-associated DAMPs can promote foam cell formation in a TLR2/4-dependent manner and that this effect is likely the result of the DAMPs' ability to reduce cholesterol efflux.

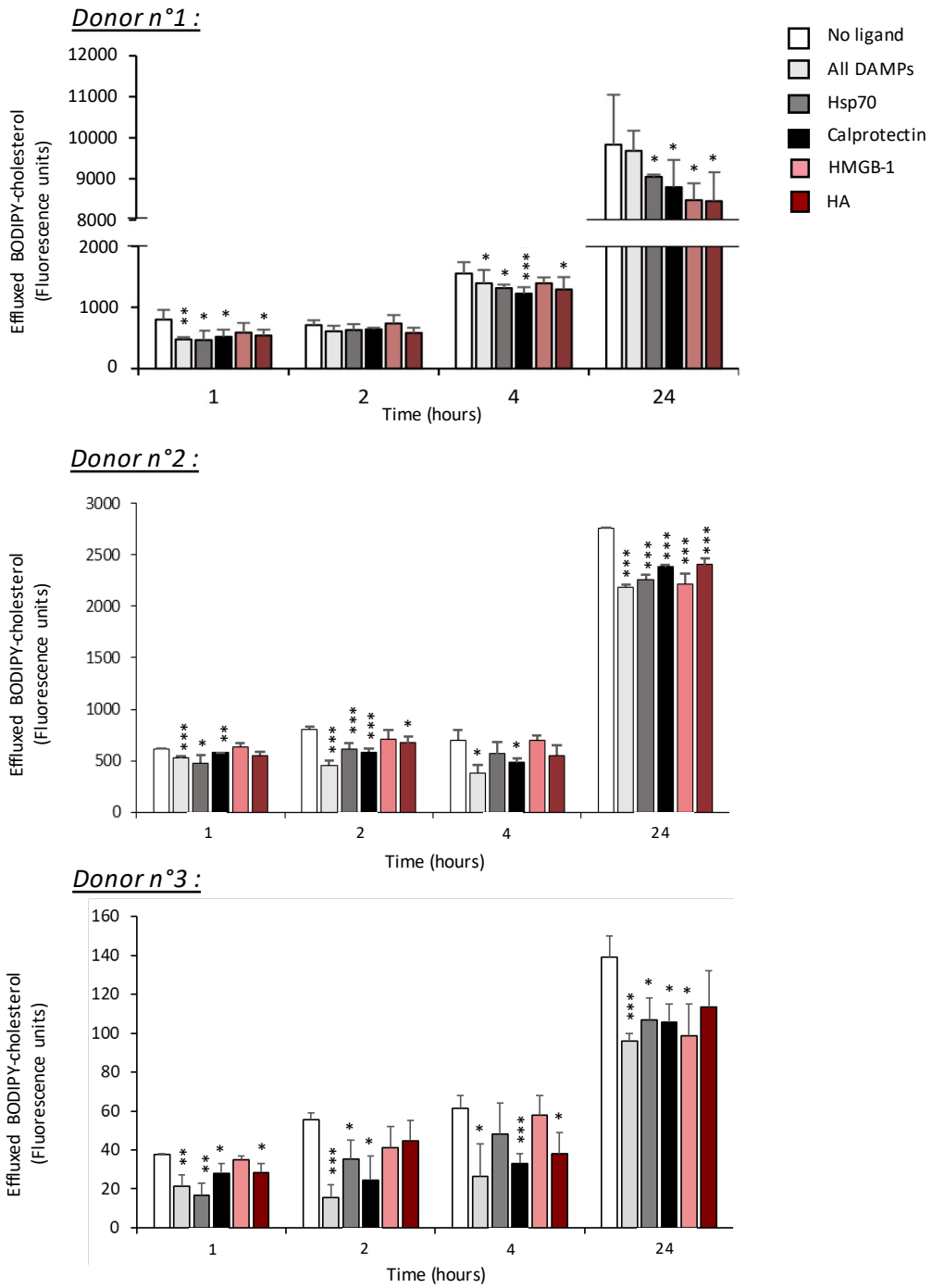


Figure 30. CKD-associated DAMPs inhibit cholesterol efflux by macrophages

Triplicate cultures of M-CSF-differentiated macrophages were loaded with BODIPY-labelled cholesterol (5 µM, 18 hours), before medium removal and exposure to the indicated DAMPs, alone or in combination (1 μg/ml). After equilibration (1 hour), medium was removed and replaced with fresh medium containing the same concentrations of DAMPs in the presence of 10% FCS as a cholesterol acceptor. BODIPY-associated fluorescence was measured in culture supernatants at the indicated time points. Results are mean +/- SD run with macrophages prepared from 3 different donors.

*, p<0.05; **, p<0.01; ***, p<0.005, DAMP Stimulation vs no stimulation.

Altogether, the data presented in this Chapter demonstrate the ability of CKD-associated DAMPs to promote several key pro-atherosclerotic functions by monocytes and macrophages, notably:

- Enhanced monocyte migratory capacity;
- Increased pro-atherogenic mediator production by both cell types;
- Modulation of a number of atherosclerosis related genes by macrophages;
- Increased foam cell formation.

These results support our hypothesis of a role for TLR DAMPs in promoting vascular inflammation and atherosclerosis progression in CKD patients, thereby contributing to the elevated CV risk in this population.

III-3.B.5. Discussion

In this Chapter, we demonstrate the ability of CKD-associated DAMPs to differentially promote a number of monocyte/macrophage responses typically associated with atherosclerosis development. These included higher pro-atherogenic cytokine production by both cell types, increased monocytic migratory capacity, increased atherosclerosis-associated gene expression as well as reduced cholesterol efflux and increased foam cell formation by macrophages. It is important to highlight that not all DAMPs affected all functions to a similar extent: while only Hsp70 induced robust cytokine production, all CKD-associated DAMPs, alone or in combination promoted monocyte migration and foam cell formation, and reduced cholesterol efflux. This differential effect suggests that different signaling pathways are involved in these responses. Because we demonstrated that TLR2/4 were major mediators for all of these responses, we hypothesise that the differences may be a result of DAMPs

interaction with their other, non-TLRs, immune receptors, which differ between DAMPs (Introduction, Section I-2.2., p.26, p.31, p.34 and p.37).

Regarding the monocytic functions tested, it is to be mentioned that primary cells, our preferred model, were used to measure cytokine production, while a cell line had to be used to evaluate migration, which may have contributed to the differential effect of the DAMPs observed between the 2 functions. The use of the MM6 monocytic cell line for migration was for technical reasons, as we found that primary monocytes isolated by adhesion of PBMCs tended to adhere to the membrane, making it difficult to count the number of cells migrated. Although cytokine production was tested in both monocytes and macrophages, it is not expected that cytokines produced by monocytes prior to their recruitment to the aortic wall will contribute directly to the atherosclerotic process itself. However, these cytokines will contribute to the establishment and/or maintenance of chronic systemic inflammation, which in turn may accelerate the atherosclerotic process, notably by promoting endothelial cell activation, priming leukocytes for recruitment and increasing LDL oxidation (345, 550).

Conversely, pro-inflammatory mediators' production by macrophages in the intima is a major driver of atherosclerosis progression. While only Hsp70 showed the ability to induce robust cytokine production, it is to be noted that we have not evaluated the effect of the combination of the 4 CKD-associated DAMPs on this readout. We expect that combining the 3 DAMPs that did not induce cytokine production with Hsp70 will lead to results similar to those observed with Hsp70 alone. Similarly, the effect of the combination of CKD-associated DAMPs on atherosclerosis-associated gene expression by macrophages remains to be evaluated.

In addition to cytokine production, a major macrophage-driven pathway promoting atherosclerosis progression is the transition from plaque macrophage to foam cell. In this

Chapter, we report the ability of all CKD-associated DAMPs, as well as their combination, to promote foam cell formation, in line with their ability to reduce cholesterol efflux. The fact that all DAMPs had the same effect on these functions suggests that this effect is a result of TLRs engagement, rather than of the engagement of other DAMPs receptors. This was confirmed in specific TLR2/4 blocking experiments. Of note, LDL, rather than OxLDL, was used to induce foam cell formation in our model. This is because OxLDL is a much more potent inducer of foam cell formation, rendering the system less sensitive to potential DAMP-induced changes. This is consistent with the previous finding that LPS-promoting effect on foam cell formation is not detectable when OxLDL, as opposed to LDL, is used to induce foam cells (446, 453, 551, 552). This can in part be explained by the fact that OxLDL is a TLR ligand in itself (Introduction, Section I-3.3., p.71). Therefore, exposure to OxLDL provides a very potent foam cell formation dual signal (high cholesterol load + TLR triggering), that cannot be easily potentiated by further TLR triggering (with LPS or DAMPs). Importantly, although OxLDL is an effective inducer of foam cell formation *in vivo*, minimally-modified or non-modified forms of LDL also drive foam cell formation in physiological conditions (551, 553). On a similar issue, fluorescent acetylated LDL, which is not physiological, was used to measure LDL uptake by macrophages. This is a well-accepted protocol which has been used ever since Brown and Goldstein found that it is avidly taken up by macrophages (554). Of note, while uptake of AcLDL tends to be larger than that of OxLDL, the same scavenger receptors are involved and studies have found that interventions typically give similar result profiles irrespective of the modified LDL form used to measure uptake (545). Of note, LDL and modified LDL can also be taken up by micropinocytosis, a potential effect of DAMPs on which we have not tested here, as it does not tend to be the main uptake route (331).

While CKD-patients' blood monocytes will be chronically exposed to the elevated plasma levels of DAMPs, determining what combination of DAMPs plaque macrophages will be exposed to is less straightforward. We carried out our *in vitro* work with macrophages under the assumption that DAMPs elevated in plasma will find their way to the intima through the endothelium rendered more permeable by chronic inflammation or pre-existing atherosclerosis in most CKD patients. However, it is likely that other DAMPs may also be found elevated at the plaque site, or that the same DAMPs may be further elevated, as previously described (555-559). Therefore, our *in vitro* work provided the proof-of-concept that TLR-DAMPs elevated in CKD have the capacity to drive a variety of cellular responses associated with atherosclerosis progression. However, it is not presented as an exhaustive list of the DAMPs that may play a role in promoting atherosclerosis progression in CKD.

In order to assess the *in vivo* relevance of the *in vitro* effects of CKD-associated TLR DAMPs described here, and to evaluate potential therapeutic strategies, we next tested in mice the therapeutic potential of a multi-TLR targeting strategy and the pharmacological inhibition of specific DAMP to reduce chronic vascular inflammation and early vascular alterations induced by CKD.

III-4. *In vivo* assessment of the capacity of CKD-DAMPs to promote vascular inflammation and early atherosclerotic responses

III-4.1. Effect of multi-TLR inhibition on systemic chronic inflammation induced by CKD in mice

A previously-described mouse model of Aristolochic Acid-induced Nephropathy (AAN) (560) was used (**Figure 31.A**) to:

- Investigate the impact of CKD on systemic inflammatory, immune and pro-atherosclerosis responses;
- Evaluate the therapeutic potential of TLR/DAMP inhibition to reduce these effects.

Repeated administration of AA induces tubular injury leading to inflammation followed by tissue remodeling and fibrosis, as well as decreased kidney function. As expected, nephropathy was confirmed by kidney histology and increased creatinine plasma levels (**Figure 31.B and C**). In line with what we observed in CKD patients, plasma levels of TLR DAMPs were found elevated following AA administration. Specifically, Calprotectin levels were significantly increased at Day 10 and remained elevated (although not statistically significant) at Day 21. HMGB-1 levels were also increased, which became statistically significant by Day 21 (**Figure 32.A**). Levels of Hsp70 and HA were not affected in this model. Species differences or differences in the stage of CKD between patients and mice may explain this finding.

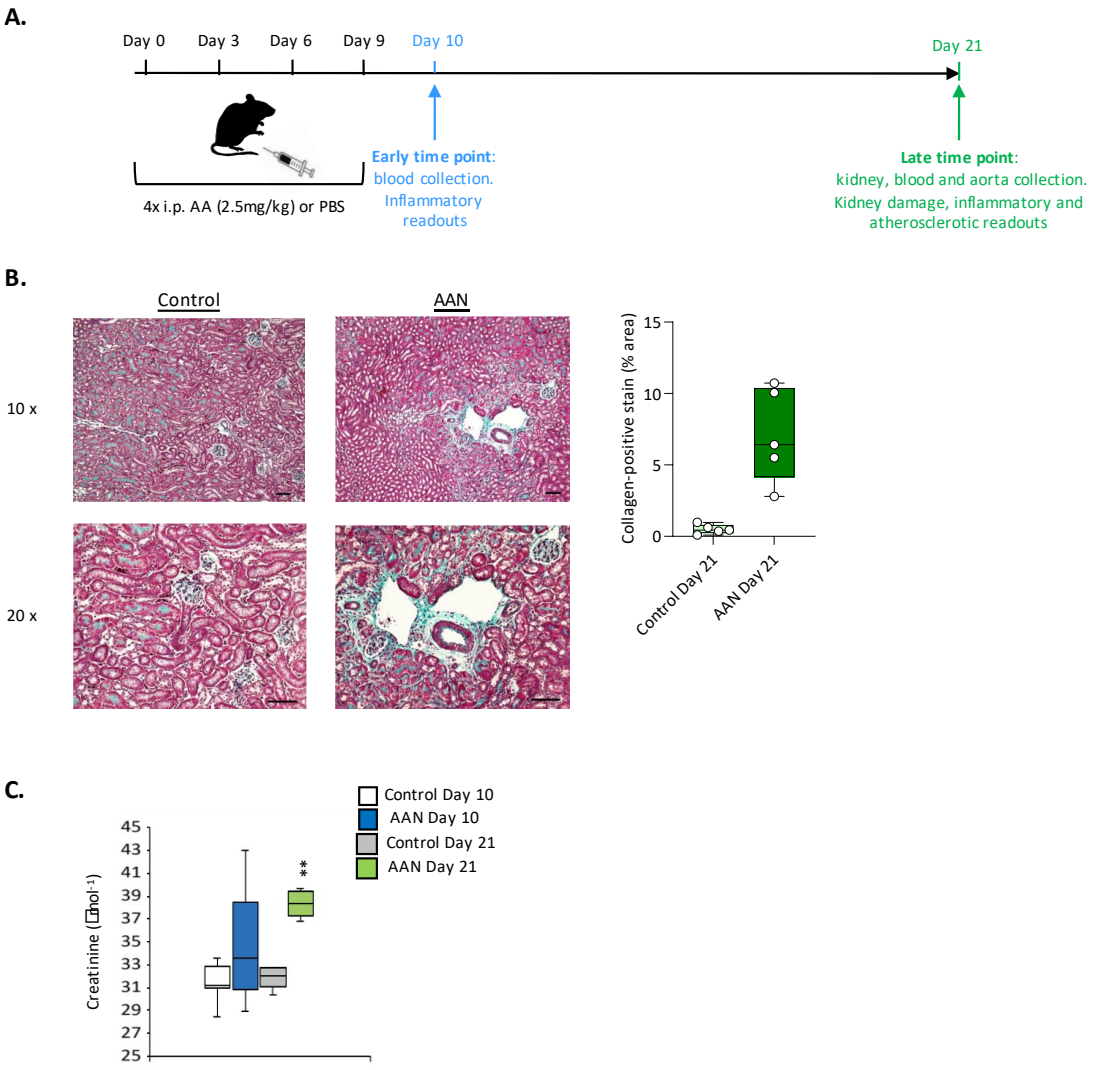


Figure 31. Kidney damage and loss of function following repeated AA injections in mice C57BL/6J mice (n=5 per group) were injected intraperitoneally with AA (2.5 mg/kg) or PBS on days 0, 3, 7, and 10 (**A**). AAN was verified at Day 21 by the development of kidney fibrosis (**B**) and a significant elevation in plasma creatinine (**C**). **B**. Representative images of Masson trichrome stain of kidneys from a healthy mouse (left panels) and a mouse with chronic AAN (right). Cytoplasm is stained red, nuclei are in dark brown and collagen is stained blue, identifying renal fibrosis. Scale bars: 100 µm. Graph shows the percentage of collagen positive stain for each group (3 non-overlapping fields of view scored for each of 5 animals/group). **C**. Creatinine measurements in plasma, **, $p<0.01$ AA Day 21 vs PBS Day 21.

To evaluate the therapeutic potential of TLR inhibition in CV risk in CKD, the effect of the soluble form of TLR2 (sTLR2) was tested in this model. sTLR2 is a natural TLR inhibitor with anti-inflammatory capacity (298, 498, 561-563). It inhibits TLR activation by i) acting as a decoy receptor, binding to TLR2 ligands and preventing their recognition by membrane TLR2, and ii) binding to the common TLR co-receptor CD14 (298). Because most TLRs rely on CD14 activity for efficient activation, sTLR2 can inhibit the activation of TLRs other than TLR2, notably TLR4 (498, 562, 563).

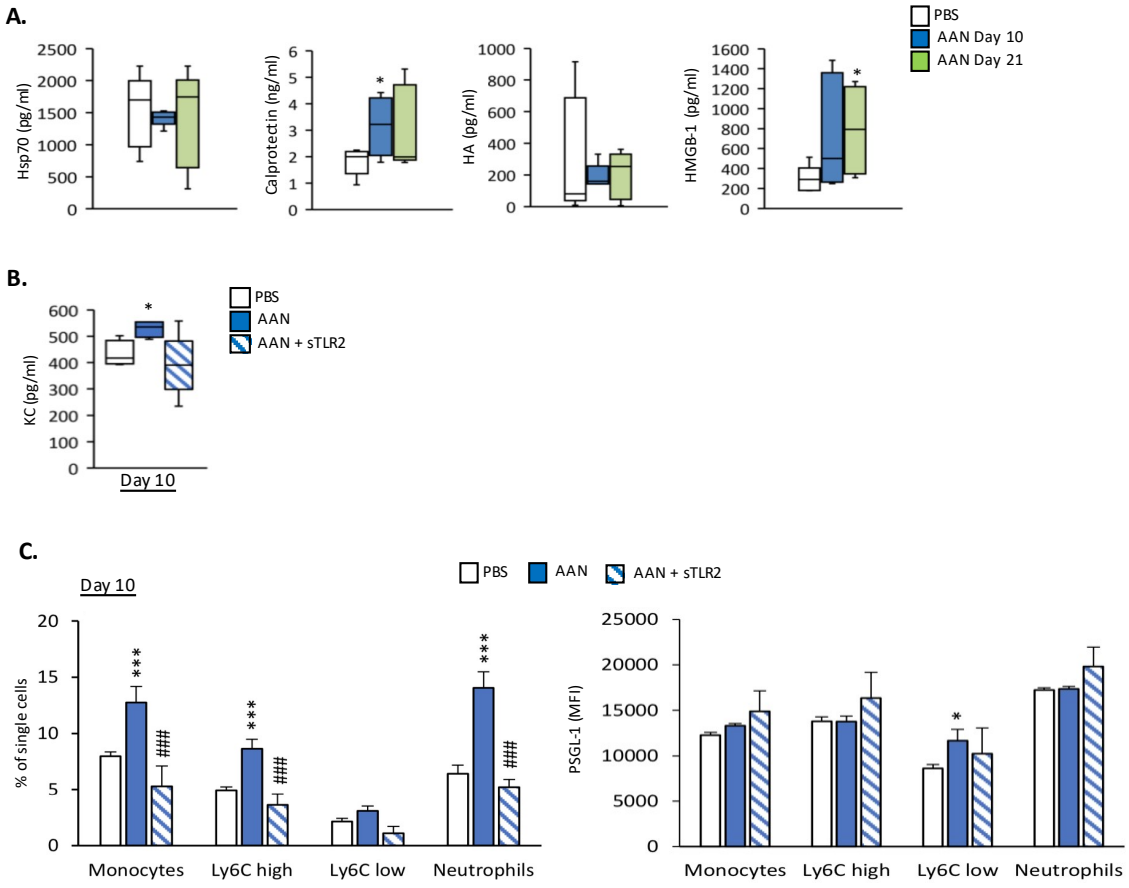


Figure 32. Effect of sTLR2 administration on AAN-induced systemic inflammatory and pro-atherosclerotic responses *in vivo*

C57BL/6J mice (n=5 per group) were injected intraperitoneally 4 times at 3 day-intervals with AA (2.5 mg/kg) or PBS, in the presence or absence of sTLR2 (250 ng/mouse). Blood was obtained at Day 10 or Day 21, Day 0 being the day of the first injection. DAMP (**A**) and cytokine (**B**) plasma levels were determined by ELISA and innate leukocyte proportions and PSGL-1 expression levels (**C**) by flow cytometry.

*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$ (*, AAN vs PBS; #, AAN +sTLR2 vs AAN).

Repeated AA injections led to an early increase (Day 10) in plasma levels of the prototypical neutrophil chemoattractant KC (murine equivalent of IL-8). KC levels were reduced by sTLR2 administration, although the changes were not statistically significant (**Figure 32.B**). Consistent with the increase in KC, CKD induction resulted in a marked increase in the proportion of neutrophils ($CD11b^{+}/Ly6G^{+}$) and monocytes ($CD11b^{+}/Ly6G^{-}$) within 24 hours of the last AA injection (**Figure 32.C**). Notably, the increase in total monocytes was mostly driven by the $Ly6C^{high}$ subset, considered as pro-inflammatory (564), being preferentially recruited to inflamed tissues, like the atherosclerotic plaque (565). Of note, higher proportions of the human equivalent "pro-inflammatory" monocytic population ($CD16^{+}/CD14^{++}$ or $CD14^{+}$) have been shown to correlate with the incidence of cardiovascular events in CKD patients and other cohorts (566-568). The proportions of neutrophils, total monocytes and $Ly6C^{high}$ monocytes were significantly reduced by administration of sTLR2. This reduction was robust, as leukocyte levels were similar to PBS-injected mice (**Figure 32.C**). Although the proportion of $Ly6C^{low}$ monocytes was not significantly affected by AAN, in this subset, there was a significant increase in the expression of PSGL-1 (CD162), which mediates monocyte adhesion to the endothelium during atherosclerosis (**Figure 32.C**). This increased expression was not significantly affected by sTLR2. To gain a better insight into the range of systemic inflammatory changes induced by AAN and the efficacy of sTLR2 as an anti-inflammatory strategy, the blood expression of 84 genes associated with inflammation and immunity was investigated. The effect of AAN on inflammation-related gene expression at Day 10 was modest with 5 genes significantly upregulated (**Figure 33.A**), coding for proteins related to inflammation (Serum amyloid P, a mice acute phase protein (569), and IL-1β), innate immunity (Nlrp3 and Tlr5) and adaptive immunity (Histocompatibility 2, Q-region-locus-10) and this profile was not markedly affected by sTLR2 administration (**Table 6**).

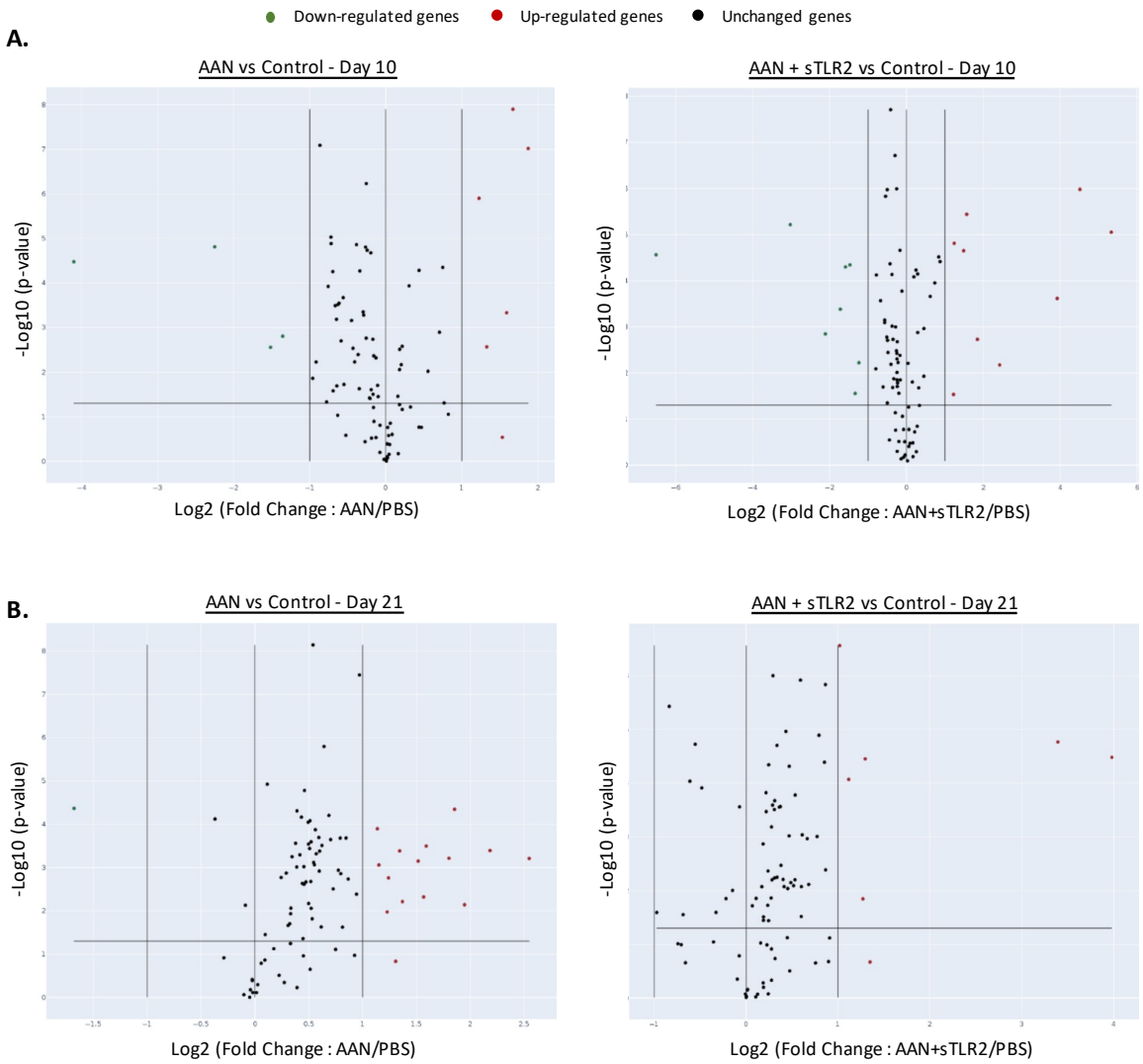


Figure 33. Effect of sTLR2 administration on AAN-induced changes on inflammation-related genes *in vivo*

C57BL/6J mice (n=5 per group) were injected intraperitoneally 4 times at 3 day-intervals with AA (2.5 mg/kg) or PBS, in the presence or absence of sTLR2 (250 ng/mouse). Blood was obtained at Day 10 or Day 21, Day 0 being the day of the first injection. Volcano plots compare the effect of AAN and AAN + sTLR2 on inflammation and immune responses-associated gene expression at Day 10 (**A**) and Day 21 (**B**). Red (upregulated, fold change ≥ 2) and green (downregulated, fold change ≤ 0.5) circles represent single genes significantly affected (p<0.05, represented by the horizontal line) compared to PBS control.

Table 6. Effect of sTLR2 on AAN-induced changes in inflammation and immunity gene expression in blood at Day 10

		AAN *		AAN + sTLR2	
Gene symbol	**Description**	**Fold Change****	***P Value*****	**Fold Change****	***P Value******
Apcs	Serum amyloid P-component	**3.7**	*0.0001*	10.2	*0.0001*
H2-q10	Histocompatibility 2, Q region locus 10	**2.3**	*0.0001*	2.8	*0.0001*
Il1b	Interleukin 1 beta	**3.2**	*0.0001*	3.0	*0.0001*
Nlrp3	NLR family, pyrin domain containing 3	**2.5**	*0.0027*	2.4	*0.0001*
Tlr5	Toll-like receptor 5	**3.0**	*0.0004*	3.6	*0.0019*
Il10	Interleukin 10	**-2.9**	*0.0028*	-1.2	*0.073*
Mpo	Myeloperoxidase	**-2.6**	*0.0016*	-3.0	*0.0001*
Rag1	Recombination activating gene 1	**-17.2**	*0.0001*	-21.3	*0.0001*
Rorc	RAR-related orphan receptor gamma	**-4.8**	*0.0001*	-8.13	*0.0001*

*Only statistically significant (P<0.05) AAN-induced ≤ 0.5 (in green) or ≥ 2 (in red) fold changes were considered.
**Compared to PBS control group.

Table 7. Effect of AA+ sTLR2 administration (compared to control) on inflammation and immunity gene expression in blood at Day 10

		AAN + sTLR2	
Gene symbol	**Description**	**Fold Change***	***P Value****
Apcs	Serum amyloid P-component	**40.2**	*0.0001*
Ccl12	Chemokine (C-C motif) ligand 12	**5.4**	*0.0067*
Ccl5	Chemokine (C-C motif) ligand 5	**2.3**	*0.0293*
Crp	C-reactive protein, pentraxin-related	**22.8**	*0.0001*
H2-q10	Histocompatibility 2, Q region locus 10	**2.8**	*0.0001*
Il1b	Interleukin 1 beta	**3.0**	*0.0001*
Mbl2	Mannose-binding lectin (protein C) 2	**15.2**	*0.0002*
Nlrp3	NLR family, pyrin domain containing 3	**2.4**	*0.0001*
Tlr5	Toll-like receptor 5	**3.6**	*0.0019*
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	**-4.3**	*0.0014*
Ifna2	Interferon alpha 2	**-3.3**	*0.0004*
Ifnb1	Interferon beta 1	**-2.5**	*0.0279*
Il5	Interleukin 5	**-2.4**	*0.0060*
Mpo	Myeloperoxidase	**-3.0**	*0.0001*
Rag1	Recombination activating gene 1	**-91.3**	*0.0001*
Rorc	RAR-related orphan receptor gamma	**-8.13**	*0.0001*
Tlr3	Toll-like receptor 3	**-2.8**	*0.0001*

*Compared to PBS control group.

At Day 21, 14 genes were upregulated by AAN (**Figure 33.B**), among them those coding for acute phase proteins (Serum amyloid P and C-reactive protein) and pro-inflammatory mediators (Colony stimulating factor 2; IFN2α; IL-2; IL-6; IL-17α; IL-23α, Myeloperoxidase). Several transcripts upregulated at this later time point were associated with the adaptive immune response: FOXP3, a transcription factor, specific marker of Tregs; IL-2, which promotes T cell proliferation and activity; IL-17 and IL-23, indicative of Th17 activity; IL-5, which promotes Th2 differentiation and IL-6, which can promote T cell differentiation towards Th17 and Tregs. Administration of sTLR2 led to a reduction in the expression of over half of the AAN-upregulated genes (**Table 8**).

Table 8. Effect of sTLR2 on AAN-induced changes in inflammation and immunity gene expression the blood at Day 21

		AAN *		AAN +sTLR2	
Gene symbol	**Description**	**Fold Change****	***P Value*****	**Fold Change****	***P Value*****
Apcs	Serum amyloid P-component	**3.0**	*0.0003*	1.3	*0.0084*
Crp	C-reactive protein, pentraxin-related	**4.5**	*0.0004*	1.7	*0.222*
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	**3.9**	*0.0072*	1.1	*0.8473*
Foxp3	Forkhead box P3	**2.4**	*0.0017*	1.8	*0.0041*
Ifna2	Interferon alpha 2	**2.9**	*0.0007*	-1.6	*0.0280*
Ifnb1	Interferon beta 1	**2.5**	*0.0004*	-1.3	*0.0904*
Il17a	Interleukin 17A	**3.5**	*0.0006*	-1.6	*0.1015*
Il2	Interleukin 2	**2.2**	*0.0009*	-1.2	*0.0256*
Il23a	Interleukin 23, alpha subunit p19	**2.3**	*0.0106*	2.7	*0.0003*
Il5	Interleukin 5	**3.6**	*0.0001*	2.5	*0.0303*
Il6	Interleukin 6	**3.0**	*0.0048*	2.6	*0.0001*
Mbl2	Mannose-binding lectin (protein C) 2	**5.8**	*0.0006*	2.9	*0.2093*
Mpo	Myeloperoxidase	**2.6**	*0.0061*	2.6	*0.0044*
Mx1	Myxovirus (influenza virus) resistance 1	**2.2**	*0.0001*	2.4	*0.0142*
Rag1	Recombination activating gene 1	**-3.2**	*0.0001*	-5.8	*0.0001*

*Only statistically significant (P<0.05) AAN-induced ≤ 0.5 (in green) or ≥ 2 (in red) fold changes were considered.
**Compared to PBS control group.

Table 9. Effect of AA + sTLR2 administration (compared to control) on inflammation and immunity blood gene expression in blood at Day 21

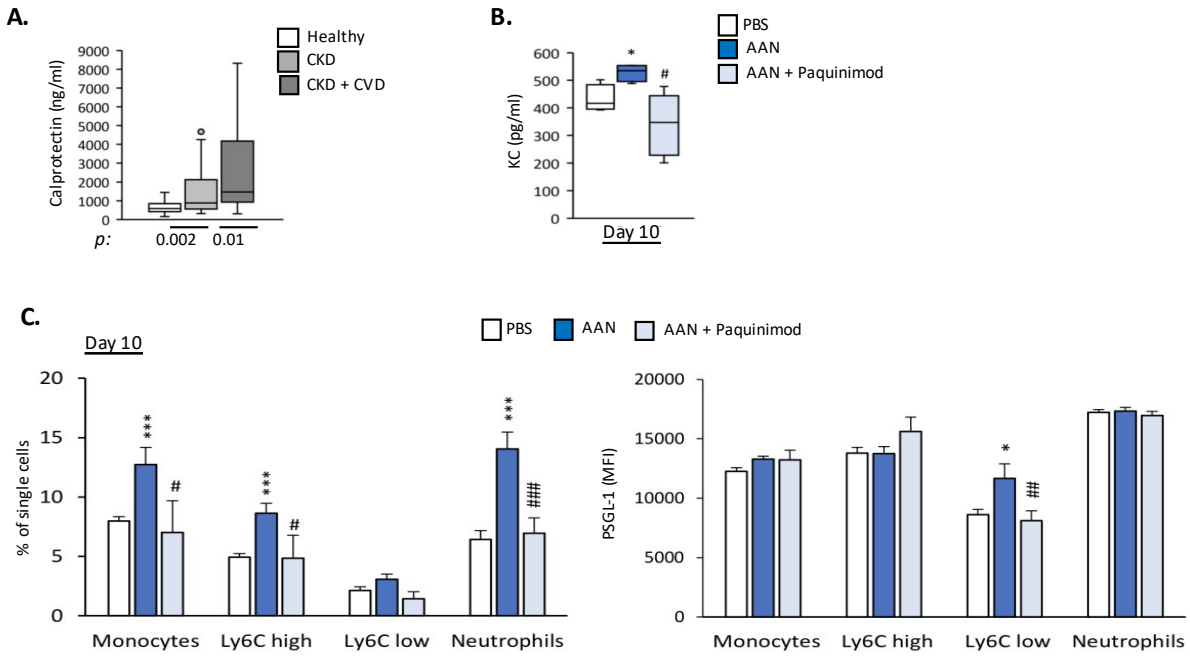
		AAN + sTLR2	
Gene symbol	**Description**	**Fold Change***	***P Value****
Cd14	CD14 antigen	**2.2**	*0.0001*
Cd80	CD80 antigen	**2.0**	*0.0000*
Il6	Interleukin 6	**2.5**	*0.0001*
Mx1	Myxovirus (influenza virus) resistance 1	**2.4**	*0.0143*
Rag1	Recombination activating gene 1	**15.8**	*0.0001*
Rorc	RAR-related orphan receptor gamma	**10.5**	*0.0001*

*Compared to PBS control group.

Thus, sTLR2 partially reduced the expression of a wide range of inflammatory/immunity genes, and showed the ability to control monocyte and neutrophil numbers and the levels of KC, all critical to the inflammation underlying CV pathology. These findings raised the question of whether specific DAMP inhibition may present an even better therapeutic alternative.

III-4.2. Effect of Calprotectin inhibition on vascular inflammation and early atherosclerosis-associated gene expression in mouse CKD

Both Calprotectin and HMGB-1 were elevated following CKD induction in mice (**Figure 32.A**). Their levels were compared in plasma from CKD patients who had not versus those who had been diagnosed with CVD at the time of sampling (the latter were excluded from the analysis in **Figure 7**). Plasma levels of Calprotectin were further elevated in the latter group (**Figure 34.A**), while HMGB-1, Hsp70 and HA levels were not (not shown). Therefore, a Calprotectin-targeting strategy was selected for *in vivo* evaluation. Paquinimod (ABR-215757) is a well-documented pharmacological inhibitor of Calprotectin, which works by interacting specifically with the S100A9 subunit, preventing its recognition by TLR4 (570, 571).



<u>Figure 34.</u> Effect of blocking Calprotectin on AAN-induced systemic inflammatory and pro-atherosclerotic responses *in vivo*.

A. Calprotectin levels in plasma from healthy donors (n=30), Stage 5 CKD patients (n=35) or Stage 5 CKD patients with diagnosed CVD (n=38). Horizontal lines in boxes denote the median value, Open circles denote outliers. *p* value*,* CKD patients *vs* healthy donors or CKD vs CKD + CVD. **B-C**. C57BL/6J mice (n=5 per group) were injected intraperitoneally 4 times at 3 day-intervals with AA (2.5 mg/kg) or PBS, in the presence or absence of Paquinimod (20 µg/mouse). Blood was obtained at Day 10. Cytokine plasma levels were determined by ELISA (**B**) and innate leukocyte proportions and PSGL-1 expression levels (**C**) by flow cytometry.

*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$ (*, AAN vs PBS*; #,* AAN +Paquinimod vs AAN).

Similar to sTLR2, Paquinimod prevented the AAN-induced increase in plasma KC levels (**Figure 34.B**) and innate immune cell proportions at Day 10 (total monocytes, Ly6C^{high} monocytes, neutrophils; **Figure 34.C**). The effect of Paquinimod on AAN-associated inflammation-related gene expression was profound at both short and longer term, as it reduced to normal levels the expression of 4 out of the 5 genes upregulated by AAN at Day 10 (**Figure 35.A**, **Table 10**) and of 10 out of 14 at Day 21 (**Figure 35.B**, **Table 12**). Interestingly, Paquinimod, as opposed

to sTLR2, did not result in a reduction in the expression of *FOXP3*, suggesting that this approach may preserve Treg anti-inflammatory activity, beneficial in the context of chronic inflammation.

Table 10. Effect of Paquinimod on AAN-induced changes in inflammation and immunity gene expression in blood at Day 10

		AAN *		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change****	***P Value****	**Fold Change****	***P Value*****
Apcs	Serum amyloid P-component	**3.7**	*0.0001*	1.6	*0.1512*
H2-q10	Histocompatibility 2, Q region locus 10	**2.3**	*0.0001*	1.8	*0.0001*
Il1b	Interleukin 1 beta	**3.2**	*0.0001*	2.5	*0.0001*
Nlrp3	NLR family, pyrin domain containing 3	**2.5**	*0.0027*	1.9	*0.0059*
Tlr5	Toll-like receptor 5	**3.0**	*0.0004*	1.9	*0.0029*
Il10	Interleukin 10	**-2.9**	*0.0028*	-2.2	*0.0030*
Mpo	Myeloperoxidase	**-2.6**	*0.0016*	-2.3	*0.0076*
Rag1	Recombination activating gene 1	**-17.2**	*0.0001*	-6.6	*0.0001*
Rorc	RAR-related orphan receptor gamma	**-4.8**	*0.0001*	-4.4	*0.0001*

*****Only statistically significant ($P<0.05$) AAN-induced ≤ 0.5 (in green) or ≥ 2 (in red) fold changes were considered.
******Compared to PBS control group.

Table 11. Effect of AA+ Paquinimod administration (compared to control) on inflammation and immunity gene expression in blood at Day 10

		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change***	***P Value****
Crp	C-reactive protein, pentraxin-related	**3.0**	*0.0044*
Il1b	Interleukin 1 beta	**2.5**	*0.0001*
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	**-4.4**	*0.0021*
Ifna2	Interferon alpha 2	**-3.1**	*0.0006*
Ifnb1	Interferon beta 1	**-5.5**	*0.0086*
Il10	Interleukin 10	**-2.2**	*0.0029*
Il1a	Interleukin 1 alpha	**-4.0**	*0.0012*
Il1r1	Interleukin 1 receptor, type I	**-2.0**	*0.0189*
Il2	Interleukin 2	**-2.5**	*0.0023*
Il5	Interleukin 5	**-4.6**	*0.0012*
Il6	Interleukin 6	**-3.3**	*0.0024*
Mpo	Myeloperoxidase	**-2.3**	*0.0076*
Rag1	Recombination activating gene 1	**-6.6**	*0.0001*
Rorc	RAR-related orphan receptor gamma	**-4.4**	*0.0001*
Tlr3	Toll-like receptor 3	**-2.1**	*0.0001*

*Compared to PBS group.

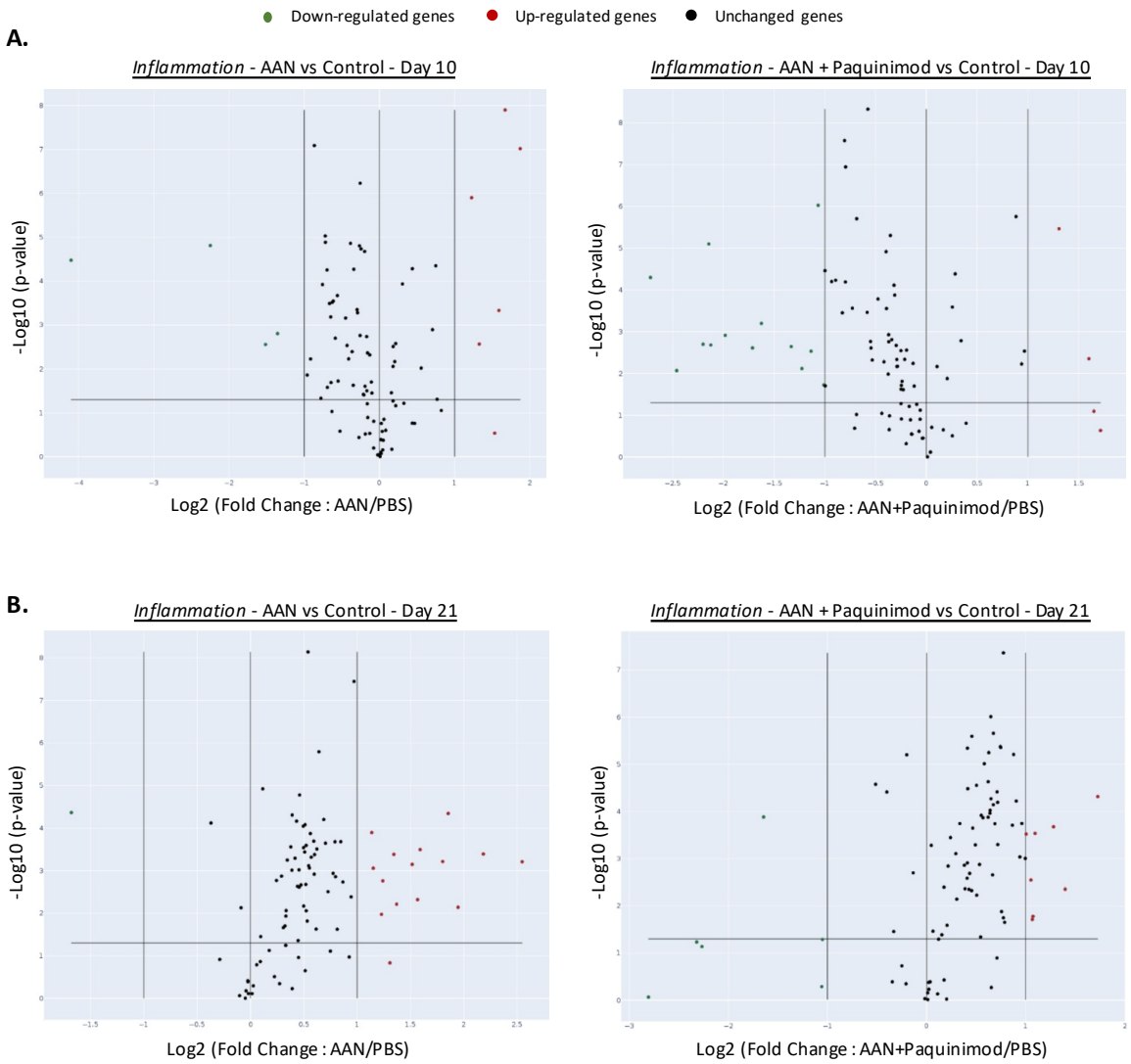


Figure 35. Effect of blocking Calprotectin administration on AAN-induced changes on inflammation-related genes *in vivo*

C57BL/6J mice (n=5 per group) were injected intraperitoneally 4 times at 3 day-intervals with AA (2.5 mg/kg) or PBS, in the presence or absence of Paquinimod (20 µg/mouse). Blood was obtained at Day 10 or Day 21. Volcano plots compare the effect of AAN and AAN + Paquinimod on inflammation and immune responses in the blood at Day 10 (**A**) and Day 21 (**B**). Red (upregulated, fold change ≥ 2) and green (downregulated, fold change ≤ 0.5) circles represent single genes significantly affected (p<0.05, represented by the horizontal line) compared to PBS control.

Table 12. Effect of Paquinimod on AAN-induced changes in inflammation and immunity blood gene expression in blood at Day 21

		AAN *		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change****	***P Value*****	**Fold Change****	***P Value*****
Apcs	Serum amyloid P-component	**3.0**	*0.0003*	-1.1	*0.0019*
Crp	C-reactive protein, pentraxin-related	**4.5**	*0.0004*	-3.1	*0.0746*
Csf2	Colony stimulating factor 2 (granulocyte-	**3.9**	*0.0072*	1.2	*0.9455*
Foxp3	Forkhead box P3	**2.4**	*0.0017*	2.4	*0.0002*
Ifna2	Interferon alpha 2	**2.9**	*0.0007*	1.0	*0.9711*
Ifnb1	Interferon beta 1, fibroblast	**2.5**	*0.0004*	1.4	*0.0456*
Il17a	Interleukin 17A	**3.5**	*0.0006*	-1.2	*0.4990*
Il2	Interleukin 2	**2.2**	*0.0009*	1.0	*0.9242*
Il23a	Interleukin 23, alpha subunit p19	**2.3**	*0.0106*	2.0	*0.0003*
Il5	Interleukin 5	**3.6**	*0.0001*	1.6	*0.1272*
Il6	Interleukin 6	**3.0**	*0.0048*	1.8	*0.0002*
Mbl2	Mannose-binding lectin (protein C) 2	**5.8**	*0.0006*	-2.1	*0.0514*
Mpo	Myeloperoxidase	**2.6**	*0.0061*	2.6	*0.0044*
Mx1	Myxovirus (influenza virus) resistance 1	**2.2**	*0.0001*	3.3	*0.0001*
Rag1	Recombination activating gene 1	**-3.2**	*0.0001*	-3.1	*0.0001*

*Only statistically significant (P<0.05) AAN-induced ≤ 0.5 (in green) or ≥ 2 (in red) fold changes were considered.
**Compared to PBS control group.

Table 13. Effect of AA+ Paquinimod administration (compared to control) on inflammation and immunity gene expression in blood at Day 21

		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change***	***P Value****
Cxcl10	Chemokine (C-X-C motif) ligand 10	**2.1**	*0.0003*
Foxp3	Forkhead box P3	**2.4**	*0.0002*
Il23a	Interleukin 23, alpha subunit p19	**2.0**	*0.0003*
Mpo	Myeloperoxidase	**2.6**	*0.0044*
Mx1	Myxovirus (influenza virus) resistance 1	**3.3**	*0.0001*
Myd88	Myeloid differentiation primary response gene 88	**2.1**	*0.0167*
Tbx21	T-box 21	**2.1**	*0.0194*
Tnf	Tumor necrosis factor	**2.1**	*0.0028*
Rag1	Recombination activating gene 1	**-3.1**	*0.0001*

*Compared to PBS control group.

Given the strong inhibitory effect of blocking Calprotectin on AAN-induced systemic inflammation, its potential effect on aortic atherosclerosis-associated gene expression was evaluated at the later time point (Day 21). Out of 84 genes tested, 23 were upregulated by AAN (**Figure 36.A**, full list of affected genes in **Table 14**), including a majority of genes involved in inflammation, endothelial activation and leukocyte recruitment to the plaque (*Ccl2, Ccl5, Ccr2, Cd44, Cxcl1, Fga, Fgb, Icam1, Il1a, Il1b, Itgax*, *Sele, Sell, Selplg, Tnf, Tnfaip3, Vcam1*), but also lipid metabolism and handling (*Apoa1, Apob, Msr1*), apoptosis (*Birc3*) and platelet activation (*Ptgs1*). Paquinimod treatment led to a robust reduction in AAN-induced aortic expression of atherosclerosis-associated genes: 17 out of the 23 genes were maintained at normal levels, while 3 were significantly reduced but remained elevated compared to the PBS control (**Figure 36.A**, full list of affected genes in **Table 14**).

Table 14. Effect of Paquinimod on AAN-induced changes in atherosclerosis-related aortic gene expression at Day 21

		AAN *		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change ****	***P Value*****	**Fold Change ****	***P Value*****
Apoa1	Apolipoprotein A-I	**33.4**	*0.0001*	21.0	*0.0001*
Apob	Apolipoprotein B	**27.5**	*0.0001*	12.0	*0.0001*
Birc3	Baculoviral IAP repeat-containing 3	**2.1**	*0.0006*	-1.1	*0.5215*
Ccl2	Chemokine (C-C motif) ligand 2	**2.8**	*0.0001*	1.2	*0.2066*
Ccl5	Chemokine (C-C motif) ligand 5	**2.6**	*0.0002*	1.0	*0.9266*
Ccr2	Chemokine (C-C motif) receptor 2	**2.1**	*0.0004*	-1.2	*0.0429*
Cd44	CD44 antigen	**2.6**	*0.0001*	1.3	*0.0009*
Cxcl1	Chemokine (C-X-C motif) ligand 1	**4.8**	*0.0046*	2.2	*0.0077*
Fga	Fibrinogen alpha chain	**25.6**	*0.0001*	10.0	*0.0001*
Fgb	Fibrinogen beta chain	**28.7**	*0.0001*	15.9	*0.0001*
Icam1	Intercellular adhesion molecule 1	**2.8**	*0.0001*	1.3	*0.0080*
Il1a	Interleukin 1 alpha	**5.4**	*0.0006*	1.7	*0.1557*
Il1b	Interleukin 1 beta	**5.8**	*0.0001*	-2.1	*0.0309*
Itgax	Integrin alpha X	**3.0**	*0.0003*	1.5	*0.0042*
Msr1	Macrophage scavenger receptor 1	**2.1**	*0.0001*	-1.3	*0.0007*
Nyp	Neuropeptide Y	**5.7**	*0.0001*	9.0	*0.0112*
Ptgs1	Prostaglandin-endoperoxide synthase 1	**2.2**	*0.0001*	-1.2	*0.0231*
Sele	Selectin, endothelial cells	**2.1**	*0.0031*	-1.4	*0.1607*
Sell	Selectin, lymphocytes	**7.8**	*0.0001*	-1.4	*0.0584*
Selplg	Selectin, platelet (p-selectin) ligand	**2.4**	*0.0001*	-1.1	*0.5787*
Tnf	Tumor necrosis factor	**15.4**	*0.0030*	3.6	*0.1639*
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	**2.3**	*0.0001*	1.6	*0.0258*
Vcam1	Vascular cell adhesion molecule 1	**4.5**	*0.0001*	1.0	*0.8135*
Il2	Interleukin 2	**-2.9**	*0.0008*	-1.9	*0.0028*
Serpinb2	Serine (or cysteine) peptidase inhibitor, mbr 2	**-4.7**	*0.0002*	-1.2	*0.2618*

*Only statistically significant ($P<0.05$) AAN-induced ≤ 0.5 (in green) or ≥ 2 (in red) fold changes were considered.
**Compared to PBS control group.

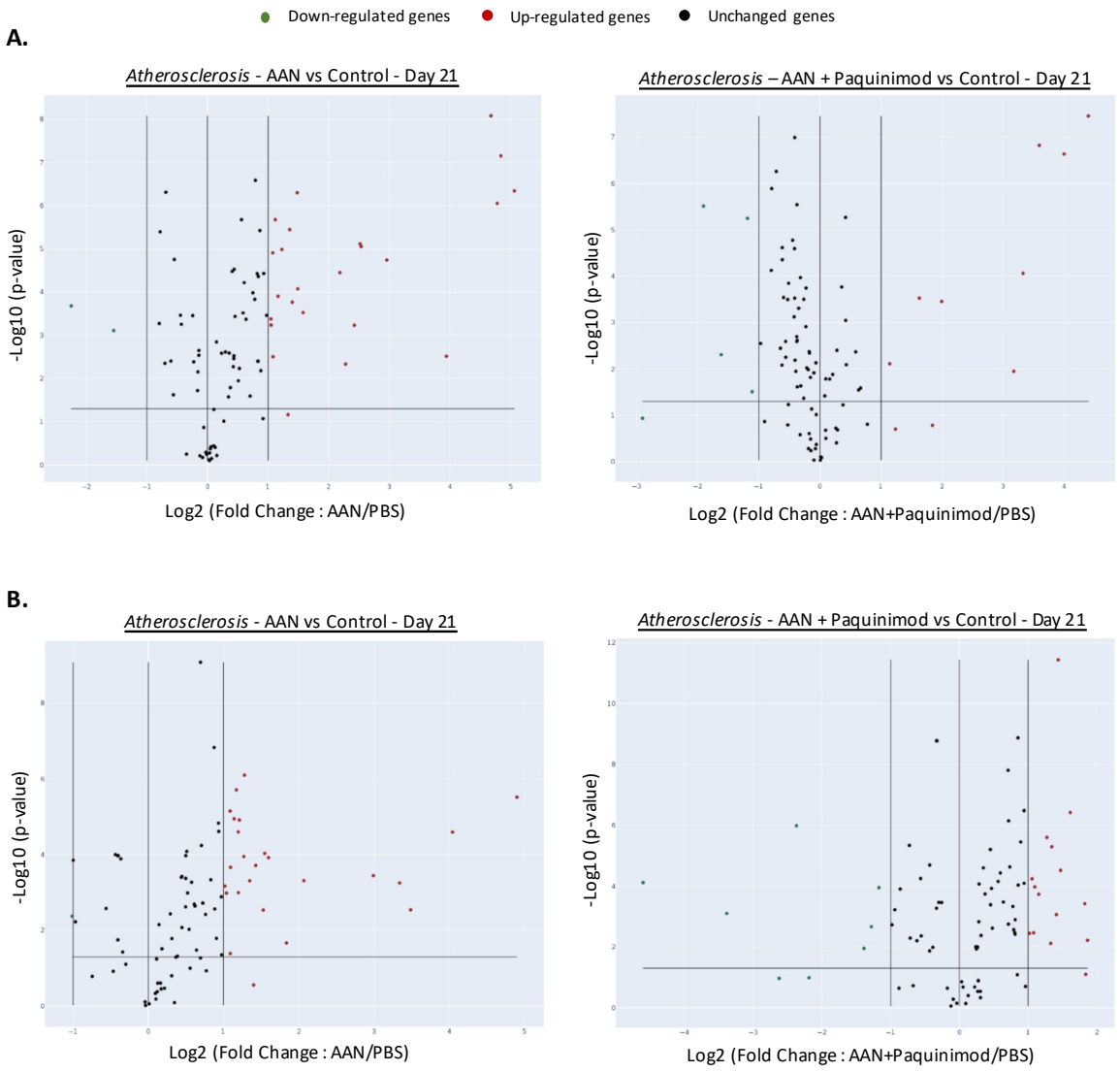


Figure 36. Effect of blocking Calprotectin on AAN-induced systemic pro-atherosclerotic responses *in vivo*

C57BL/6J mice (n=5 per group) were injected intraperitoneally 4 times at 3 day-intervals with AA (2.5 mg/kg) or PBS, in the presence or absence of Paquinimod (20 µg/mouse). Aortas (**A**) and blood (**B**) were obtained at Day 21. Volcano plots compare the effect of AAN and AAN + Paquinimod on atherosclerosis-associated gene expression. Red (upregulated, fold change ≥ 2) and green (downregulated, fold change ≤ 0.5) circles represent single genes significantly affected (p<0.05, represented by the horizontal line) compared to PBS control.

Table 15. Effect of AA + Paquinimod administration (compared to control) on atherosclerosis-related aortic gene expression at Day 21

		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change***	***P Value****
Apoa1	Apolipoprotein A-I	**21.0**	*0.0001*
Apob	Apolipoprotein B	**12.0**	*0.0001*
Cxcl1	Chemokine (C-X-C motif) ligand 1	**2.2**	*0.0077*
Fga	Fibrinogen alpha chain	**10.0**	*0.0001*
Fbg	Fibrinogen beta chain	**15.9**	*0.0001*
Nyp	Neuropeptide Y	**9.0**	*0.0112*
Serpine1	Serine (or cysteine) peptidase inhibitor, mbr 1	**3.1**	*0.0003*
Spp1	Secreted phosphoprotein 1	**4.0**	*0.0003*
Il1b	Interleukin 1 beta	**-2.2**	*0.0310*
Il4	Interleukin 4	**-3.1**	*0.0050*
Itga5	Integrin alpha 5 (fibronectin receptor alpha)	**-2.3**	*0.0000*
Klf2	Kruppel-like factor 2 (lung)	**-3.8**	*0.0000*

***Compared to PBS control group.

Of note, AAN also led to the upregulation of atherosclerosis-associated genes in blood cells at Day 21, which could also be inhibited by treatment with Paquinimod (**Figure 36.B** and **Table 16**). Specifically, out of 84 genes tested, 24 were upregulated by AAN and half of these genes were maintained at normal levels when Paquinimod was co-administered with AA. These included genes encoding notably Apolipoprotein B, the primary apolipoprotein in LDL; CCR1, a chemokine receptor involved in leukocyte recruitment to the intima; ICAM-1, which is upregulated on activated leukocytes and contributes to various immune cell effector functions; Integrin α5, the knock-down of which reduces atherosclerosis; PDGFB, which, together with hypercholesterolaemia, promotes atherosclerosis development; Serpin-e1 (or Plasminogen activator inhibitor-1), a key regulator of fibrinolysis and promoter of cell migration in atherosclerosis; and TNFα-induced protein 3, which is involved in negative feedback loop to end TNFα-induced responses, notably in atherosclerosis. Interestingly, only

1 gene was found downregulated in AAN after 21 days, Platelet-derived growth factor α, and its expression was not affected by Paquinimod treatment.

Table 16. Effect of Paquinimod on AAN-induced changes in atherosclerosis-related gene expression in blood at Day 21

		AAN *		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change****	***P Value****	**Fold Change****	***P Value****
Ace	Angiotensin I converting enzyme 1	**2.1**	*0.0001*	3.1	*0.0000*
Apoa1	Apolipoprotein A-I	**11.2**	*0.0029*	-4.6	*0.1038*
Apob	Apolipoprotein B	**16.5**	*0.0001*	-5.2	*0.0001*
Bcl2	B-cell leukemia/lymphoma 2	**2.1**	*0.0002*	1.9	*0.0002*
Birc3	Baculoviral IAP repeat-containing 3	**2.4**	*0.0001*	2.1	*0.0001*
Ccr1	Chemokine (C-C motif) receptor 1	**2.3**	*0.0001*	1.7	*0.0001*
Ccr2	Chemokine (C-C motif) receptor 2	**2.9**	*0.0002*	2.8	*0.0001*
Cdh5	Cadherin 5	**4.2**	*0.0005*	3.6	*0.0061*
Eng	Endoglin	**2.3**	*0.0001*	1.2	*0.0042*
Fga	Fibrinogen alpha chain	**10.1**	*0.0006*	-24.3	*0.0002*
Fgb	Fibrinogen beta chain	**29.9**	*0.0001*	-6.2	*0.1081*
Icam1	Intercellular adhesion molecule 1	**2.3**	*0.0010*	1.7	*0.0027*
Il1b	Interleukin 1 beta	**3.0**	*0.0001*	2.1	*0.0034*
Itga5	Integrin alpha 5 (fibronectin receptor alpha)	**2.1**	*0.0010*	1.8	*0.0038*
Itgax	Integrin alpha X	**2.2**	*0.0001*	2.4	*0.0001*
Klf2	Kruppel-like factor 2	**2.7**	*0.0002*	1.8	*0.0013*
Ldlr	Low density lipoprotein receptor	**2.3**	*0.0001*	2.5	*0.0001*
Lif	Leukemia inhibitory factor	**2.1**	*0.0409*	2.5	*0.0076*
Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	**2.4**	*0.0001*	2.0	*0.1988*
Ppara	Peroxisome proliferator activated receptor alpha	**8.0**	*0.0004*	2.7	*0.0000*
Ppard	Peroxisome proliferator activated receptor delta	**2.0**	*0.0007*	1.7	*0.0005*
Serpine1	Serine (or cysteine) peptidase inhibitor, mbr 1	**3.6**	*0.0216*	1.2	*0.2897*
Tnf	Tumor necrosis factor	**2.9**	*0.0029*	3.5	*0.0004*
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	**2.5**	*0.0005*	1.9	*0.0000*
Pdgfa	Platelet derived growth factor, alpha	**-2.0**	*0.0042*	-2.4	*0.0022*

*Only statistically significant ($P<0.05$) AAN-induced ≤ 0.5 (in green) or ≥ 2 (in red) fold changes were considered.
**Compared to PBS control group.

Table 17. Effect of AA + Paquinimod administration (compared to control) on atherosclerosis-related gene expression in the blood at Day 21

		AAN + Paquinimod	
Gene symbol	**Description**	**Fold Change***	***P Value****
Ace	Angiotensin I converting enzyme 1	**3.1**	*0.0000*
Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	**2.1**	*0.0001*
Birc3	Baculoviral IAP repeat-containing 3	**2.1**	*0.0001*
Ccr2	Chemokine (C-C motif) receptor 2	**2.8**	*0.0001*
Cdh5	Cadherin 5	**3.6**	*0.0061*
Fgf2	Fibroblast growth factor 2	**2.0**	*0.0040*
Il1b	Interleukin 1 beta	**2.1**	*0.0034*
Itgax	Integrin, alpha X	**2.4**	*0.0000*
Ldlr	Low density lipoprotein receptor	**2.5**	*0.0000*
Lif	Leukemia inhibitory factor	**2.5**	*0.0076*
Ppara	Peroxisome proliferator activated receptor alpha	**2.7**	*0.0000*
Pparg	Peroxisome proliferator activated receptor gamma	**2.7**	*0.0009*
Sele	Selectin, endothelial cell	**2.2**	*0.0002*
Apob	Apolipoprotein B	**-5.2**	*0.0000*
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	**-2.6**	*0.0111*
Cxcl1	Chemokine (C-X-C motif) ligand 1	**-10.5**	*0.0008*
Fga	Fibrinogen alpha chain	**-24.3**	*0.0001*
Pdgfa	Platelet derived growth factor, alpha	**-2.4**	*0.0022*
Selp	Selectin, platelet	**-2.3**	*0.0001*

***Compared to PBS control group.

Taken together, the *in vivo* findings demonstrated that:

- CKD induces a range of systemic inflammatory, immune and atherosclerosis-promoting responses that are expected to drive CV risk;
- Chronic nephropathy also led to a significant upregulation of atherosclerosis promoting-genes in the aortas;
- TLR inhibition with sTLR2 is able to control chronic inflammation induced by CKD;
- Pharmacologic inhibition of Calprotectin prevents a wide range of CKD-induced pro-inflammatory and atherogenic responses, both in the blood and vessels.

Therefore, both DAMP/TLR pathway-targeting strategies represent promising therapeutic avenues to reduce atherosclerosis development in CKD.

III-4.3. Discussion

Following the description of the capacity of CKD-associated DAMPs to promote key cellular functions driving atherosclerosis *in vitro*, the work presented in this last Chapter focused on verifying our hypothesis of a role of DAMPs in promoting CV risk in CKD, and atherosclerosis in particular, *in vivo.* For this purpose, we selected an *in vivo* model of chronic nephropathy developed locally in the Welsh Kidney Research Unit (560). In this model, local inflammation and tissue damage develop in the kidney immediately after repeated injections of AA, while kidney fibrosis (increase in collagen deposition and α-smooth muscle actin expression) and increased plasma creatine (indicative of renal dysfunction) appear in the subsequent 2 weeks, modelling a transition from acute kidney injury to chronic kidney disease. Other available models of CKD include notably administration of other nephrotoxins (e.g. cisplatin (572), folic acid (573)) or surgical models (I/R injury (574), 5/6 nephrectomy (573)), which we considered but did not select due to:

- Clinical relevance: Exposure to nephrotoxins, notably medication, is a leading cause of CKD. In particular, consumption of AA for its anti-inflammatory properties led to an epidemy of CKD, notably in the Balkans, over the last 20-30 years (575). While I/R injury also is a common cause of CKD, kidney removal cannot be considered as relevant.
- Appropriateness to our hypothesis: The AAN model used here reproduces both kidney damage and loss of kidney function sufficient to induce uremia, 2 factors that may lead to DAMPs production in CKD patients. On the contrary, our in-house model of I/R

injury does not induce significant loss of kidney function and 5/6 nephrectomy is based on kidney removal rather than damage. Therefore, we speculate that DAMPs may not be produced at sufficient levels to test our hypothesis in either of these models.

- Model severity and complexity: The AAN model is both simpler and less severe than surgical models of CKD or administration of other nephrotoxins. Notably, although widely reported, cisplatin injections lead to repeated animal deaths in our hands.

To investigate the systemic and vascular consequences of AAN, readouts were evaluated at 2 times points, one immediately following the AA injections (Day 10) and one at the start of loss of kidney function (Day 21). Of note, inflammatory responses, such as cytokine production or increase in inflammatory leukocyte proportions were stronger at the early time point, in line with the fact that the model mimics an acute kidney damage episode, highly pro-inflammatory, progressing over time towards chronic nephropathy. However, inflammatory gene expression changes were maintained at the later time point, suggestive of a state of chronic inflammation. Interestingly, DAMPs blockade, either with sTLR2 or Paquinimod, led to a reduction of inflammation at both time points, suggesting that TLR DAMPs start mediating chronic inflammation early in CKD progression.

One limitation of the model is that a potential direct effect of AA on the systemic readouts tested here could not be investigated. We verified that a single injection of AA, which does not lead to CKD, did not induce any changes in our readouts of interest, compared to the PBS control group (not shown). Nevertheless, it could be argued that, while a single injection has no direct effects, repeated administration does. However, given the reported anti-inflammatory properties of AA (576), it is unlikely that the pro-inflammatory responses

observed here are the result of a direct systemic effect of AA rather than that of AA-induced nephropathy.

Although TLR2 and TLR4 were found to be the main mediators of the DAMPs-induced inflammatory and atherogenic responses *in vitro*, the multi-TLR inhibitor sTLR2 was not as efficient as the single DAMP (Calprotectin) inhibitor Paquinimod at reducing AAN-induced systemic inflammation. This may in part be because sTLR2-mediated inhibition of DAMPs ligands for TLRs other than TLR2 relies entirely on CD14 blocking (298, 498, 562). However, to what extent DAMPs recognition by TLRs requires CD14 is expected to vary with the DAMP and existing literature is limited. It is also possible that timing and dosage of sTLR2 administration could be optimised for a more robust effect.

Another interesting difference between Paquinimod and sTLR2 treatments is that Paquinimod did not impact on the extent of AAN, as judged by maintained creatinine plasma levels while sTLR2 did (**Figure 37**). This observation would need to be confirmed by quantifying kidney fibrosis as well as verifying creatinine levels at a later time point in this model (e.g. Day 28), when a bigger rise in plasma creatinine is expected. If confirmed, this would suggest that Calprotectin is not involved in inducing kidney damage in this model while other TLR-DAMPs are. This is in line with previous reports indicating that Paquinimod was not efficient at reducing nephrotoxin-induced kidney injury, although different models were used (577). Therefore, this would also suggest that the effect of Paquinimod described here is mediated via a true inhibition of the systemic consequences of CKD, while the effect of sTLR2 may in part be the result of a reduction in kidney damage itself.

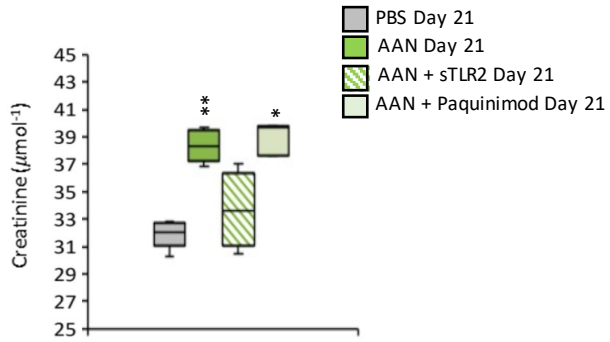


Figure 37. Creatinine measurements in plasma at Day 21
C57BL/6J mice (n=5 per group) were injected intraperitoneally with AA (2.5 mg/kg) or PBS on days 0, 3, 7, and 10. Plasma for each condition was extracted from blood at Day 21 and creatinine levels were measured.
*, $p<0.05$; **, $p<0.01$; *Stimulation vs PBS.

IV- GENERAL DISCUSSION

From the early stages, CKD is associated with markedly increased cardiovascular morbidity and mortality. Atherosclerosis, a chronic inflammatory condition of the arterial wall, underlies the majority of cardiovascular events, and the atherosclerotic burden is increased in CKD (22, 578, 579). Established treatments for atherosclerotic disease in non-CKD patients, including antiplatelet- and lipid-lowering therapy and percutaneous coronary angioplasty, are less effective in CKD patients (578). These suboptimal responses are believed to be underpinned by the unique state of chronic inflammation present in CKD patients, which may explain why treatment strategies that simply correct the biochemical abnormalities have not improved patient outcomes. Therefore, improved insights into the mechanisms that drive chronic inflammation and atherosclerosis in CKD are required before effective treatment strategies can be developed.

Here, we identified specific TLR DAMPs as important contributors to chronic systemic inflammation and early atherosclerosis-associated responses in CKD and revealed the potential of a specific DAMP and a multi-TLR inhibitory strategy to prevent or reduce systemic inflammation and atherosclerosis-associated gene expression induced by chronic nephropathy in mice.

The therapeutic potential of targeting TLR DAMPs to reduce CV risk in CKD was first highlighted by their increased levels in plasma from CKD patients. Our study has not investigated the source(s) of these DAMPs, as it is not of critical relevance to their function and potential as therapeutic targets. Nonetheless, it can be speculated that the DAMPs elevated in CKD plasma may originate from:

- The damaged kidneys: as increased local DAMP production in CKD is well described (85, 580, 581). From the kidneys, these DAMPs could cross to the bloodstream and to the systemic circulation.
- The dialysis treatment: We have demonstrated that exposure to PD solutions leads to the release of DAMPs in the peritoneal cavity in mice and in PD patients (563). It is possible that some of these DAMPs also cross to the bloodstream via the peritoneal membrane. Similarly, HD can induce increased DAMP production (582, 583), although we have not look here at this particular patient population.
- The damaged endothelium: Chronic exposure to inflammatory mediators and uremic toxins activates and damages the endothelium (584) and may induce DAMP production by endothelial cells, therefore contributing to the pool of elevated plasma DAMPs in CKD.
- The activated leukocytes: As for the endothelium, circulating leukocytes are activated by uremic toxins and chronic inflammation, which may also lead to increased DAMPs release. Notably, Calprotectin expression is mostly restricted to phagocytes and makes up to 50% of the soluble protein content of neutrophils (585, 586).
- Reduced plasma clearance: due to kidney failure
- A combination of all of the above: Different plasma DAMPs are also likely to have different origins.

Although increased local (aorta) or systemic TLR-DAMPs levels have been correlated with increased risk of CV event (Introduction, Section I-1.4, p.19), it has not been made clear whether the DAMPs released play a role in worsening CVD or are simply a consequence of increased chronic inflammation and vascular injury. While these reports highlight a strong

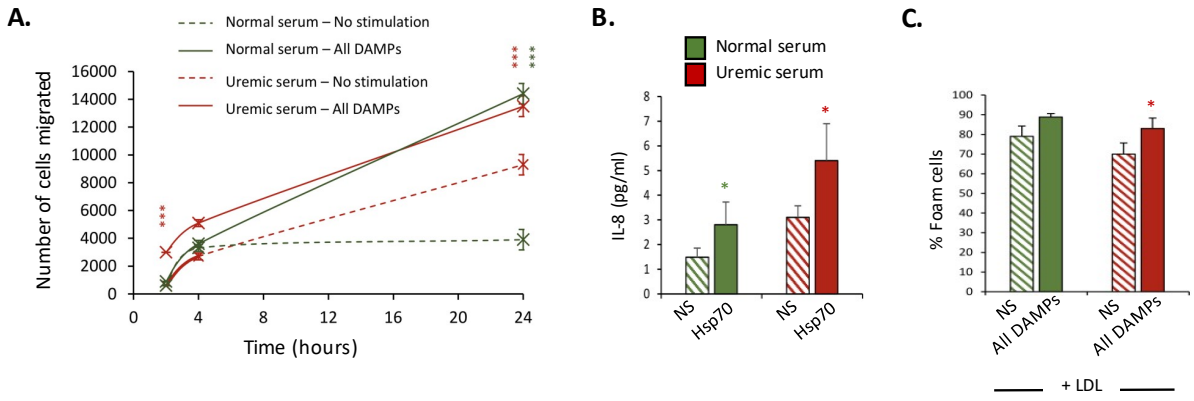
potential for DAMPs as biomarkers and predictors of CV risk, further studies are necessary to better characterise the pathological functions of the DAMPs and their potential as therapeutic targets. Therefore, following the identification of four CKD-associated DAMPs, we demonstrated their ability to promote a number of cellular responses typically associated with atherosclerosis development. These included loss of endothelial barrier function, increased monocytic migratory capacity, higher pro-atherogenic cytokine production and gene expression by macrophages as well as reduced cholesterol 'efflux and increased foam cell formation. Of note, the cellular responses to the DAMPs found elevated in CKD could not be anticipated, as different TLR ligands may induce qualitatively and quantitatively different responses (501-503), and different DAMPs may involve different TLR co-receptors, and have different additional immune receptors other than TLRs (504-506). Accordingly, while only Hsp70 was found to induce cytokine production by macrophages, all of the CKD-associated DAMPs could promote monocyte migration and foam cell formation, suggesting the engagement of different signaling pathways.

In addition to the cellular functions tested here, which are key to the development of atherosclerosis, a potential effect of the DAMPs on cellular responses associated with vascular calcification should also be considered and addressed. As mentioned before, atherosclerotic plaques tend to be more calcified in CKD patients than in the general population (578, 587). Vascular calcification induces a loss of vessel elasticity and is strongly associated with CV events. Excessive and premature calcification in CKD is thought to be driven a combination of metabolic disorders (e.g. elevated blood levels of calcium and phosphorous) and chronic exposure to inflammatory mediators. This combination of factors induces the trans-differentiation of vascular smooth muscle cells (VSMCs) in the intima media to resemble bone formative osteoblasts. This transition leads to well-characterised changes

in morphology, mediator production and gene expression profile (588). For example, VSMCs down-regulate production of smooth muscle specific genes, such as alpha-smooth muscle actin (α-SMA) and lose their contractile properties. Simultaneously they up-regulate markers of osteochondrogenesis such as Runx2, Osteopontin and alkaline phosphatase, and generate calcium and phosphate-rich matrix vesicles that mineralise the vascular wall. Recently, TLR2 activation with bacterial ligands or Hsp60 has been shown to promote VSMC transformation to an osteochondrogenic phenotype (589). Therefore, an effect of CKD-associated DAMPs on VSMC-to-osteoblast transition may be worth investigating. In addition, endothelial cells are important regulators of VSMC differentiation: in health, they provide a barrier that prevents VSMC exposure to cytokines and produce Osteoprotegrin, an inhibitor of vascular calcification. In disease, they generate Bone Morphogenetic Protein-2, a potent osteoblastic differentiation factor that propagates the osteochondrogenic cascade in VSMCs. Therefore, a potential effect of the CKD DAMPs on the production by endothelial cells of mediators that promote or inhibit VSMC-to-osteoblast transition would also need clarifying.

One limitation of this study is that healthy cells were used to carry out these experiments, as it was not possible to obtain endothelial cells from CKD patients or to obtain the large number of macrophages required to carry out foam cell formation experiments from the volume of CKD whole blood that could be drawn (up to 50 ml). However, we confirmed that uremic conditions, reproduced by 48 hours' cell pre-exposure to 25% uremic patient serum, did not affect the ability of the cells to respond to DAMP stimulation, as judged by monocyte migration, pro-inflammatory mediators' production by macrophages and foam cell formation (**Figure 38**). Of note, the proportions of foam cells were much higher in these experimental conditions than in the absence of pre-exposure to serum, either form CKD patients or healthy individuals, probably due to the high levels of LDL/modified LDL present in the serum. This

higher background proportion of foam cells likely underlies the fact that the effect of the combined DAMPs, although statistically significant, was smaller than previously observed (**Figure 27**).



<u>Figure 38.</u> Culture in uremic conditions does not affect the effect of the DAMPs on monocyte migration, cytokine production and foam cell formation by macrophages
Triplicate cultures of MM6 monocytes (**A**) or M-CSF-differentiated macrophages (**B and C**) were cultured for 48 hours in 25% normal AB serum (green) or serum from 5 pooled Stage 5 CKD patients (red) prior to assessing the effect of the indicated CKD-associated DAMPs on monocyte migration (**A**), IL-8 production by macrophages (**B**) and foam cell formation (**C**). These experiments were performed as previously described (Figures 18.B, 25.A and 27.A, respectively).
*, $p<0.05$; ***, $p<0.005$, DAMPs stimulation vs no stimulation (NS), normal (green) or uremic (red) condition.

The potential of TLRs and DAMPs as therapeutic targets was demonstrated by the ability of a multi-TLR inhibitor (sTLR2) and a specific Calprotectin inhibitor (Paquinimod) to prevent inflammation induced by AAN in mice, at early and late time points. The fact that both sTLR2 and Paquinimod were able to inhibit long-term AAN-induced changes while only being administered during CKD induction suggests that DAMPs are produced early in the CKD process and that early targeting may be beneficial to reduce chronic inflammation and overall CV risk in the long term. Although TLR2 and TLR4 were found to be the main mediators of the

DAMPs-induced inflammatory and atherogenic responses *in vitro*, the pan-TLR inhibitor sTLR2 was not as efficient as the single DAMP (Calprotectin) inhibitor Paquinimod at reducing AAN-induced systemic inflammation. While blocking TLR activity with specific anti-TLR neutralising antibodies may bring a more robust inhibition of AAN's inflammatory consequences, full TLR blockade carries a risk of increased susceptibility to infections, whereas we demonstrated that sTLR2's anti-inflammatory activity does not compromise bacterial clearance *in vivo* (498). This is particularly relevant when considering the long-term treatment or management of a patient population prone to infections (590).

Of critical relevance to this study, AAN induced a range of atherosclerosis-promoting gene expression changes in the aortas, which were robustly inhibited by Calprotectin blockade. The potential of Calprotectin to inhibit CKD-associated development of full-blown atherosclerosis needs to be verified in mice prone to atherosclerosis development, which will require the development of a combined CKD and atherosclerosis model, for example inducing AAN in $LDLR^{-/-}$ mice on a high-fat diet. Nonetheless, it is reasonable to expect that maintained reduction of vascular chronic inflammation and arterial atherosclerosis-associated gene expression will result in a lower atherosclerotic burden in susceptible animals.

Of note, Paquinimod co-administration with AA did not reduce the extent of AAN while sTLR2 appeared to (**Figure 37**), at least as judged by creatinine levels. Therefore, a combination of Paquinimod and sTLR2 would be worth evaluating as it may bring a dual beneficial effect on lowering chronic inflammation and CV risk in CKD by: i) directly reducing the systemic and vascular consequences of CKD and ii) reducing kidney damage itself, although this effect of sTLR2 needs to be further demonstrated and likely depends on the primary cause and the extent of CKD.

Paquinimod was originally developed for the treatment of Systemic Lupus Erythematosus (591) and has shown efficiency in other immune diseases, such as diabetes (592), asthma (592) and systemic sclerosis (593). It was granted orphan drug status by the EU and US for the treatment of the latter. Notably, administration of Paquinimod has been shown to lead to the reduction of the atherosclerotic burden in diabetic mice via a reduction of diabetes-induced thrombocytosis (594).

Targeting TLR DAMPs in CKD with a pharmaceutical inhibitor that has already been employed in humans could facilitate the translation of our findings into patient benefit. However, although Calprotectin was selected here as a proof-of-concept DAMP, it is possible that others among the CKD-associated DAMPs identified here, or DAMPs that we have not tested, could also be promising therapeutic targets to reduce CV risk in CKD. As DAMPs have also been found to drive CKD-associated pathologies other than CVD, such as diabetes (595), Alzheimer's disease (596) or rheumatoid arthritis (557), targeting the DAMP/TLR pathway may help to better treat or manage multimorbidites in the CKD cluster.

V- MATERIAL AND METHODS

V-1. Patients samples

Blood samples from aged-matched healthy individuals and Stage 5 CKD patients on PD were obtained in accordance with the institutional review board of Cardiff University and the local National Health Service Research Ethics Committee. Written informed consent was obtained from all donors. Samples from CKD patients with diabetes or malignancy were excluded from the analysis. For patients with prior CVD at the time of sampling (analysed in Figure 34.A), CVD diagnosis included ischemic heart disease, left ventricular dysfunction and peripheral vascular disease. Plasma samples were prepared by centrifugation (15 minutes, 2,000 rpm), aliquoted (200 μl) and promptly frozen at -80°C before testing. At the time of testing, samples were thawed and kept on ice for the duration of the preparation, then analysed by ELISA according to the manufacturer's instructions.

V-2. Cell culture and macrophage differentiation

Human aortic endothelial cells (HAEC, NBS Biologicals) were cultured in M200 supplemented with 2% Large Vessel Endothelial Supplement (Fisher Scientific) and 5% low endotoxin foetal calf serum (FCS, HyClone; < 0.06 U/mL endotoxin).

Human Umbilical Arterial Endothelial Cells (HUAEC, Promo Cell) were cultured in endothelial low serum cell growth medium (Promo Cell).

The human monocytic cell line, MonoMac6 (MM6), was cultured in RPMI-1640 medium supplemented with 10% FCS, 1% insulin (Fisher Scientific), 1% Non-essential Amino Acids (Fisher Scientific) and 1% pyruvate (Fisher Scientific).

Human peripheral blood mononuclear cells (PBMC) were obtained from buffy coats from healthy donors (Welsh Blood Bank Services) through Histopaque 1077 (Merck) density-gradient, as previously described (298).

Blood was diluted 1:2 with room temperature PBS, and 25 ml were cautiously overlaid on top of 15 ml room temperature Histopaque 1077. Blood was then centrifuged at 2,300 rpm for 30 minutes at room centrifuge without brake. After centrifugation, 4 phases are obtained (from bottom to top): the pellet containing red cells and neutrophils, cell-free Histopaque, the white ring containing PBMC, and plasma. PBMC were collected, washed 5 times with PBS at 4°C to remove platelets (1st wash 1,200 rpm for 7 minutes without brake, subsequent washes 1,200 rpm for 7 minutes with brake). PBMC were then cultured in RPMI-1640 medium supplemented with 10% low endotoxin FCS and 1% penicillin/streptomycin. Macrophages were differentiated from blood monocytes obtained by PBMC adhesion (2 hours in RPMI-1640 supplemented with 1% FCS and 1% penicillin/streptomycin). Non-adherent cells were removed and adherent cells (monocytes) were rinsed 2 times with warm sterile PBS before being cultured for at least 7 days in complete medium (RPMI-1640, 10% FCS, 1% penicillin/streptomycin) supplemented with human M-CSF (10 ng/ml, Peprotech) or GM-CSF (80 IU/ml, Peprotech). M-CSF or GM-CSF was replenished every 3 days for the duration of the culture. Purity of the culture was evaluated by flow cytometry based on forward and side scatter profile as well as CD14, TLR2 and TLR4 expression and was always > 97%.

Foam cells, if not stated otherwise, were differentiated from M-CSF macrophages by addition of an excess concentration (50 µg/ml) of OxLDL (InVitrogen) in culture medium for 48 hours. Cell viability was routinely assessed by Trypan Blue staining and was always > 90%.

V-3. Cytokine production assays

For activation experiments, cells were resuspended in their respective complete medium (Material and Methods, Section V-2.) and were seeded in round bottom 96-well plates (PBMC) or flat bottom 96-well plates (HAEC and macrophages, to favour adhesion).

Triplicate aliquots of HAEC (5 x 10^3 cells/well), PBMC (1.5 x 10^5 cells/well) or monocytes-derived (1.5 x 10^4 cells/well) macrophages were cultured (18 hours, 37°C) in the presence of the indicated concentrations of ultra-pure LPS (E. coli O111:B4 strain; Invivogen), the synthetic bacterial lipopeptide Pam_3-CSK_4 (EMC microcollections), recombinant human Hsp70 (active, functional grade, Abcam), recombinant human Calprotectin (S100A8/A9, functional grade, Biolegend), recombinant human HMGB-1 (functional grade, R&D) or hyalorunic acid (low - 15-40 kDa - and medium - 75-350 kDa - molecular weights, functional grade, R&D), or a combination of the 4 DAMPs. The activation step was performed in the absence or presence of OxLDL (10 μg/ml, Invitrogen) for co-exposure experiments (Figure 12 and Figure 25). For pre-exposure experiment in Figure 11, cells were pre-exposed to recombinant human TNFα (10 ng/ml, Abcam) prior to stimulation.

For endotoxin control experiments, cells were co-exposed to the indicated DAMPs or LPS, with or without Polymixin B (5 μg/ml, Merck, Figure 8.A) or before and after Hsp70 denaturation by boiling (10 minutes, 95°C; Figure 8.B).

For TLR blocking experiments, cells were pre-incubated for 1 hour (37°C) with anti-TLR2 (clone T2.5, Invivogen, 10 μg/ml) or anti-TLR4 (clone 3C3, Invivogen, 10 μg/ml) neutralising monoclonal antibodies, alone or combined, or an isotype control (clone MOPC21, 10 μg/ml) prior to addition of 10 ng/ml of ultra-pure LPS (E. coli O111:B4 strain; Invivogen) or a combination of 4 DAMPs at 1 μg/ml (recombinant human Hsp70 (active, functional grade,

Abcam), recombinant human Calprotectin (S100A8/A9, functional grade, Biolegend), recombinant human HMGB-1 (functional grade, R&D) or hyalorunic acid (low and medium molecular weight, functional grade, R&D)) in Figure 18.D. or 1,000 ng/ml of recombinant human Hsp70 (active, functional grade, Abcam) in Figure 25.C. for 18 hours.
Cell viability was routinely assessed at the end of the experiments by Trypan Blue staining. It was always > 90% and was not affected by treatment.

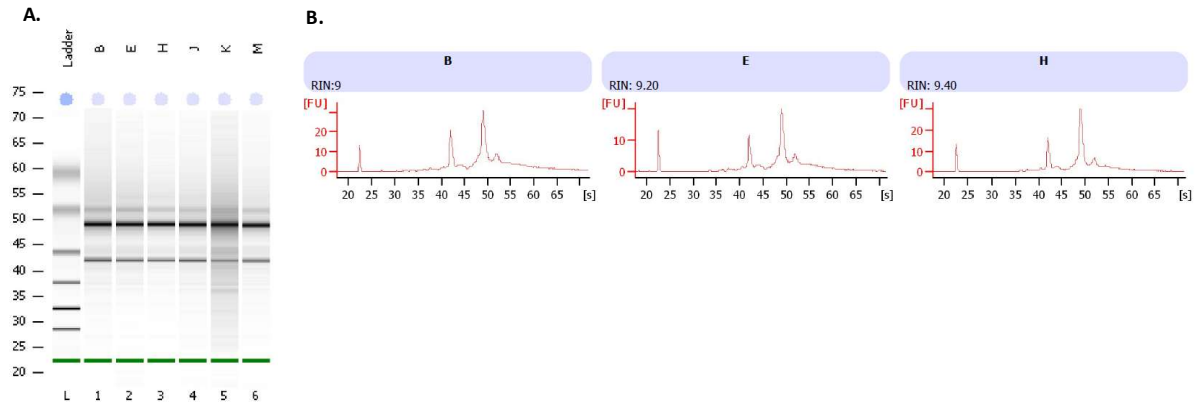
Culture supernatants were collected following incubation, and analysed by ELISA for the levels of MCP-1, IL-8, IL-6, IL-10 or TFIII according to the manufacturer's instructions (all kits are from R&D).

V-4. Focused gene arrays

V-4.1. *In vitro*

For activation, monocytes derived-macrophages or HAEC were seeded at a density of 0.75 x 10^6 cells/well in a 6-well plate or T25 respectively. Macrophages were cultured for 18 hours (37°C) in the absence or presence of 1,000 ng/ml of recombinant human Hsp70. HAEC were cultured for 6 hours in the absence or presence of 10 ng/ml of ultra-pure LPS (E. coli O111:B4 strain; Invivogen) or a combination of 4 DAMPs at 1 μg/ml: recombinant human Hsp70 (active, functional grade, Abcam), recombinant human Calprotectin (S100A8/A9, functional grade, Biolegend), recombinant human HMGB-1 (functional grade, R&D) or hyalorunic acid (low and medium molecular weight, functional grade, R&D). Following activation, cells were collected and lysed with Red Blood Cells lysis buffer (Pharm Lyse, BD Biosciences). Total RNA was extracted and purified using a Qiagen RNeasy mini kit (Qiagen), following the manufacturer's instructions. Final RNA concentration was quantified via

Nanodrop, and stored at -80°C until further use. When in use, purified RNA was thawed and kept on ice. Prior to RNA use in qPCR gene arrays, RNA quality was determined by evaluating the RNA integrity number (RIN) on an Agilent 2100 Bioanalyzer (Central Biotechnology Services, CBS, Cardiff University). RIN for all RNA preparations subsequently used in gene arrays was >8, indicating high quality RNA and low degradation (examples in **Supplementary Figure 1**).



<u>Supplementary Figure 1.</u> RNA quality determination using the Agilent 2100 Bioanalyzer
RNA integrity was evaluated by electrophoresis of 2µl of total RNA preparations on microchips (**A**) Images typically show 2 main bands comprising the 28S (upper) and 18S (lower) and RNI was determined by the ratio between these 2 bands, using the values given by the electropherograms (**B**). RNA is considered of high quality when RIN > 8. 6 (**A**) and 3 (**B**) of our total RNA samples are shown here as an example.

Purified RNA (250 ng/condition for macrophages, and 500 ng/condition for HAEC) was converted into cDNA by reverse transcription (RT) using RT^2 First Strand kit (Qiagen). Quantitative real-time PCR (qPCR) was then performed in triplicate for each experimental condition using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen), combined with the human

Atherosclerosis RT^2 Profiler PCR Array (Qiagen, 384 wells, Cat. No. PAHS-038Z) plate. 84 genes were profiled in addition to 5 housekeeping genes (Full list in **Supplementary Table 1**) as well as reverse transcription and positive PCR and human genomic DNA contamination controls). Thermocycler program was as recommended: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C on a QuantStudio 12K Flex Real-Time PCR thermocycler. Reference genes were selected automatically from the housekeeping gene panel (based on least Ct variation between groups) and relative gene expression was calculated using the ΔΔCt method using the Qiagen Geneglobe analysis tool.

Supplementary Table 1. Genes analysed by human atherosclerosis-focused gene array
House-keeping genes are shown in blue

Gene symbol	Description
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1
Ace	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
Apoa1	Apolipoprotein A-I
Apob	Apolipoprotein B (including Ag(x) antigen)
Apoe	Apolipoprotein E
Bax	BCL2-associated X protein
Bcl2	B-cell CLL/lymphoma 2
Bcl2a1	BCL2-related protein A1
Bcl2l1	BCL2-like 1
Bid	BH3 interacting domain death agonist
Birc3	Baculoviral IAP repeat containing 3
Ccl2	Chemokine (C-C motif) ligand 2
Ccl5	Chemokine (C-C motif) ligand 5
Ccr1	Chemokine (C-C motif) receptor 1
Ccr2	Chemokine (C-C motif) receptor 2
Cd44	CD44 molecule (Indian blood group)
Cdh5	Cadherin 5, type 2 (vascular endothelium)
Cflar	CASP8 and FADD-like apoptosis regulator
Col3a1	Collagen, type III, alpha 1
Csf1	Colony stimulating factor 1 (macrophage)
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
Ctgf	Connective tissue growth factor
Egr1	Early growth response 1
Eln	Elastin
Eng	Endoglin
Fabp3	Fatty acid binding protein 3, muscle and heart (mammary-derived growth
Fas	Fas (TNF receptor superfamily, member 6)
Fga	Fibrinogen alpha chain
Fgf2	Fibroblast growth factor 2 (basic)
Fn1	Fibronectin 1
Hbegf	Heparin-binding EGF-like growth factor
Icam1	Intercellular adhesion molecule 1
Ifnar2	Interferon (alpha, beta and omega) receptor 2
Ifng	Interferon, gamma
Il1a	Interleukin 1, alpha
Il1r1	Interleukin 1 receptor, type I
Il1r2	Interleukin 1 receptor, type II
Il2	Interleukin 2
Il3	Interleukin 3 (colony-stimulating factor, multiple)
Il4	Interleukin 4
Il5	Interleukin 5 (colony-stimulating factor, eosinophil)
Itga2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
Itga5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)

Gene symbol	Description
Itgax	Integrin, alpha X (complement component 3 receptor 4 subunit)
Itgb2	Integrin, beta 2 (complement 3 receptor 3 and 4 subunit)
Kdr	Kinase insert domain receptor (a type III receptor tyrosine kinase)
Klf2	Kruppel-like factor 2 (lung)
Lama1	Laminin, alpha 1
Ldlr	Low density lipoprotein receptor
Lif	Leukemia inhibitory factor (cholinergic differentiation factor)
Lpa	Lipoprotein, Lp(a)
Lpl	Lipoprotein lipase
Mmp1	Matrix metallopeptidase 1 (interstitial collagenase)
Mmp3	Matrix metallopeptidase 3 (stromelysin, progelatinase)
Msr1	Macrophage scavenger receptor 1
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
Nos3	Nitric oxide synthase 3 (endothelial cell)
Npy	Neuropeptide Y
Nr1h3	Nuclear receptor subfamily 1, group H, member 3
Pdgfa	Platelet-derived growth factor alpha polypeptide
Pdgfb	Platelet-derived growth factor, beta polypeptide
Pdgfrb	Platelet-derived growth factor receptor, beta polypeptide
Plin2	Perilipin 2
Ppara	Peroxisome proliferator-activated receptor alpha
Ppard	Peroxisome proliferator-activated receptor delta
Pparg	Peroxisome proliferator-activated receptor gamma
Ptgs1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and
Rxra	Retinoid X receptor, alpha
Sele	Selectin E
Sell	Selectin L
Selplg	Selectin P ligand
Serpinb2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
Serpine1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type
Sod1	Superoxide dismutase 1, soluble
Spp1	Secreted phosphoprotein 1
Tgfb1	Transforming growth factor, beta 1
Tgfb2	Transforming growth factor, beta 2
Thbs4	Thrombospondin 4
Tnc	Tenascin C
Tnf	Tumor necrosis factor
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3
Vcam1	Vascular cell adhesion molecule 1
Vegfa	Vascular endothelial growth factor A
Vwf	Von Willebrand factor
Actb	Actin, beta
B2m	Beta-2-microglobulin
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Hprt1	Hypoxanthine phosphoribosyltransferase 1
Rplp0	Ribosomal protein, large, P0

V-4.2. *In vivo*

For gene arrays on mouse blood, blood from 3 animals from a same group were pooled to have enough volume to perform RNA extraction. Total RNA was extracted from mouse blood using QIAamp RNA Blood Mini Kit according to the manufacturer's instructions, quantified by Nanodrop, and kept frozen (-80°C) until further use. For aortic gene arrays, thoracic portions of aortas were obtained at the time of culling, and frozen immediately on dry ice before storage at -80°C until further use. Aortic tissue was homogenised on a bead TissueLyser II (Qiagen, via CBS) by 1 minute disruption at 30 Hz. Total RNA was then extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions, quantified by Nanodrop, and kept frozen (-80°C) until further use. High-quality (RIN>8, see above and **Supplementary Figure 1**) purified RNA (325 ng/condition for blood, and 500 ng/condition for aortas) was converted into cDNA by reverse transcription (RT^2 First Strand kit, Qiagen). Focused transcriptomic analysis was then performed by qPCR of the cDNA using a mouse Atherosclerosis (for aortas, Qiagen, 384 wells, Cat. No. PAMM-038ZE-4, full list of gene in **Supplementary Table 2**) or mouse Innate and Adaptive responses RT^2 Profiler PCR Array (for blood, Qiagen, 384 wells, Cat. No. PAMM-052ZE-4, full list of gene in **Supplementary Table 3**). qPCR was then performed in triplicate for each experimental condition and 84 genes per array were profiled. qPCR conditions and data analysis were performed as described above for *in vitro* samples (Section V-4.1.).

Supplementary Table 2. Genes analysed by mouse atherosclerosis-focused gene array
House-keeping genes are shown in blue

Gene symbol	Description
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1
Ace	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
Apoa1	Apolipoprotein A-I
Apob	Apolipoprotein B
Apoe	Apolipoprotein E
Bax	BCL2-associated X protein
Bcl2	B-cell leukemia/lymphoma 2
Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a
Bcl2l1	Bcl2-like 1
Bid	BH3 interacting domain death agonist
Birc3	Baculoviral IAP repeat containing 3
Ccl2	Chemokine (C-C motif) ligand 2
Ccl5	Chemokine (C-C motif) ligand 5
Ccr1	Chemokine (C-C motif) receptor 1
Ccr2	Chemokine (C-C motif) receptor 2
Cd44	CD44 antigen
Cdh5	Cadherin 5
Cflar	CASP8 and FADD-like apoptosis regulator
Col3a1	Collagen, type III, alpha 1
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
Ctgf	Connective tissue growth factor
Cxcl1	Chemokine (C-X-C motif) ligand 1
Eln	Elastin
Eng	Endoglin
Fabp3	Fatty acid binding protein 3, muscle and heart
Fas	Fas (TNF receptor superfamily, member 6)
Fga	Fibrinogen alpha chain
Fgb	Fibrinogen beta chain
Fgf2	Fibroblast growth factor
Fn1	Fibronectin 1
Hbegf	Heparin-binding EGF-like growth factor
Icam1	Intercellular adhesion molecule 1
Ifng	Interferon gamma
Il1a	Interleukin 1 alpha
Il1b	Interleukin 1 beta
Il1r1	Interleukin 1 receptor, type I
Il1r2	Interleukin 1 receptor, type II
Il2	Interleukin 2
Il3	Interleukin 3
Il4	Interleukin 4
Il5	Interleukin 5
Itga2	Integrin alpha 2
Itga5	Integrin alpha 5 (fibronectin receptor alpha)

Gene symbol	Description
Itgax	Integrin alpha X
Itgb2	Integrin beta 2
Kdr	Kinase insert domain protein receptor
Klf2	Kruppel-like factor 2 (lung)
Lama1	Laminin, alpha 1
Ldlr	Low density lipoprotein receptor
Lif	Leukemia inhibitory factor
Lpl	Lipoprotein lipase
Lypla1	Lysophospholipase 1
Mmp1a	Matrix metallopeptidase 1a (interstitial collagenase)
Mmp3	Matrix metallopeptidase 3
Msr1	Macrophage scavenger receptor 1
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1,
Npy	Neuropeptide Y
Nr1h3	Nuclear receptor subfamily 1, group H, member 3
Pdgfa	Platelet-derived growth factor, alpha
Pdgfb	Platelet-derived growth factor, B polypeptide
Pdgfrb	Platelet-derived growth factor receptor, beta polypeptide
Plin2	Perilipin 2
Ppara	Peroxisome proliferator activated receptor alpha
Ppard	Peroxisome proliferator activated receptor delta
Pparg	Peroxisome proliferator activated receptor gamma
Ptgs1	Prostaglandin-endoperoxide synthase 1
Rxra	Retinoid X receptor alpha
Sele	Selectin, endothelial cell
Sell	Selectin, lymphocyte
Selp	Selectin, platelet
Selplg	Selectin, platelet (p-selectin) ligand
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2
Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1
Sod1	Superoxide dismutase 1, soluble
Spp1	Secreted phosphoprotein 1
Tgfb1	Transforming growth factor, beta 1
Tgfb2	Transforming growth factor, beta 2
Thbs4	Thrombospondin 4
Tnc	Tenascin C
Tnf	Tumor necrosis factor
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3
Vcam1	Vascular cell adhesion molecule 1
Vegfa	Vascular endothelial growth factor A
Vwf	Von Willebrand factor homolog
Actb	Actin, beta
B2m	Beta-2 microglobulin
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gusb	Glucuronidase, beta
Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1

Supplementary Table 3. Genes analysed by mouse innate and adaptive responses-focused gene array
House-keeping genes are shown in blue

Gene symbol	Description
Apcs	Serum amyloid P-component
C3	Complement component 3
C5ar1	Complement component 5a receptor 1
Casp1	Caspase 1
Ccl12	Chemokine (C-C motif) ligand 12
Ccl5	Chemokine (C-C motif) ligand 5
Ccr4	Chemokine (C-C motif) receptor 4
Ccr5	Chemokine (C-C motif) receptor 5
Ccr6	Chemokine (C-C motif) receptor 6
Ccr8	Chemokine (C-C motif) receptor 8
Cd14	CD14 antigen
Cd4	CD4 antigen
Cd40	CD40 antigen
Cd40lg	CD40 ligand
Cd80	CD80 antigen
Cd86	CD86 antigen
Cd8a	CD8 antigen, alpha chain
Crp	C-reactive protein, pentraxin-related
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)
Cxcl10	Chemokine (C-X-C motif) ligand 10
Cxcr3	Chemokine (C-X-C motif) receptor 3
Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
Fasl	Fas ligand (TNF superfamily, member 6)
Foxp3	Forkhead box P3
Gata3	GATA binding protein 3
H2-Q10	Histocompatibility 2, Q region locus 10
H2-T23	Histocompatibility 2, T region locus 23
Icam1	Intercellular adhesion molecule 1
Ifna2	Interferon alpha 2
Ifnar1	Interferon (alpha and beta) receptor 1
Ifnb1	Interferon beta 1, fibroblast
Ifng	Interferon gamma
Ifngr1	Interferon gamma receptor 1
Il10	Interleukin 10
Il13	Interleukin 13
Il17a	Interleukin 17A
Il18	Interleukin 18
Il1a	Interleukin 1 alpha
Il1b	Interleukin 1 beta
Il1r1	Interleukin 1 receptor, type 1
Il2	Interleukin 2
Il23a	Interleukin 23, alpha subunit p19

Gene symbol	Description
Il4	Interleukin 4
Il5	Interleukin 5
Il6	Interleukin 6
Irak1	Interleukin-1 receptor-associated kinase 1
Irf3	Interferon regulatory factor 3
Irf7	Interferon regulatory factor 7
Itgam	Integrin alpha M
Jak2	Janus kinase 2
Ly96	Lymphocyte antigen 96
Lyz2	Lysozyme 2
Mapk1	Mitogen-activated protein kinase 1
Mapk8	Mitogen-activated protein kinase 8
Mbl2	Mannose-binding lectin (protein C) 2
Mpo	Myeloperoxidase
Mx1	Myxovirus (influenza virus) resistance 1
Myd88	Myeloid differentiation primary response gene 88
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-
Nlrp3	NLR family, pyrin domain containing 3
Nod1	Nucleotide-binding oligomerisation domain containing 1
Nod2	Nucleotide-binding oligomerisation domain containing 2
Rag1	Recombination activating gene 1
Rorc	RAR-related orphan receptor gamma
Slc11a1	Solute carrier family 11 (proton-coupled divalent metal ion
Stat1	Signal transducer and activator of transcription 1
Stat3	Signal transducer and activator of transcription 3
Stat4	Signal transducer and activator of transcription 4
Stat6	Signal transducer and activator of transcription 6
Tbx21	T-box 21
Ticam1	Toll-like receptor adaptor molecule 1
Tlr1	Toll-like receptor 1
Tlr2	Toll-like receptor 2
Tlr3	Toll-like receptor 3
Tlr4	Toll-like receptor 4
Tlr5	Toll-like receptor 5
Tlr6	Toll-like receptor 6
Tlr7	Toll-like receptor 7
Tlr8	Toll-like receptor 8
Tlr9	Toll-like receptor 9
Tnf	Tumor necrosis factor
Traf6	Tnf receptor-associated factor 6
Tyk2	Tyrosine kinase 2
Actb	Actin, beta
B2m	Beta-2 microglobulin
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gusb	Glucoronidase, beta
Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1

V-5. Trans-endothelial resistance measurements

Trans-endothelial electrical resistance (TER) was measured in collaboration with the team of Prof. Claus P. Schmitt (Heidelberg University, Germany) as previously described by them (597). Briefly, primary HUAEC were seeded on 0.4 μm polyester transwell-inserts placed in 24 well plates (50,000 cells/insert, low serum cell growth medium). TER was measured daily with an EVOM volt/ohm meter with STX-2 electrodes (World Precision Instruments) until it reached a plateau, indicating the formation of a confluent, well-polarised monolayer. Baseline TER was measured, and medium was exchanged for treatment solution containing LPS (10 ng/ml) or the indicated concentrations of DAMPs, alone or in combination. TER was then measured at the indicated time points. Plotted TER values were calculated by subtracting blank well measurement from control wells with cells and multiplying it by 0.33 (area) with resulting unit of $\Omega.cm^2$. A ratio was calculated between the TER values after the treatment at each time points and the baseline TER (0 hour) values to calculate % of initial TER for each condition.

For ZO-1 visualisation, cells were fixed with 100% EtOH for (5 minutes, -20°C), followed by permeabilisation with 0.5% TritonX (10 minutes, RT). Cells were then incubated with 5% BSA for 60 minutes at room temperature before staining with an AF555-conjugated anti-ZO-1 mAb (Invitrogen, MA3-39100-A555, 1:500, 1h, RT). Cells were washed 3 times and nuclei were stained with DAPI (Invitrogen, 30 nM, 15 minutes, RT). Transwell filters were cut out from the plastic and fixed in Prolong Gold (Thermo Fisher, 10 μl) on a glass slide. Slides were allowed to harden for 24 hours. Cells were visualised using Acquifer widefield microscope (ACQUIFER Imaging GmbH). For each condition, z-stack images (10 slices, 3 μm slice distance) were acquired using a 20X objective. The Fiji software was used for image analysis. Greyscale

images were used to create z-stack projections using maximum intensity method to obtain a clear signal from cell membrane areas. 3 stacks were used for every condition. 5 different areas were randomly selected for each condition where the cell membranes were annotated manually, and ZO-1 signal intensity was measured at cell-to-cell junctions and corrected for analysed area to obtain the values shown.

V-6. Monocyte migration

For activation, MM6 (1 x 10^6 cells/well) were cultured (18 hours, 37°C) in the presence of the indicated concentrations of ultra-pure LPS (10 ng/ml), or recombinant human Hsp70, recombinant human Calprotectin, recombinant human HMGB-1 or hyalorunic acid (low and medium molecular weight), alone or in combination. For TLR blocking, cells were pre-incubated for 1 hour (37°C) with a combination of anti-TLR2 (clone T2.5, 10 μg/ml) and anti-TLR4 (clone 3C3, 10 μg/ml) neutralising monoclonal antibodies or an isotype control (clone MOPC21, 10 μg/ml) prior to addition of 10 ng/ml of ultra-pure LPS or a combination of the 4 CKD-associated DAMPs, each at 1 μg/ml for 18 hours.

MM6 were then starved in serum-free medium for 1 hour prior to seeding (200,000 cells, in triplicates) in the top chamber of 8 μM pores trans-wells. The bottom compartment was filled with RPMI + 10% FCS + recombinant human MCP-1 (CCL2, active, functional grade, R&D, 50 ng/ml). Cell numbers were counted in the bottom compartment at the indicated time points, typically 2, 4, 6 and 24 hours.

V-7. Foam cells formation and Oil Red-O staining

Monocytes-derived macrophages were seeded in 8-well microscopy slide (175,000 cells/well) and starved in serum-free RPMI-1640, 0.2% fatty-acid free BSA for 24 hours. Following this starvation step, cells were activated by culturing (37°C) in serum-free medium in the presence, or not, of human LDL (Invitrogen, 25 µg/ml), alone or in addition of ultra-pure LPS or recombinant human Hsp70, recombinant human Calprotectin, recombinant human HMGB-1 or hyalorunic acid (low and medium molecular weight), separately or in combination (1 µg/ml) for 24 hours. For TLR blocking, cells were pre-incubated in serum-free RPMI-1640, 0.2% fatty-acid free BSA for 1 hour (37°C) with anti-TLR2 (clone T2.5, 10 µg/ml) or anti-TLR4 (clone 3C3, 10 µg/ml) neutralising monoclonal antibodies or an isotype control (clone MOPC21, 10 µg/ml) prior to addition, or not, of 1 µg/ml of all 4 DAMPs in the presence, or absence, of human LDL (25 µg/ml) for 24 hours.

Following foam cell formation, cells were fixed using 10% phosphate buffered formalin (10 minutes), then rinsed briefly in PBS then in 60% isopropanol to facilitate the staining of neutral lipids. Oil Red-O (Sigma) was used to stain and visualise intracellular neutral lipids. Staining was performed following Xu S. and al. (544) improved protocol, with a 30-minute incubation time (37°C in the dark) with Oil Red-O pigments. Cells were then destained briefly in 60% isopropanol and rinsed twice in PBS (3 minutes each). Slides were fixed and mounted using mounting medium with DAPI (Fluoroshield ab104139, Abcam) to preserve fluorescence and visualise cell nucleus. Microscopy photos were taken at 20X magnification on a Leica DM LA microscope, and 5 non-overlapping fields view were selected for each condition. To quantify foam cell formation, 20 cells were selected from the top left corner of each picture, each cell with detectable red staining was considered a foam cell, while cells

without staining were considered normal cells. The process was repeated with the 5 pictures taken for each condition (total of 100 cells counted/condition). The percentage of foam cells per condition was then used as a measure of foam cell formation.

V-8. Modified cholesterol uptake

For modified LDL uptake experiments, monocytes-derived macrophages were seeded in 24-well plates at a density of 40,000 cells/well, and starved in serum-free RMPI-1640 supplemented with 0.2% fatty-acid free BSA for 18 hours (37°C) in the presence or absence of ultra-pure LPS (10 ng/ml) or recombinant human Hsp70, recombinant human Calprotectin, recombinant human HMGB-1 or hyalorunic acid (low and medium molecular weight), alone or in combination (each at 1 μg/ml). Dil (554/571)-conjugated acetylated LDL (Dil-AcLDL, Alfa Aesar) was added to the culture medium at a concentration of 10 μg/ml, unless stated otherwise. After 24 hours, the Dil-AcLDL-containing medium was removed, the cells were rinsed twice with sterile PBS, and internalised Dil-AcLDL was quantified by flow cytometry on a FACS Canto II cytometer (at least 10,000 events/condition, Mean Fluorescence Intensity, MFI, shown).

V-9. Modified cholesterol efflux

Macrophages were seeded in 24-well plates at a density of 250,000 cells/well and starved in serum-free RPMI-1640 supplemented with 0.2% fatty-acid free BSA for 18 hours prior to experiment. Cells were then loaded with BODIPY-labelled cholesterol (5 μM, Cayman Chemical Company) in the same serum-free medium (0.2% fatty-acid free BSA) for 24 hours before medium removal. Cells were rinsed with PBS before addition of equilibrium medium

for 1 hour (RPMI-1640 + 0.2% fatty-acid free BSA) with or without exposure to ultra-pure LPS (10 ng/ml), or recombinant human Hsp70, recombinant human Calprotectin, recombinant human HMGB-1 or hyalorunic acid, alone or in combination (1 μg/ml). After equilibration, medium was removed and replaced with fresh RPMI-1640 containing the same concentrations of LPS or DAMPs in the presence of 10% FCS as a cholesterol acceptor for the rest of the experiment. Supernatents were collected after 1, 2, 4, and 24 hours and transferred in a white bottom plate in order to detect fluorescence. BODIPY-associated fluorescence was then measured as described (598), using OMEGAstar software (excitation: 485 nm, emission: 520 nm, gain: 500).

V-10. Cell surface staining

V-10.1. *In vitro*

Expression levels of cell surface antigens were determined by flow cytometry. Cell suspensions (0.15-1 x 10^6 cells/staining) were prepared in sterile PBS and Fc receptors were blocked by incubation in 50% normal rabbit serum (10 minutes, room temperature) before incubation (45 minutes, 4°C) with directly conjugated monoclonal antibodies (**Supplementary Table 4**), at a concentration recommended by the manufacturer (typically 5 μg/ml in 100 μl incubation volume). Cells were rinsed twice with PBS before immediate flow cytometry analysis on a FACS Canto II cytometer. Cellular debris and doublets were excluded based on their FSC-A/SSC-A and FSC-H/FSC-A scatter profiles, respectively.

HAECs were stained for TLR2, TLR4, CD14, ICAM-1 and VCAM-1. MM6 were stained for CCR2. Monocytes were stained for PSGL-1, CD11b and α4. GM-CSF and M-CSF-differentiated macrophages were stained for TLR2, TLR4, CD14, CD36 and SR-A. M-CSF foam

cells were stained for CD36 and SR-A. All antibody clones, fluorophores and manufacturers are detailed in **Supplementary Table 4**.

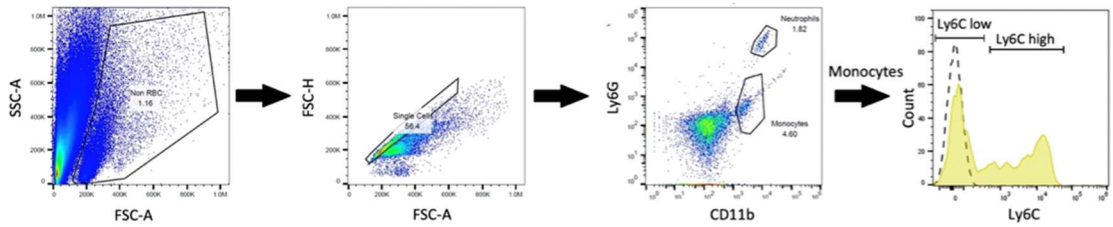
Supplementary Table 4. List of antibodies used for flow cytometry analysis of *in vitro* experiments

Antigen	Antibody clone	Fluorophore	Manufacturer
TLR2	11G7	Brilliant Violet 421	BD Biosciences
TLR4	HTA125	APC	InVitrogen
CD14	61D3	FITC	eBioscience
ICAM-1	HA58	FITC	BioLegend
VCAM-1	STA	APC	BioLegend
CCR2	K036C2	Brilliant Violet 421	BioLegend
PSGL-1	688101	Alexa 488	R&D
CD11b	M1/79	PerCP Cy5.5	BioLegend
α4	7.2R	Alexa 647	R&D
CD36	5-271	FITC	BioLegend
SR-A	7C9C20	APC	BioLegend

V-10.2. *In vivo*

Mouse blood was obtained by intra-cardiac puncture at cull points and immediately placed on ice until further processing. 30 µl of blood/condition was used for flow cytometry analysis. Samples underwent red blood lysis (Pharm Lyse, BD Biosciences) for 15 minutes at room temperature, before centrifugation (450 x *g*, 5 minutes) and pellet resuspended in sterile PBS. Fc receptors were blocked (5 minutes, 4°C) by incubation with mouse Fc block (clone 2.4G2, BD Biosciences, 1:2,500) before incubation (45 minutes, 4^{o}C) with a mix of directly conjugated monoclonal antibodies (see **Supplementary Table 5**), at concentrations recommended by the manufacturer (1:40 dilution for antibodies, 1:500,000 for live-dead stain, 100 µl incubation volume). Cells were rinsed twice with PBS before immediate flow cytometry analysis using an Attune NxT cytometer and software. Cellular debris and doublets

were excluded based on their FSC-A/SSC-A and FSC-H/FSC-A scatter profiles, respectively. Prior to fluorescent signal analysis, compensation, or the process of correcting for fluorescence spillover by removing the signal of any given fluorochrome from all detectors except the one devoted to measuring that dye, was done manually following acquisition. Single cell viability was always >95% and only live cells were included in the analysis (Fixable yellow negative). As described in the literature, neutrophils were defined as $Ly6G^+$ and $CD11b^+$ and monocytes as $Ly6G^-$ and $CD11b^+$ (599, 600). Among total monocytes, $Ly6C^{high}$ and $Ly6C^{low}$ populations were determined based on their Ly6C expression (**Supplementary Figure 2**).



Supplementary Figure 2. Gating strategy for defining leukocyte populations in blood
Cellular debris and doublets were excluded based on their FSC-A/SSC-A and FSC-H/FSC-A scatter profiles, respectively. Neutrophils were defined as $Ly6G^+$ and $CD11b^+$ and monocytes as $Ly6G^-$ and $CD11b^+$. Among total monocytes, $Ly6C^{high}$ and $Ly6C^{low}$ populations were determined based on their Ly6C expression.

Supplementary Table 5. List of antibodies used for flow cytometry analysis of *in vivo* experiments

Antigen	Antibody clone	Fluorophore	Manufacturer
Ly6C	HK1.4	AF488	BioLegend
Ly6G	1A8	AF647	BioLegend
CD11b	M1/70	PerCP Cy5.5	BioLegend
PSGL-1	2PH1	Brilliant Violet 421	BioLegend
Live-dead	-	Fixable yellow	Life Tech.

V-10.3. Use of isotype controls

Isotype controls were used to verifying the efficacy of Fc receptor blocking and in experiments aimed at quantitative evaluation of expression levels (Figure 9, Figure 14.A, Figure 21.B). However, as recommended in specialised flow cytometry reports(601-603), they were not used when:

- Populations were gated based on clear bi-model distribution of an antigen(601, 603), where one population provides an internal negative control for the other. For example, neutrophils express Ly6G while monocytes in the same sample do not (e.g. Leukocyte proportions in Figures 32.C, 34.C; Supplementary Figure 2).
- Biological comparison samples are present(601, 603). For example, in stimulation assays, the unstimulated sample provides the reference level of antigen expression, and the aim is to evaluate changes in expression rather than actual expression levels (e.g. VCAM-1 and ICAM-1 expression in Figure 14.B; PSGL-1, CD11b and Integrin α4 expression in Figures 19, 20; CD36 and SR-A expression in Figures 28.B, 29.B; PSGL-1 expression in Figures 32.C, 34.C).

V-11. Animal work

All procedures were carried out under Home Office project license (PA4A9D766). Inbred 7 to 9-week-old wild-type C57/BL6J mice were obtained from Charles River. Mice (n=5/group) were intraperitoneally injected with PBS (500 µl), Aristolochic Acid (AA, Sigma, 2.5 mg/kg), AA + sTLR2 (250 ng/mouse) or AA + Paquinimod (1 mg/kg). Concentrations of AA, sTLR2 and Paquinimod were selected following previous reports, by ourselves or others (298,

498, 560, 604). Injections were repeated every 3 days for a total of 4 times and mice were sacrificed 1 day (Day 10) or 10 days (Day 21) after the last injection. Blood was obtained by cardiac puncture and analysed by flow cytometry as described above. Following centrifugation (15 minutes, 800 x *g*, 4°C), plasma was kept frozen (-80°C) until analysis by ELISA (cytokines and DAMP levels) or for creatinine levels (Cardiff and Vale UHB, Medical Biochemistry services). RNA was extracted as described above from the total blood cell fraction. The heart was flushed with PBS (10 ml) prior to aorta and kidney isolation. The thoracic portion of the aorta was immediately snap-frozen prior to RNA extraction and the right kidney was halved lengthwise, transferred to a histology cassette, and fixed in 10% Neutral-Buffered Formalin (24 hours). Cassettes were then transferred to 70% ethanol and kept at 4°C prior to embedding, sectioning (8 μm) and Masson's trichrome staining (Bioimaging Hub, Cardiff University).

V-12. Statistical analysis

Box-and-whiskers plots were used to visualise results of experiments where variability was an important factor, such as when data from different donors or animals were pooled (Figure 7, Figure 17, Figure 25.A, Figure 31.B, Figure 32, Figure 34 and Figure 37). For experiments where data were not normally distributed, *p*-values were calculated using a Mann-Whitney U test for independent samples (Figure 7, Figure 31.B, Figure 32, Figure 34 and Figure 37) the non-parametric Wilcoxon signed-rank test for paired samples (Figure 17 and Figure 25.A). For all other figures shown *p* values were calculated using an unpaired, 2-tailed, Student's *t* test. Significance levels were set at $p<0.05$.

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